

Microarray Analysis of Differentially Expressed Genes in Rat Frontal Cortex Under Chronic Risperidone Treatment

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Long-term administration of antipsychotic drugs can induce differential expression of a variety of genes in the brain, which may underscore the molecular mechanism of the clinical efficacy and/or side effects of antipsychotic drugs. We used cDNA microarray analysis to screen differentially expressed genes in rat frontal cortex under 4 weeks' treatment of risperidone (1 mg/kg). Using real-time quantitative PCR, we were able to verify eight genes, whose expression were significantly upregulated in rat frontal cortex under chronic risperidone treatment when compared with control animals. These genes include receptor for activated protein kinase C, amida, cathepsin D, calpain 2, calcium-independent receptor for α -latrotoxin, monoamine oxidase B, polyubiquitin, and kinesin light chain. In view of the physiological function of these genes, the results of our study suggest that chronic risperidone treatment may affect the neurotransmission, synaptic plasticity, and proteolysis of brain cells. This study also demonstrates that cDNA microarray analysis is useful for uncovering genes that are regulated by chronic antipsychotic drugs treatment, which may help bring new insight into the molecular mechanism of antipsychotic drugs.

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INTRODUCTION

Risperidone is an atypical antipsychotic drug for the acute and long-term treatment of patients with schizophrenia (Pajonk, 2004). It is a potent antagonist of both dopamine and serotonin receptors (Janssen *et al*, 1988; Schotte *et al*, 1996). However, apart from its receptor-binding profile, little is known about the underlying molecular mechanism of the drug action of risperidone. It usually takes couples of weeks to see clinical improvement after taking antipsychotic drugs. The molecular mechanism underlying the slow therapeutic effects of antipsychotic drugs is still not clear. It is generally believed that antipsychotic drugs may affect various genes expression in the brain, which subsequently leads to change of synaptic structure and function and neurogenesis in the brain (Hyman and Nestler, 1996; Konradi and Heckers, 2001). The time course of the altered genes expression in the brain corresponds to the clinical effects of antipsychotic drugs and may underscore the

molecular mechanism of antipsychotic drugs. Hence, study of differentially expressed genes regulated by antipsychotic drugs may help uncover the molecular underpinnings of the clinical efficacy of antipsychotic drugs.

Many studies have reported that either acute or chronic administration of typical or atypical antipsychotic drugs can differentially regulate a variety of genes expression in rat brain, such as *c-fos*, *zif268* (Nguyen *et al*, 1992; Rogue and Vincendon 1992; Semba *et al*, 1996), Homer (de Bartolomeis *et al*, 2002; Polese *et al*, 2002), nuclear receptor genes (Beaudry *et al*, 2000; Langlois *et al*, 2001), neurotensin (Merchant *et al*, 1992, 1994), brain-derived neurotrophic factor (BDNF) (Chlan-Fourney *et al*, 2002), fibroblast growth factor 2 (Riva *et al*, 1999), glutamate receptor subunit genes (Riva *et al*, 1997; Toyoda *et al*, 1997), regulator of G-protein signalling 2 (Robinet *et al*, 2001), and so on. The studies of the altered gene expression pattern contribute substantially to our understanding of the mechanism of antipsychotic drug action.

Microarray-based gene expression profiling is a functional genomic method that allows simultaneous assessment of multiple genes expression (Brown and Botstein, 1999; Schena *et al*, 1995). This technology can be used to investigate the drug response of brain cells under chronic administration of psychoactive drugs at molecular level (Palfreyman *et al*, 2002). Microarray technology has been used in studying the brain pathology of psychiatric

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disorders such as schizophrenia and bipolar affective disorders (Bahn *et al.*, 2001; Mirnics *et al.*, 2001; Bunney *et al.*, 2003; Iwamoto *et al.*, 2004). Recently, researchers started to use cDNA microarray technology to investigate global gene expression profiles in rats after chronic treatment of antipsychotic drugs such as clozapine and haloperidol (Chong *et al.*, 2002; Kontkanen *et al.*, 2002), and found that chronic haloperidol and clozapine treatment can alter transcription levels of genes involved in neurotransmission, signal transduction, oxidative stress, cell adhesion, apoptosis, and proteolysis (Thomas *et al.*, 2003).

The purpose of this study was to employ cDNA microarray analysis to identify novel genes, whose expression are differentially regulated by the chronic risperidone treatment. We hope that identification of these differentially expressed genes may help uncover the molecular mechanism of the drug action of risperidone.

MATERIALS AND METHODS

Animals and Drug Treatment

Male Sprague-Dawley rats were housed in a temperature and humidity-controlled environment with a 12-h light/dark cycle and had free access to food and water. All experimental procedures were approved by the Ethical Committee on Animal Experiments of the Institute, and all the animals were taken care of according to the Karolinska Institute's Animal Care Guidelines. The animals were used in two experiments. In microarray experiment, animals weighing about 120–150 g received intraperitoneally injection of risperidone (1 mg/kg) ($n = 4$) or vehicle ($n = 4$) once daily for 4 weeks. In real-time quantitative PCR experiment, animals weighing about 120–150 g received intraperitoneally injection of risperidone (1 mg/kg) or vehicle once daily for 1, 2, 3, and 4 weeks, respectively. In both experiments, animals were killed 24 h after the last injection under CO₂ anesthesia. Dissected cerebral cortex were stored in RNAlater solution (Ambion, Inc., Austin, TX) at 4°C for 1 day and stored at –152°C until use.

Total RNA Preparation

Total RNA from rat cerebral cortex was extracted using TRIzol Total RNA Isolation Reagent according to the manufacturer's instruction (Invitrogen Life Technologies, Carlsbad, CA). In brief, rat cerebral cortex was homogenized directly in 1 ml TRIzol denaturing reagent per 50–100 mg tissue. Homogenates were incubated on ice for 5 min, vortexed vigorously, and centrifuged at 12 000g for 10 min at 4°C. Supernatant was transferred to a new tube. For each ml of supernatant, 200 µl chloroform was added, mixed vigorously, incubated on ice for 5 min, and centrifuged at 12 000g for 10 min at 4°C. The upper water phase was transferred to a new tube and chloroform extraction procedure was repeated. The aqueous phase was collected, and mixed with 0.7 volume of isopropyl alcohol. The mixtures were incubated at –20°C for 1 h, centrifuged at 12 000g for 30 min at 4°C. RNA pellets were washed with 75% ethanol twice, air dried, and dissolved in RNase-free water.

DNase Digestion

Aliquots of total RNA were digested with RQ1 RNase-Free DNase (Promega Corporation, Madison, WI) to remove trace contaminated genomic DNA according to the protocol from the manufacturer. After digestion, equal volume of chloroform was added, mixed, and centrifuged at 12 000g for 5 min at 4°C. The supernatant was removed to a new tube, and chloroform extraction was repeated twice. After final extraction, the purified RNA was precipitated with 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate. The mixtures were centrifuged at 12 000g for 30 min at 4°C. RNA pellets were washed with 70% ethanol, air-dried, and dissolved in the RNA Storage Solution (Ambion, Inc., Austin, TX). The RNA concentration was determined using spectrophotometry, the stock concentration of RNA ranged from 3 to 5 µg/µl.

RNA Quality Assurance

The integrity of 28S and 18S rRNA were checked by electrophoresis of 2 µg of total RNA in 1.2% agarose gel containing 2.2 M formaldehyde and in a running buffer containing 0.2 M of MOPS (pH 7.0), 20 mM of sodium acetate and 10 mM of EDTA (pH 8.0). To ensure the total RNA is free from genomic DNA after DNase digestion, aliquots of total RNA were subjected to PCR amplification in a volume of 20 µl containing 1 µg of total RNA, 1 µM each of sense (5'-accacagtcctatgccatcac-3') and antisense primer (5'-tccaccacctgttgctga-3'), 0.2 mM of dNTP, 50 mM of KCl, 1.5 mM of MgCl₂, 0.1% vol/vol of Triton X-100, 10 mM Tris-HCl (pH 9.0), and 2.5 U Taq polymerase. PCR conditions were as follows: initial denaturation at 95°C 5 min, followed by 35 cycles of 95°C 1 min, 60°C 1 min, and 72°C 1 min. The presence of a 450 bp PCR products of rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene indicates incomplete DNase digestion.

cDNA Preparation

cDNA was prepared by reverse transcription using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA). A 12 µl reaction mixture containing 2 µg of total RNA and 2 µl (50 µM) of random hexamers (Applied Biosystems) were first heated at 65°C for 5 min, and quickly chilled on ice. After brief centrifugation to collect the content, 4 µl of 5 × first-strand buffer, 2 µl of 0.1 M DTT, 1 µl (20 U) of RNase Inhibitor (Applied Biosystems) and 1 µl (200 U) of Superscript II Reverse transcriptase were added into the tube. The mixtures were incubated at 42°C for 60 min, and the reaction was stopped by heat inactivation at 70°C for 15 min.

Microarray Hybridization

GeneMap Rat Clone Arrays from Genomic Solutions Inc. (Ann Arbor, WI) were used in this study. Each microarray gene chip contains duplicated cDNA fragments of 1536 genes. The cDNA microarray hybridization experiment followed the protocol from the manufacturer. In brief, fluorescent cDNA probes were prepared from 50 µg of total RNA labeled with Cy3-dCTP or Cy5-dCTP by reverse

transcription. The fluorescent cDNA probes were purified using G-50 Sephadex spin column. Equal volumes of Cy3- and Cy5-labeled probes were mixed with hybridization solution with the final volume of 110 μ l. The mixtures were heated at 75°C for 5 min, chilling on ice prior to application to the array. Hybridization was performed in humidified chamber at 50°C for at least 16 h. Following hybridization, the slides were washed with wash solutions provided by the manufacturer. Arrays were immediately scanned at a resolution of 10 μ m using a confocal scanner ScanArray 4000XL from Packard BioChip Technologies (Billerica, MA). We have eight animals to form four experiment-control pairs of animals. In two pairs of animals, risperidone-treated and control samples were labeled with Cy3-dCTP and Cy5-dCTP, respectively, while in the other two pairs of animals, the risperidone-treated and control samples were labeled with Cy5-dCTP and Cy3-dCTP, respectively.

Microarray Data Analysis

The image data were extracted using QuantArray software from Packard BioChip Technologies (Billerica, MA). Spots of poor quality were excluded, and the fluorescent intensity of each spot was calculated by subtracting local background intensity. The image data were subject to further analysis using BRB-ArrayTools v3.1, which was developed by Dr Richard Simon and Amy Peng Lam and is available from <http://linus.nci.nih.gov/BRB-ArrayTool.html>. The data were first examined by M-A plot, and then normalized by log transformation of intensity before comparing the differential gene expression between experiment and control animals. Identification of differentially expressed genes was carried out using class comparison function of the BRB-ArrayTool v3.1, which computes an F-test separately for each gene using the normalized log-ratios. Genes showing significantly differential expression ($p < 0.001$) were selected for further verification in another experiment with a larger sample size using real-time quantitative PCR.

Real-Time Quantitative PCR Conditions

Real-time quantitative PCR was performed using ABI PRISM 7700 Sequence Detection System in combination with continuous SYBR Green detection (Applied Biosystems). Real-time PCR was performed in a 25 μ l reaction volume containing 2.5 μ l of cDNA, 12.5 μ l of SYBR Green PCR Master Mix (Applied Biosystems), 2.5 μ l each of sense and antisense primers (10 μ M), and 5 μ l of H₂O. The general PCR conditions were as follows: polymerase activation at 95°C for 10 min, followed by 50 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. After amplification, a melting curve was acquired to determine the optimal PCR conditions. Primer sequences for PCR amplification were designed using online Primer3 software (http://biocore.unl.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of primer, length of PCR amplicons, and optimal annealing temperature are listed in Table 1.

Quantification and Normalization

Relative standard curve method was used for quantification of mRNA expression (User Bulletin #2 ABI PRISM 7700 Sequence Detection System). In this method, serial dilutions of known amount of total RNA from a reference sample were used to generate external standard curve. For each unknown sample the relative amount was calculated using linear regression analysis from their respective standard curves. The mRNA expression levels of each genes were normalized by the geometric mean of three housekeeping genes, that is, GAPDH mRNA, cyclophilin A mRNA, and 18S rRNA (Vandesompele *et al*, 2002). Both GAPDH and cyclophilin A mRNAs were assayed using real-time quantitative PCR and SYBR Green detection as described. Primer sequences and PCR conditions for GAPDH and cyclophilin A mRNA quantification are listed in Table 1. The 18S rRNA reference gene was measured using Pre-Developed TaqMan Assay Reagents 18S rRNA MGB according to the manufacturer's protocol (Applied Biosystems). All experiments were performed in duplicates.

Statistical Analysis

Differences of the normalized mRNA expression levels of selected genes between experiment and control animals were assessed using Student's *t*-test. Significant differences are defined as those with a *p*-value smaller than 0.05. All the calculations were implemented using SPSS for windows version 11.5.

RESULTS

In microarray experiment, 17 out of 1536 genes in the gene chips were found to have significant differential expression in rat frontal cortex under chronic risperidone treatment using class comparison function of the BRB-ArrayTools v3.1 (Table 1). To verify these findings, we further compared the gene expression levels of these 17 genes in another experiment with a larger sample size using real-time quantitative PCR. Eight genes out of these 17 genes were found to be significantly upregulated in the rat frontal cortex under 4 weeks' risperidone treatment when compared to control animals ($p < 0.05$). These genes include receptor for activated protein kinase C (PKC), amida, cathepsin D, calpain 2, calcium-independent receptor for α -latrotoxin, monoamine oxidase B, polyubiquitin, and kinesin light chain. The mean mRNA expression level of each gene as normalized by the geometric mean of 18S rRNA, cyclophilin, and GAPDH in both experiment and control animals are summarized in Table 2.

Figure 1 illustrates the time course of gene expression of these eight genes in the rat frontal cortex under risperidone treatment for 1, 2, 3, and 4 weeks, respectively. There are no significant differences of gene expression of these eight genes between risperidone-treated and control animals after 2 weeks' treatment. Cathepsin D, polyubiquitin, calpain 2, calcium-independent receptor for α -latrotoxin, kinesin light chain genes were found to have significant differential expression between risperidone-treated and control animals after 3 weeks' treatment, while the expression levels of Amida, receptor for activated PKC, and monoamine oxidase

Table 1 Primer Sequences, Length of PCR Products, and Optimal Annealing Temperature for Each Gene Assayed in Real-Time Quantitative PCR

Genes	GenBank		Primer sequences (5'-3')	Size (bps)	T _a (°C)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_017008	Sense	AAACCCATCACCATCTTCCA	198	60
		Antisense	GTGGTTCACACCCATCACAA		
Cyclophilin A	NM_017101	Sense	AGCACTGGGGAGAAAGGATT	174	60
		Antisense	GATGCCAGGACCTGTATGCT		
Monoamine oxidase B (MAOB)	NM_013198	Sense	TGGGAAGATTCCAGAGGATG	150	60
		Antisense	GCTGACAAGATGGTGGTCAA		
Amida	AB029495	Sense	AGATTCCTCATGCGAGTGCT	153	60
		Antisense	TGGGGAAAGTCCCTCTTTCT		
Protein kinase C receptor (RACK1)	U03390	Sense	GAAGCTGACCAGGGATGAGA	150	60
		Antisense	GTGAGATCCCAGAGGCGTAG		
Kinesin light chain I (Klc1)	XM_343114	Sense	TGGCTATCCGTGAGAAAACC	233	60
		Antisense	CTCCACCTCCTCGTACTTGC		
Myosin I heavy chain (MYR3)	X74815	Sense	TTGGGTTGAGGGACAGTCTT	157	60
		Antisense	TAGGCACTAGGTTGGAACG		
Polyubiquitin	BC060312	Sense	GACACCATCGAGAACGTGAA	150	60
		Antisense	GACAAGGTGCAGGTTGACT		
Calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G)	NM_031601	Sense	GAGCTGAGCTGGATTTCAGG	150	60
		Antisense	AATGGAGTGTCTCCGCTGTT		
Superoxide dismutase 3 (Sod3)	NM_012880	Sense	TGCACTCCACAGACCAACTC	149	60
		Antisense	ATTTCGACCTCTGGGGTAAG		
Telomerase protein component 1 (Tlp1)	U89282	Sense	GAAAGGTTCAACATGCACCA	149	60
		Antisense	CGGGCTTCATCTGTAGCAAT		
Tyrosine kinase receptor (Ptk-3)	L26525	Sense	ACTGCTTGTTGGGAAAAT	155	60
		Antisense	TGTTGTGAACCTCCCCATGA		
Galanin (Gal)	NM_033237	Sense	GAATGCCAACAAAGGAGAAG	150	60
		Antisense	GTCTCCCTTCCTCCACTTCC		
GSK-3beta interacting protein rAxin (Axin)	NM_024405	Sense	CCCTGAAGCAATACCAGGAG	142	60
		Antisense	CCAATGAGGTGCAGTGGATA		
Epithelin 1 and 2	X62322	Sense	TGGCTCTAAGGTTGGGAATG	148	65
		Antisense	GAAGCCAATGGGACAACAGT		
Cathepsin D	BC062032	Sense	GGCATGGGCTACCCTTTTAT	182	60
		Antisense	GACAGCTCCCCGTGGTAGTA		
Phosphatidylinositol 3-kinase (PIK3C3)	NM_022958	Sense	AGCCACAGGTGAAAATACGG	199	65
		Antisense	TTTTCTTTCCGCAACAGCTT		
Calcium-independent alpha-latrotoxin receptor (CIRL)	U72487	Sense	GGTGACAAGGAGTACAGCA	184	55
		Antisense	GGACGACTCTGTCTGCTTCC		
Calpain 2 (Capn2)	NM_017116	Sense	AGCCAATGAGGAGGACATTG	195	60
		Antisense	CTCCCATCTTCATCCAGCAT		

T_a: optimal annealing temperature.

B genes showed significant differences after 4 weeks' treatment. These data support that the upregulation of these eight genes are indeed induced by the chronic administration of risperidone.

Representative equations of simple linear regression describing the standard curve of each gene assayed in this study are listed in Table 3. There is a large dynamic range with high determination coefficient of each gene, and small variation of

slopes among each equation represents similar PCR amplification efficiency of each gene in PCR quantification.

DISCUSSION

Several studies have demonstrated that chronic treatment of antipsychotic drugs can change the gene expression of

Table 2 Normalized Relative Expression Level of Genes that were Upregulated in the Rat Cerebral Cortex after 4 Weeks' Treatment of Risperidone

Gene	Risperidone (n)	Control (n)	p-value
Monoamine oxidase B	0.517±0.048 (7)	0.347±0.089 (8)	0.001
Amida	1.416±0.219 (7)	0.946±0.226 (7)	0.002
Protein kinase C receptor	1.423±0.522 (7)	0.921±0.149 (7)	0.031
Kinesin light chain I	1.628±0.416 (8)	1.037±0.190 (8)	0.003
Polyubiquitin	0.126±0.035 (8)	0.082±0.025 (8)	0.012
Cathepsin D	0.783±0.198 (8)	0.514±0.193 (7)	0.020
Calcium-independent alpha-latrotoxin receptor	0.073±0.019 (8)	0.048±0.015 (7)	0.016
Calpain 2	0.989±0.344 (8)	0.540±0.161 (7)	0.008

biogenic amine-synthetic enzymes such as tyrosine hydroxylase (Tejedor-Real *et al*, 2003) and aromatic L-amino acid decarboxylase (Cho *et al*, 1997), and biogenic amine-binding receptors such as dopamine receptor (Buckland *et al*, 1993; Damask *et al*, 1996) and serotonin receptor (Frederick and Meador-Woodruff 1999). However, there is little information about the gene expression of biogenic amine-catabolic enzymes following long-term antipsychotic drugs treatment. Monoamine oxidase B (MAOB) is one of the catabolic enzymes of biogenic amine neurotransmitters, and has been implicated in several neuropsychiatric diseases. In 1970s, researchers reported reduced MAOB activity in platelets from schizophrenic patients, and proposed MAOB activity as a genetic maker for vulnerability to schizophrenia (Murphy and Wyatt, 1972; Wyatt *et al*, 1973). However, later studies showed that reduced platelet MAO activity could be attributed to chronic treatment of haloperidol (DeLisi *et al*, 1981; Kemali *et al*, 1985). Later study further suggested that haloperidol metabolites, rather than haloperidol itself, contribute to the reduction of platelet MAOB activity in schizophrenic patients undergoing long-term haloperidol treatment (Fang *et al*, 1995). There is little literature about whether risperidone can affect the MAOB enzyme activity to our knowledge. One speculation for the increased MAOB gene expression may be due to the compensation of inhibitory MAOB enzyme activity of risperidone. The increased MAOB gene expression after chronic risperidone treatment as found in this study suggests that risperidone may also affect the genes involved in the degradation of biogenic amine neurotransmitters.

Amida was first identified as an associated protein of Arc (activity-regulated cytoskeleton-associated protein) from rat hippocampus by the yeast two-hybrid system (Irie *et al*, 2000). Study showed that overexpression of Amida can induce apoptosis in cultured cells. When cotransfected with Arc, Amida interacts with Arc and transports it into the nucleus, which in turn interferes with the apoptosis triggered by Amida (Irie *et al*, 2000). These results suggest that Amida together with Arc is involved in modulating the programmed cell death in the brain. Except for these findings, little is known about the function of Amida in the brain. Arc is encoded by a nontranscription factor

immediate-early gene and is thought to play a significant role in activity-dependent synaptic plasticity of dendrites (Lyford *et al*, 1995; Steward *et al*, 1998; Guzowski *et al*, 2000; Steward and Worley, 2001). Previous studies have revealed that psychoactive drugs such as antidepressant (Pei *et al*, 2003), methamphetamine (Kodama *et al*, 1998), amphetamine, cocaine (Tan *et al*, 2000), phencyclidine (Nakahara *et al*, 2000), and haloperidol (Fosnaugh *et al*, 1995) can induce increased expression of Arc mRNA. Studies also showed that atypical antipsychotic drugs such as clozapine, olanzapine, and risperidone prevented phencyclidine (PCP)-induced Arc expression in rat brain (Nakahara *et al*, 2000). These data suggest that Arc is involved in the molecular mechanism of psychoactive drugs action. In this study, we found that chronic risperidone treatment induces increased expression of Arc associated protein gene-Amida in rat cerebral cortex. The significance of this finding needs further clarification due to the limited knowledge about the function of Amida in the brain. However, in view of the important role of Arc in the activity-regulated synaptic plasticity, our finding suggests that long-term administration of risperidone may change the synaptic plasticity via the regulating the Amida/Arc pathway.

Abnormalities in PKC signaling have been reported to be involved in the pathophysiology of bipolar disorder. Mood stabilizers such as lithium and valproic acid can inhibit the PKC activity (Wang *et al*, 2001). Upon activation, PKC binds to receptors for activated C-kinase (RACKs) in the cytoplasm, and translocates from cytoplasm to cell membrane. Patients with bipolar affective disorder were found to have increased association of PKC and RACK1 (Ron *et al*, 1994) in frontal cortex (Wang and Friedman 2001). Recent study showed that RACK1 is also part of messenger ribonuclearprotein complexes that bind to polyA-mRNAs and are involved in the activity-triggered control of protein synthesis in neurons (Angenstein *et al*, 2002). The increased RACK1 mRNA expression in rat brain after repetitive administration of risperidone suggests that risperidone may not only interfere with the PKC signaling (Feng *et al*, 2001) but also associate with the long-lasting changes in synaptic functions via altered protein synthesis.

Kinesin is a large family of proteins that function as microtubule-based molecular motors for transporting membranous and nonmembranous cargoes such as vesicles, organelles, protein complexes, and mRNA in neuronal and non-neuronal cells (Goldstein and Yang, 2000; Seog *et al*, 2004). Conventional kinesin is a heterotetramer protein complex consisted of two heavy chains and two light chains. Kinesin heavy chain has a motor domain that binds to microtubules; an α -helical coiled coil stalk domain that forms dimer with another heavy chain; and a C-terminal globular tail domain that interacts with the kinesin light chain, and binds to some cargoes (Gunawardena and Goldstein, 2004). Kinesin light chain has an N-terminal coiled coil domain that interacts with kinesin heavy chain and a C-terminal domain that contains six tetratricopeptide motifs. The tetratricopeptide domain of kinesin light chain has been suggested to mediate the selective binding to its cargo (Verhey *et al*, 2001; Gunawardena and Goldstein, 2004). Neuronal cells are highly specialized cells for information processing, and the intraneuronal transportation of molecules such as signaling proteins, neurotrophic

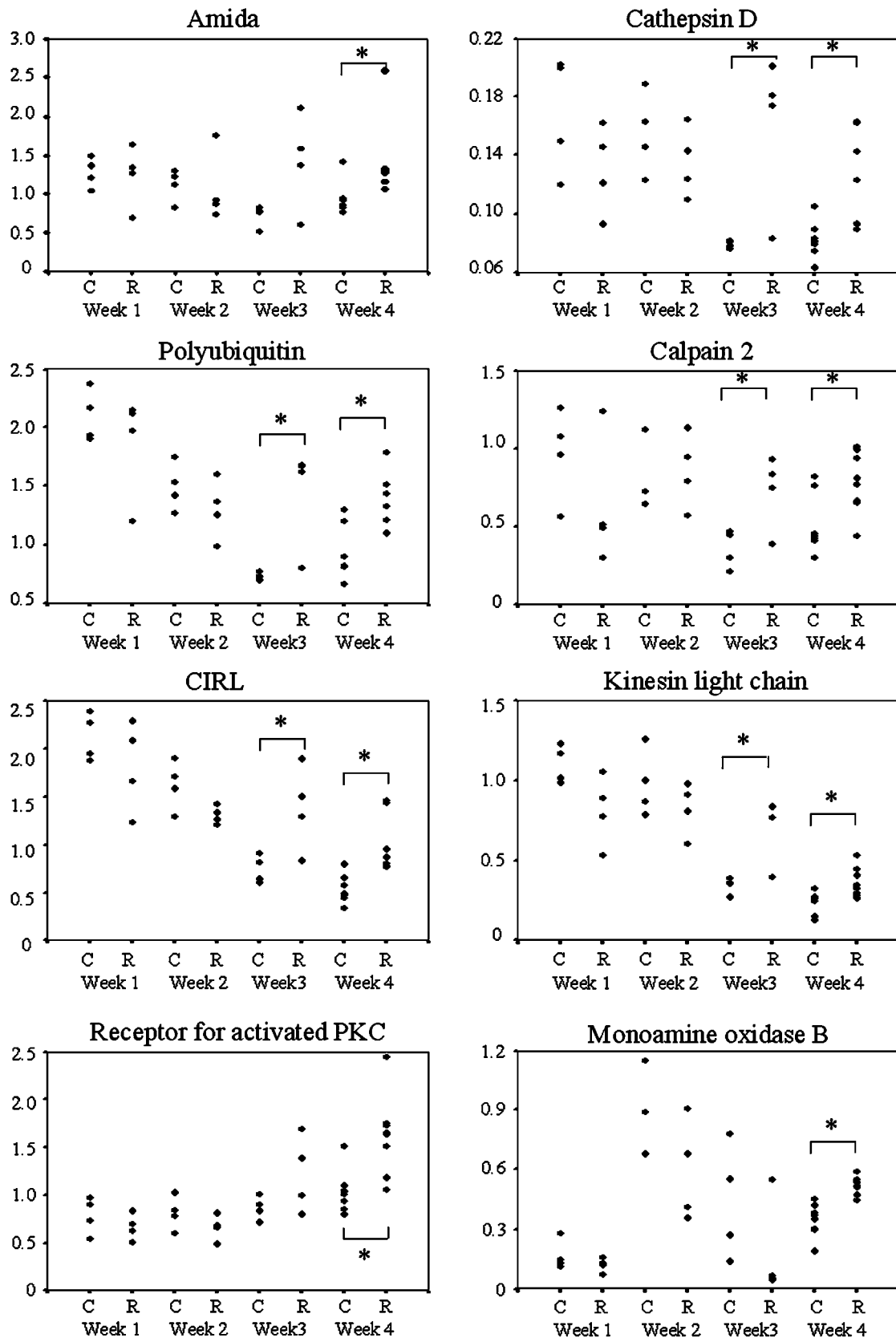


Figure 1 Expression levels of eight genes in rat frontal cortex after intraperitoneal injection of risperidone (1 mg/kg) or vehicle for 1, 2, 3, and 4 weeks, respectively. Y-axis indicates relative gene expression ratio as normalized by the geometric mean of the expression levels of GAPDH, cyclophilin A, and 18S rRNA. X-axis indicates time interval of experiment, C indicates control animals, R indicates animals treated with risperidone. *Indicates $p < 0.05$, CIRL indicates calcium-independent α -latrotoxin receptor.

Table 3 Equations Describing the Standard Curves of the Genes in this Study

Gene	Regression equation	Coefficient of determination
18S rRNA	$Y = -3.69X \pm 29.37$	$R^2 = 0.999$
GAPDH	$Y = -3.23X \pm 34.32$	$R^2 = 0.996$
Cycliphilin A	$Y = -3.40X \pm 34.60$	$R^2 = 0.998$
Monoamine oxidase B	$Y = -3.29X \pm 40.04$	$R^2 = 0.993$
Amida	$Y = -3.37X \pm 41.10$	$R^2 = 0.976$
Protein kinase C receptor	$Y = -3.42X \pm 39.27$	$R^2 = 0.998$
Kinesin light chain I	$Y = -3.58X \pm 38.27$	$R^2 = 0.997$
Myosin I heavy chain	$Y = -3.29X \pm 42.95$	$R^2 = 0.998$
Polyubiquitin	$Y = -3.45X \pm 33.14$	$R^2 = 0.998$
Calcium channel, voltage-dependent, T type, alpha 1G subunit	$Y = -3.38X \pm 40.27$	$R^2 = 0.989$
Superoxide dismutase 3	$Y = -3.89X \pm 44.96$	$R^2 = 0.994$
Telomerase protein component 1	$Y = -5.21X \pm 49.53$	$R^2 = 0.960$
Tyrosine kinase receptor	$Y = -3.96X \pm 43.83$	$R^2 = 0.985$
Galanin	$Y = -3.49X \pm 41.13$	$R^2 = 0.992$
GSK-3beta interacting protein rAxin	$Y = -3.03X \pm 41.27$	$R^2 = 0.999$
Epithelin	$Y = -3.47X \pm 41.47$	$R^2 = 0.997$
Cathepsin D	$Y = -3.08X \pm 39.44$	$R^2 = 0.983$
Phosphatidylinositol 3-kinase	$Y = -3.36X \pm 42.10$	$R^2 = 0.993$
Calcium-independent alpha-latrotoxin receptor	$Y = -3.45X \pm 40.59$	$R^2 = 0.997$
Calpain 2	$Y = -3.77X \pm 46.62$	$R^2 = 0.996$

factors, vesicles, and cytoskeletal proteins is essential for the neuronal functionality (Gunawardena and Goldstein, 2004). The increased kinesin light chain mRNA expression in rat brain as found in this study suggests that chronic treatment of risperidone may affect the transportation machinery of important signal molecules in the brain cells.

In this study, three protease-related genes were upregulated by chronic risperidone treatment, these genes are calpain 2, cathepsin D, and polyubiquitin, respectively. Calpains are a large family of calcium-dependent cysteine proteases that participate in a variety of cellular processes including remodeling of cytoskeletal/membrane attachments, different signal transduction pathways, and apoptosis (Suzuki *et al*, 2004). Pathogenic activation of calpain has been found to be associated with many diseases such as stroke, spinal cord injury (Ray *et al*, 2003), and Alzheimer's disease, whereas loss-of-function mutations of calpain genes are associated with gastric cancer, type II diabetes, and limb-girdle muscular dystrophy 2A (Vanderklish and Bahr, 2000; Huang and Wang, 2001). Many studies have shown that increased calpain activity is associated with neuronal apoptosis (Ray *et al*, 2003). Cathepsins are also a large family of cysteine proteases that were originally thought as nonselective degrading enzymes in lysosomes. Recent studies suggested that cathepsins are also involved in many specific physiological functions, including bone remodeling, processing of MHC class II antigen presentation, enzyme

and prohormone processing, and apoptosis (Turk *et al*, 2002). Cathepsin D is one member of the cathepsin family that plays a role in mediating apoptosis in addition to its general housekeeping function. Recent study showed that microinjection of cathepsin D into the cytosol of human fibroblast cells induces apoptosis, which was mediated by cytochrome *c* release and caspase activation (Roberg *et al*, 2002).

Ubiquitin is a small protein that can be covalently linked to proteins in single molecule or polyubiquitin form. The ubiquitin-tag proteins are destined to 26S proteasome for degradation. However, recent studies have demonstrated that ubiquitin and related proteins have additional functions in the cells, including plasma membrane protein internalization, delivery of membrane-protein to multi-vesicular bodies, and transportation of protein into nucleus (Aguilar and Wendland, 2003). Ubiquitination is also involved in the regulation of apoptosis pathway, many regulator proteins of apoptosis pathway are subject to modification by ubiquitination (Lee and Peter, 2003).

Previous study showed that acute administration of haloperidol increases the apoptotic death of neurons in rat striatum and substantia nigra, which might lead to tardive dyskinesia after long-term use of haloperidol (Mitchell *et al*, 2002). In cell culture study, the *N*-methyl-4-phenylpyridinium ion (MPP⁺)-induced apoptosis can be prevented by atypical antipsychotic drugs but not by haloperidol (Qing *et al*, 2003). These data suggest that antipsychotic drugs treatment may affect the intricate regulation of apoptosis in the brain. In this current study, three protease-related genes were upregulated in the rat brain after chronic administration of risperidone. Our data suggest that long-term use of risperidone may affect proteolysis in the brain cells, while whether there is an association of the apoptosis with risperidone treatment needs further clarification.

Calcium-independent receptors of α -latrotoxin (CIRL) are involved in the massive exocytosis of synaptic vesicles at synapses caused by α -latrotoxin poisoning. CIRL proteins have large extra- and intracellular domains as well as seven transmembrane domains, which indicate that they might couple with G-protein and function as signal transducer (Sudhof, 2001). CIRL proteins were originally thought to be located at presynaptic areas. However, recent studies indicated that CIRL proteins may also locate at postsynaptic area (Kreienkamp *et al*, 2002). The natural endogenous ligands for the CIRLs have not been identified yet, CIRL receptors may function as cell-adhesion molecules coupled with signal transduction (Kreienkamp *et al*, 2002). In this study, the increased expression of the CIRL gene in rat brain suggests that long-term administration of risperidone may change neurotransmission and/or intercellular interaction in the brain.

Hyman and Nestler (1996) proposed an 'initiation and adaptation' model to explain the mechanism of action of psychotropic drugs, including antipsychotic drugs. In this model, they suggested that chronic psychotropic drug administration could drive the adaptations in postreceptor signaling pathways, including regulation of neural gene expression. The differentially expressed genes can turn into the structural and functional modifications of neural cells in the brain, which underscore the clinical efficacy of

antipsychotic drugs treatment. Hence, study of differential regulation of genes and proteins expression in the brain cells as induced by chronic antipsychotic drugs treatment may not only promote our understanding of the molecular mechanisms of antipsychotic drugs but also help uncover the neurobiology of mental illnesses, especially schizophrenia. In this study, we were able to screen and verified eight genes that were upregulated in rat cerebral cortex under chronic treatment of risperidone. In view of the physiological functions of these eight genes, the results of this current study suggest that chronic administration of risperidone may affect the neurotransmission, synaptic plasticity, and proteolysis of brain cells.

The results of this study are compatible with the model of initiation and adaptation of psychoactive drugs action as proposed (Hyman and Nestler, 1996). This study also demonstrates that cDNA microarray analysis is a useful novel approach to bring new information about the psychoactive drug action at molecular level. However, this study has several limitations. First, the study is limited by the small number of genes spotted on the chips, which prohibits from finding related genes that are co-regulated by risperidone treatment. Hence, the information obtained from the small number of genes as identified in this study is only suggestive. Second, there are many other ways to verify differential gene expression, such as Northern blot analysis, nuclease protection assay, *in situ* hybridization, which may add further information in addition to quantitative PCR as used in this study. Third, the differential gene expression as shown in this study needs further proof at protein level, because cross hybridization is sometimes an issue at RNA level study.

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