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Effects of lithium chloride on the gene expression profiles in *Drosophila* heads

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Abstract

To gain insight into the basic neurobiological processes regulated by lithium—an effective drug for bipolar disorder—we used Affymetrix Genome Arrays to examine lithium-induced changes in genome-wide gene expression profiles of head mRNA from the genetic model organism *Drosophila melanogaster*. First, to identify the individual genes whose transcript levels are most significantly altered by lithium, we analyzed the microarray data with stringent criteria (fold change > 2; p <0.001) and evaluated the results by RT-PCR. This analysis identified 12 genes that encode proteins with various biological functions, including an enzyme responsible for amino acid metabolism and a putative amino acid transporter. Second, to uncover the biological pathways involved in lithium's action in the nervous system, we used less stringent criteria (fold change >1.2; FDR <0.05) and assigned the identified 66 lithium-responsive genes to biological pathways using DAVID (Database for Annotation, Visualization and Integrated Discovery). The gene ontology categories most significantly affected by lithium were amino acid metabolic processes. Taken together, these data suggest that amino acid metabolism is important for lithium's actions in the nervous system, and lay a foundation for future functional studies of lithium-responsive neurobiological processes using the versatile molecular and genetic tools that are available in *Drosophila*.

Keywords

Drosophila; lithium; microarray; RT-PCR; gene expression; nervous system

Introduction

The alkaline metal lithium affects various developmental and physiological processes in evolutionarily diverse organisms (Phiel and Klein, 2001). In particular, lithium's actions in the nervous system have attracted special attention because lithium is highly effective in the prophylaxis and treatment of bipolar affective disorder (Schou, 2001). In addition, recent studies suggest that chronic lithium treatment is efficacious in preventing apoptosis-dependent neuronal death (Chuang, 2004), which raises the interesting possibility that lithium might be effective in treating or preventing brain damage, either following injury or over the course of

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progression of neurodegenerative diseases. Despite the proven clinical usefulness and the further potential of this drug, the molecular mechanisms underlying its actions in the nervous system are still only poorly understood.

At therapeutically relevant concentrations, lithium inhibits several enzymes, including glycogen synthase kinase 3β (GSK3β) as well as inositol monophosphatase (IMPase) and related enzymes (Berridge et al., 1989; Klein and Melton, 1996). These lithium-sensitive enzymes are intimately involved in the regulation of various intracellular molecular cascades, such as the Wnt and inositol phosphate signaling pathways (Chen et al., 2000; Ding et al., 2000). Lithium-based perturbation of these signaling pathways has a significant impact on global gene expression profiles (Rowe and Chuang, 2004), and this may contribute to the therapeutic as well as toxic actions of lithium. In order to elucidate the importance of gene regulation in lithium's actions in the nervous system, we need to identify genes whose expression is influenced by therapeutic concentrations of lithium, and to study their roles in the lithium-responsive neurobiological processes at the molecular, cellular and organismal levels.

The fruit fly *Drosophila melanogaster* has been a valuable genetic model system for examining fundamental problems in neurobiology. In part, this is due to the fact that *Drosophila* and higher vertebrates share genetic pathways for cellular signaling (Miklos and Rubin, 1996; Rubin et al., 2000). In addition, many human genes involved in brain functions and neurological disorders have fly counterparts (Reiter et al., 2001; Davis, 2005; Hamet and Tremblay, 2006). Importantly, the genetic pathways involved in lithium's actions in the nervous system appear to be shared by *Drosophila* and vertebrates. For example, the administration of lithium to fruit flies and vertebrates has a similar effect on circadian clocks, and in both cases this effect involves the inhibition of glycogen synthase kinase 3β (GSK3β) (Padiath et al., 2004; Dokucu et al., 2005; Iitaka et al., 2005). Additionally, as in vertebrates, lithium has neuroprotective effects in transgenic flies that over-express either human tau proteins or a mutant form of huntingtin (Mudher et al., 2004; Berger et al., 2005). Furthermore, lithium improves the physiological, behavioral and developmental mutant phenotypes characteristic of a mouse model of Fragile X syndrome (Min et al., 2009), and likewise rescues such defects in a *Drosophila* model of this disease (McBride et al., 2005). These results strongly suggest that studies of the genes responsible for lithium's actions in the *Drosophila* nervous system would provide important insights into the basis of lithium's neurobiological effects in vertebrates.

In this study, we carried out a microarray-based gene expression profiling analysis of *Drosophila* head mRNA, to identify the genes and biological pathways of the nervous system that are significantly influenced by lithium treatment in adult animals. This study lays the foundation for future functional studies using the versatile molecular and genetic tools available in *Drosophila* to understand the lithium-responsive neurobiological processes.

Materials and Methods

Drosophila stock

Flies were reared at 25°C at 65% humidity, in a 12 hr:12 hr light:dark cycle, on a conventional cornmeal-based medium containing glucose, yeast and agar supplemented with the mold inhibitor methyl 4-hydroxybenzoate (0.05 %). The Canton-S (CS) strain was used as the wild-type control.

RNA extraction and microarray experiment

Newly eclosed 0–1 day old wild-type female flies were grouped into sets of 20 and placed into a vial containing regular fly food with or without 50 mM LiCl. Flies in five vials (total of 100

flies) were combined as one biological sample, and three biological replicates were prepared for each treatment condition. The fly heads were removed from bodies on a dry ice block after 24-hour treatment, and kept frozen at −80°C until used. Total RNAs were extracted from the fly heads using Trizol solution (Invitrogen, Carlsbad, CA), followed by further purification using RNasy column (Qiagen, Valencia, CA). The quality of the purified total RNA was verified using Agilent Bioanalyzer (Agilent Technologies, Stockport, Cheshire, UK). cRNA labeling and microarray experiments were carried out at the Translational Genomics Research Institute (Phoenix, AZ), using Affymetrix *Drosophila* Genome 2.0 Arrays (Affymetrix, Santa Clara, CA).

Microarray data analysis

Image data were quantified using the genechip-operating software Affymetrix GCOS v1.4. Gene expression data were normalized using the robust multi-array average (RMA) statistical algorithms (Irizarry et al., 2003). Besides six sets of data from wild-type flies (three biological replicates for each condition, with or without lithium treatment), additional six data sets created under the same conditions from *Shudderrer* mutant flies (which display neurological phenotypes that are improved by lithium treatment) (Williamson, 1982) were included in the normalization process. In this report, we have focused on the wild-type data to lay a foundation for future genetic studies on lithium-responsive processes. A heat-map was generated for the expression data corresponding to a subset of genes with fold change >1.2; FDR<0.05 (Supplementary data) using Partek GS version 6.4. (Partek Inc., St. Louis, MO). Correlation coefficients were calculated using the GeneSpring software. Cluster euclidean distance analysis (Dougherty et al., 2002) was carried out using Ward's method, via Partek GS software (Partek Inc., St. Louis, MO). Comparisons between signals for lithium-treated and untreated groups were made using one way ANOVA. Bonferoni's multiple comparison corrections were applied to obtain the false discovery rate (FDR). Genes were annotated and biological processes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) (Dennis et al., 2003).

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from 30 fly heads after 24-hour treatment with or without 50mM LiCl. Four biological replicates were analyzed for each experimental condition. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and subjected to semi-quantitative PCR amplification using gene-specific primers, most of which were designed to overlap with the locations of Affymetrix probes for the corresponding genes. The primers used for the RT-PCR were listed in Table 1. The PCR products corresponding to 1–5 ng of the original total RNA were analyzed on an agarose gel for quantification. *Rp49* (a.k.a., *RpL32*), which encodes the ribosomal protein L32, is commonly used as the internal standard for quantitative analysis of mRNA levels in *Drosophila* (Gabler et al., 2005). The *Rp49* transcripts were amplified from the same cDNA samples using *Rp49*-specific primers and used as the internal standard. PCR conditions were optimized for each gene, such that the endpoint of each PCR was in the linear range of amplifications. Transcript levels were quantified by analyzing pixel intensity of the bands using Image J software (NIH, Bethesda, MD). A fold-change for each gene was determined by calculating a ratio of the band signal intensities between lithium-treated and untreated samples. Averages of the fold-changes were presented with the standard error of the mean (SEM).

RESULTS

In order to determine the transcriptome response to lithium treatment in *Drosophila* heads, wild-type female flies were treated for 24 hours with or without 50 mM LiCl. We chose this concentration of lithium because it results in an internal lithium level that is comparable to the

therapeutic serum concentrations (μM ~ mM) in bipolar disorder patients treated with lithium (Padiath et al., 2004; Dokucu et al., 2005). These lithium concentrations do not cause obvious toxic effects on adult wild-type flies after 24 hr treatment, but do have significant effects on circadian rhythm in wild-type flies (Padiath et al., 2004; Dokucu et al., 2005), as well as on cellular or behavioral phenotypes in certain mutants and transformants (Williamson, 1982; Mudher et al., 2004; Berger et al., 2005; McBride et al., 2005).

Three biological replicates were analyzed for each condition, i.e., with or without lithium treatment, using the Affymetrix *Drosophila* Genome 2.0 Arrays. The signal intensities of each chip were normalized using RMA algorithms, and the lithium-treated and untreated groups were compared as described in the Material and Methods section. The correlation coefficients for the three biological replicates were greater than 0.93 under both conditions, indicating that the gene expression data obtained in this study were highly reproducible. The biological replicates were well clustered when a method of hierarchical clustering based on Ward's distance was applied to all 6 wild-type data sets (3 treated with lithium and 3 not treated with lithium; Fig. 1). Consistency among the biological replicates was visualized by generating a heat-map of expression data for the genes that are differentially regulated in response to lithium (fold change >1.2; FDR <0.05) (Figure 1).

In this study, we took two approaches to investigating lithium-induced changes in genome-wide expression profiles of *Drosophila* head mRNA. In the first, we identified individual genes whose transcript levels are most significantly altered in response to lithium treatment, using stringent criteria (fold change >2; p <0.001) to select differentially regulated genes. In the second, we uncovered the biological pathways that are likely involved in lithium's actions in the nervous system, using less stringent criteria (fold change >1.2; FDR <0.05) to identify lithium-responsive genes and then assigning these genes to relevant biological pathways by DAVID.

Genes whose transcript levels are most significantly altered in response to lithium treatment

Of 18,500 *Drosophila* genes analyzed using the Affymetrix *Drosophila* Genome 2.0 Arrays, 16 genes that were represented by 18 Affymetrix probe sets responded to lithium treatment by changes of >2 (p <0.001) in their expression, in wild-type flies. Among these 16 genes, 2 and 14 were down- and up-regulated, respectively, in response to lithium treatment. The results of the microarray experiment were confirmed by RT-PCR analysis on four RNA samples independently prepared from wild-type fly heads that had or had not been treated with 50 mM lithium for 24 hr. RT-PCR analysis confirmed that the transcript levels of 12 genes differed significantly between lithium-treated and lithium-untreated samples (one down-regulated and 11 up-regulated in lithium-treated samples) (Fig. 2, Table 2). Four genes (*CG31781*, *CG11654*, *CG11425* and *CG18522*) of the original 16 were either not amplified by RT-PCR or yielded results that were not consistent with those of the microarray analysis (data not shown). For 10 of the 12 genes identified both by microarray and RT-PCR analyses, the fold-change based on RT-PCR analysis was smaller than that estimated by microarray analysis (Table 2). This is probably because the linear range of RT-PCR analysis is narrower than that for the Affymetrix microarray method. The 12 genes identified as undergoing significant change in expression encode a variety of proteins, including ones involved in the transport (*CG15088*) and metabolism (*CG1673*) of amino acids, and others required for detoxification (*GstD2*) and the stress response (*Arc1*) (Table 2).

Biological pathways that are significantly affected by lithium-induced alterations in gene expression

In less stringent analysis of the microarray data obtained from lithium-treated and untreated flies (fold change >1.2; FDR<0.05), we identified 71 probe sets representing 66 differentially

regulated genes, including 28 that were down-regulated and 38 up-regulated (Supplementary data). These genes were assigned to biological pathways using a DAVID functional annotation tool (http://david.abcc.ncifcrf.gov). This web-accessible program annotates each gene and performs enrichment analysis that identifies the most relevant biological meanings associated with a given gene list, with output in the form of a "functional annotation chart". Although the functional annotation chart is useful for identifying biological terms or pathways in which the analyzed genes are significantly enriched, these terms often overlap due to inherent redundancy in annotations. To improve the usefulness of the functional annotation analysis, we carried out DAVID clustering analysis. The functional annotation chart was integrated with the clustering analyses to identify biological pathways that are significantly affected by lithium-induced alterations in genome-wide gene expression.

Analysis of the identified 66 lithium-responsive genes with the DAVID functional annotation tool resulted in the identification of 13 term entries as significantly "enriched terms/pathways", with $p < 0.001$ (Table 3). Among these, the gene ontology (GO) term "branched chain family amino acid metabolic process (GO: 0009081)" showed the highest fold enrichment (69.1-fold). In addition, "valine, leucine and isoleucine degradation (KEGG PATHWAY)" was identified as the term with the third highest fold enrichment (15.9-fold).

We also performed the DAVID clustering analysis under the high stringency conditions on the list of lithium-responsive genes obtained (Huang da et al., 2007). Only three clusters met the statistical criteria ($p < 0.01$), and all of these were related to amino acid metabolism (Table 4). Each of these clusters was composed of 3–4 GO terms, with a fold enrichment of 3.30–49.3. For example, functional cluster 2 is composed of four GO terms that apply to identical sets of seven genes. These terms were "amino acid metabolic process (GO:0006520)", "amino acid and derivative metabolic process (GO:0006519)", "amine metabolic process (GO:0009308)" and "nitrogen compound metabolic process (GO:0006807)". As shown in Figure 3, these seven genes displayed increases in gene expression of 1.20–2.36-fold in response to lithium treatment. A fraction of the genes in functional cluster 2 also formed functional clusters 1 and 3. Three of the seven genes (*CG3267*, *CG6638* and *CG1673*) were involved in the branched amino acid (BCAA) degradation pathway.

In addition to identifying the terms related to amino acid metabolic pathways, the DAVID-based functional annotation analysis identified the term "stress response" with the second highest enrichment (43.8-fold), with $p = 9.23E{-}05$ (Table 3). Two GO terms that were revealed in this analysis with highest statistical significance were "organic acid metabolism" and "carboxylic acid metabolism" ($p = 8.2E{-}06$) (Table 3). For the *Drosophila melanogaster* genes, these two terms share identical components. Eleven genes were assigned to these GO terms, showing a significant enrichment (6.0 times) in the corresponding biological processes.

Discussion

Characteristic features of the individual genes whose expression is significantly affected by lithium

In this study, we examined the lithium-induced alterations in genome-wide gene expression profiles in the adult head of the genetic model organism, *Drosophila melanogaster*. Using stringent analysis criteria, we identified 12 genes whose transcript levels are most significantly altered by lithium (Table 2). These genes can be categorized into 4 groups based on their biological function or characteristic features: (i) amino acid transport and metabolism, (ii) detoxication, stress response or self-defense reactions, (iii) psychiatric or neurological disorders, and (iv) others.

(i) Genes involved in amino acid transport and metabolism (*CG15088* and *CG1673*)—Of the 18,500 *Drosophila* genes analyzed, the expression of *CG15088* was most significantly affected by lithium treatment, as assessed by the *p*-value (*p*=1.12E−09 and 6.97E −13 for two Affymetrix probe sets recognizing *CG15088*). *CG15088* encodes a putative nutrient amino acid transporter of the solute carrier 6 (SLC6) Na^+/Cl^--dependent transporter family (Thimgan et al., 2006; Romero-Calderon et al., 2007; Miller et al., 2008). We have recently investigated the role of *CG15088* in lithium-responsive biological processes. Using transformants expressing *CG15088*-specific RNAi and flies deficient in *CG15088*, we showed that down-regulation or complete elimination of *CG15088* function results in a remarkable increase in the susceptibility of adult flies to lithium's toxic effects, demonstrating the importance of this transporter for resistance to lithium's toxicity. We have also obtained evidence that this is likely a consequence of its transporter activity in glia of the CNS (Kasuya et al., submitted). This finding strongly supports the expectation that our microarray analysis is effective in revealing new genes of functional significance to the lithium-responsive pathways. Further genetic studies on *CG15088* are expected to lead to useful information regarding the mechanisms that underlie lithium-dependent gene regulation and resistance to lithium toxicity.

CG1673 encodes a cytosolic isoform of branched-chain-amino-acid transaminase (BCATc; EC 2.6.1.42). BCAT catalyzes transamination, which is the first reaction in the common degradation pathway for three essential branched-chain amino acids (valine, leucine, and isoleucine). BCAT is also involved in the synthesis of L-glutamate (an important excitatory neurotransmitter both in vertebrates and invertebrates) from these three branched-chain amino acids.

(ii) Genes involved in detoxication, the stress response and self-defense reactions (*CG5999*, *GstD2, Bin1* and *Arc1*)—Of the 12 genes confirmed to be lithium-responsive in this analysis, *CG5999* displayed the largest fold increase in expression, both by microarray and RT-PCR analyses. *CG5999* encodes UDP-glucuronosyl/UDP-glucosyltransferase, which catalyzes the addition of the glycosyl group from a UTP-sugar to a small hydrophobic molecule (e.g. an apolar xenobiotic). This is an important step for detoxication, as it converts potentially toxic molecules to less harmful and more water-soluble forms.

GstD2 (*CG4181*) encodes an isoform of glutathione S transferase (Gst), GstD2 (EC 2.5.1.18) and is up-regulated by lithium. Gst is one of the major detoxication enzymes and plays an essential role in protecting cells against oxidative stress by conjugating the antioxidant glutathione to various oxidized molecules to convert them to nontoxic forms. There are 38 glutathione S transferase genes in the *Drosophila* genome (Tu and Akgul, 2005). In addition to *GstD2*, five other Gst genes (*GstD1*, *D10*, *E6*, *E7* and *E9*) were also up-regulated in a lithium-dependent manner, with *p*-values of 0.005 or less, although fold changes for these genes were less than 2.

Bicoid interacting protein 1 (*Bin1*) (*CG6046*) is also highly up-regulated by lithium. *Bin1* is an ortholog of the human SAP18 (Sin3 associated polypeptide p18) gene. It encodes a component of the histone acetylase complex that causes transcriptional repression by modifying histones (Zhu et al., 2001). In *Arabidopsis*, *SAP18* loss-of-function mutants are unusually sensitive to NaCl, indicating that this protein is involved in transcriptional repression of genes under conditions of salt stress (Song and Galbraith, 2006).

A lithium-inducible gene, *Activity-regulated cytoskeleton-associated protein 1* (*Arc1* or *CG12505*) has been recognized as an immediate early gene whose expression in mammalian CNS neurons is enhanced by synaptic activity (Lyford et al., 1995). This suggests that it may

play a role in neuronal plasticity. *Arc1* expression is also modulated by stress. The stress-dependent modulation of *Arc1* is impaired in the hippocampus-specific glucocorticoid receptor-deficient mouse (GR+/−), a genetic model of predisposition to depression (Molteni et al., 2009). In *Drosophila*, *Arc1* expression is up-regulated by seizure (Guan et al., 2005). *Drosophila Arc1* mutants have recently been shown to be more resistant to starvation than wild-type flies, and their ability to survive longer in the absence of food than wild type flies (Mattaliano et al., 2007) may be related to the fact that they lack normal starvation-induced hyperlocomotor activity. Thus, *Arc1* plays a role in behavioral responses to metabolic stress in *Drosophila*.

(iii) Genes potentially related to psychiatric or neurological disorders (*Nmdmc, CG9377*, and *Lsp1γ*)—*Nmdmc*** (*NAD-dependent methylenetetrahydrofolate dehydrogenase*) or *CG18466* encodes nicotinamide adenine dinucleotide (NAD)-dependent tri-functional enzyme, and is up-regulated in response to lithium. This enzyme has distinct functional domains for each of its substrates: 5,10-methylenetetrahydrofolatedehydrogenase, 5,10-methenyltetrahydrofolatecyclohydrolase and 10-formylotetrahydrofolatesynthetase. It catalyzes three sequential reactions in the conversion of 1-carbon derivatives of tetrahydrofolate, which are substrates for biosynthesis of the purine nucleotides thymidylate and methionine (Hum et al., 1988). Recent genotype analysis of patients for bipolar disorder and schizophrenia suggests that there is a genetic association between risk of these disorders and polymorphisms in one of the human *Nmdmc* orthologs (Kempisty et al., 2007).

CG9377 encodes a serine proteinase and is the only down-regulated gene that has been positively scored by both microarray and RT-PCR analyses. Serine proteases and serine proteinase homologs have been suggested to participate in various defense responses (Jiang and Kanost, 2000; Kanost et al., 2001). Cytosolic serine peptidase, which hydrolyzes relatively short proline-containing peptides (prolyl oligopeptidase), has been found to be significantly down-regulated in bipolar disorder patients undergoing lithium treatment (Breen et al., 2004), although the functional significance of prolyl oligopeptidase suppression by lithium is not known.

Lithium treatment increased the expression of *Larval serum protein 1 gamma* (*Lsp1γ*) or *CG6821*. Lsp1γ is one of the major hemolymph proteins in larvae that serve as a nutrient reservoir for the production of adult cuticle structures. Although several different isoforms of the larval serum proteins are expressed in adult, especially in the adipose cells of the head, their function in adult flies remains unknown. Lsp1γ protein is down-regulated significantly (by ~40%) according to proteome analysis of transgenic flies expressing human wild-type α-synuclein; this implies a potential connection between Lsp1γ and a *Drosophila* model of Parkinson's disease (Xun et al., 2008).

(iv) Other genes (*CG15794*, *Cyp309a1* and *CG7763*)—*CG15794*** is significantly up-regulated in response to lithium. However, its function is not known. A 1.8kb transcript is expected to encode a polypeptide composed of 554 amino acids. It is presumably a soluble protein, and possesses 2 regions of 22 serine-residue stretches separated by ~220 amino acids. Several repetitive motifs (e.g., GXXX or HG repeats) are found throughout the protein. A blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicates that similar proteins are present in other species of *Drosophila*, but not in vertebrate organisms.

Lithium treatment also leads to up-regulated expression of *Cyp309a1* (*CG9964*). *Cyp309a1* encodes a member of cytochrome P450 enzymes, which catalyze a diverse range of chemical reactions including the oxidization of xenobiotic compounds and the synthesis of endogenous hormones. Notably, the expression levels of several cytochrome P450 enzyme genes also increase with lithium treatment in the human lung carcinoma cell line A549 (Allagui et al.,

2007). Although this enzyme may play a role in the detoxication of certain molecules, its specific functions are not yet identified. Among the 87 cytochrome P450 enzyme genes on the chip, this analysis identified *cyp309a1* as the only one whose expression is significantly influenced by lithium.

The function of another up-regulated gene, *CG7763* is unknown. The predicted gene product contains C-type lectin domain, suggesting that it may encode a type of sugar-binding C-type lectin. Several C-type lectins are involved in immune-system functions in vertebrates (Mukherjee et al., 2009).

Potential significance of amino acid metabolic processes in the genomic response to lithium

Our dual approaches — a direct analysis of lithium-regulated genes and an analysis of biological pathways affected by lithium treatment — have revealed the potential significance of amino acid metabolism and stress response/self-defense reaction in the genomic response to lithium. Several groups have previously performed microarray-based expression profiling in mammalian systems to examine the effects of lithium on gene regulation (Wang et al., 2004; Rojek et al., 2005; Zhang et al., 2005; Blair et al., 2006; Yazlovitskaya et al., 2006; Youngs et al., 2006; McQuillin et al., 2007; Chetcuti et al., 2008; Seelan et al., 2008). In these studies, mRNA samples were isolated from different sources (e.g., primary brain cell cultures, neuronal cell lines and brain tissues of mice, rats or humans) and various conditions were used for lithium treatment. Nevertheless, certain genes and biological pathways were identified as being lithium-responsive in multiple studies.

When mouse and human neuronal cell lines were chronically exposed to lithium, genes that positively and negatively regulate apoptosis were down- and up-regulated, respectively (Yazlovitskaya et al., 2006; Seelan et al., 2008). However, our current analysis did not show any significant change in the expression levels of pro- and anti-apoptotic genes. This discrepancy could be partially due to different durations of lithium treatment. In the mammalian studies, cells were treated with lithium for 7–33 days, whereas flies were exposed to lithium for 24 hr in our experiment.

Consistent with our results, some mammalian microarray studies revealed that genes involved in stress response/self-defense reaction and amino acid metabolism are differentially regulated by lithium. For example, antioxidant genes, such as *Gst* or *Peroxiredoxin 2* (*PRDX2*), were found to be up-regulated by lithium in primary cultured rat cerebral cortical cells or human neuronal cells (Wang et al., 2004; Seelan et al., 2008). Our study also showed that the levels of several Gst transcripts are increased after lithium treatment, suggesting that up-regulation of antioxidant genes may be an evolutionarily conserved genomic response to lithium. Lithium's influence on antioxidative systems through the activation of Gst expression may protect the brain from oxidative stress and contribute to its therapeutic action (Wang et al., 2004). In addition, the expression of mammalian genes that are related to amino acid metabolic pathways is significantly affected by lithium treatment. These include genes encoding two aminotrasferases (McQuillin et al., 2007), glutamate ammonia ligase (glutamine synthase) (Chetcuti et al., 2008) and aminobutyl aminotransferase (GABA transaminase) (Zhang et al., 2005). These findings further suggest that amino acids are important to the physiological responses to lithium in different animal species.

Our study shows that the degradation pathways for branched amino acids are specifically enhanced by lithium treatment. What are the implications of this enhanced degradation of branched amino acids for lithium's effects on nervous system functions? One of the possibilities is its influence on the brain serotonin level. Reduced serotonergic neuronal activity is thought to contribute to the pathogenesis of bipolar affective disorder (van Praag and de Haan, 1980) and, because serotonin is synthesized from tryptophan in neurons, brain

tryptophan levels play a critical role in the availability of serotonin. All large neutral amino acids, such as tryptophan and branched amino acids, are transported into the brain across the blood-brain barrier by a single transport system, which means that all of these amino acids compete for the carrier proteins (Oldendorf and Szabo, 1976). Thus, lower levels of branched amino acids result in higher tryptophan — and consequently also higher serotonin — levels in the brain. It has been reported that the tryptophan/neutral amino acid ratio is significantly lower in unipolar and bipolar patients than in control subjects (Lucca et al., 1992) and that lithium decreases plasma levels of isoleucine, leucine, and valine (Leighton et al., 1983). These observations are consistent with the hypothesis that modulation of amino acid profiles contributes to lithium's therapeutic action. It will therefore be interesting to investigate whether in *Drosophila* lithium influences brain functions and behaviors by increasing levels of tryptophan and serotonin therein.

The present genome-wide gene expression study has established an important foundation for functional analyses of the mechanisms underlying lithium's action in the nervous system, using the genetic model organism *Drosophila melanogaster*. As exemplified by our analysis of the lithium-inducible *CG15088*, *Drosophila* offers versatile molecular and genetic tools for functional investigation of the evolutionarily conserved genes in lithium-responsive processes. Similar studies of other genes identified in this microarray analysis are expected to reveal their functional significance in lithium's biological actions at the molecular, cellular and organismal levels, which should provide novel insights into the basic neurobiological processes regulated by lithium in higher vertebrates including humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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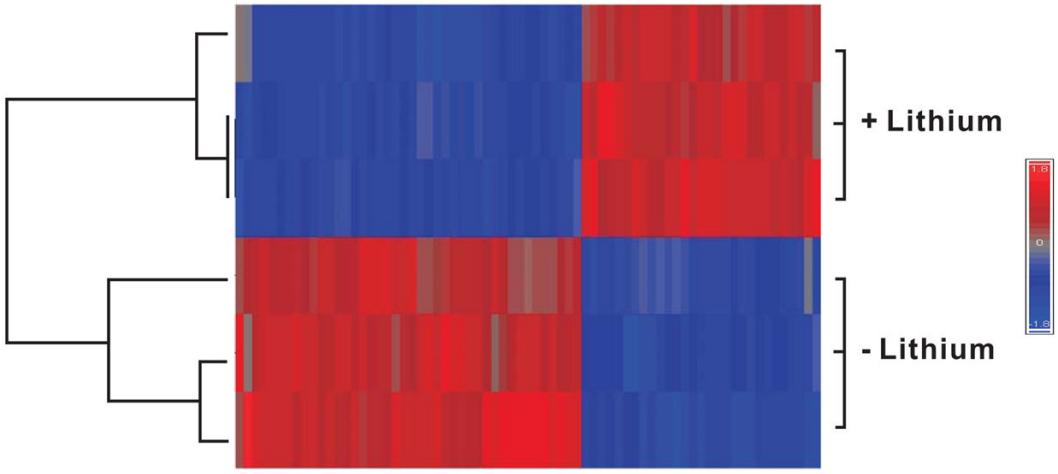

Figure 1. Correlation of microarray data obtained from lithium-treated and untreated samples

Euclidean distance clustering analysis was performed using Ward's method. Three biological replicates (+Lithium or −Lithium) were well clustered. Consistency of the data is visualized by the heat-map representation of expression data for the 66 genes (represented by 71 probe sets) that are differentially regulated in response to lithium treatment (fold change >1.2; FDR <0.05).

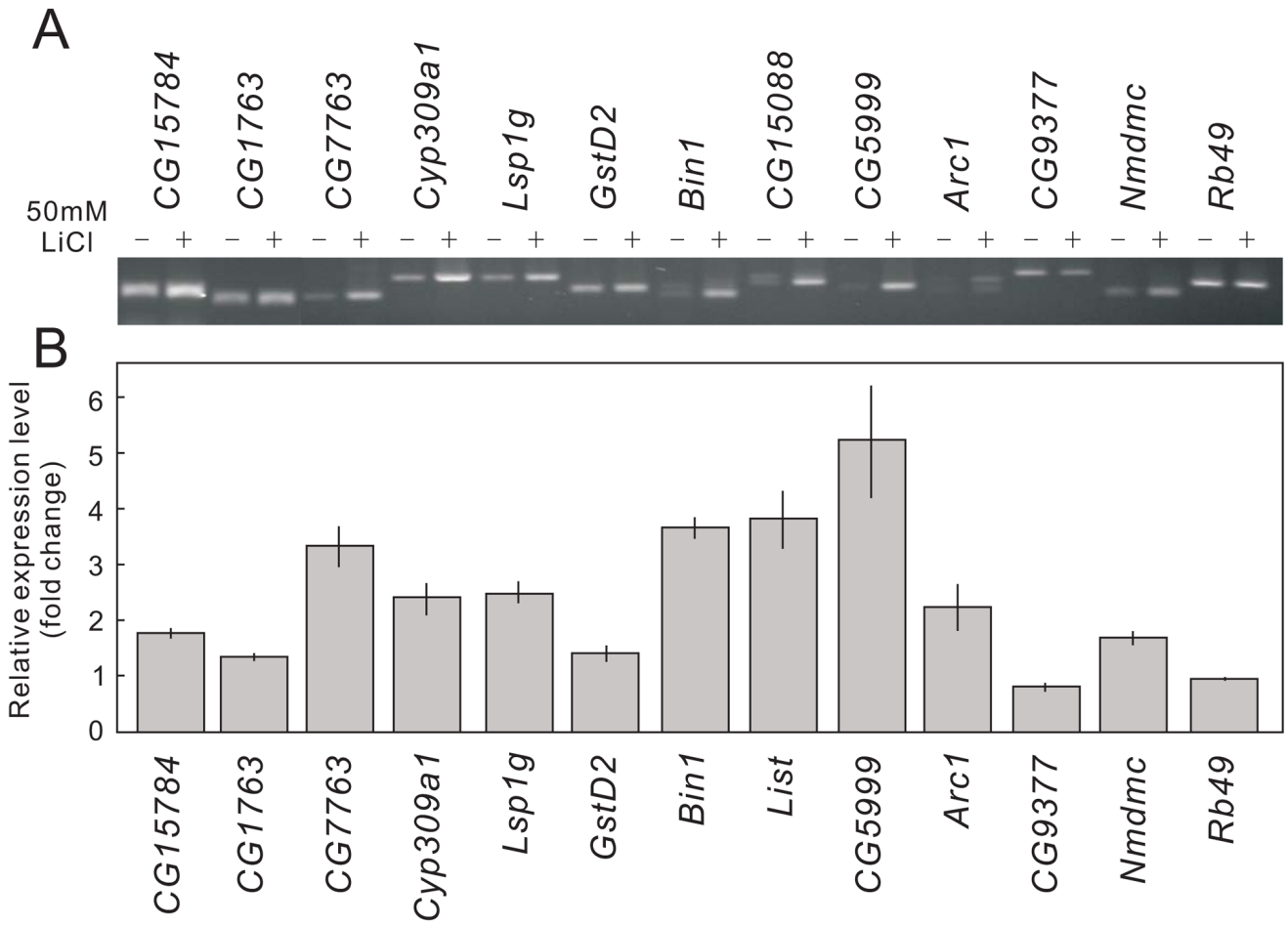

Figure 2. RT-PCR analysis of the genes whose expression is significantly affected by lithium treatment

(A) A representative result of the RT-PCR analysis (carried out in quadruplicate). The PCR products were quantitated based on the pixel intensity of the corresponding bands using Image J software. (B) Ratios of signal intensity of lithium-treated and untreated samples. Average ± SEM, *N*=4.

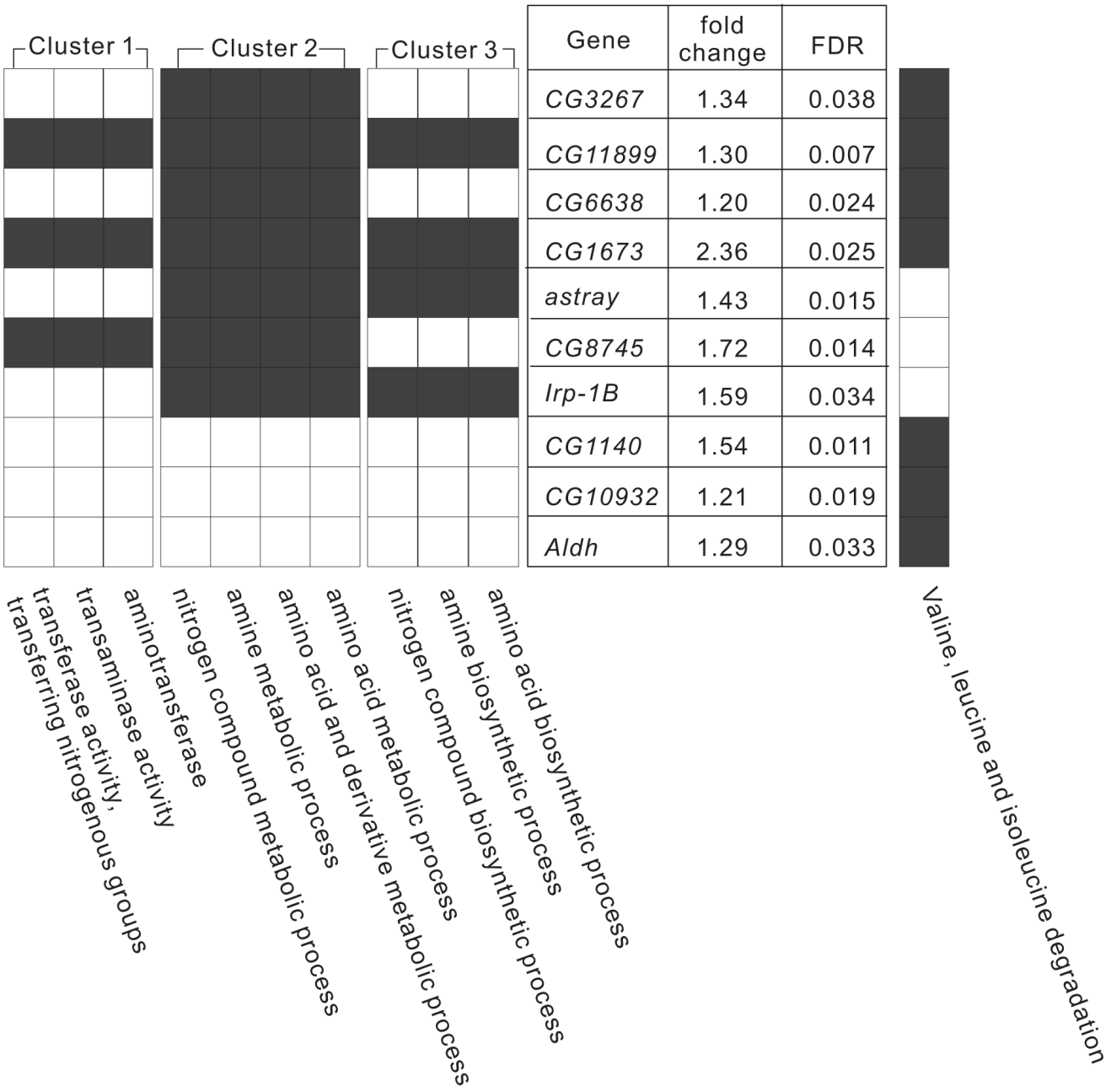

Figure 3. Biological pathways significantly affected by lithium treatment

Biological pathways identified by DAVID clustering analysis, based on the genes whose expression is affected by lithium treatment. Genes that were assigned to these pathways are also shown with values for fold change in expression and false discovery rate (FDR) in response to lithium treatment. Genes that are involved in “valine, leucine, isoleucine degradation pathway (KEGG PATHWAY)” are indicated at right.

Table 1

Primers used for RT-PCR analysis

Gene	5′-primer	3′-primer
CG9377	TGATCACCACCGCGCACTGC	ACATCAGCGGCACAGCGGTCA
GST D2	CATCGCCGTCTATCTGGTGGA	GGCATTGTCGTACCACCTGG
Arc1	CTACAGTGGGCGTGAGCCGGGCA	AGTTGATGGCGCACGGTGCAAG
CG1673	TGCGCTTTTACTTCCAAGCAGCA	GGGCCTAGGTTCTACTGACGGGT
Nmdmc	ATCGATGGCAAGGCCATAGC	ACCAGTATCCCCGTGACCTGG
CG7763	TTCGCAACAACTGCTGACAC	GATATCCACACAATTGGCCTTCG
CG15784	TCCAGCTCCGATGGCGAAGTG	AGAGAACGACTGCGACGACCTG
Cyp309a1	GATTCGAAACATCTGGAGCCGT	CCTGGTAATTTGAGAGGATGTGC
CG15088	TTTCTTCCTCATGCTATTCGTCTTAGGCAT	GAGCGGACATATCGAGCAGCATGT
Lsp 1γ	ACTATCAAGCGCAGCTCCAAG	ACTAAGAGCTAGATCCAGTGTGG
Bin 1	ACCGCAGAATAGGGAGACATG	ACTCCGGAAGTTGGGGTACA
CG5999	TATTCTTTACTCGAAGTGGCTTCCGCA	CTCCGTCCAGTAGAGGAATGATTC
Rp49	GATCGATATGCTAAGCTGTCGC	CGACCACGTTACAAGAACTCT′

Table 2

Genes differentially expressed in response to lithium[a].

Probe set ID	Gene	Microarray				RT-PCR	Description
		Normalized Signal intensity (log2) [b]		Fold Change	p-value	Fold Change[c]	
		− LiCl	+ LiCl				
Down-regulated gene							
1631512_at	*CG9377*	7.17 ± 0.09	6.05 ± 0.10	−2.17	1.54E−04	−1.30 ± 0.06	Serine type endopeptidase
Up-regulated genes							
1630258_at	*Glutathione S transferase D2*	6.27 ± 0.21	7.40 ± 0.30	2.19	6.17E−03	1.39 ± 0.14	Glutathione S transferase
1639180_at 1639694_s_at	*Arc1*	10.01 ± 0.08 8.83 ± 0.06	11.19 ± 0.35 10.00 ± 0.32	2.27 2.25	4.80E−03 3.45E−03	2.18 ± 0.43	zinc ion binding; nucleic acid binding
1623770_at	*CG1673*	7.10 ± 0.09	8.30 ± 0.04	2.31	2.60E−05	1.30 ± 0.10	Branched chain amino acid transaminase
1640775_a_at	*NAD dependent methylenetetrahydrof olate dehydrogenase*	8.49 ± 0.11	9.86 ± 0.37	2.59	3.62E−03	1.68 ± 0.12	methenyltetrahydrofolate cyclohydrolase/dehydrogenase/dehydrogenase
1626144_at	*CG7763*	5.61 ± 0.54	7.20 ± 0.25	3.02	9.42E−03	3.28 ± 0.35	C type lectin/sugar binding
1640884_at	*CG15784*	9.16 ± 0.02	10.84 ± 0.57	3.22	7.06E−03	1.84 ± 0.02	unknown
1626324_at	*Cyp309a1*	7.69 ± 0.28	9.39 ± 0.37	3.25	3.22E−03	2.36 ± 0.28	monooxygenase
1636594_at		7.31 ± 0.07	9.28 ± 0.07	3.91	4.91E−06		Sodium:neurotransmitter
1632986_a_at	*CG15088*	6.75 ± 0.09	9.26 ± 0.02	5.68	1.12E−06	3.74 ± 0.49	symporter
1626429_at	*Larval serum protein 1 γ*	4.50 ± 0.17	6.69 ± 0.22	4.55	1.71E−04	2.41 ± 0.16	oxygen transporter
1632565_at	*Bicoid interacting protein 1*	7.64 ± 0.03	10.06 ± 0.73	5.32	4.73E−03	3.61 ± 0.18	transcription co repressor
1638182_at	*CG5999*	6.79 ± 0.32	10.46 ± 1.11	12.7	5.32E−03	5.12 ± 0.96	UDP glucuronosyltransferase

[a] Genes were selected based on the following criteria: 2.0 fold or greater changes in the normalized signal intensities with p-value with 0.01 or less in Student's t-test.

[b] Signal intensities of each chip were normalized values using RMA algorithm and presented in log2.

[c] Fold change values estimated from RT-PCR analyses were shown as Ave ± SEM, n=4.

Table 3

Functional annotation chart of the selected lithium-responsive genes analyzed by DAVID.

	Term	p-value[a]	Fold Enrichment[b]
1	branched chain family amino acid metabolic process (GO:0009081)	7.57E−04	69.1
2	stress response (SP PIR keywords)	9.23E−05	43.8
3	valine, leucine and isoleucine degradation (KEGG pathway: dme00280)	1.69E−05	15.9
4	organic acid metabolic process[c] (GO:0006082)	8.20E−06	5.99
5	carboxylic acid metabolic process[c] (GO:0019752)	8.20E−06	5.99
6	oxidoreductase (SP PIR keywords)	3.18E−05	5.83
7	mitochondrion (GO:0005739)	9.09E−05	4.29
8	amino acid metabolic process (GO:0006520)	8.05E−04	6.11
9	oxidoreductase activity (GO:0016491)	9.69E−04	3.16
10	hydrolase (SP PIR keywords)	4.03E−04	3.15
11	cytoplasmic part (GO:0044444)	1.66E−04	2.25
12	cytoplasm (GO:0005737)	8.09E−04	1.92
13	catalytic activity (GO:0003824)	3.48E−05	1.71

[a] Modified Fisher Exact p-value (EASE score),

[b] Enrichment factor for the lithium responsive genes (fold change >1.2; FDR<0.05),

[c] The identical genes are assigned to these terms for *Drosophila* genome.

Table 4

Functional annotation clustering of the selected lithium-responsive genes.

Functional cluster 1			
Category	**Term**	**p-value**[a]	**Fold Enrichment**[b]
SP PIR keywords	aminotransferase	0.0016	49.3
GO:0008483	transaminase activity	5.79E−03	25.7
GO:0016769	transferase activity, transferring nitrogenous groups	7.29E−03	22.8
Functional cluster 2			
GO:0006520	amino acid metabolic process	8.05E−04	6.11
GO:0006519	amino acid and derivative metabolic process	0.0020	5.10
GO:0009308	amine metabolic process	0.0145	3.39
GO:0006807	nitrogen compound metabolic process	0.0165	3.30
Functional cluster 3			
GO:0008652	amino acid biosynthetic process	0.0030	13.4
GO:0009309	amine biosynthetic process	0.0070	9.96
GO:0044271	nitrogen compound biosynthetic process	0.0075	9.69

[a] Modified Fisher Exact p-value (EASE score),

[b] Enrichment factor for the lithium-responsive genes (fold change >1.2; FDR<0.05) in each category.