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Molecular Cloning of a Novel Isoform of Diphosphoinositol Polyphosphate Phosphohydrolase:

A Potential Target of Lithium Therapy

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The mechanisms underlying the therapeutic effects of lithium are largely unknown but may involve progressive adaptive alterations at the level of gene expression. Using differential display PCR, we identify a novel cDNA fragment, the expression of which was increased in the rat frontal cortex after 5 weeks of lithium administration. A full-length cDNA (2954-nt) was cloned by arrayed cDNA library screening, and sequencing of the clone revealed an open reading frame of 537-bp encoding a 179-residue protein. Amino acid sequence comparisons revealed that our clone is a member of the Nudix hydrolase family, with the highest percentage of homology (95%) being with a subtype of human diphosphoinositol polyphosphate phosphohydrolase, hDIPP2. Northern blot analysis revealed that chronic lithium treatment significantly increased rDIPP2 mRNA levels in frontal cortex, but not in hippocampus, midbrain, and cerebellum. The effect of lithium on rDIPP2 mRNA expression was not shared by two other anticonvulsant mood stabilizers, carbamazepine and valproate. Time-course studies showed that 1-week of lithium had no effect on rDIPP2 mRNA abundance in the frontal cortex. Our results suggest that DIPP2 may represent a biologically relevant target of lithium therapy, further supporting the notion that abnormalities in inositol phosphate metabolism may be significant in the pathophysiology and pharmacotherapy of bipolar disorder.
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KEY WORDS: *mRNA Differential display; Lithium; Gene expression; Nudix hydrolase; Diphosphoinositol polyphosphates*

Although lithium salts have been widely prescribed for many years as an effective treatment for acute mania and the prophylaxis of manic depressive (bipolar) disorder, the cellular and molecular changes that underlie their therapeutic effectiveness (or side effects) are still poorly understood. Recent progress in the search for the cellular and molecular basis of action of lithium, however, indicates that modulation of intracellular signaling mechanisms and regulation of gene expression may be significant with regard to the mood-stabilizing properties of this agent (Jope 1999; Manji and Lenox 1999; Li et al. 2000).

An important signal transduction pathway that may be involved in the therapeutic effects of lithium is the phosphoinositide second messenger system (Manji and

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Lenox 1999). In this pathway, receptor-mediated activation of phospholipase C generates two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which release Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively (Berridge 1987). Inositol 1,4,5-trisphosphate is subsequently metabolized either by dephosphorylation to Ins(1,4)P_2 or by phosphorylation to Ins(1,3,4,5)P_4, followed by recycling of these compounds back to inositol. The complexity of inositol phosphate pathway is further revealed by the presence of numerous other inositol polyphosphates, including the inositol pentakisphosphate ($InsP_5$), inositol hexakisphosphate ($InsP_6$), and diphosphoinositol polyphosphates, the functions of which are less clear (Shears 1998).

Despite much evidence that lithium can modulate the metabolism of inositol phosphates (InsPs, Ins(1,4)P_2, and Ins(1,3,4)P_3), there is little or no information concerning the effect of lithium on the more highly phosphorylated members of the inositol derivatives. Studies in vivo have shown that lithium, at therapeutically relevant concentrations, uncompetitively inhibits inositol monophosphatase (Hallcher and Sherman 1980), and to a lesser extent, inositol polyphosphate 1-phosphatase (Inhorn and Majerus 1988), leading to a depletion of intracellular *myo*-inositol levels. This has led to the suggestion that the acute lowering of *myo*-inositol content by lithium may initiate a cascade of secondary adaptive changes in the phosphoinositide signaling pathway, which would lead to alterations in PKC and gene expression in the brain (Manji et al. 1995). This idea is supported by several lines of data, among them, isozyme-selective reduction in the protein levels of PKC α and PKC ε, as well as MARCKS (myristoylated alanine rich C kinase substrate), following chronic lithium treatment via a *myo*-inositol dependent mechanism (Manji et al. 1996; Manji and Lenox 1999). More recently, lithium has been shown to modulate transcription factor binding to AP-1 and cyclic AMP responsive element in rat brain (Ozaki and Chuang 1997; Yuan et al. 1998; Wang et al. 1999). The lithium-induced changes in AP-1 DNA binding are likely mediated, in part, via activation of c-jun N-terminal kinases by a PKC-mediated and *myo*-inositol-dependent mechanism (Yuan et al. 1999).

The delayed time course of clinical efficacy of lithium is consistent with progressive adaptive changes in neuronal function that may involve changes at the level of gene expression (Jope 1999). Recently, diverse effects of lithium on several gene transcripts have been described in rat brain and cultured cell models; these transcripts include those for c-fos (Miller and Mathe 1997), G protein α-subunits (Colin et al. 1991; Li et al. 1993), adenylyl cyclases (Colin et al. 1991), dopamine receptors (Dziedzicka-Wasylewska and Wedzony 1996), neuropeptides (Sivam et al. 1988, 1989), and other cellular regulatory proteins (Wang and Young 1996; Feinstein 1998; Shamir et al. 1998; Chen and Chuang 1999; Chen et al. 1999; Hua et al. 2000). To gain a more complete picture of lithium-regulated changes in gene expression, we used differential display polymerase chain reaction (ddPCR), an mRNA-based screening technique to search for candidate genes whose expression is influenced by chronic lithium administration (Hua et al. 2000). We report herein the identification of a candidate cDNA fragment (LRG2) the expression of which was significantly increased in the rat frontal cortex after 5 weeks, but not 1 week, of lithium treatment, an effect that was not shared by chronic carbamazepine (CBZ) or valproate (VPA) administration. A cDNA clone encoding a novel Nudix (**Nu**cleoside **di**phosphate linked to some other moiety, ***X***) hydrolase was isolated from a rat brain cDNA library. The deduced amino acid sequence of the novel clone displays high homology to a family of recently identified enzymes in human, diphosphoinositol polyphosphate phosphohydrolases (DIPP), which catalyze the dephosphorylation of diphosphoinositol pentakisphosphate (PP-$InsP_5$) and bis-diphosphoinositol tetrakisphosphate ($[PP]_2InsP_4$) to $InsP_6$ (Safrany et al. 1998; Caffrey et al. 2000). These findings suggest that the enzyme catalyzing the dephosphorylation of "high energy" diphosphorylated inositol phosphates may represent a novel biologically relevant target of lithium therapy and emphasize the important role of inositol polyphosphate metabolism in the mechanism of action of lithium.

METHODS

Chemicals

Lithium carbonate (Li_2CO_3), CPZ, and sodium VPA were obtained from Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]dCTP (~3000 Ci/mmol) and [α-^{33}P]dATP (~2000Ci/mmol) were purchased from DuPont, New England Nuclear (Boston, MA). Other reagents were acquired as molecular biological grade from commercial sources.

Animals and Treatments

Male Wistar rats (275–300 g; Charles-River, St. Constant, Quebec) were individually housed in a temperature-controlled room (21°C ± 1°C) and maintained on a 12-h light/dark cycle with free access to food and water for at least 1 week before experiments. Animals were fed rat chow in pellet form containing Li_2CO_3 (2.2 g/kg diet; Bioserve, Frenchtown, NJ) for 1 or 5 weeks. Water and 2.6% saline were provided ad libitum to all animals. Rats receiving CBZ were fed with food pellets containing 0.25% CBZ for the first 4 days followed by 0.5% CBZ (Bioserve) for the next 31 days. In the VPA comparison group, animals were maintained on chow

pellets containing 0.4% VPA (Bioserve) for 5 weeks. Separate control groups of rats were fed with the regular rat chow for the indicated period. Imipramine (10 mg/kg), and haloperidol (1 mg/kg) were injected intraperitoneally once daily for 5 weeks. Control animals received injection of an equivalent volume of saline. Animals receiving mood-stabilizing drugs, imipramine, or haloperidol had weight gains similar to those of control animals (data not shown). Animals used in this study were cared for in strict accordance with guidelines of the Canadian Council on Animal Care, and the study was approved by the local Animal Care Committee.

At the end of the experiments, animals were decapitated. The brain was rapidly removed, and gross brain regions were dissected over ice using natural lines of demarcation, and frozen on dry ice and stored at −70°C until use. Blood samples were collected immediately after decapitation from the cervical trunk into heparinized test tubes. Plasma was separated by centrifugation (900 *g*, 20 min) for subsequent determination of drug levels. Plasma lithium levels were determined using the *Vitros* Li Slides (Johnson & Johnson, Mississauga, ON), a colorimetric assay for lithium-crown-ether dye complex, and ranged from 0.62 to 0.92 mM in the lithium-treated rats. Mean (±SEM) plasma CBZ concentrations were 16 ± 4 μM, as determined by the *Vitros* CRBM Slides (Johnson & Johnson, Mississauga, ON), an immunoassay using anti-CBZ antibody. Plasma VPA levels were measured by the TDxFLx valproic acid assay system using fluorescence polarization immunoassay (Abbott Lab., Abbott Park, IL), and were 13 ± 4 μM in the VPA-treated rats.

mRNA Differential Display

Differential display was carried out essentially as previously described (Hua et al. 2000). Briefly, total RNA from frontal cortex was isolated by guanidinium isothiocyanate-cesium chloride method and the residual chromosomal DNA removed using the MessageClean Kit (GenHunter, Nashville, TN). The DNase-treated total RNA (0.2 μg) was reverse transcribed in three separate pools using superscript II reverse transcriptase (GIBCO-BRL) and one of the 3′ composite anchor primers H-T_{11}M (H = 5′AGGC, M = A, C, or G). Two μl of the cDNA within each pool 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 (RNAimage kit; GenHunter, Nashville, TN), 0.2 μM of the respective anchor primer, 0.3 μl of [α-^{33}P]dATP (2000Ci/mmol) and 1 U of AmpliTaq DNA polymerase (Perkin–Elmer, Branchburg, NJ). The PCR was conducted using the following program on an 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 s, 40°C for 2 min and 72°C for 30 s, then a final extension at 72°C for 5 min, and rapid cooling at 4°C. The amplified cDNA fragments were then separated on a 6% denaturing polyacrylamide gel. The gel was vacuum-dried and exposed to Kodak X-Omat AR film for 1 to 2 days. To minimize the occurrence of false positives, two lithium-treated animals and two control animals were analyzed separately, but in parallel, for each amplification. Autoradiographic bands that were visually different in intensity in both lithium-treated animals compared with controls were excised, rehydrated, and reamplified by PCR with the appropriate set of primers.

The reamplified fragments were subcloned into the pGEM-T vector (Promega, Madison, WI), followed by transformation into INVαF′ competent cells (Invitrogen, Carlsbad, CA). Plasmids harboring inserts were identified by either restriction enzyme digestion using sites on the vector flanking the cloning sites or PCR screening using primers across the cloning sites. Inserts were manually sequenced on both strands using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech., Baie d'Urfe, Quebec).

cDNA fragments isolated from plasmids were labeled with [α-^{32}P]dCTP using the Strip-EZ DNA probe synthesis and removal kit (Ambion Inc., Austin, Texas) to a specific activity of 1×10^9 cpm/μg, and used as probes for northern blot analysis.

Northern Blot Analysis

Ten micrograms of heat-denatured total RNA from individual control and experimental animals were separated by electrophoresis on 1% agarose/formaldehyde gels and transferred to GeneScreen Plus membranes (New England Nuclear, Boston, MA) as previously described (Li et al. 1993; Hua et al. 2000). The membranes were hybridized overnight with a ^{32}P-labeled, random-primed cDNA probe (1×10^6 cpm/ml) prepared from a gel-purified insert. After high-stringency washing, hybridization signals were obtained by autoradiography using phosphoscreens. The levels of LRG2 mRNA were normalized against cyclophilin mRNA, determined by reprobing the same membrane with ^{32}P-labeled cyclophilin cDNA.

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 (LRG2)-specific primer (5′-CCATTTCTTTACGCCGCCACACAAGTC-3′) in accordance to manufacturer's protocol. The positive subplate (3H) was rescreened by PCR with the

same set of primers to identify positive subwells. Positive subwell (10G) stocks were plated out onto LB-Ampicillin (100 μg/ml) plates, and colonies were screened to identify positive clones. Nucleotide sequence analysis of these cDNA inserts was performed automatically using a dye-terminator sequencing kit followed by analysis 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. The nucleic acid and amino acid sequences were compared with other known genes and proteins in the GenBank database using the BLAST algorithms (Altschul et al. 1990).

Statistical Analysis

Results were expressed as mean ± SEM. Statistical analysis of the data was performed using two-tailed unpaired Student's *t*-test. *P*-values $< .05$ were considered statistically significant.

RESULTS

mRNA Differential Display

The changes in gene expression induced by chronic lithium treatment were analyzed using ddPCR. A cDNA fragment of about 630 bp was amplified to a greater extent in the frontal cortex samples from rats treated chronically with lithium as compared with control animals (Figure 1A). This ~630 bp cDNA fragment (designated as LRG2) was excised, reamplified, radiolabeled, and used to probe several regions of individual rat brains of both the lithium and control treatment groups. A single mRNA species of ~3.0 kb was detected in each brain region examined, including frontal cortex (Figure 1B), hippocampus, midbrain, and cerebellum (data not shown). Figure 2A shows that the abundance of mRNA for LRG2 was significantly increased (26%) in the frontal cortex ($t = 2.79$, $df = 14$, $p < .01$) following chronic lithium treatment. However, there were no apparent differences between control and lithium-treated animals for LRG2 mRNA levels in the hippocampus, midbrain, cerebellum, and the rest of cortex (i.e., whole cortex minus frontal cortex) (Figure 2B). In addition, short-term (1-week) lithium treatment did not significantly affect ($t = .26$, $df = 10$, $p = .8$) the amount of frontocortical LRG2 mRNA (Figure 2A).

Effects of Chronic Treatment of Anticonvulsant Mood Stabilizers and Psychotropic Drugs on LRG2 mRNA Levels

To assess whether the regulation of LRG2 mRNA level was an effect unique to lithium or common to other anticonvulsant mood stabilizing agents, the effects of chronic lithium, VPA, and CBZ treatment on the levels of LRG2 mRNA were investigated. Figure 3 shows that 5 weeks of VPA or CPZ administration did not significantly influence the expression of LRG2 mRNA in the rat frontal cortex, in contrast to the increase that was observed in response to lithium treatment ($t = 3.11$, $df = 8$,

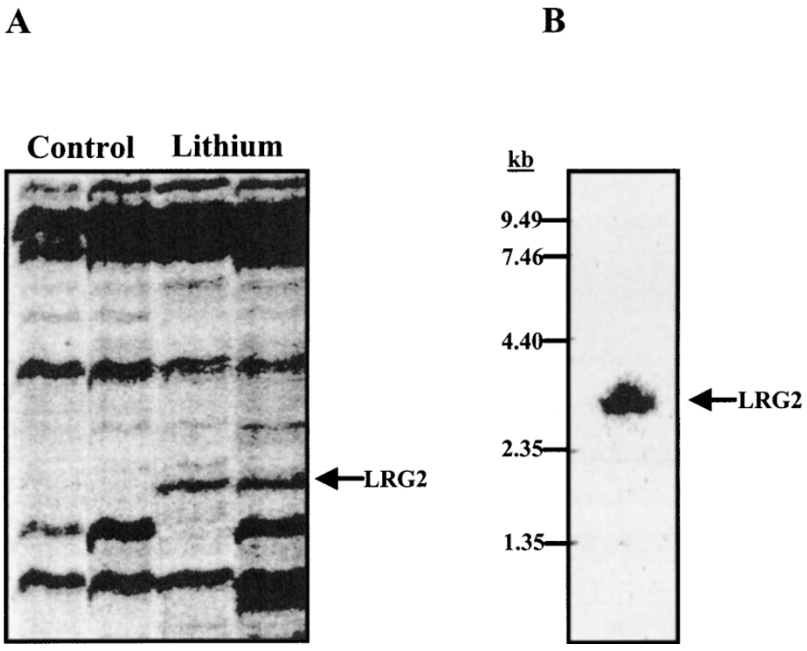


Figure 1. (**A**) Differential display of mRNA comparing gene expression in the frontal cortex from representative control and lithium-treated rats. The autoradiogram of [α-^{33}P]dATP-labeled differentially displayed products, using H-T_{11}C as 3′ primer and H-AP3 (5′-AGGCTTTGGTCAG-3′) as 5′ primer, 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.

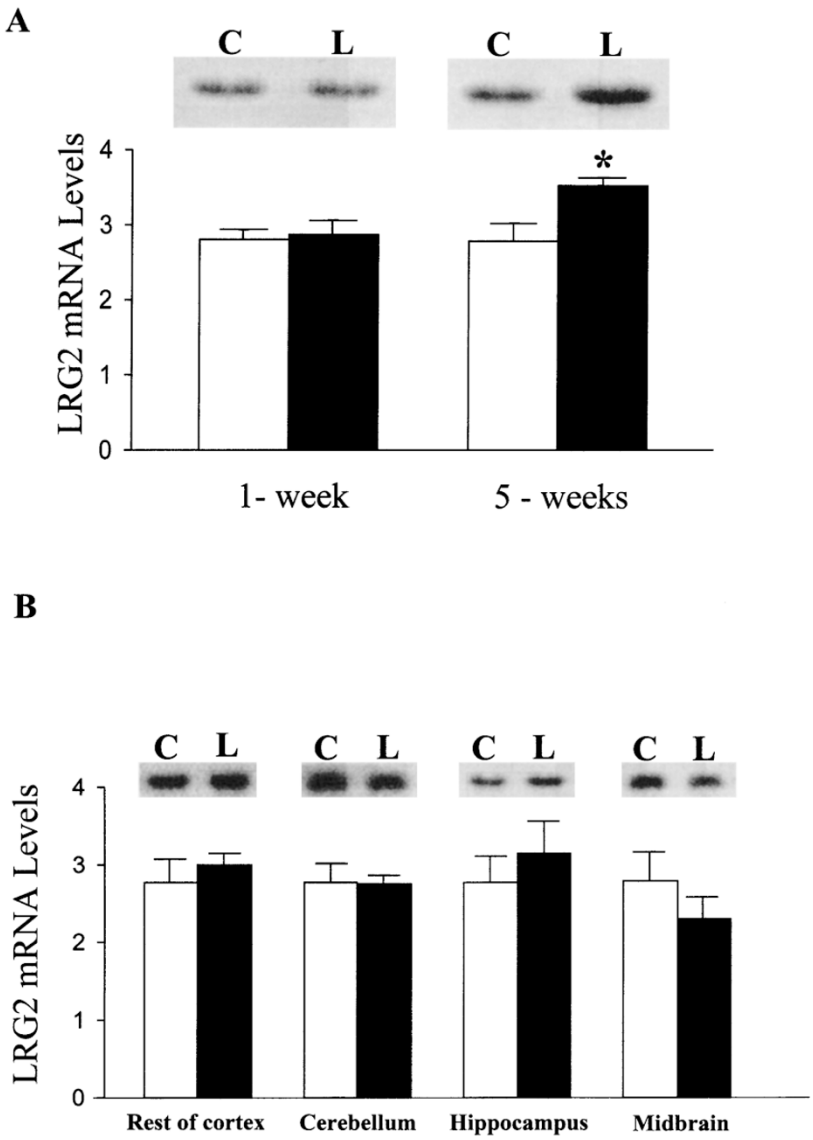


Figure 2. Effect of lithium administration on the expression of LRG2 mRNA in the rat. (**A**) Time course of the mRNA levels of LRG2 in frontal cortex of rats fed with 0.22% Li_2CO_3 (filled columns) or regular rat chow (open columns) for 1 or 5 weeks. (**B**) Effect of chronic (5-weeks) lithium treatment on the mRNA levels of LRG2 in different rat brain regions. Representative northern blots of mRNA from control (**C**) or lithium-treated (L) rats are shown in the top panels of each bar graph. The signal for LRG2 mRNA was normalized to cyclophilin mRNA. The results are expressed as the mean ± SEM for six to nine animals. * $p < .01$ compared with control animals (Student's *t*-test).

$p < .05$). Likewise, chronic administration of imipramine or haloperidol (5 weeks) did not increase the levels of LRG2 mRNA in the frontal cortex (vehicle, 100 ± 8; imipramine, 100 ± 4; haloperidol, 100 ± 6; mean ± SEM percent of vehicle; $n = 7$ per group).

Sequence Analysis of LRG2 cDNA Fragment

Results from the DNA sequencing revealed that LGR2 contained 633 bp and had the expected primers at its 5′ and 3′ ends. LRG2 contained a putative polyadenylation signal which was located 26 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 algorithm to search the GenBank database, the sequence of LRG2 displayed a near perfect homology (>98%) to the nucleotide sequence (nt 3-623) for an expressed sequence tag (EST; GenBank accession No. U95001.1) (Figure 4, denoted in shading).

Isolation of a Putative Full-Length Clone for LRG2

We screened an adult rat brain cDNA library by PCR to isolate a full-length cDNA clone using a sense primer that is specific to the vector (pCMV-XL4) and an antisense primer that is specific to the LRG2 cDNA fragment. We obtained five positive clones that contained an insert of ~3.0 kb. Sequence analysis of one of the cloned cDNA (clone C10) revealed a 537-bp open reading frame (ORF) with ATG start codon at nucleotide position 111 and TAG termination codon at nucleotide position 648 (Figure 4). We presume that translation of C10 initiates where indicated, because this is the first

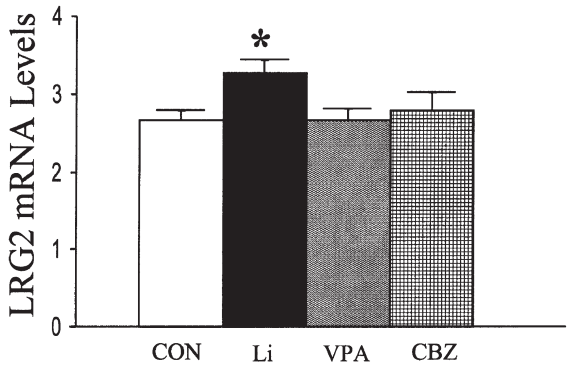


Figure 3. Effect of chronic lithium (Li), valproate (VPA), or carbamazepine (CBZ) administration on the expression of LRG2 mRNA in the rat frontal cortex. Rats were fed with regular chow (CON), or chow containing lithium Li, VPA, or CBZ for 5 weeks as described in Materials and Methods. Levels of LRG2 mRNA were determined in the frontal cortex by northern blot analysis and normalized to cyclophilin mRNA. The results are expressed as the mean ± SEM for five animals. * $p < .05$, compared with control animals (Student's *t*-test).

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 17 CpG doublets, and it does not contain an in-frame stop codon upstream of the start codon. The 3′-UTR contained three polyadenylation signals (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 for a predicted protein of 179 amino acids in length with a deduced molecular mass of 20,138 Da, 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 hDIPP1 (Safrany et al. 1998), hDIPP2 (Caffrey et al. 2000), and the human unnamed protein (hUnP; Accession #AK001490). Figure 5A shows the comparison of the deduced amino acid sequence of C10 with those from other members of Nudix hydrolase family, which has 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 and hUnP was 91%, in contrast to the 78% identify between C10 and hDIPP1 or rDIPP1 (Figure 5B). 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 seems 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 (Figure 6). Corresponding to the size of cloned cDNA, a single transcript of ~3 kb was observed in various 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 a relatively higher amount in the heart (Figure 6).

DISCUSSION

The main finding of our study is that chronic lithium administration increases the expression of a candidate cDNA fragment of ~630 bp (LRG2) in rat frontal cortex, but not in other brain regions examined. The effect of lithium is dependent on chronic administration, because 5, but not 1, weeks of lithium treatment increased the abundance of LRG2 mRNA. In addition, regulation of LRG2 expression seems to be specific to lithium, because chronic administration of VPA, CBZ, imipramine, or haloperidol did not alter the levels of LRG2 mRNA. These findings suggest that lithium increases the expression of LRG2 mRNA in a time-dependent, regionally specific, and pharmacologically selective manner, which may be relevant to its mode of action (or side effects).

Subsequent screening of a cDNA library showed that the clone isolated here is likely the rat homologue of DIPP2 recently identified in human (Caffrey et al. 2000). It is likely that we have isolated the complete cDNA sequence, because the size of the insert (2954 bp) is similar to that of the LRG2/rDIPP2 transcript detected by northern blot analysis (~3 kb; Figures 1, 6). Although there are three polyadenylation signals in the 3′-UTR, it seems 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 because of the use of alternate polyadenylation signals (Caffrey et al. 2000). On the

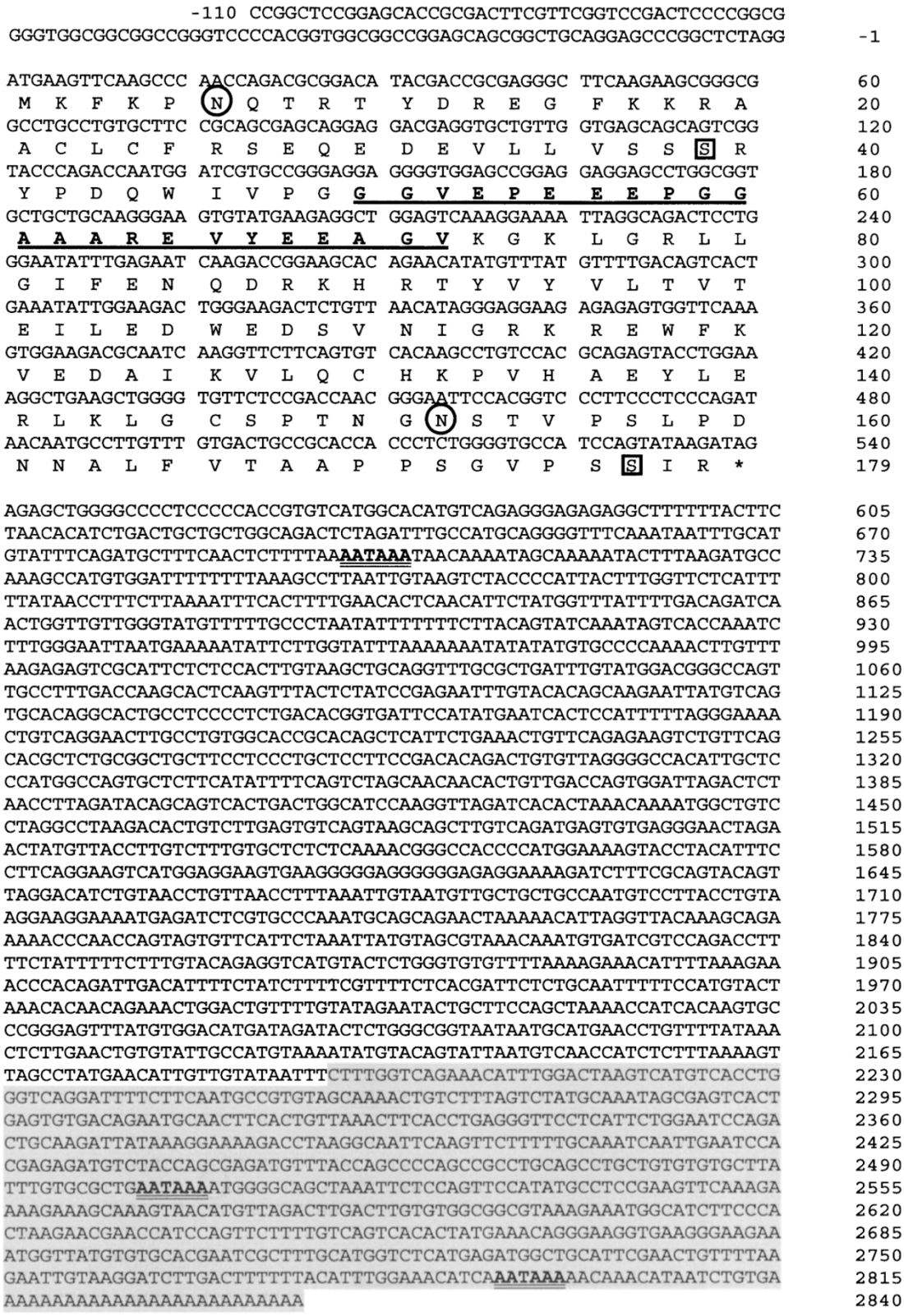
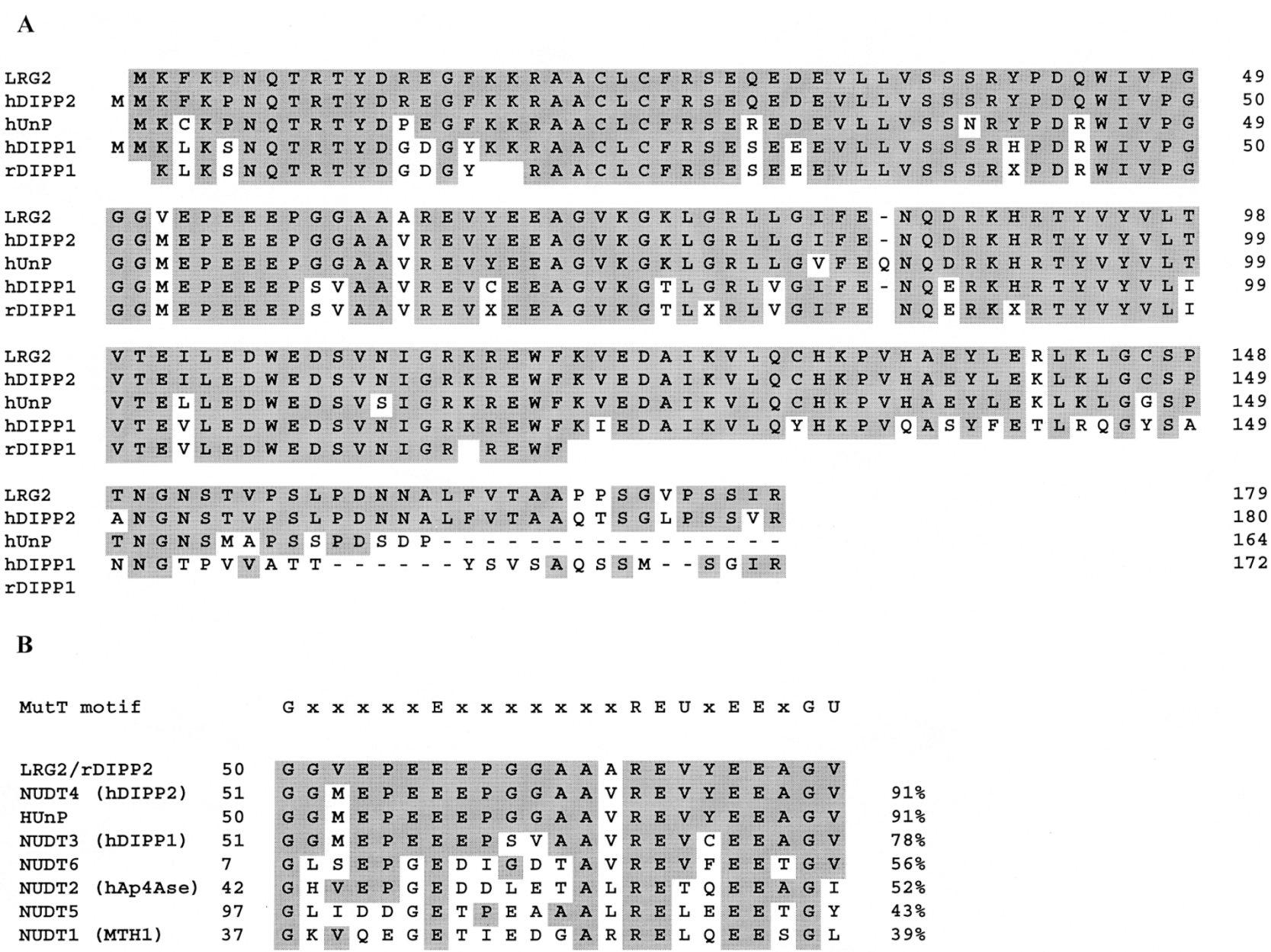


Figure 4. The nucleotide and deduced amino acid sequence (single letter code) of the cDNA encoding rat DIPP2. Numbers indicate the positions of nucleotides or amino acid residues are relative to the ATG initiation codon. Deduced amino acid sequence is shown below the nucleotide sequence. The predicted termination codon is indicated by an asterisk. Potential phosphorylation sites for protein kinase C are boxed, and 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 (Accession number AF253473).


A
LRG2 MKFKPNQTRTYDREGFKKRAACLCFRSEQEDEVLLVSSSRYPDQWIVPG 49
hDIPP2 MMKFKPNQTRTYDREGFKKRAACLCFRSEQEDEVLLVSSSRYPDQWIVPG 50
hUnP MKCKPNQTRTYDPEGFKKRAACLCFRSEREDEVLLVSSNRYPDRWIVPG 49
hDIPP1 MMKLKSNQTRTYDGDGYKKRAACLCFRSESEEEVLLVSSSRHPDRWIVPG 50
rDIPP1 KLKSNQTRTYDGDGY RAACLCFRSESEEEVLLVSSSRXPDRWIVPG
LRG2 GGVEPEEEEPGGAAAREVYEEAGVKGKLGRLLGIFE-NQDRKHRTYVYVLT 98
hDIPP2 GGMEPEEEEPGGAAVREVYEEAGVKGKLGRLLGIFE-NQDRKHRTYVYVLT 99
hUnP GGMEPEEEEPGGAAVREVYEEAGVKGKLGRLLGVFEQNQDRKHRTYVYVLT 99
hDIPP1 GGMEPEEEEPSVAAVREVCEEAGVKGTLGRLVGIFE-NQERKHRTYVYVLI 99
rDIPP1 GGMEPEEEEPSVAAVREVXEEAGVKGTLXRLVGIFE NQERKXRTYVYVLI
LRG2 VTEILEDWEDSVNIGRKREWFKVEDAIKVLQCHKPVHAEYLERLKLGCSP 148
hDIPP2 VTEILEDWEDSVNIGRKREWFKVEDAIKVLQCHKPVHAEYLEKLKLGCSP 149
hUnP VTELLEDWEDSVSIGRKREWFKVEDAIKVLQCHKPVHAEYLEKLKLGGSP 149
hDIPP1 VTEVLEDWEDSVNIGRKREWFKIEDAIKVLQYHKPVQASYFETLRQGYSA 149
rDIPP1 VTEVLEDWEDSVNIGR REWF
LRG2 TNGNSTVPSLPDNNALFVTAAPPSGVPSSIR 179
hDIPP2 ANGNSTVPSLPDNNALFVTAAQTSGLPSSVR 180
hUnP TNGNSMAPSSPDSDP---------- 164
hDIPP1 NNGTPVVATT------YSVSAQSSM--SGIR 172
rDIPP1
B
MutT motif GxxxxxExxxxxxxREUxEExGU
LRG2/rDIPP2 50 GGVEPEEEEPGGAAAREVYEEAGV
NUDT4 (hDIPP2) 51 GGMEPEEEEPGGAAVREVYEEAGV 91%
HUnP 50 GGMEPEEEEPGGAAVREVYEEAGV 91%
NUDT3 (hDIPP1) 51 GGMEPEEEEPSVAAVREVCEEAGV 78%
NUDT6 7 GLSEPGEDIGDTAVREVFEETGV 56%
NUDT2 (hAp4Ase) 42 GHVEPGEDDLETALRETQEEAGI 52%
NUDT5 97 GLIDDGETPEAAALRELEEETGY 43%
NUDT1 (MTH1) 37 GKVQEGETIEDGARRELQEESGL 39%

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, (1) the presence of multiple CpG doublets that are characteristic of "housekeeping" enzymes (Bird 1986); and (2) the high G/C content, which may be important in regulating translational efficiency (Kozak 1996). We also found other putative regulatory elements in this region, including sequences similar to AP-2 (TCCCCGGCGG, position −74) and AP-4 (GCAGCGGCTG, position −28) binding sites.

The assignment of C10 as a clone containing rDIPP2 cDNA is based on the significant homology (95%) of its deduced amino acid sequence with that of the human homologue (Caffrey et al. 2000). Homologies are especially noteworthy in several regions, including the Nudix motif (Figure 5), 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 in amino acid sequence likely reflects the constraints against evolutionary divergence in protein structure dictated by the unique function of this protein.

We queried the database of rat ESTs maintained by the GenBank with the nucleotide sequence of C10 using the BLAST algorithm (Altschul et al. 1990). The dbEST sequences returned 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 inframe 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 the existence of another rDIPP2 variant with an extra amino acid may also occur in the rat.

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).

The pharmacological significance of lithium regulation of rDIPP2 mRNA expression is presently unknown, but 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; Shears 1998). Electrophysiolgical studies in *Drosophila* have provided evidence of aberrant synaptic transmission in response to lithium exposure, which is attributable 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 lithium modulation of synaptic function may be mediated partly by its influences 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. 1999). It is of interest in this regard that long-term lithium treatment has recently been shown to exert neuroprotective effects against cell death induced by a variety of insults both in cultured neurons and in intact animals (Nonaka and Chuang 1998, Nonaka et al. 1998). It is tempting to suggest that lithium's regulation of rDIPP2 expression may also

Figure 5. (**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**) 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.

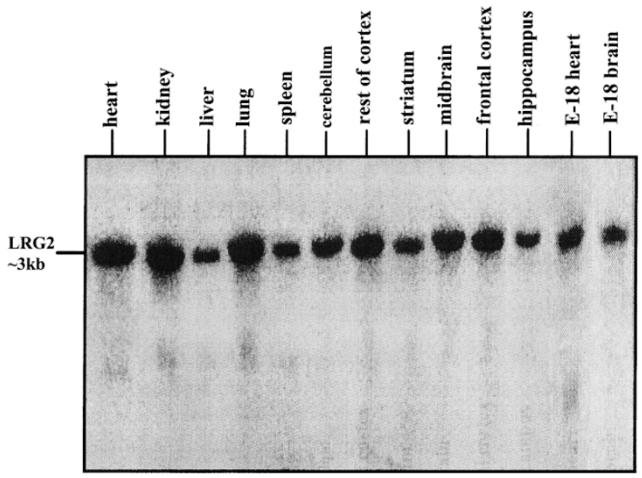


Figure 6. Tissue distribution of rDIPP2 mRNA in the rat. Northern blot containing 10 μg of total RNA from different adult rat tissues and the brain and heart of embryonic day (E) 18 rat, was hybridized with a [^{32}P]dCTP labeled rDIPP2 cDNA. A singe mRNA species of ~3 kb is detected from all tissues. Size markers (kilobases) are on the left.

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), seems to underscore the importance of lithium regulation of this enzyme in the maintenance of cell integrity and viability.

The increase in rDIPP2 mRNA expression following long-term lithium, but not CBZ or VPA administration, supports the pharmacological specificity of this effect to lithium. A variety of evidence suggests mood-stabilizing agents may exert their therapeutic effects through such convergent mechanisms as targeting specific signal transduction cascades (Manji et al. 1995; Jope 1999; Li et al. 2000) or cellular responses (e.g., neuroprotection) (Nonaka et al. 1998; Chen et al. 1999). There is also increasing recognition, however, of differential clinical response to lithium and anticonvulsant mood stabilizers as well as putative augmenting effects of combinations of these agents in managing more treatment refractory bipolar patients (Shelton et al. 1998). Such clinical observations suggest selective effects of lithium, such as those identified here, may distinguish its therapeutic spectrum of actions. In this regard, it would be of interest to determine whether changes in DIPP2 expression occur in bipolar patients and are of clinical utility in predicting lithium responsiveness.

mRNA differential display has been used by other groups to identify transcriptionally regulated genes in response to long-term lithium treatment. One study revealed upregulation of 2′,3′-cyclic nucleotide 3′-phosphodiesterase type II in rat C6 glioma cells incubated with 1 mM of lithium for one week (Wang and Young 1996). In other studies, altered mRNA levels of the transcription factor polyomavirus enhancer-binding protein 2β (Chen et al. 1999), nitrogen permease regulator 2 (Wang et al. 1999) and aldolase A (Hua et al. 2000) were reported in rat frontal cortex following chronic lithium administration. 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.

In summary, we report herein the novel observations that lithium, when administered in a therapeutically relevant paradigm, increases the expression of rDIPP2, an enzyme involved in the metabolism of diphosphoinositol polyphosphates, in rat frontal cortex. Our results suggest that one of the DIPP isoforms (i.e., DIPP2) may represent a putative biologically relevant target of lithium therapy, further supporting the notion that abnormalities in the inositol phosphate metabolism and/or phosphoinositide signaling pathway may be significant in the pathophysiology and pharmacotherapy of bipolar disorder (Jope 1999; Li et al. 2000). Additional experiments are necessary to study the regulation of function and/or protein levels of rDIPP2 to understand the potential significance of these findings for its therapeutic mechanism of action (or side effects) in bipolar disorder.

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REFERENCES

Acharya JK, Labarca P, Delgado R (1998): Synaptic defects and compensatory regulation of inositol metabolism in inositol polyphosphate 1-phosphatase mutants. Neuron 20:1219–1229

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

Baker JC, Jacobson MK (1986): Alteration of adenyl dinucleotide metabolism by environmental stress. Proc Natl Acad Sci USA 83:2350–2352

Berridge MJ (1987): Inositol trisphosphate and diacylglycerol: Two interacting second messengers. Ann Rev Biochem 56:159–163

Bessman MJ, Frick DN, O'Handley SF (1996): The MutT proteins or "Nudix" hydrolases, a family of versatile,

widely distributed "housecleaning" enzymes. J Biol Chem 271:25059–25062

Bird AP (1986): CpG-rich islands and the function of DNA methylation. Nature 321:209–213

Caffrey JJ, Safrany ST, Yang X, Shears SB (2000): Discovery of molecular and catalytic diversity among human diphosphoinositol-polyphosphate phosphohydrolases: An expanding NUDT family. J Biol Chem 275:12730z7–12736

Chen RW, Chuang D-M (1999): Long-term lithium treatment suppresses p53 and Bax expression but increases bcl-2 expression. J Biol Chem 274:6039–6042

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

Colin SF, Chang HC, Mollner S, Pfeuffer T, Reed RR, Duman RS, Nestler EJ (1991): Chronic lithium regulates the expression of adenylate cyclase and G_i-protein α-subunit in rat cerebral cortex. Proc Natl Acad Sci USA 88:10634–10637

De Camilli P, Emr SD, McPherson PS, Novick P (1996): Phosphoinositides as regulators in membrane traffic. Science 271:1533–1539

Dixon JF, Los GV, Hokin LE (1994): Lithium stimulates glutamate "release" and inositol 1,4,5-triphosphate accumulation via activation of the N-methyl-D-aspartate receptor in monkey and mouse cerebral cortex slices. Proc Natl Acad Sci USA 91:8358–8362

Dziedzicka-Wasylewska M, Wedzony K (1996): The effect of prolonged administration of lithium on the level of dopamine D_2 receptor mRNA in the rat striatum and nucleus accumbens. Acta Neurobiol Exp 56:29–34

Feinstein DL (1998): Potentiation of astroglial nitric oxide synthase type-2 expression by lithium chloride. J Neurochem 71:883–886

Fukuda M, Mikoshiba K (1997): The function of inositol high polyphosphate binding proteins. Bioessays 19:593–603

Hallcher LM, Sherman WR (1980): The effect of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. J Biol Chem 255:10896–10901

Hua LV, Green M, Warsh JJ, Li PP (2000): Lithium regulation of aldolase A expression in rat frontal cortex: Identification by differential display. Biol Psychiat 48:58–64

Inhorn RC, Majerus PW (1988): Properties of inositol polyphosphate 1-phosphatase. J Biol Chem 263:14559–14565

Jope RS (1999): Anti-bipolar therapy: Mechanism of action of lithium. Mol Psychiat 4:117–128

Kisselev LL, Justesen J, Wolfson AD, Frolova LY (1998): Diadenosine oligophosphates (ApnA), a novel class of signaling molecules? FEBS Lett 427:157–163

Kozak M (1996): Interpreting cDNA sequences: Some insights from studies on translation. Mamm Genome 7:563–574

Li PP, Young LT, Tam YK, Warsh JJ (1993): Effects of chronic lithium and carbamazepine treatment of G-protein subunit expression in rat cerebral cortex. Biol Psychiat 34:162–170

Li PP, Andreopoulos S, Warsh JJ (2000): Signal transduction abnormalities in bipolar affective disorder. In Reith MEA (ed), Cerebral Signal Transduction: From First to Fourth Messengers. New Jersey, Humana Press, pp 283–309

Manji HK, Potter WZ, Lenox RH (1995): Signal transduction pathways: Molecular targets for lithium's action. Arch Gen Psychiat 52:531–543

Manji HK, Bersudsky Y, Chen G, Belmaker RH, Potter WZ (1996): Modulation of protein kinase C isozymes and substrates by lithium: The role of myo-inositol. Neuropsychopharmacology 15:370–381

Manji HK, Lenox, RH (1999): Protein kinase C signaling in the brain: Molecular transduction of mood stabilization in the treatment of manic-depressive illness. Biol Psychiat 46:1328–1351

Miller JC, Mathe AA (1997): Basal and stimulated c-fos mRNA expression in the rat brain: Effect of chronic dietary lithium. Neuropsychopharmacology 16:408–418

Miras-Portugal MT, Gualix J, Pintor J (1998): The neurotransmitter role of diadenosine polyphosphates. FEBS Lett 430:78–82

Nonaka S, Chuang D-M (1998): Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. Neuroreport 9:2081–2084

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

Ozaki N, Chuang D-M (1997): Lithium increases transcription factor binding to AP-1 and cyclic AMP-responsive element in cultured neurons and rat brain. J Neurochem 69:2336–2344

Safrany ST, Caffrey JJ, Yang X, Bembenek ME, Moyer MB, Burkhart WA, Shears SB (1998): A novel context for the "MutT" module, a guardian of cell integrity, in a diphosphoinositol polyphosphate phosphohydrolase. EMBO J 17:6599–6607

Safrany ST, Ingram SW, Cartwright JL, Falck JR, McLennan AG, Barnes LD, Shears SB (1999): The diadenosine hexaphosphate hydrolases from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* are homologues of the human diphosphoinositol polyphosphate phosphohydrolase. J Biol Chem 274:21735–21740

Shamir A, Ebstein RP, Nemanov L, Zohar A, Belmaker RH (1998): Inositol monophosphatase in immortalized lymphoblastoid cell lines indicates susceptibility to bipolar disorder and response to lithium therapy. Mol Psychiat 3:481–482

Shears SB (1998): The versatility of inositol phosphates as cellular signals. Biochim Biophys Acta 1436:49–67

Shelton RC, Thase ME, Kowatch R, Baldessarini RJ (1998): Update on the management of bipolar illness. J Clin Psychiat 59:484–495

Sivam SP, Takeuchi K, Li S, Douglass J, Civelli O, Calvetta L, Herbert E, McGinty JF, Hong JS (1988): Lithium increases dynorphin A (1-8) and prodynorphin mRNA levels in the basal ganglia. Mol Brain Res 3:155–164

Sivam SP, Krause JE, Takeuchi K, Li S, McGinty JF, Hong JS (1989): Lithium increases rat striatal beta- and gamma-

preprotachykinin messenger RNAs. J Pharmacol Exp Ther 248:1297–1301

Wang HY, Friedman E (1988): Chronic lithium: Desensitization of autoreceptors mediating serotonin release. Psychopharmacology 94:312–314

Wang JF, Young LT (1996): Differential display PCR reveals increased expression of 2′3′-cyclic nucleotide 3′-phosphodiesterase by lithium. FEBS Lett 386:225–229

Wang JF, Asghari V, Rockel C, Young LT (1999): Cyclic AMP responsive element binding protein phosphorylation and DNA binding is decreased by chronic lithium but not valproate treatment of SH-SY5Y neuroblastoma cells. Neuroscience 91:771–776

Yang X, Safrany ST, Shears SB (1999): Site-directed mutagenesis of diphosphoinositol polyphosphate phosphohydrolase, a dual specificity NUDT enzyme that attacks diadenosine polyphosphates and diphosphoinositol polyphosphates. J Biol Chem 274:35434–35440

Yuan PX, Chen G, Huang LD, Manji HK (1998): Lithium stimulates gene expression through the AP-1 transcription factor pathway. Mol Brain Res 58:225–230

Yuan PX, Chen G, Manji HK (1999): Lithium activates the c-Jun NH2-terminal kinases in vitro and in the CNS in vivo. J Neurochem 73:2299–2309