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Microarray gene expression profiling of mouse brain mRNA in a model of lithium treatment

Albert Chetcuti[a,b,c,d], Linda J. Adams[a,b], Philip B. Mitchell[d,e] and Peter R. Schofield[a,b,c,d]

Objectives Even after five decades of use, the mood stabilizer lithium continues to be the mainstay of treatment for bipolar disorder in many countries. The mechanism of action for lithium, however, remains unclear.

Methods In this study, microarray analysis was used to identify genes and cellular pathways that are altered in the mouse brain after treatment with lithium at human therapeutic concentrations. Mice received daily injections of lithium chloride for 7 consecutive days. Whole-brain total RNA was used as a template for microarray gene expression profiling.

Results This study has identified 19 transcripts that are differentially expressed by four-fold when compared with control untreated mice. The altered expression of these genes was validated by quantitative PCR analysis with five genes showing significant differential expression. Lithium was found to significantly decrease the expression of metallothionein 3 (MT3), ATPase, Na^+/K^+ transporting, α1 polypeptide (ATP1A1), transcription elongation factor B (SIII)-polypeptide 2 (TCEB2), proteasome subunit β type 5 (PSMB5), and guanine nucleotide binding protein β1 (GNB1).

Conclusion These genes are involved in a diverse range of biological functions, including maintaining metal ion homeostasis and chemical/electrical gradients across membranes, regulating RNA polymerase II, protein degradation, and G-protein-coupled signal transduction. These results indicate that lithium can regulate a large number of different cellular pathways in the brain. Understanding the molecular and cellular mechanisms by which lithium achieves its therapeutic action represents a valuable step in clarifying the pathophysiology of bipolar disorder. *Psychiatr Genet* 18:64–72 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The discovery of lithium's efficacy as a mood-stabilizing agent revolutionized the treatment of patients with bipolar disorder. In 1949, Cade reported that lithium had a calming influence on guinea pigs, and that it lessened manic symptoms in humans (Cade, 1949; Mitchell and Hadzi-Pavlovic, 1999). After five decades of use, lithium continues to be the mainstay of treatment for bipolar disorder in many countries, both for acute mania and as prophylaxis for recurrent manic and depressive states. From a clinical perspective, it has been appreciated that the therapeutic effects of lithium can take 1–2 weeks to occur. It is clear that the effect of lithium must be occurring at the transcriptional level rather than only at the biochemical level (Manji *et al.*, 2001).

Extensive research has identified a number of proteins and pathways that are regulated by lithium. It has been demonstrated that lithium reduces glycogen synthase kinase (GSK)-3β activity in two ways, both directly and by increasing the inhibitory phosphorylation of GSK-3β (Klein and Melton, 1996; Jope, 2003). Lithium has also been identified as an inhibitor of the enzyme-converting inositol monophosphates to myoinositol. Lithium-treated rats exhibit a significant increase in the concentration of inositol monophosphates and a significant decrease in myoinositol concentration compared with saline-treated controls (O'Donnell *et al.*, 2003).

Many studies have replicated the finding that lithium increases the gene expression of the antiapoptotic gene Bcl-2. This was first described in a differential display experiment used to identify concordant changes in gene expression induced by two mood-stabilizing agents, lithium and valproate (Chen *et al.*, 1999). Both treatments robustly increased the levels of Bcl-2 in the frontal cortex of rats. In addition, both treatments increased mRNA levels of the transcription factor polyomavirus enhancer-binding protein 2β and the DNA binding activity of polyomavirus enhancer-binding protein2 αβ, which can

transcriptionally regulate Bcl-2 expression. Interestingly, the increase in Bcl-2 mRNA has also been described for the antipsychotics, clozapine and olanzapine (Bai *et al.*, 2004).

Although a large number of studies have treated rodents with lithium, very few have investigated altered lithium gene expression in brain tissue using high-density microarray analysis. This experimental design should complement other microarray studies, which have investigated altered gene expression using postmortem brain tissue derived from bipolar patients (Iwamoto *et al.*, 2004), or lithium-treated lymphocytes derived from bipolar patients (Su *et al.*, 2004). Several studies have treated rats with lithium and examined altered gene expression in brain tissue using differential display (Chen *et al.*, 1999; Wang *et al.*, 1999; Hua *et al.*, 2000). Only one study has reported the effects of treating rats with lithium and investigated altered gene expression in brain tissue using microarray analysis (Bosetti *et al.*, 2002). This study treated rats with lithium for 7 and 42 days before hybridizing whole rat brain total RNA onto human complementary DNA (cDNA) microarrays containing 4000 genes. As alternatives to in-vivo animal models, studies have treated yeast cells or cultured human chondrocyte T/C28a cells with lithium and examined altered gene expression using microarray analysis (Bro *et al.*, 2003; Zhang *et al.*, 2005).

This study describes the analysis of altered gene expression in the whole mouse brain after treatment with lithium at serum concentrations that matched the human therapeutic level. In this study we have treated mice with the mood-stabilizing drug lithium and investigated altered gene expression in brain tissue using high-density microarray technology. Identifying the molecular and cellular mechanisms by which lithium achieves its therapeutic action represents a valuable step in developing new drug targets and clarifying the pathophysiology of bipolar disorder.

Materials and methods

Animals and lithium serum quantitation

Male C57BL/6 mice (ARC, Perth, Australia) were used for all experiments. Animals were maintained with standard chow, water, and 1.5% sodium chloride *ad libitum* in a controlled temperature and humidity environment with a 12-h day/night cycle. Animal studies were approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Experimentation Ethics Committee (New South Wales, Australia, AEEC #00/01). All animals were weighed before each injection and were 8–10 weeks of age at the commencement of each study. Lithium chloride (Sigma-Aldrich, Sydney, Australia) was administered via intraperitoneal injection once daily at a rate of 8.0 mmol/kg/day for 7 consecutive days. Lithium chloride was prepared in 0.9% saline solution. Control mice received intraperitoneal injections once daily of 10 ml/kg of sterile 0.9% saline solution for 7 consecutive days. Four hours after the last injection on the seventh day of treatment, control and lithium-treated mice were euthanized under 4% halothane anesthesia. A cardiac puncture procedure was used to collect 1 ml of blood for lithium serum quantitation. The whole brain was immediately removed, snap-frozen in liquid nitrogen, and stored at −80°C until required. Mouse bloods were assayed for lithium concentration by the SydPath Diagnostic Testing Facility (St Vincent's Hospital, Sydney, Australia).

Whole-brain total RNA extraction

Total RNA was prepared by extracting whole mouse brain tissue in TRI Reagent (Sigma-Aldrich, Sydney, Australia) following the manufacturer's instructions. Total RNA pellets were resuspended in 100 µl of RNase-free water and the concentration was determined by measuring the absorbance at 260 nm using a GeneQuant UV spectrophotometer (Pharmacia Biotech, Sydney, New South Wales, Australia). The purity of extracted total RNA was assessed by the 260/280 nm ratio (> 1.8) measured. The quality and integrity were checked by visualization of total RNA samples on 1.0% formaldehyde-denaturing agarose gel.

Microarray sample preparation and hybridization

A total of 30 mice were treated with 8.0 mmol/kg/day of lithium daily for 7 days. The resultant blood lithium concentration ranged from 0.5 to 1.2 mmol/l, with a mean of 0.824 ± 0.205 mmol/l (mean ± SD). These lithium concentrations are within the maintenance and acute antimanic therapeutic ranges for human treatment (Mitchell, 2001). To minimize interindividual variability, the 30 mice were randomly divided into six groups containing five mice each. The whole-brain total RNA for each group of five mice was pooled in equal proportion and hybridized onto a single Affymetrix GeneChip Murine Genome U74Av2 microarray (Santa Clara, California, USA). The mean blood lithium concentration for the five samples used on each microarray was 0.60 ± 0.10, 0.64 ± 0.06, 0.70 ± 0.12, 0.94 ± 0.17, 0.96 ± 0.17, and 1.04 ± 0.11 mmol/l. Fifteen control, saline-treated, mice were randomly divided into three groups each containing five mice. The whole-brain total RNA for each group of five mice was pooled in equal proportion and hybridized onto a single microarray chip.

Microarray biotinylated copy RNA (cRNA) probes were prepared as outlined in the Affymetrix GeneChip Expression Analysis technical manual and as previously described (Chetcuti *et al.*, 2006). Briefly, probes were prepared using BioArray High Yield RNA Transcription Labeling Kit (Affymetrix, Santa Clara, California, USA), hybridized to Affymetrix GeneChip Murine Genome U74Av2 microarrays, washed using GeneChip Fluidics Station 400 (Affymetrix), and scanned using an Agilent

GeneArray Scanner (Agilent Technologies, Palo Alto, California, USA). Before hybridization to Affymetrix GeneChip Murine Genome U74Av2 microarrays, the quality of each prepared cRNA probe was assessed by hybridizing prepared cRNA probes to Affymetrix Test3 microarrays. Prepared cRNA probes were only hybridized to Murine Genome U74Av2 microarrays if the 3′/5′ signal intensity ratio for the mouse genes GAPDH (M32599) and β-actin (M12481) was less than 3.0.

Microarray data analysis

Using Affymetrix Microarray Analysis Suite software v5.0 (Santa Clara, California, USA), the fluorescent signal intensities of all 12 488 transcripts contained on the Affymetrix GeneChip Murine Genome U74Av2 microarrays were determined. The quality of hybridization was assessed by examining the microarray hybridization intensity image and 3′/5′ ratios of the mouse housekeeping genes GAPDH (M32599) and β-actin (M12481). The raw intensity values were normalized to the mean intensity of all probe sets. Genes were initially filtered for a 'present' call as assigned by the MAS software for each microarray. Genes were only selected for further analysis if they were 'present' on all nine microarrays (three control and six lithium treatment). Differentially expressed genes were determined by calculating the mean signal intensity for each gene in control and treated groups. The fold change signal ratio was then calculated for control versus lithium treatment and used to rank genes based on the magnitude of fold difference. Genes were further selected that had a $\geq$ four-fold difference between control and lithium treatment microarrays.

Reverse transcription and quantitative PCR analysis

Altered gene expression identified using microarray analysis was validated using quantitative PCR analysis. Before reverse transcription, total RNA was incubated for 15 min at room temperature with two units of DNase I (Invitrogen, Sydney, Australia) in 1 × DNase I reaction buffer (Invitrogen) to remove any genomic DNA contamination. Reverse transcription was performed using 2 μg of total RNA. Total RNA was denatured for 10 min at 65°C in the presence of oligo dT_{12-18} (500 ng, Invitrogen). After denaturing, cDNA was synthesized in a 50 μl reaction volume containing 1 × First Strand buffer (Invitrogen), deoxyribonucleotide triphosphate (400 μmol/l each), dithiothreitol (10 mmol/l), RNaseOUT ribonuclease inhibitor (80 units, Invitrogen), Superscript III Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (400 units, Invitrogen) and incubated for 60 min at 50°C.

All quantitative PCR amplifications were performed simultaneously on all 15 individual control mouse cDNA samples and all 30 individual lithium-treated mouse cDNA samples. Each quantitative PCR experiment included seven serial diluted DNA standards of known concentration and a no template control. These standards were used to calculate the mRNA copy number for each gene in every sample and were amplified in duplicate arrangement. For quantitative PCR amplification, 1 μl of 2/5 diluted cDNA was added to a 10 μl PCR reaction containing 1 × Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and forward/reverse primers (200 nmol/l each). PCR primers were designed using MacVector v6.5.3 software (Accelrys, Cambridge, UK) and synthesized by Sigma Genosys (Sydney, Australia) (Table 1). Amplification conditions included the following: uracil-DNA glycosylase treatment at 50°C for 2 min, denaturation at 95°C for 2 min, and 45 cycles of amplification (denaturation at 95°C for 5 s, annealing for 15 s, and extension for 15 s at 72°C). After amplification,

Table 1 Quantitative PCR oligonucleotide primers used for amplifying specific genes using mouse brain cDNA

Gene	Forward primer	Reverse primer	Size (bp)	Annealing temperature (°C)
ACTB	5′-TGGGAATGGGTCAGAAGGACTC-3′	5′-GGTCATCTTTTCACGGTTGGC-3′	227	58
EEF1A2	5′-AAGTCCCTGAAGTCTGGTGATGC-3′	5′-TGTGCGGATTTTGTGACCTTG-3′	195	65
1110008P14Rik	5′-ACCTGGAGGAGAAGAATGACCAC-3′	5′-AAGGGTGATGGGCAGTGAATGG-3′	281	65
GNB1	5′- CCTGAAAACTCGTGAAGGGAATG-3′	5′- TCGTGTCCTGTAAAGGTCTGCC -3′	303	58
TCEB2	5′-TTTACGGACGCCAAGGAGTC-3′	5′-GAAGGTGTCATCTGCTCGGAAG-3′	213	55
SST	5′-AGGACGAGATGAGGCTGGAG-3′	5′-TATGGGGTTTGGGGGAGAG-3′	170	65
MT3	5′-CCCTGTCCTACTGGTGGTTC-3′	5′-GGCACACTTCTCACATCCG-3′	119	55
PSMB5	5′-CCCAGACGGTGAAGAAAGTAATAG-3′	5′-AGTTTAGAGGCTGCTGCGAC-3′	163	55
HK1	5′-GAGCATCACATAGAATACCACCCG-3′	5′-CCCAGAAGACAGTCCAAGAGAAGG-3′	203	65
ATP1A1	5′-TTCACCTGCCATACAGCGTTC-3′	5′-CACAAAGATGAGAAGGGAGTAGGG-3′	232	65
NFIX	5′-TCACAGACTCCAGTTGCGAC-3′	5′-TCCCAGGATAGAACACATCATC-3′	202	65
MT1	5′-ATAGTATCGGACCAACCCAGCGTC-3′	5′-CCAAAGACATTACCCCTCTGTGAG-3′	231	66
MRPS34	5′-GTCTGGTTACCCGCAAGTCC-3′	5′-CTCTGGTTTCGCAGTGAACG-3′	242	70
NDUFB6	5′-TACCGCTCCAGTCTCTTCGC-3′	5′-TCAGGAAAATCTCTCATTGGTGG-3′	182	65
CSNK1G2	5′-CTCTTCACTGACCTCTTTGACCG-3′	5′-GCAGCGATTTTCTCTTTCTCCTC-3′	304	67
CX3CL1	5′-CTGACAAAGCCTGAATCCGC-3′	5′-AGTAGTGGACACCTGAGGAGATGG-3′	318	65
PTOV1	5′-CTATGTGAACCAGGGCGAGAAC-3′	5′-AGGAGAAATGTGGGGGAAGAGC-3′	223	65
UQCRC1	5′-AGGGGTGGGGGTAGAGAGATAC-3′	5′-TTGGAGCAAATGTCACGCAG-3′	219	65
GLUL	5′-CTGGAGTCAAGATTACGGGGAC-3′	5′-TGGTGCTGAAGTTGGTGTGGG-3′	219	65
GAPDH	5′-AACTTTGGCATTGTGGAAGGG-3′	5′-TCATCATACTTGGCAGGTTTCTCC-3′	279	58

cDNA, complementary DNA.

samples were dissociated by incremental heating between 72 and 99°C, at a rate of 0.2°C/1 s. SYBR Green I fluorescence intensity was measured at the end of the annealing step and during the melt dissociation step. Amplification was performed in 100 µl tubes (Corbett Research, Sydney, Australia) using a Rotor-Gene 3000 PCR machine (Corbett Research). The validity of each quantitative PCR experiment was determined by checking for amplification in the no template control, uniformity of standard replicates, individual sample amplification as determined by the incremental dissociation step, and the R^2 value for the standard curve.

The relative expression for each gene of interest (GOI) was expressed as a ratio of the number of GOI mRNA copies to the number of copies of the housekeeping gene GAPDH mRNA for each sample. GAPDH was used as an internal reference gene, as previously described and showed no statistical difference between treatment groups (Wang *et al.*, 1999; Chetcuti *et al.*, 2006). The GOI : GAPDH mRNA ratio means for control and lithium treatment cDNAs were then calculated. Statistical analysis was performed using StatView software v5.47 (Abacus Concepts, Berkeley, California, USA). An unpaired Student's *t*-test was used and a two-tailed *P* value of less than 0.05 was considered statistically significant for all analyses.

Results

Microarray data analysis and differentially expressed genes

Pooled cRNA mouse brain samples from control and lithium-treated mice were prepared and hybridized to Affymetrix U74Av2 microarrays. The number of transcripts present on control versus lithium treatment microarrays indicated that lithium had a global effect of downregulating gene expression, with approximately 15% less transcripts 'present' after lithium treatment. The mean number of transcripts present was 6024 ± 80 and 5125 ± 372 for control and Lithium treatment microarrays, respectively (mean ± SD). A statistically significant reduction in the number of transcripts expressed on the lithium treatment microarrays when compared with control microarrays was observed ($P = 0.0052$). Within both treatment groups (control or lithium), greater than 78% of expressed transcripts (90.9 and 78.9% for control and lithium treatment microarrays, respectively) showed common transcript expression. Of the 12 488 transcripts contained on Affymetrix U74Av2 microarrays, a total of 3952 transcripts were present across all nine microarrays. Data analysis was performed on the 3952 transcripts to identify genes with ≥ four-fold differential expression between control and lithium treatment microarrays. A total of 19 transcripts (18 known genes and one unknown transcript) were identified (Table 2). Of these, one gene was upregulated and 18 genes were downregulated by lithium treatment.

Verification of differential gene expression by quantitative PCR analysis

The validity of the 19 differentially expressed genes identified by microarray analysis was evaluated by quantitative PCR analysis. A total of 15 control brain cDNA samples and 30 lithium-treated brain cDNA samples were used as a template to perform quantitative PCR analysis. These were the 45 samples used for microarray analysis. The change in expression (upregulation or downregulation) was confirmed by quantitative PCR analysis for 15 out of 19 genes (Fig. 1). Only five genes, metallothionein 3 (MT3), ATPase, Na^+/K^+ transporting α1 (ATP1A1), transcription elongation factor B (SIII) polypeptide 2 (TCEB2), proteasome (prosome, macropain) subunit β5 (PSMB5), and guanine nucleotide binding protein β1 (GNB1) showed statistically significant differences in expression profiles ($P < 0.05$).

To further validate this animal model of lithium treatment, we examined the expression of Bcl-2 and GSK-3β using quantitative PCR analysis. Bcl-2 is known to be upregulated and GSK-3β is known to be downregulated by lithium treatment, respectively. Bcl-2 expression was shown to increase by 13% and GSK-3β expression decreased by 15% after lithium treatment, although both changes were not statistically significant (data not shown).

Discussion

This study has used an animal model of lithium treatment to identify lithium-regulated genes in the whole mouse brain. A number of published studies have also investigated lithium-regulated genes using in-vitro cell culture and in-vivo animal models. These in-vitro studies have used cell lines, including HEK293 cells (Su *et al.*, 2004), SH-SY5Y neuroblastoma cells (Yuan *et al.*, 1999), rat cerebellar granule neurons (Kopnisky *et al.*, 2003), cerebral cortical cells (Pardo *et al.*, 2003), and yeast cells (Vaden *et al.*, 2001). The majority of in-vivo animal model studies has used rats (Chen *et al.*, 1999; Wang *et al.*, 1999; Yuan *et al.*, 1999; Gandarias *et al.*, 2000; Hua *et al.*, 2000; Semba *et al.*, 2000; Spencer and Houpt, 2001; O'Donnell *et al.*, 2003; Vasconcellos *et al.*, 2003; Maayan *et al.*, 2004; Wood *et al.*, 2004; Basselin *et al.*, 2005; Constantinou *et al.*, 2005), whereas very few have used mice (Sarno *et al.*, 2002; Beaulieu *et al.*, 2004; Su *et al.*, 2004). Each study has used a different experimental design. Lithium treatment was implemented by using lithium-containing rodent chow or via daily injections. The duration of treatment ranged from 4 h for acute studies to 4 weeks for chronic studies. Our experimental design entailed treating mice with the same dose of lithium chloride for 7 days, and then measuring blood lithium concentration at the end point. Accumulating evidence in the literature suggesting that a greater improvement is seen within the first 2 weeks of drug treatment, compared with any other 2-week period

Table 2 Differential gene expression in mice treated with lithium versus control mice by microarray analysis

Gene name	Affymetrix probe	Protein product	Gene ontology[a]	Fold change
Decreased expression in mice treated with Lithium				
ACTB	101578_f_at	Actin, β, cytoplasmic	Structural constituent of cytoskeleton Motor activity Nucleotide binding	7.479
EEF1A2	94429_at	Eukaryotic translation elongation factor 1 α 2	Translation elongation factor activity Nucleotide binding	5.001
1110008P14Rik	96709_at	RIKEN cDNA 1110008P14 gene	Unknown	4.933
GNB1	97458_at	Guanine nucleotide binding protein, β 1	GTPase activity Signal transduction	4.839
TCEB2	160402_at	Transcription elongation factor B (SIII), polypeptide 2	Regulation of transcription Protein modification Ubiquitin cycle	4.789
SST	95436_at	Somatostatin	Regulation of cell migration	4.706
MT3	95340_at	Metallothionein 3	Metal ion homeostasis Negative regulation of neurogenesis	4.636
PSMB5	101558_s_at	Proteasome (prosome, macropain) subunit, β type 5	Ubiquitin-dependent protein Catabolism	4.591
HK1	99335_at	Hexokinase 1	Hexokinase activity ATP binding	4.565
ATP1A1	93797_g_at	ATPase, Na^+/K^+ transporting, α 1 polypeptide	Metal ion binding ATPase activity	4.451
NFIX	101930_at	Nuclear factor I/X	DNA replication Regulation of transcription	4.375
MT1	93573_at	Metallothionein 1	Metal ion homeostasis Nitric oxide-mediated signal transduction	4.365
MRPS34	95730_at	Mitochondrial ribosomal protein S34	Structural constituent of ribosome	4.350
NDUFB6	92615_at	NADH dehydrogenase (ubiquinone) 1β subcomplex 6	NADH dehydrogenase activity Mitochondrial electron transport	4.195
CSNK1G2	96284_at	Casein Kinase 1, γ 2	Protein serine/threonine kinase Activity ATP binding	4.116
CX3CL1	98008_at	Chemokine (C-X3-C motif) ligand 1	Immune response Cell adhesion Signal transduction	4.091
PTOV1	99599_s_at	Prostate tumor over expressed gene 1	Unknown	4.079
UQCRC1	101989_at	Ubiquinol-cytochrome C reductase core protein 1	Mitochondrial electron transport	4.033
Increased expression in mice treated with Lithium				
GLUL	94852_at	Glutamate-ammonia ligase (glutamine synthase)	Glutamine biosynthesis Nitrogen metabolism/fixation	5.650

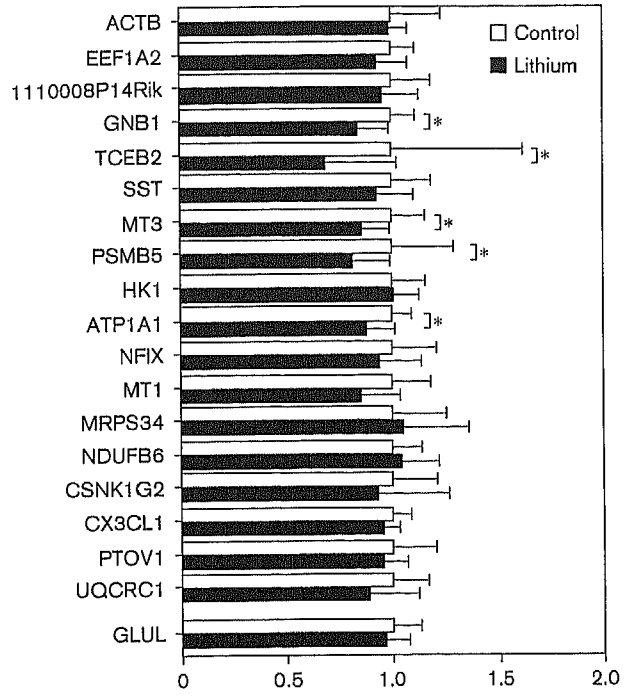
cDNA, complementary DNA; NADH, nicotinamide adenine dinucleotide.
[a]Gene Ontology as determined by QuickGO Gene Ontology Browser, European Bioinformatics Institute (*www.ebi.ac.uk/ego*).

thereafter, is present (Agid *et al.*, 2006). This has been observed for antipsychotic drugs and would indicate that treatment of mice for 1 week would be sufficient to observe biochemical changes attributed to the direct molecular mechanism of lithium. Our results show that the resultant blood lithium concentration after treating 30 mice with the same dose of lithium ranged from 0.5 to 1.2 mmol/l of lithium. Despite this range, the resultant differential lithium serum concentrations did not have a substantial affect on global brain gene expression. This was evident because the six microarrays hybridized to cRNA probes prepared from mice treated with lithium showed greater than 78% similarity in the overall number of genes expressed. Another aspect of our experimental design is the fact that we used whole mouse brain tissue to investigate altered gene expression using microarray and quantitative PCR analyses. A number of other studies that have also used whole rodent brain tissue to investigate altered brain gene expression after treatment with neurotrophic drugs are present (O'Donnell *et al.*, 2003; Takahashi *et al.*, 2006).

To confirm whether the treatment regime used in this study was sufficient to cause significant gene expression alteration in the mouse brain, we examined the mRNA expression of Bcl-2 and GSK-3β using quantitative PCR analysis. Although statistically significant differences were not found, a 13% increase in Bcl-2 expression and a 15% decrease in GSK-3β expression were found, respectively. This would indicate that the level and duration of treatment in this study were sufficient to induce expression changes as documented in the literature.

Microarray experiments generate substantial lists of genes that require further confirmation using alternative techniques. Using quantitative PCR analysis, only five of the 19 genes identified by microarray analysis were

Fig. 1



Quantitative PCR analysis of the steady state mRNA level (mean ± SD) of genes altered in the brains of mice treated with lithium. The relative expression level for each gene has first been normalized to GAPDH expression. The level of expression in control samples has been normalized to 1. *=$P<0.05$.

confirmed as statistically significantly altered in relative expression levels. Before hybridization onto each microarray, cRNA probes were prepared by pooling together in equal proportion five individual whole mouse brain total RNA samples. This pooling technique equilibrated any small variations in gene expression between individual mice that were pooled onto the same microarray (Peng *et al.*, 2003). For the control group, a total of three independent microarray hybridizations were performed, and for lithium treatment six independent microarray hybridizations were performed. These microarray hybridizations represent the cumulative gene expression data from 15 to 30 individual mice, respectively. One of the limitations of this study is that gene expression was compared between 15 control mice and 30 treated mice. The difference in the number of mice used for control and treated groups could explain why only a small number of genes were identified with relatively low differential expression. The control sample group is likely to have more variance than the treated group because less control samples were included in the study. This limitation in the study design may explain why only five genes were confirmed using quantitative PCR analysis. For quantitative PCR analysis, amplification was performed on 45 individual mouse brain cDNA samples (15 controls and 30 lithium treated). More variability in gene expression observed using quantitative PCR analysis compared with microarray analysis was seen (data not shown). This variability explains why the fold change in gene expression was less by quantitative PCR analysis compared with microarray analysis. This observation also explains why only five of the 19 genes identified were confirmed as statistically significantly altered using quantitative PCR analysis. These results highlight the importance of repeating quantitative PCR experiments on individual samples to ensure the accuracy and reproducibility of quantitative PCR analysis and also the difficulty in confirming microarray gene expression using PCR.

This study has identified and confirmed that MT3, ATP1A1, TCEB2, PSMB5, and GNB1 mRNA expression levels are significantly altered by the mood-stabilizing drug lithium. This study has identified that MT3 is downregulated in lithium-treated mice. MT3 is a small cysteine-rich metal binding protein that was originally discovered unexpectedly as a neuronal growth inhibitory factor that was depleted in people with Alzheimer's disease (Uchida *et al.*, 1988). Uchida and coworkers established that brain extracts from Alzheimer's disease patients stimulated the survival and neurite growth of rat neuronal cultures to a greater degree than normal brain extracts. Unlike MT1 and MT2, MT3 is relatively unresponsive to cytokines, hormones, and metals indicating that the amounts of metallothioneins are regulated differently in cells (Giralt *et al.*, 2001). MT3 participates in the processes of heavy metal detoxification, metal homeostasis regulation, protection from oxidative free radicals damage, and defense against nitrogen species (Hidalgo *et al.*, 2001). Studies have shown that MT3 may be a key gene involved in facilitating the antidepressant-like activities of eugenol (Iriea *et al.*, 2004). Using mice treated with eugenol or imipramine, Iriea and coauthors demonstrated that MT3 expression was induced in the hippocampus of mice treated with eugenol only. The induction of MT3 expression was not seen for imipramine treatment or for MT1 (Iriea *et al.*, 2004). Our finding that MT3 expression is downregulated by lithium treatment may be a physiological response to increased levels of the metal ion lithium in the brain. Notwithstanding, MT3 may be related to the mechanism of action of the antidepressant eugenol and thus may contribute to the therapeutic action of lithium. Clearly, MT3 performs many important biological functions essential in brain tissue and it is unclear which one of these functions relates to the possible mechanism of action of lithium.

This study has shown that ATP1A1 is downregulated in the brains of mice treated with lithium. ATP1A1 encodes the $\alpha 1$ subunit of the ubiquitously expressed ouabain-sensitive Na^+, K^+ ATPase pump (Lingrel and Kuntzweiler, 1994). Na,K-ATPase is an integral membrane protein that functions by transporting Na^+ out of the cell

and K^+ into the cell, using ATP as the energy source. This transport produces both a chemical and an electrical gradient across the cell membrane. The electrical gradient is essential for maintaining the resting potential of cells and for the excitable activity of muscle and nerve tissue (Lingrel and Kuntzweiler, 1994). Gene knockout studies in mice have shown that ATP1A1 heterozygous knockouts are viable (Lingrel *et al.*, 2003), whereas homozygous ATP1A1 knockouts do not develop past the blastocyst stage. This indicates that ATP1A1 is essentially required during embryonic development (Barcroft *et al.*, 2003). Clearly, ATP1A1 plays an important part in how neurons function normally and may possibly explain the antimanic effect of lithium. There have been a number of clinical studies indicating an effect of lithium on Na,K-ATPase, for example, contributing to the effect of lithium on renal function (Laursen *et al.*, 2004).

This study has demonstrated that TCEB2 and PSMB5 are downregulated after lithium treatment. TCEB2, commonly referred to as Elongin B, is a subunit of the heterodimeric Elongin BC complex, which was originally identified as a positive regulator of RNA polymerase II elongation factor (Elongin A) (Bradsher *et al.*, 1993). The Elongin BC complex has been identified as a component of the multiprotein von Hippel–Lindau tumor suppressor complex (Duan *et al.*, 1995), and regulates the expression of hypoxia-inducible genes (Iliopoulos *et al.*, 1996) and the suppressor of cytokine signaling-1 (SOCS1) complex (Kamura *et al.*, 1998). PSMB5 is one of the subunits (b5) that forms the central 'b' ring in the 20S proteasome. In mammalian cells, the 26S proteasome is a proteinase complex consisting of a 20S core complex and a 19S regulatory complex (Glickman and Ciechanover, 2002). The 20S proteasome comprises a cylindrical stack of four rings, two outer rings formed by seven 'a' subunits (a1–a7) and two inner rings of seven 'b' subunits (b1–b7). The proteasome facilitates the degradation of many cellular proteins and plays a role in cell cycle progression, development, cell death, and elimination of abnormal proteins by mutation and oxidation (Demasi and Davies, 2003). The accumulation of oxidized/misfolded proteins within cells is known to be associated with degenerative diseases such as Parkinson's disease (Holtz and O'Malley, 2003). Both TCEB2 and PSMB5 participate in cellular functions that are ubiquitously necessary for cells to function normally. At present it is, however, unclear whether the decrease in TCEB2 or PSMB5 contributes to the mechanism of action of lithium.

This study has confirmed the downregulation of GNB1 expression in the brains of mice treated with lithium. The product of the GNB1 gene, the b1 subunit of heterotrimeric GTP-binding protein (Gb1), is a component of a transducer between the cell surface receptors and intracellular effectors, such as phospholipases and protein kinases (Kitanaka *et al.*, 2003). GNB1 was first discovered in experiments to identify genes that initiate the development of behavioral sensitization after administration of psychostimulant drugs, such as cocaine and amphetamine (Wang *et al.*, 1997). These studies showed that in rats that received a single injection of d-amphetamine or cocaine, GNB1 mRNA expression was transiently induced by up to two-fold in the striatum compared with the baseline expression level after 4 h (Kitanaka *et al.*, 2003). This observation has also been demonstrated in mice, where a significant increase in GNB1 mRNA expression was observed in the mouse striatum 2–4 h after a single injection of methamphetamine. No change in the expression level of GNB1 mRNA was observed in the cerebral cortex, hippocampus or thalamus/hypothalamus. In contrast, chronic administration of methamphetamine did not alter the GNB1 expression level (Kitanaka *et al.*, 2003). The regulation of GNB1 by cocaine and amphetamine in dopamine-rich striatal and accumbens regions would be consistent with the role of dopamine D1 and D2 receptors in psychostimulant-induced locomotion and reward (Hooks *et al.*, 1994). Conceivably, these drug-induced Gb1 expression changes could change the molecular balances between 'b', 'g', and 'a' G-protein subunits and/or alter quantitative features of the ratios between G-linked receptors and G-proteins. This would potentially change signal transduction pathways mediated by 'a' and/or 'bg' subunits, including those activated by D1 and D2 family receptors. Such mechanisms could contribute to the neuroplastic changes likely to underlie behavioral alterations noted after chronic psychostimulant administration (Wang *et al.*, 1997). The transient increase in the expression level of GNB1 in the mouse striatum after acute administration of methamphetamine suggested the physiological role of the GNB1 product in the modulation of intercellular or intracellular signal transduction pathways in the striatum. G-protein-coupled signal transduction is an important cellular function and thus the downstream effect of dysregulated GNB1 expression requires further functional analysis. Certainly there has been considerable interest over the last decade in the role of G proteins in both the therapeutic mechanism of lithium and the pathophysiology of bipolar disorder (Mitchell *et al.*, 1997; Manji and Lenox, 2000).

In conclusion, this study has identified several genes, including GNB1, TCEB2, MT3, PSMB5, and ATP1A1, whose expression is decreased by lithium, and are involved in a number of cellular functions, including maintaining metal ion homeostasis and chemical/electrical gradients across membranes, regulating RNA polymerase II, protein degradation, and G-protein-coupled signal transduction. Significantly, MT3 has been implicated as a possible gene involved in the mechanism of action of the antidepressant drug eugenol. Further investigation is required to confirm the altered gene expression identified in this study. This would include

gene expression analysis on postmortem brain tissue from bipolar disorder patients and controls. Such analysis would enable comparison of the expression between an animal model of lithium treatment and tissue from affected bipolar disorder patients. In addition, it would be important to investigate, using in-situ techniques, the anatomical expression of these genes in this mouse model and human bipolar disorder brain tissue specimens.

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References

Agid O, Seeman P, Kapur S (2006). The 'delayed onset' of antipsychotic action- an idea whose time has come and gone. *J Psychiatry Neurosci* **31**:93–100.

Bai O, Zhang H, Li XM (2004). Antipsychotic drugs clozapine and olanzapine upregulate Bcl-2 mRNA and protein in rat frontal cortex and hippocampus. *Brain Res* **1010**:81–86.

Barcroft LC, Moseley AE, Lingrelc JB, Watson AJ (2003). Deletion of the Na/K-ATPase a1-subunit gene (Atp1a1) does not prevent cavitation of the preimplantation mouse embryo. *Mech Dev* **121**:417–426.

Basselin M, Chang L, Seemann R, Bell JM, Rapoport SI (2005). Chronic lithium administration to rats selectively modifies 5-HT(2A/2C) receptor-mediated brain signaling via arachidonic acid. *Neuropsychopharmacology* **30**: 461–472.

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG (2004). Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci* **101**:5099–5104.

Bosetti F, Seemann R, Bell JM, Zahorchak R, Friedman E, Rapoport SI, Manickam P (2002). Analysis of gene expression with cDNA microarrays in rat brain after 7 and 42 days of oral lithium administration. *Brain Res Bull* **57**:205–209.

Bradsher JN, Jackson KW, Conaway RC, Conaway JW (1993). RNA polymerase II transcription factor SIII 1. Identification, purification, and properties. *J Biol Chem* **268**:25587–25593.

Bro C, Regenberg B, Lagniel G, Labarre J, Montero-Lomeli M, Nielsen J (2003). Transcriptional, proteomic, and metabolic responses to lithium in galactose-grown yeast cells. *J Biol Chem* **278**:32141–32149.

Cade JF (1949). Lithium salts in the treatment of psychotic excitement. *Med J Aust* **2**:349–352.

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.

Chetcuti A, Adams LJ, Mitchell PB, Schofield PR (2006). Altered gene expression in mice treated with the mood stabilizer sodium valproate. *Int J Neuropsychopharmacol* **9**:267–276.

Constantinou C, Bolaris S, Valcana T, Margarity M (2005). Acute LiCl-treatment affects the cytoplasmic T4 availability and the expression pattern of thyroid hormone receptors in adult rat cerebral hemispheres. *Neurosci Lett* **51**: 235–241.

Demasi M, Davies KJA (2003). Proteasome inhibitors induce intracellular protein aggregation and cell death by an oxygen-dependent mechanism. *FEBS Lett* **542**:89–94.

Duan DR, Pause A, Burgess WH, Aso T, Chen DY, Garrett KP, *et al.* (1995). Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science* **269**:1402–1406.

Gandarias JMd, Acebes I, Echevarria E, Vegas L, Abecia LC, Casis L (2000). Lithium alters mu-opioid receptor expression in the rat brain. *Neurosci Lett* **279**:9–12.

Giralt M, Carrasco J, Penkowa M, Morcillo MA, Santamarý J, Campbell IL, Hidalgo J (2001). Astrocyte-targeted expression of interleukin-3 and interferon-alpha causes region-specific changes in metallothionein expression in the brain. *Exp Neurol* **168**:334–346.

Glickman MH, Ciechanover A (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**:373–428.

Hidalgo J, Aschner M, Zatta P, Vasak M (2001). Roles of the metallothionein family of proteins in the central nervous system. *Brain Res Bul* **55**:133–145.

Holtz WA, O'Malley KL (2003). Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem* **278**:19367–19377.

Hooks MS, Juncoq JL, Justice JB, Meiergerd SM, Povlock SL, Schenk J, Kalivasl PW (1994). Individual locomotor response to novelty predicts selective alterations in D1 and D2 receptors and mRNAs. *J Neurosci* **14**:6144–6152.

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

Iliopoulos O, Levy AP, Jian C, Kaelin WG, Goldberg MA (1996). Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci U S A* **93**:10595–10599.

Iriea Y, Itokazua N, Anjikia N, Ishigea A, Watanabea K, Keung WM (2004). Eugenol exhibits antidepressant-like activity in mice and induces expression of metallothionein-III in the hippocampus. *Brain Res* **1011**:243–246.

Iwamoto K, Bundo M, Kato T (2004). Altered expression of mitochondria-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. *Hum Mol Genet* **14**:241–253.

Jope RS (2003). Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends Pharmacol Sci* **24**:441–443.

Kamura T, Sato S, Haque D, Liu L, Kaelin WG, Conaway RC, Conaway JW (1998). The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev* **12**:3872–3881.

Kitanaka N, Kitanaka J, Walther D, Wang XB, Uhl GR (2003). Comparative inter-strain sequence analysis of the putative regulatory region of murine psychostimulant-regulated gene GNB1 (G protein b1 subunit gene). *DNA Seq* **14**:257–263.

Klein PS, Melton DA (1996). A molecular mechanism for the effect of lithium on development. *PNAS* **93**:8455–8459.

Kopnisky KL, Chalecka-Franaszek E, Gonzalez-Zulueta M, Chuang DM (2003). Chronic lithium treatment antagonizes glutamate-induced decrease of phosphorylated CREB in neurons via reducing protein phosphatase 1 and increasing MEK activities. *Neuroscience* **116**:425–435.

Laursen UH, Pihakaski-Maunsbach K, Kwon TH, Jensen EO, Nielsen S, Maunsbach AB (2004). Changes of rat kidney AQP2 and Na,K-ATPase mRNA expression in lithium-induced nephrogenic diabetes insipidus. *Nephron Exp Nephrol* **97**:e1–e16.

Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, *et al.* (2003). Functional roles of the alpha isoforms of the Na,K-ATPase. *Ann NY Acad Sci* **986**:354–359.

Lingrel JB, Kuntzweiler T (1994). Na+,K+-ATPase. *J Biol Chem* **269**: 19659–19662.

Maayan R, Shaltiel G, Poyurovsky M, Ramadan E, Morad O, Nechmad A, *et al.* (2004). Chronic lithium treatment affects rat brain and serum dehydroepiandrosterone (DHEA) and DHEA-sulphate (DHEA-S) levels. *Int J Neuropsychopharmacol* **7**:71–75.

Manji HK, Lenox RH (2000). Signaling: cellular insights into the pathophysiology of bipolar disorder. *Biol Psychiatry* **48**:518–530.

Manji HK, Moore GJ, Chen G (2001). Bipolar disorder: leads from the molecular and cellular mechanisms of action of mood stabilizers. *Br J Psychiatry* **178**:s107–s119.

Mitchell PB (2001). Therapeutic drug monitoring of psychotropic medications. *Br J Pharmacol* **52**:45S–54S.

Mitchell PB, Hadzi-Pavlovic D (1999). John Cade and the discovery of lithium treatment for manic depressive illness. *Med J Aust* **171**:262.

Mitchell PB, Manji HK, Chen G, Jolkovsky L, Smith-Jackson E, Denicoff K, *et al.* (1997). High levels of GSA in platelets of euthymic patients with bipolar affective disorder. *Am J Psychiatry* **154**:218–223.

O'Donnell T, Rotzinger S, Nakashima TT, Hanstock CC, Ulrich M, Silverstone PH (2003). Chronic lithium and sodium valproate both decrease the concentration of myoinositol and increase the concentration of inositol monophosphates in rat brain. *Eur Neuropsychopharmacol* **13**:199–207.

Pardo R, Andreolotti AG, Ramos B, Picatoste F, Claro E (2003). Opposed effects of lithium on the MEK-ERK pathway in neural cells: inhibition in astrocytes and stimulation in neurons by GSK3 independent mechanisms. *J Neurochem* **87**:417–426.

Peng X, Wood CL, Blalock EM, Chen KC, Landfield PW, Stromberg AJ (2003). Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics* **4**:26.

Sarno PD, Li X, Jope RS (2002). Regulation of Akt and glycogen synthase kinase-3beta phosphorylation by sodium valproate and lithium. *Neuropharmacology* **43**:1158–1164.

Semba J, Watanabe H, Suhara T, Akanuma N (2000). Chronic lithium chloride injection increases glucocorticoid receptor but not mineralocorticoid receptor mRNA expression in rat brain. *Neurosci Res* **38**:313–319.

Spencer CM, Houpt TA (2001). Dynamics of c-fos and ICER mRNA expression in rat forebrain following lithium chloride injection. *Brain Res Mol Brain Res* **93**:113–126.

Su Y, Ryder J, Li B, Wu X, Fox N, Solenberg P, *et al.* (2004). Lithium, a common drug for bipolar disorder treatment, regulates amyloid-beta precursor protein processing. *Biochemistry* **43**:6899–6908.

Takahashi Y, Washiyama K, Kobayashi T, Hayashi S (2006). Gene expression in the brain from fluoxetine-injected mouse using DNA microarray. *Ann NY Acad Sci* **1074**:42–51.

Uchida Y, Ihara Y, Tomonaga M (1988). Alzheimer's disease brain extract stimulates the survival of cerebral cortical neurons from neonatal rats. *Biochem Biophys Res Commun* **150**:1263–1267.

Vaden DL, Ding D, Peterson B, Greenberg ML (2001). Lithium and valproate decrease inositol mass and increase expression of the yeast INO1 and INO2 genes for inositol biosynthesis. *J Biol Chem* **276**:15466–15471.

Vasconcellos AP, Tabajara AS, Ferrari C, Rocha E, Dalmaz C (2003). Effect of chronic stress on spatial memory in rats is attenuated by lithium treatment. *Physiol Behav* **79**:143–149.

Wang JF, Bown C, Young LT (1999). Differential display PCR reveals novel targets for the mood-stabilizing drug valproate including the molecular chaperone GRP78. *Mol Pharmacol* **55**:521–527.

Wang JF, Chen B, Young LT (1999). Identification of a novel lithium regulated gene in rat brain. *Brain Res Mol Brain Res* **70**:66–73.

Wang XB, Funada M, Imai Y, Revay RS, Ujike H, Vandenbergh DJ, Uhl GR (1997). rGb1: a psychostimulant-regulated gene essential for establishing cocaine sensitization. *J Neurosci* **17**:5993–6000.

Wood GE, Young LT, Reagan LP, Chen B, McEwen BS (2004). Stress-induced structural remodeling in hippocampus: prevention by lithium treatment. *Proc Natl Acad Sci* **101**:3973–3978.

Yuan P, 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.

Zhang WV, Jullig M, Connolly AR, Stott NS (2005). Early gene response in lithium chloride induced apoptosis. *Apoptosis* **10**:75–90.