

# Chronic Olanzapine Treatment Causes Differential Expression of Genes in Frontal Cortex of Rats as Revealed by DNA Microarray Technique

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Recent emerging biochemical data indicate that several important neuroregulatory genes and proteins may be involved in the etiology of schizophrenia and bipolar disorder. Additionally, the same genes appear to be targets of several psychotropic medications that are used to treat these disorders. Recent DNA microarray studies show that genes involved in synaptic neurotransmission, signal transduction, and glutamate/GABA regulation may be differentially regulated in brains of subjects with schizophrenia. We hypothesized that chronic administration of olanzapine to rats would alter expression of various genes that may be involved in the etiology of schizophrenia and mood disorders. Rats were administered olanzapine ( $N = 20$ , 2 mg/kg/day) or sterile saline intraperitoneally ( $N = 20$ ) daily for 21 days. Control and olanzapine-treated frontal cortices were analyzed using cDNA microarray technology. The results showed significant downregulation of 31 genes and upregulation of 38 genes by greater than two-fold in the drug-treated brains vs controls. Our results provide evidence for altered regulation of genes involved with signal transduction and cell communication, metabolism and energy pathways, transport, immune response, nucleic acid metabolism, and neuronal growth factors. Real-time quantitative RT-PCR analysis verified the direction and magnitude of change in six genes of interest: calbindin 3, homer 1, regulator of G-protein signaling (RGS) 2, pyruvate kinase, Reelin and insulin 2. Western blotting showed significant upregulation in protein products for Reelin 410 and Reelin 180 kDa and downregulation for NMDA3B and RGS2. Our results show for the first time that olanzapine causes changes in levels of several important genes that may be involved in the etiology and treatment of schizophrenia and other psychiatric disorders.

*Neuropsychopharmacology* (2006) 31, 1888–1899. doi:10.1038/sj.npp.1301002; published online 11 January 2006

**Keywords:** olanzapine; DNA microarray; schizophrenia; bipolar disorder; Reelin; insulin

## INTRODUCTION

Olanzapine is a second-generation antipsychotic agent that, like clozapine, exhibits greater 5HT<sub>2A</sub> than D<sub>2</sub> antagonism (Fatemi and Meltzer, 2000), but does not share clozapine's propensity for agranulocytosis. Olanzapine, similar to clozapine, has high affinity for a number of other receptors, that is, 5HT<sub>2A</sub>, 5HT<sub>2C</sub>, 5HT<sub>1A</sub>, 5HT<sub>6</sub>, 5HT<sub>7</sub>, 5HT<sub>3</sub>, muscarinic,  $\alpha$ 1-adrenergic, and histamine-H<sub>1</sub> receptors (Fatemi and Meltzer, 2000; Bymaster *et al*, 1997). Additionally, olanzapine facilitates NMDA neurotransmission via effects on glutamatergic NMDA and AMPA receptors

(Tarazi *et al*, 2003; Wang *et al*, 2000). However, apart from its receptor-binding profile, little is known about the molecular mechanism of olanzapine drug action. Olanzapine has been used successfully to treat schizophrenia, bipolar disorder, and some forms of depression and anxiety. It has been hypothesized that antipsychotic agents affect various brain genes leading to changes in synaptic structure and function that may underlie clinical response (Chen and Chen, 2005).

Previous similar microarray studies investigating the effects of chronic administration of antipsychotics in brains of rodents are limited and yet informative (Chen and Chen, 2005; Chong *et al*, 2002; Kontkanen *et al*, 2002; MacDonald *et al*, 2005; Thomas *et al*, 2003). Chong *et al* (2004) showed that chronic administration of clozapine in rats for 28 days upregulated mRNA and protein for rat glia-derived nexin in brains of exposed animals. The authors suggest a possible neuroprotective role for nexin, a protease inhibitor, in clozapine treatment, most likely in the prevention of neuronal proteolytic degradation (Chong *et al*, 2004). In a

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Received 16 May 2005; revised 17 October 2005; accepted 24 October 2005

Online publication: 2 November 2005 at <http://www.acnp.org/citations/Npp110205050324/default.pdf>

different microarray-based study, Chong *et al* (2002) administered haloperidol in rats for 28 days and discovered that it upregulated both mRNA and protein for synapsin II gene by >50% in the striatum of exposed rats. Recent studies have also shown involvement of synapsin in schizophrenic pathology (Mirnics *et al*, 2000). In a different study, Thomas *et al* (2003) used total gene expression analysis to examine gene expression profiles of mouse striatum and frontal cortex following exposure to 14 days of haloperidol or clozapine. The authors showed that both drugs increased genes associated with lipid metabolism, such as apolipoprotein D, the mouse homolog of oxysterol-binding protein-like protein 8, and lysophosphatidic acid acyltransferase (Thomas *et al*, 2003). MacDonald *et al* (2005) investigated gene profiles in the frontal cortex and striatum of rats in response to chronic exposure to haloperidol and clozapine. Several genes coding for presynaptic proteins including VAMP1, syntaxin 1A, and SNAP 25 were upregulated in the frontal cortex, potentially increasing synaptic plasticity and synaptic activity (MacDonald *et al*, 2005). Finally, in the most recent publication, Chen and Chen (2005) analyzed gene expression profiles in the frontal cortex of rats administered risperidone for 1–4 weeks. Several genes were upregulated in rat frontal cortex including three genes encoding catabolic enzymes of biogenic amine neurotransmitters, which may partly account for risperidone's clinical efficacy (Chen and Chen, 2005). Taken together, these studies indicate that multiple metabolic pathways are affected by the actions of antipsychotic medications, which affect gene families dealing with synaptic plasticity, neuroprotection, presynaptic neurotransmission, and lipid metabolism.

In the absence of DNA-microarray-based studies of the effects of olanzapine, we used microarray technology to investigate brain response to olanzapine following chronic treatment in rats and to identify potential genes of interest involved in the etiology of schizophrenia and bipolar disorder.

## MATERIALS AND METHODS

### Animals and Drug Treatment

Male Sprague–Dawley albino rats (Charles River) initially weighing approximately 250 g were housed in a temperature and humidity-controlled environment with a 12-h light/dark cycle and had access to food and water *ad libitum*. The University of Minnesota Animal Care Committee approved all experimental procedures. Rats were randomly assigned to one of two groups: (1) olanzapine (2 mg/kg/day, intraperitoneally (i.p.),  $N=20$ ); or (2) sterile saline ( $N=20$ ), and administered drug or diluent in 1 ml volume for 21 days. The dosage for olanzapine was chosen based on values obtained from the literature that correspond to clinically relevant human-equivalent dosage (Kusumi *et al*, 2000; Timmerman *et al*, 1999). Kusumi *et al* (2000) injected olanzapine at 1 or 2 mg/kg, i.p., into rats for 3 weeks and showed no increase in D2 receptor density in the striatum. There is also published evidence that olanzapine at 4 mg/kg causes catalepsy (Hoffman and Donovan, 1995). Thus, our choice of 2 mg/kg dose was based on data that showed this

dose to be sufficient enough to produce human-equivalent clinical efficacy but did not cause extrapyramidal side effects in rats. Animals were killed 24 h after the last injection under deep anesthesia using ketamine (90 mg/kg) and Nembutal (40 mg/kg). Brains were removed and the frontal cortex ( $N=4$ /group) was dissected and flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for future assays.

### DNA Microarray

Frontal cortical tissues were homogenized by standard procedures in Trizol (Gibco-BRL) for total mRNA extraction. Total RNA (10  $\mu\text{g}$ ) from each sample was used to generate a high fidelity cDNA, which was modified at the 3' end to contain an initiation site for T7 RNA polymerase as per the manufacturer's protocol (SuperChoice, Gibco-BRL). Upon completion of cDNA synthesis, 1  $\mu\text{g}$  of product was used in an *in vitro* transcription (IVT) reaction that contained biotinylated UTP and CTP, which was utilized for detection following hybridization to the microarray as per the manufacturer's protocol (ENZO). Full-length IVT product (20  $\mu\text{g}$ ) was subsequently fragmented in 200 mM Tris-acetate (pH 8.1), 500 mM KOAc, and 150 mM MgOAc at  $94^{\circ}\text{C}$  for 35 min. Following fragmentation, all components generated throughout the processing procedure (cDNA, full-length cRNA, and fragmented cRNA) were analyzed by gel electrophoresis to assess the appropriate size distribution prior to microarray analysis. All samples generated were subject to gene expression analysis by the Affymetrix rat genome 230 2.0 array set, which contained sequences from approximately 28 000 known rat genes and ESTs at the University of Rochester Microarray Core Facility. Each gene on the array was represented by 16–20 pairs of 25mer oligonucleotides that spanned the coding region for each gene represented. Each probe pair consisted of a perfect match sequence that was complementary to the cRNA target and a miss match sequence that had a single base pair mutation in a region critical for target hybridization; this sequence served as a control for nonspecific hybridization. Hybridization, staining, and washing of all arrays were performed in the Affymetrix fluidics module as per the manufacturer's protocol. Streptavidin phycoerythrin stain (SAPE, Molecular Probes) was the fluorescent conjugate used to detect hybridized target sequences. The detection and quantitation of target hybridization was performed with a GeneArray Scanner (Hewlett Packard/Affymetrix) set to scan each array twice at a factory set PMT level and resolution. In addition, all arrays were scanned pre- and post-antibody amplification to address potential issues with respect to the dynamic range of the scanner. All arrays referred to in this study were assessed for 'array performance' prior to data analysis. This process involves the statistical analysis of control transcripts that are spiked into the hybridization cocktail to assess array performance. In addition, several genes have been identified on each array to help assess the overall quality of signal intensity from all arrays. The result of this analysis has demonstrated that each array is within a 0.2-fold difference of each other at baseline. This analysis affords the necessary confidence needed to apply a global scaling approach to data normalization in the subsequent analyses.

## RNA Extraction and cDNA Synthesis

Total RNA was extracted from brain tissues using a combination of Trizol for organic extraction followed by the RNeasy total RNA extraction kit with modifications made by the URM C FGC (detailed protocol at <http://fgc.urmc.rochester.edu/>). RNA quality was assessed by electrophoresis using the Agilent Bioanalyzer 2100 and spectrophotometric analysis prior to cDNA synthesis. Between 1 and 10 µg of total RNA from each sample was used to generate a high fidelity cDNA using either oligo dT<sub>(12–18)</sub> primers or random hexamers for reverse transcriptase primer. Superscript II was used for the reverse transcriptase and the manufacturer's protocol was used for the same procedure.

## Validation of Microarray by Real-Time Quantitative RT-PCR

Gene expression was examined using TaqMan chemistry with probes and primers selected by the Applied Biosystems Assay on Demand Program. The following dye combinations were used for detection and data normalization: FAM (for the genes of interest), HEX (for normalizer genes, see below), BHQ1 (non-fluorescent quencher) and ROX (reference). Following probe and primer optimization, all cDNAs were diluted and used in a 10 µl PCR reaction containing 5 µl of ABI 2 × Universal Master Mix, 1 × of AOD, and RNAase/DNAase free water. The expression analysis of transcripts was carried out with predesigned and optimized TaqMan Gene Expression Assays with B2m ( $\beta$ -2 microglobulin) or GAPDH used as an endogenous control. All reactions were performed in triplicate and the experiments replicated three times. All reactions were run in an ABI 7900 with the following cycle parameters: 1 cycle of 50°C (2 min), followed by 95°C (10 min), 40 cycles of 95°C (15 s), followed by 60°C (1 min). Data were collected at every temperature phase during each cycle. Raw data were analyzed using the Sequence Detection Software (ABI, Foster City, CA) while relative quantitation using the comparative threshold cycle ( $C_T$  method) was performed in Microsoft Excel (ABI Technote#2: Relative Gene Expression Quantitation). All genes presented were analyzed as described above.

## Data Analysis

Multiple data analysis approaches were used to identify interesting genes within this experimentally tractable approach to studying the effects of psychotropic agents on neuronal tissue. The Microarray Analysis Suite 5.0 (Affymetrix) was employed to generate one approach to comparative analysis presented in this study. Distinct algorithms were used to determine the detection signal, which distinguished the presence or absence of a transcript, the differential change in gene expression (increase (I), decrease (D), marginal increase (MI), marginal decrease (MD), and no change (NC)), and the magnitude of change, which is represented as fold change. The fold change of any transcript between the baseline and experimental is calculated following global scaling. All data represented from this first approach were from pairwise comparison

analysis. The second approach to identifying differentially expressed genes utilized GeneSpring (Silicon Genetics).

Following each normalization approach, all genes differentially expressed were clustered based on biological relevance. Genes were assessed based on primary biological function and grouped accordingly. All data have been organized in a central database in the University of Rochester Functional Genomics Center and are accessible through a variety of web-based solutions (<http://fgc.urmc.rochester.edu/data2.html>). Additional information on normalization methods can be found on the Neuropsychopharmacology website published as a supplement to this manuscript.

## SDS-PAGE and Western Blotting

Brain tissue (~25 mg) per rat was cut and placed on ice in lysis buffer (3 ml/g of tissue) (20 mM Tris, pH 8.0, 0.2 mM EDTA, 150 mM NaCl, 3% Igelal/NP40 (v/v), 1% sodium deoxycholate (w/v), 0.1% SDS (w/v), 50 µg/ml leupeptin, 0.2 mM PMSF, 1 mM sodium orthovanadate, and 30 µl/ml buffer Aprotinin (Sigma A6279)). Tissue samples were homogenized using a Kontes hand pestle while the temperature was maintained at 4°C. Following homogenization, an additional 1 µl of PMSF (0.2 mM) was added to each sample, and the samples were set on ice for 30 min. The homogenates were centrifuged for 20 min at 10 000g at 4°C. Supernatants were collected and assayed for total protein using the Bradford method (BioRad). Samples were stored at –86°C until used.

Samples were mixed with denaturing SDS sample buffer (20% glycerol, 100 mM Tris, pH 6.8, 0.05% w/v bromophenol blue, 2.5% SDS (w/v), 5%  $\beta$ -mercaptoethanol) and denatured by heating at 100°C for 5 min. For  $\beta$ -actin and synaptotagmin IV, 10% SDS-polyacrylamide gels were prepared with standard Laemmli solutions (BioRad). In all, 60 µg of protein per lane was loaded onto the gel and electrophoresed, initially for 15 min at 75 V and then for 50 min at 150 V at room temperature (RT). The proteins were electroblotted onto nitrocellulose paper for 2 h at 300 mA at 4°C. Protein blots were blocked overnight at 4°C with 0.2% I-Block (Tropix) in PBS with 0.3% Tween-20. The blots were then incubated with primary antibody for 1 h at RT (mouse anti- $\beta$ -actin, Sigma A5441, 1:5000; or rabbit anti-synaptotagmin IV, a gift from Dr Fukuda, 1:1000) followed by secondary antibody incubation for 1 h at RT (goat anti-mouse or goat anti-rabbit IgG, HRP conjugated, 1:80 000, both from Sigma). Between each step, the immunoblots were rinsed with PBS-T. The immune complexes were visualized using the ECL Plus detection system (Amersham Pharmacia Biotech) and exposed to CL-Xposure film (Pierce). Sample densities were analyzed using a Bio-Rad densitometer and Quantity One software.

NMDA3B was analyzed as above, with the following changes: an 8% SDS-polyacrylamide gel was used; after transfer, the blot was blocked at RT for 1 h, followed by overnight incubation with primary antibody (rabbit anti-NMDA3B, Upstate 07–351, 1:1000) at 4°C. Secondary antibody was (goat-anti-rabbit IgG, HRP conjugated, 1:80 000) applied for 1 h at RT, and all washes and visualization took place as described above.

Reelin was analyzed as above (Fatemi *et al*, 2005a,b), with the following changes: 6% SDS-polyacrylamide gel was used; gels were run at 75 V for 15 min at RT, as above, but then run at 150 V for 60 min. Gels were transferred to PVDF membrane (prewet in methanol for 1 min, rinsed in dH<sub>2</sub>O, and soaked in transfer buffer for at least 20 min prior to assembling transfer sandwich) at 150 mA for 15 h at 4°C. Blots were blocked for 6 h at RT and primary antibody (mouse anti-Reelin, Chemicon MAB5366, 1:1000) was applied for 22 h at 4°C, and secondary antibody (goat anti-mouse IgG, HRP conjugated, Sigma, 1:1000) was applied for 1 h at RT. All washes and visualization took place as described above.

Regulator of G-protein signaling (RGS) 2 utilized a Tricine SDS-PAGE technique, a modified version of that has been described by Schagger and von Jagow (1987). Briefly, 16% SDS-polyacrylamide gels were prepared and 60 µg of protein was loaded onto the gel in 2 × loading buffer (Invitrogen) and electrophoresed (1 M tricine, 1 M TRIS base, 0.1% SDS) at 30 V for 40 min, then at 110 V for 110 min, both at RT. Gels were transferred onto 0.22 µm PVDF-Plus (Osmonics, Inc., GE Healthcare) membrane (prewet as above) at 300 mA for 120 min at 4°C. The blot was then rinsed in dH<sub>2</sub>O and blocked for 1 h at RT and incubated in primary antibody (rabbit anti-RGS2, a gift from Dr Siderovski, 1:1000) overnight at 4°C. Following rinses in PBS-T, secondary antibody (goat anti-rabbit IgG, HRP conjugated, Sigma, 1:80 000) was applied for 1 h at RT, and all washes and visualization took place as described above.

The densities of approximately 410, 330, and 180 kDa (Reelin), 97 kDa (NMDA3B), 50 and 25 kDa (RGS2), 48 kDa (synaptotagmin IV), and 46 kDa ( $\beta$ -actin), immunoreactive bands were quantified with background subtraction. Control samples (30–90 µg) were immunoblotted to ascertain that all samples fell within the linear range of the densitometric curve (not shown). Results obtained are based on between two and eight independent experiments with  $n=3$  or  $n=4$  rats per gel. For each experiment, control and olanzapine samples were run on the same gel and processed simultaneously to avoid variability due to intragel differences.

## Statistical Analysis

For microarray data, all statistical analyses were performed using SPSS. Differences of the normalized mRNA expression levels of selected genes between drug-treated and saline-treated rats were assayed using Student's *t*-test. Significant differences are defined as those with a *P*-value < 0.05.

For Western blots, differences of the protein levels of selected genes between drug-treated and saline-treated rats were normalized against  $\beta$ -actin and assayed using a Student's *t*-test. Significant differences are defined as those with a *P*-value < 0.05.

## RESULTS

Olanzapine treatment of rats for 21 days resulted in significant ( $P<0.05$ ) downregulation of 31 genes and

significant ( $P<0.05$ ) upregulation of 38 genes in the frontal cortex of drug-treated rats (Table 1). We used multiple types of data analytic approaches (RMA, GCRMA, and MAS5) for the current microarray studies because different approaches analyze data in fundamentally different ways, allowing for increased data mining (Fatemi *et al*, 2005c; Irizarry *et al*, 2003; Wu *et al*, 2004; Hubbell *et al*, 2002). For this data set, RMA, GCRMA, and MAS5 analyses were combined to provide an optimal number of statistically significant changes in gene expression. Data presented represent all genes with altered expression at significant levels. Additionally, the genes were compiled on the basis of statistical significance using standard *t*-test and at gene expression levels of 2-fold or more vs saline-treated rat brains following previous methodology (Elshatory *et al*, 2003; Fatemi *et al*, 2005c).

Of note, examples of some of the upregulated genes of interest included *homer 1*, *sarcosin*, *timeless*, *calgranulin A and B*, *cocaine and amphetamine-regulated transcript (CART)*, *ileal peptidase*,  $\gamma$ -*synuclein* and *calpain 8* (Table 1). Examples of some of the downregulated genes included *calbindin 3*, *prolactin-like protein A*, *RGS2*, *fibroblast growth factor receptor 2*, *insulin-like growth factor binding protein 2 (IGFBP2)*, *sucrase-isomaltase*, *methionine synthase*, and *NMDA3B* (Table 1). Finally, real-time quantitative RT-PCR (QPCR) verified magnitude and direction of change in several significantly affected genes of interest, including *calbindin 3*, *homer 1*, *pyruvate kinase (liver and RBC)*, *RGS2*, and *insulin 2* (Table 2).

Tabulation of the significantly affected genes, based on functional relevance (Table 3), showed involvement of genes from multiple biological pathways, such as signal transduction/cell communication, transport, protein metabolism, and cell growth/maintenance, in the olanzapine mechanism of drug response (Table 3).

We also measured protein levels for Reelin, NMDA receptor 3B, synaptotagmin IV, and RGS2 using Western blotting. Reelin molecules appeared on SDS-PAGE as multimeric bands ranging from  $\approx 410$  to  $\approx 330$  to  $\approx 180$  kDa (Figure 1a) and showed significant upregulation in the 410 and 180 kDa isoforms normalized against  $\beta$ -actin in olanzapine-treated rat frontal cortex ( $P=0.0033$  and  $0.0001$ , respectively; Figure 1b and d; Table 4). The 330 kDa isoform of Reelin showed a nonsignificant upregulation (Figure 1c; Table 4). NMDA3B appeared as a single band at  $\approx 97$  kDa and showed significant downregulation in olanzapine-treated rat frontal cortex ( $P=0.018$ ; Table 4). The RGS2 antibody recognized RGS2 as a dimer band at  $\approx 50$  kDa and a monomer band at  $\approx 25$  kDa (F.S. Willard and D.P. Siderovski, unpublished observations). The RGS2 dimer band showed a significant downregulation in olanzapine-treated rat frontal cortex ( $P=0.0061$ ; Table 4), as did the total RGS2 protein (dimer and monomer bands taken together;  $P=0.0039$ ), while the 25 kDa monomer band showed a nonsignificant downregulation (Table 4). Synaptotagmin IV appears as a single band at  $\approx 48$  kDa and showed a nonsignificant downregulation in olanzapine-treated rat frontal cortex (Table 4). There were no significant differences in levels of  $\beta$ -actin in olanzapine-treated brains vs controls (Table 4) indicating absence of any major treatment-related effects on protein stability.

**Table 1** Effects of Chronic Administration of Olanzapine on Rat Frontal Cortex

Upregulated genes	Downregulated genes
Aldehyde oxidase 1 <sup>a</sup>	Activating transcription factor 3 <sup>a</sup>
Angiopoietin-like 2 <sup>b</sup>	Calbindin 3 <sup>b</sup>
Apolipoprotein A-V <sup>b</sup>	Calcium activated potassium channel beta 4 subunit <sup>a</sup>
Calpain 8 <sup>b</sup>	Calcium-independent alpha-latrotoxin receptor homolog 3 <sup>a</sup>
Chemokine-binding protein 2 <sup>b</sup>	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3 <sup>b</sup>
Chondroitin sulfate proteoglycan 3 <sup>a</sup>	Chymotrypsinogen B <sup>b</sup>
Cocaine and amphetamine regulated transcript (CART) <sup>a</sup>	Cytochrome P450IIB3 <sup>a</sup>
Cold shock domain protein A <sup>b</sup>	Cytochrome P450, subfamily 11A <sup>b</sup>
Cysteinyl leukotriene receptor 1 <sup>a</sup>	D site albumin promoter binding protein <sup>a</sup>
Endothelial and smooth muscle cell-derived neuropilin-like protein <sup>b</sup>	Dentin sialophosphoprotein <sup>a</sup>
Fibroblast growth factor 3 <sup>b</sup>	Fibroblast growth factor receptor 2 <sup>a</sup>
Galanin receptor 1 <sup>b</sup>	Glutamate receptor, ionotropic, NMDA3B <sup>a</sup>
Glutamate receptor interacting protein 1 <sup>a</sup>	Glycoprotein hormone alpha 2 <sup>a</sup>
Heme oxygenase 1 <sup>b</sup>	Insulin-like growth factor binding protein 2 <sup>a</sup>
Homer, neuronal immediate early gene, 1 <sup>a</sup>	Interleukin 1 receptor accessory protein <sup>a</sup>
Insulin 2 <sup>b</sup>	Interleukin 2 receptor, beta chain <sup>a</sup>
Mast cell protease 8 <sup>b</sup>	Interleukin 23, alpha subunit p19 <sup>a</sup>
MT-protocadherin <sup>a</sup>	Lecithin-retinol acyltransferase <sup>b</sup>
Muscle glycogen phosphorylase <sup>b</sup>	Methionine synthase <sup>a</sup>
N-acetylated alpha-linked acidic dipeptidase-like 1 (ileal peptidase 1100) <sup>b</sup>	Prolactin-like protein A <sup>b</sup>
Pancreatic polypeptide receptor 1 <sup>b</sup>	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform <sup>a</sup>
Phospholipase A2, group IVA (cytosolic, calcium-dependent) <sup>a</sup>	Regulator of G-protein signaling protein 2 (RGS2) <sup>b</sup>
Procollagen, type I, alpha 2 <sup>a</sup>	Solute carrier family 22, member 3 <sup>a</sup>
Pyruvate kinase, liver and RBC <sup>a</sup>	Solute carrier family 24, member 3 <sup>a</sup>
Rat mRNA for RT1.D beta chain similar to Class II MHC RT1.D(n) beta chain precursor <sup>b</sup>	Sucrase-isomaltase <sup>a</sup>
Regulator of G-protein signaling protein 19 (RGS19) <sup>b</sup>	Synaptotagmin 4 <sup>a</sup>
RT1 class Ib gene, locus M3 <sup>a</sup>	Thyroid stimulating hormone receptor <sup>a</sup>
S100 calcium-binding protein A8 (calgranulin A) <sup>a</sup>	Tripartite motif protein 17 <sup>a</sup>
S100 calcium-binding protein A9 (calgranulin B) <sup>b</sup>	UDP glycosyltransferase 2 family, polypeptide B <sup>a</sup>
Sarcomeric muscle protein (Sarcosin) <sup>b</sup>	Uncoupling protein UCP-4 <sup>a</sup>
Sodium channel, nonvoltage-gated, type I, alpha polypeptide <sup>b</sup>	Zinc finger, C3HC4 type (RING finger) <sup>a</sup>
Solute carrier family 12, member 1 <sup>b</sup>	
Solute carrier family 38, member 1 <sup>b</sup>	
Synuclein, gamma <sup>b</sup>	
Thrombomodulin <sup>a</sup>	
Timeless (Drosophila) homolog <sup>b</sup>	
Tissue-type vomeronasal neurons putative pheromone receptor V2R2 <sup>b</sup>	
Transmembrane 4 superfamily member 3 <sup>a</sup>	

All genes shown are significantly ( $P < 0.05$ ) changed by greater than two-fold vs saline-treated controls.

<sup>a</sup>Fold change of at least 2.0.

<sup>b</sup>Fold change of at least 4.0.

## DISCUSSION

Our studies are the first to demonstrate, at the molecular level, the chronic effects of olanzapine on multiple gene families of interest in the frontal cortex of the rat, spanning genes involved in signal transduction, cell communication, metabolism, energy pathways, transport, immune response, nucleic acid metabolism, and neuronal growth factors (Table 3). Additionally, scrutiny of the affected genes

identified several genes/proteins implicated in the pathology of schizophrenia and mood disorders (Table 5).

## Genes Upregulated due to Olanzapine Treatment

A total of 38 genes were upregulated significantly by olanzapine treatment. One of these upregulated genes is CART, which is involved in feeding, drug reward, and stress.

**Table 2** QPCR Results for Selected Genes Involved in Olanzapine Treatment

Protein	Gene ID	Fold change	Two-tailed p-value
Calbindin 3*	CALB3	−1.46	0.0004
Homer, neuronal immediate early gene, 1	HOMER1	+1.08	0.5677
Insulin 2*	INS2	+3.68	0.0100
Pyruvate kinase, liver and RBC	PKLR	+1.47	0.0601
Reelin*	RELN	+1.65	0.0046
Regulator of G-protein signaling protein 2*	RGS2	−2.32	0.0002

\* Statistically significant ( $P < 0.05$ ).

CART peptides are distributed in the brain, gut, pituitary, adrenals, and pancreas (Hunter and Kuhar, 2003). A previous report suggested an association between a polymorphism in intron 1 of *CART* gene and alcoholism (Jung et al, 2004). In homozygous circadian clock mutant mice, which have an attenuated diurnal feeding rhythm and which are hyperphagic and obese and develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia, *CART* expression is significantly reduced (Turek et al, 2005). *CART*, *ghrelin*, and *orexin* all contain clock responsive E-box elements (Turek et al, 2005). Interestingly, *CART*, which also increases glucose and regulates body weight, is upregulated significantly by olanzapine (Tables 1, 3) potentially providing a mechanistic insight into increases in body weight observed in schizophrenic patients treated with this drug.

*Fibroblast growth factors* (FGF) are members of a family of trophic factors whose expression are regulated by neurotransmitters or learning (Ovalle et al, 2001). *FGF* has previously been shown to be increased in serum of schizophrenic patients (Hashimoto et al, 2003). Additionally, members of the *FGF* gene family have been known to be altered in depression (Evans et al, 2004). Olanzapine increased mRNA for *FGF3* and decreased *FGF* receptor 2 in the frontal cortex of exposed rats, presumably facilitating signal transduction, cell communication, and neuronal growth (Tables 1, 3). *FGFs* appear to be targets of several psychotropic medications including clozapine (Riva et al, 1999), E-5842 (Ovalle et al, 2001), and olanzapine. Riva et al (1999) showed increases in levels of *FGF2* mRNA and protein in rat striatum following administration of clozapine. Ovalle et al (2001) showed that E-5842, a preferential sigma-1 receptor agonist and ligand and a putative atypical antipsychotic, causes upregulation of *FGF2* mRNA in the prefrontal cortex (PFC) and striatum and downregulation of *FGF2* mRNA in the hypothalamus of rat brains following 21-day chronic exposure. Thus, it appears that *FGF2* could play a role as a neuroprotective agent, which is upregulated by olanzapine, clozapine, and sigma-1 receptor ligand E-5842 (Ovalle et al, 2001; Riva et al, 1999). Olanzapine is also capable of upregulating levels of *nerve growth factor* and downregulating levels of *BDNF* in various areas of the rat brain (Angelucci et al, 2005) attesting to varied effects of olanzapine on different trophic factors in the brain.

Impairment of glycolytic pathways has been investigated in schizophrenia (Stone et al, 2004). Stone et al (2004) identified positive associations between schizophrenia and

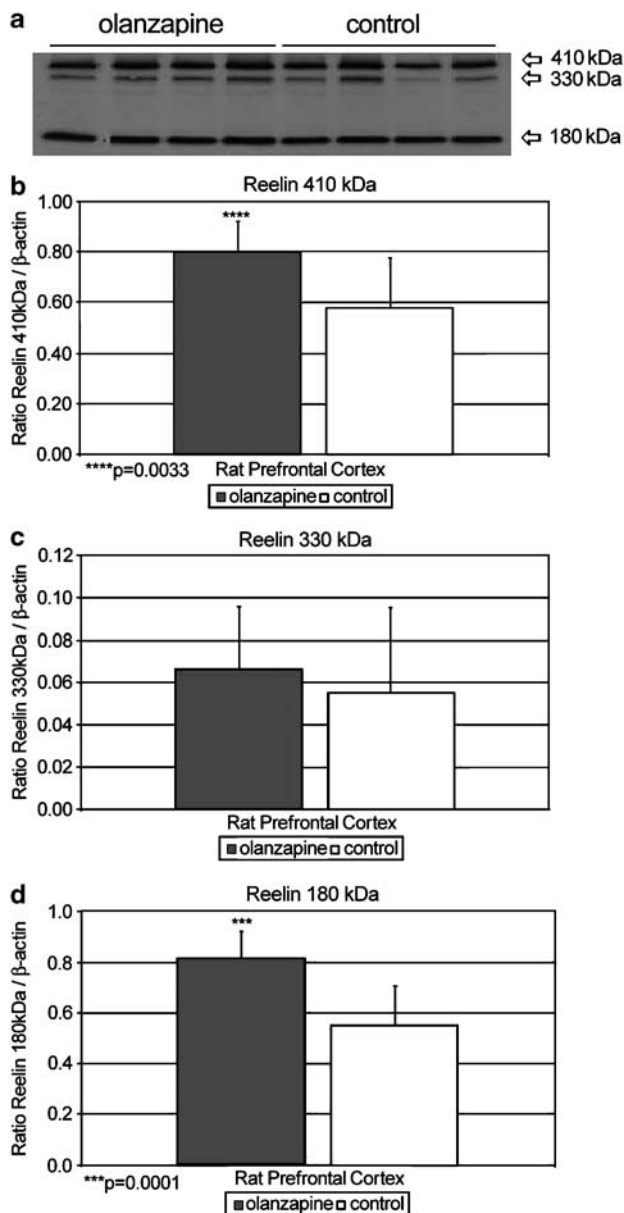
several enzymes involved in glycolysis, including *6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 2*, *hexokinase 3*, and *pyruvate kinase 3*. Similarly, olanzapine treatment of rats caused significant alterations in several key glycolytic enzymes, such as increases in muscle glycogen phosphorylase and in *RBC and liver pyruvate kinase* (which also showed a near significant increase based on our QPCR results; Table 2) and a decrease in *sucrase-isomaltase* (Tables 1, 3).

The *Homer* family of genes and proteins are scaffold-related molecules that are involved in the modulation of glutamate receptors and  $Ca^{2+}$  stores and affect signal transduction cascades (VanderVorst, 2004). The *Homer* protein family also assists in assembly of glutamate receptors and  $Ca^{2+}$  channels in the plasma membrane (VanderVorst, 2004). Norton et al (2003) identified one SNP (*Homer 1* IVS4+18A>G) to be associated with schizophrenia, confirmed by genotyping. Govek et al (2004) also reported absence of a *Homer* interacting protein, namely oligophrenin-1 (a Rho-GTPase activating protein) in a family affected with X-linked mental retardation. Olanzapine in rats increased levels of *Homer 1* significantly (Tables 1, 3), a result that was confirmed through QPCR, albeit nonsignificantly (Table 2). Other studies have shown haloperidol increasing *Homer* in both the caudate putamen and nucleus accumbens of rats (Polese et al, 2002). Clozapine, however, increased *Homer* in the accumbens only (Polese et al, 2002). Thus, it appears that haloperidol, clozapine, and olanzapine all increase *Homer* mRNA levels in various brain sites of rodents modulating glutamate metabotropic system.

Olanzapine increased expression of *insulin 2* significantly (Tables 1, 3) in exposed rat frontal cortex. There are two insulin isomers in rat, namely *insulin 1* and *insulin 2*, with the latter species being more responsive to changes in glucose levels (Toriumi and Imai, 2003). *Insulin 2* is the probable analogue of human insulin in rat (Toriumi and Imai, 2003). The increase in *insulin 2* gene expression was verified by QPCR, showing a significant increase ( $p < 0.01$ ) in *insulin 2* mRNA by  $\approx 4$ -fold in olanzapine-treated rats (Table 2). In contrast, *IGFBP2* was reduced by olanzapine. Transgenic mice, which overexpress *IGFBP1*, display lateral ventricular enlargement (Doublier et al, 2000). In addition to effects on brain development, perturbations of insulin/insulin-like growth factors have been associated with susceptibility to schizophrenia (Gunnell and Holly, 2004; DeLisi et al, 2002). Olanzapine-induced increases in levels of *insulin 2* and decreases in level of *IGFBP2* may reflect the

**Table 3** Biochemical Pathways Involved in Olanzapine Action

Biological function	Genes upregulated by olanzapine	Genes downregulated by olanzapine
Signal transduction; cell communication	Chondroitin sulfate proteoglycan 3 Cysteinyl leukotriene receptor 1 Endothelial and smooth muscle cell-derived neuropilin-like protein Galanin receptor 1 Glutamate receptor interacting protein  Homer, neuronal immediate early gene, 1 MT-protocadherin Pancreatic polypeptide receptor 1 Regulator of G-protein signaling protein 19 (RGS19) S100 calcium-binding protein A8 (calgranulin A) S100 calcium-binding protein A9 (calgranulin B) Transmembrane 4 superfamily member 3	Calcium-independent alpha-latrotoxin receptor homolog 3 Fibroblast growth factor receptor 2 Glycoprotein hormone alpha 2  Insulin-like growth factor binding protein 2 Protein phosphatase 2, regulatory subunit A (formerly 2A), regulatory subunit A (PR 65), alpha isoform Regulator of G-protein signaling protein 2 (RGS2) Synaptotagmin 4 Thyroid stimulating hormone, receptor
Metabolism; energy pathways	Aldehyde oxidase 1 Calpain 8 Heme oxygenase 1 Muscle glycogen phosphorylase Phospholipase A2, group IVA (cytosolic, calcium-dependent) Pyruvate kinase, liver and RBC Synuclein, gamma Insulin 2	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3 Chymotrypsinogen B Cytochrome P45011B3 Cytochrome P450, subfamily 11A Lecithin-retinol acyltransferase Methionine synthase Prolactin-like protein A Sucrase-isomaltase UDP glycosyltransferase 2 family, polypeptide B
Transport	Apolipoprotein A-V Sodium channel, nonvoltage-gated, type I, alpha polypeptide Solute carrier family 12, member 1 Solute carrier family 38, member 1	Calbindin 3 Calcium activated potassium channel beta 4 subunit Glutamate receptor, ionotropic, NMDA3B Solute carrier family 22, member 3 Solute carrier family 24, member 3
Immune response	Chemokine-binding protein 2 Rat mRNA for RT1.D beta chain similar to Class II MHC RT1.D(n) beta chain precursor RT1 class Ib gene, locus M3 Thrombomodulin	Interleukin 1 receptor accessory protein Interleukin 2 receptor, beta chain  Interleukin 23, alpha subunit p19
Nucleic acid metabolism	Cocaine and amphetamine regulated transcript (CART) Cold shock domain protein A Timeless (Drosophila) homolog	Activating transcription factor 3 D site albumin promoter binding protein Zinc finger, C3HC4 type (RING finger)
Growth factors	Angiopoietin-like 2 Fibroblast growth factor 3	
Miscellaneous	Mast cell protease 8 N-acetylated alpha-linked acidic dipeptidase-like 1 (ileal peptidase 1100) Procollagen, type I, alpha 2 Sarcomeric muscle protein (Sarcosin) Tissue-type vomeronasal neurons putative pheromone receptor V2R2	Dentin sialophosphoprotein Tripartite motif protein 17  Uncoupling protein UCP-4



**Figure 1** (a) Reelin bands of 410, 330, and 180 kDa from the PFC homogenates (70  $\mu$ g protein per lane) of representative olanzapine-treated and control rats are shown. Mean Reelin 410 (b), 330 (c), and 180 (d) kDa/ $\beta$ -actin ratios for olanzapine-treated (filled histogram bars) and control rats (unfilled histogram bars) are shown. Levels of Reelin 410 kDa/ $\beta$ -actin (b) and Reelin 180 kDa/ $\beta$ -actin (d) were significantly increased vs controls ( $P=0.0033$  and  $0.0001$ , respectively). Reelin 330 kDa/ $\beta$ -actin (c) was nonsignificantly increased vs controls.

antiapoptotic action of olanzapine and its multifarious positive roles in neurogenesis, myelination, synaptogenesis, and dendritic branching (Gunnell and Holly, 2004).

Calgranulin B, a calcium binding protein that is found in a wide range of cells, has been shown to be involved in a number of cellular processes, such as cell cycle progression and differentiation. Levels of *calgranulin B* blood cell-derived RNA have recently been reported to be significantly increased in schizophrenic subjects (Tsuang et al, 2005). Interestingly, olanzapine significantly increased *calgranulin A* and *B* levels in rats treated with this agent.

Finally, while the DNA array only showed a trend for increase in *Reln* gene, its protein product, Reelin, was significantly upregulated in rat brain. Reelin glycoprotein has been implicated in pathology of schizophrenia, mood disorders, and autism (Fatemi et al, 2000, 2005c; Guidotti et al, 2000). Our lab has previously demonstrated decreased levels of Reelin in brains of patients with autism, bipolar disorder, major depression and schizophrenia (Fatemi et al, 2000, 2001a,b, 2002). We were therefore interested in examining the effect of olanzapine on Reelin levels. Our protein measurements in the frontal cortex showed a significant upregulation for Reelin 410 and 180 kDa proteins in the drug-exposed rats (Figure 1b and d). By the same token, QPCR experiments showed significant upregulation in Reelin mRNA by olanzapine (Table 2). These increases in Reelin mRNA and protein product by olanzapine suggest a possible mechanism for olanzapine's efficacy in modulating synaptic plasticity.

### Genes Downregulated due to Olanzapine Treatment

A total of 31 genes were downregulated significantly by olanzapine treatment. One of these genes was *RGS2*. *RGS* proteins enhance hydrolysis of GTP to GDP by G-protein  $\alpha$ -subunits, thereby reducing the  $\alpha$ -subunit activation of downstream effectors (Kehrl and Sinnarajah, 2002). *RGS* proteins are therefore known as GTPase activating proteins. *RGS2*, first identified in a screen for activation genes in human lymphocytes (Siderovski et al, 1994), blocks Gq  $\alpha$ -mediated signaling and inhibits Gs signaling to certain adenylyl cyclases as well (Kehrl and Sinnarajah, 2002). *RGS2* mRNA has been shown to have widespread expression in cerebral cortical layers, several thalamic and hypothalamic nuclei, and hindbrain regions (Grafstein-Dunn et al, 2001). Targeted mutations of *RGS2* in mice increase anxiety levels and alter synaptic development in hippocampal CA1 neurons (Kehrl and Sinnarajah, 2002). Kehrl and Sinnarajah (2002) suggest that *RGS2* could negatively regulate metabotropic glutamate receptors and thus affect long-term synaptic plasticity in the hippocampus. Levels of *RGS4* are known to be decreased in both schizophrenia and Alzheimer's disease (Mirnics et al, 2001; Muma et al, 2003). Reductions in levels of *RGS4* lead to increases in levels of *RGS2* as a compensatory response (Taymans et al, 2003). Interestingly, olanzapine significantly decreased *RGS2* gene expression (Tables 1, 3). This decrease in *RGS2* was verified by QPCR measurement (Table 2), showing a significant 2.32-fold decrease in *RGS2* mRNA in exposed rat frontal cortex (Table 2). Additionally, olanzapine significantly decreased *RGS2* protein product in rat frontal cortex vs controls (Table 4).

The glutamatergic involvement in pathology of schizophrenia and bipolar disorder is well known. Glutamate receptor *NMDA3B* is purported to be involved in schizophrenia (Lipsky and Goldman, 2003). Olanzapine significantly reduced glutamate ionotropic *NMDA3B* receptor expression while increasing glutamate receptor interacting protein (*GRIP*) expression (Tables 1, 3), suggesting an effect on glutamate transmission. QPCR experiments also showed a significant reduction in levels of *NMDA3B* mRNA ( $p<0.018$ ) in drug-treated rat frontal cortex (Table 4).



**Table 4** Western Blotting Results for Selected Proteins Involved in Olanzapine Treatment

Protein	Olanzapine-treated (mean $\pm$ SD)	Control (mean $\pm$ SD)	% change	P-value <sup>a</sup>
Reelin 410 kDa/ $\beta$ -actin	0.799 $\pm$ 0.118	0.577 $\pm$ 0.202	38% $\uparrow$	0.0033
Reelin 330 kDa/ $\beta$ -actin	0.066 $\pm$ 0.033	0.056 $\pm$ 0.041	18% $\uparrow$	NS
Reelin 180 kDa/ $\beta$ -actin	0.811 $\pm$ 0.112	0.553 $\pm$ 0.159	47% $\uparrow$	0.0001
NMDA3 $\beta$ / $\beta$ -actin	0.154 $\pm$ 0.047	0.232 $\pm$ 0.078	34% $\downarrow$	0.0180
RGS2 50 kDa Dimer/ $\beta$ -actin	0.356 $\pm$ 0.163	0.602 $\pm$ 0.194	41% $\downarrow$	0.0061
RGS2 25 kDa Monomer/ $\beta$ -actin	0.0156 $\pm$ 0.0181	0.0225 $\pm$ 0.022	31% $\downarrow$	NS
Total RGS2/ $\beta$ -actin	0.371 $\pm$ 0.154	0.625 $\pm$ 0.189	41% $\downarrow$	0.0039
Synaptotagmin IV/ $\beta$ -actin	0.270 $\pm$ 0.0064	0.385 $\pm$ 0.154	30% $\downarrow$	NS
$\beta$ -Actin	10.2 $\pm$ 1.78	10.4 $\pm$ 1.03	2% $\downarrow$	NS

<sup>a</sup>Two-tailed unpaired *t*-test.

NS, nonsignificant.

 $\uparrow$  Increases. $\downarrow$  Decreases.**Table 5** Positive Associations between Olanzapine-Expressed Genes and Neuropsychiatric Disorders

Gene	Schizophrenia	Mental retardation	Alcoholism	Depression	Alzheimer's	Bipolar	Autism
FBGF	$\uparrow$ Serum <sup>1</sup>	—	—	$\downarrow$ <sup>12</sup>	—	—	—
Homer-linked oligophrenin-I	—	$\downarrow$ <sup>2</sup>	—	—	—	—	—
Calgranulin B	$\uparrow$ Serum <sup>3</sup>	—	—	—	—	—	—
Homer I	Polymorphism <sup>4</sup>	—	—	—	—	—	—
Synaptotagmin IV	$\downarrow$ <sup>5</sup>	—	—	—	—	—	—
RGS4	$\downarrow$ <sup>6</sup>	—	—	—	$\downarrow$ <sup>7</sup>	—	—
Pyruvate kinase 3	Polymorphism <sup>8</sup>	—	—	—	—	—	—
CART	—	—	Polymorphism <sup>9</sup>	—	—	—	—
Calbindin	$\downarrow$ <sup>10</sup>	—	—	—	—	—	—
NMDA3B	$\downarrow$ <sup>11</sup>	—	—	—	—	—	—
Reelin	$\downarrow$ Brain <sup>13</sup>	—	—	$\downarrow$ Brain <sup>13</sup>	—	$\downarrow$ Brain <sup>13</sup>	$\downarrow$ Brain and blood <sup>14</sup>

1. Hashimoto *et al* (2003); 2. Govek *et al* (2004); 3. Tsuang *et al* (2005); 4. Norton *et al* (2003); 5. Ferguson *et al* (2001); 6. Mimics *et al* (2001); 7. Muma *et al* (2003); 8. Stone *et al* (2004); 9. Jung *et al* (2004); 10. Iritani *et al* (1999); 11. Lipsky and Goldman (2003); 12. Evans *et al* (2004); 13. Fatemi *et al* (2000); Guidotti *et al* (2000); 14. Fatemi *et al* (2005b).

 $\uparrow$  Increases. $\downarrow$  Decreases.

Levels of synaptotagmin IV have been reported to be significantly decreased in schizophrenic subjects (Ferguson *et al*, 2001). Interestingly, we found that olanzapine significantly reduced *synaptotagmin IV* mRNA levels in rats treated with this agent (Tables 1, 3) as well as a 20% decrease in protein levels (Table 4), questioning the cause for change in the same protein purported to be due to schizophrenic pathology.

### Olanzapine Effects on Synaptic Plasticity

Several lines of evidence show that glutamatergic hypofunction plays a role in pathology of schizophrenia (Javitt, 2004). Thus, improving the glutamate neurotransmission may help in treatment of some of the deficits present in schizophrenia. Olanzapine clearly affects several genes that modulate glutamate neurotransmission, for example, *homer 1*, *NMDA3B*, *GRIP*, *Reelin*, and *RGS2*. One of the important genes upregulated by olanzapine is *Reelin*, which helps in

normal lamination of the brain during embryogenesis and affects synaptic plasticity in adulthood (Beffert *et al*, 2005; Fatemi, 2005). Reelin deficiency has been shown to be present in schizophrenia (Fatemi *et al*, 2000; Guidotti *et al*, 2000). Recent reports show that Reelin receptor ApoER2 interacts with, and alters the conformation of NMDA receptors NR2A and NR2B (Beffert *et al*, 2005). Additionally, Reelin induces tyrosine phosphorylation of NR2A and NR2B receptors in hippocampal tissue (Beffert *et al*, 2005), thus modulating NMDA receptor activity and synaptic plasticity in the hippocampus. As such, olanzapine helps to improve cognition/memory processing by upregulating Reelin expression, potentially via a complex interaction that may involve *NMDA3B*, *Homer 1*, *RGS2*, and *GRIP*. Supporting evidence for the potential role in olanzapine enhancing neuroplasticity was recently shown by Lieberman *et al* (2005) who demonstrated a cessation of brain grey matter loss in brains of patients with schizophrenia treated with olanzapine for 12 weeks and not in those

treated for the same time period with haloperidol. Additionally, Wang and Deutch (2004) also showed that olanzapine prevented decreases in spine density of basilar dendrites on layers II, III, and IV of the PFC pyramidal neurons in rats lesioned with 6-hydroxydopamine. The propensity to affect glutamate neurotransmission is not restricted to olanzapine, since other atypical agents like clozapine, risperidone, and quetiapine are capable of affecting the same neurotransmitter system (Wang *et al*, 2000; Tarazi *et al*, 2003).

### Olanzapine Effects on Glucose Production

Olanzapine affects glucose production via participation of several gene families involved in various metabolic pathways (Table 2). Olanzapine upregulates *pyruvate kinase*, an enzyme that catalyzes the formation of pyruvate and ATP from phosphoenolpyruvate and ADP. Pyruvate kinase polymorphisms have been discovered in subjects with schizophrenia (Stone *et al*, 2004) and Type II diabetes (Wang *et al*, 2002), and olanzapine increases the risk for diabetes in subjects with schizophrenia (Meyer and Nasrallah, 2003). Olanzapine also upregulates *glycogen phosphorylase*, a key enzyme in glycogen degradation, leading to removal of a terminal glucose residue from the nonreducing end of a glycogen chain providing more glucose for local brain energy expenditure. Indeed, glycogen phosphorylation is localized primarily to astrocytes in the brain alluding to a direct effect by olanzapine on glial cells (Pfeiffer-Guglielmi *et al*, 2003). Antipsychotics can affect brain concentrations of glycogen phosphorylases (Ktenas *et al*, 1978; Iriye and Simmonds, 1971a,b); however, the majority of typical antipsychotics reduce the levels of *glycogen phosphorylase* while antidepressants and olanzapine increase the activity of this enzyme (Iriye and Simmonds, 1971a). Olanzapine also increases *insulin 2* expression, which may be in response to increased glucose production or potentially due to other triggers. By the same token, olanzapine can also reduce the concentration of sucrase-isomaltase to conserve glucose in disaccharide forms.

In summary, chronic administration of olanzapine to rats caused significant alterations in gene expression in 69 genes involved in signal transduction, cell communication, energy and metabolism, transport, immune response, cell growth, and nucleic acid metabolism. Specifically, olanzapine appears to enhance glutamate neurotransmission, modulate synaptic plasticity, and increase energy production via glycolytic pathways in rat frontal cortex. Future studies should correlate expression of genes following exposure to antipsychotic agents and those genes involved in pathology of psychiatric disorders in order to gain insights into the etiology and treatment of these disorders.

### ACKNOWLEDGEMENTS

We appreciate the grant support provided by Stanley Medical Research Institute (SHF), the gift of olanzapine by Eli Lilly and Company, and the secretarial assistance by Ms L Iversen. Some of the data published in this manuscript were presented at International Congress of Schizophrenia,

held at Savannah, Georgia and the Pharmacogenomics in Psychiatry Meeting at the Zucker Hillside Hospital in Glen Oaks, NY, in April of 2005.

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Supplementary Information accompanies the paper on Neuropsychopharmacology website (<http://www.nature.com/npp>).