Neuroscience Research 64 (2009) 413–420

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

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

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ARTICLE INFO

Article history:
Received 6 January 2009
Received in revised form 3 April 2009
Accepted 23 April 2009
Available online 3 May 2009

Keywords:
Drosophila
Lithium
Microarray
RT-PCR
Gene expression
Nervous system

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*.

1. 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 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

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doi:10.1016/j.neures.2009.04.015

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.

2. Materials and methods

2.1. Drosophila stock

Flies were reared at 25 °C at 65% humidity, in a 12:12 h 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.

2.2. RNA extraction and microarray experiment

Newly eclosed 0 to 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-h treatment, and kept frozen at −80 °C until used. Total RNA was extracted from the fly heads using Trizol solution (Invitrogen, Carlsbad, CA), followed by further purification using RNeasy 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).

2.3. 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://www.david.abcc.ncifcrf.gov) (Dennis et al., 2003).

2.4. Reverse transcription PCR (RT-PCR)

Total RNA was extracted from 30 fly heads after 24-h treatment with or without 50 mM 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

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
Lsp1γ	ACTATCAAGCGCAGCTCCAAG	ACTAAGAGCTAGATCCAGTGTGG
Bin 1	ACCGCAGAATAGGGAGACATG	ACTCCGGAAGTTGGGGTACA
CG5999	TATTCTTTACTCGAAGTGGCTTCCGCA	CTCCGTCCAGTAGAGGAATGATTC
Rp49	GATCGATATGCTAAGCTGTCGC	CGACCACGTTACAAGAACTCT′