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A Comparative Proteomics Analysis of Rat Mitochondria from the Cerebral Cortex and Hippocampus in Response to Antipsychotic Medications

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An increasing number of experiments have found anomalies in mitochondria in the brains of psychotics, which suggests that mitochondrial dysfunction or abnormal cerebral energy metabolism might play an important role in the pathophysiology of schizophrenia (SCZ). We adopted a proteomic approach to identify the differential effects on the cerebral cortex and hippocampus mitochondrial protein expression of Sprague–Dawley (SD) rats by comparing exposure to typical and atypical antipsychotic medications. Differential mitochondrial protein expressions were assessed using two-dimensional (2D) gel electrophoresis for three groups with Chlorpromazine (CPZ), Clozapine (CLZ), quetiapine (QTP) and a control group. A total of 14 proteins, of which 6 belong to the respiratory electron transport chain (ETC) of oxidative phosphorylation (OXPHOS), showed significant changes in quantity including NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10 (Ndufa10), NADH dehydrogenase (ubiquinone) flavoprotein 2 (Ndufv2), NADH dehydrogenase (ubiquinone) Fe–S protein 3 (Ndufs3), F1-ATPase beta subunit (Atp5b), ATPase, H⁺ transporting, lysosomal, beta 56/58 kDa, isoform 2 (Atp6v1b2) and ATPase, H⁺ transporting, V1 subunit A, isoform 1 (Atp6v1a1). The differential proteins subjected to 2D were assessed for levels of mRNA using quantitative real time PCR (Q-RT-PCR), and we also made partial use of Western blotting for assessing differential expression. The results of our study may help to explain variations in SD rats as well as in human response to antipsychotic drugs. In addition, they should improve our understanding of both the curative effects and side effects of antipsychotics and encourage new directions in SCZ research.

Keywords: schizophrenia • comparative proteomics • mitochondrial dysfunction • complex I • oxidative phosphorylation • antipsychotic medications

Introduction

Schizophrenia (SCZ), the most severe of psychiatric disorders, affects 1% of the world population with broadly equal prevalence throughout diverse cultures and geographic areas.

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The symptoms of SCZ are generally divided into two categories, namely, ‘positive’ symptoms, such as hallucinations, delusions, disorganization of thought, bizarre and incongruous behavior, and ‘negative’ symptoms, such as loss of motivation, restricted range of emotional experience and expression, alogia and reduced hedonic capacity.¹ Although a great deal of work has been done in this area, the molecular mechanism triggering SCZ has, so far, remained elusive. The etiology of SCZ appears to be multifaceted, with genetic, nutritional, environmental, and developmental factors all implicated.² Antipsychotic drugs remain the current standard of care for mental disorders including schizophrenia.³ Chlorpromazine (CPZ) was the first of the first-generation of antipsychotics (the so-called “typical antipsychotics”). It operates on central dopaminergic pathways, and although effective, it has serious side effects.⁴ A number

of new antipsychotic drugs (the so-called “atypical antipsychotics”) have been introduced since 1990. Clozapine (CLZ) is the first atypical antipsychotic drug, so designated because it has more antipsychotic effects without the adverse mobility effects of the first-generation drugs.⁴ Subsequent antipsychotic drugs have followed, such as quetiapine (QTP), risperidone and olanzapine, and so forth. The atypical antipsychotic drugs usually act on both dopamine receptors and serotonin receptors.^{5,6}

There is increasing evidence that mitochondrial energy metabolism might be disturbed by antipsychotic drugs. The research results of Burkhardt et al. showed that the typical and atypical antipsychotics have a direct inhibitory effect on the respiratory electron transport chain (ETC), especially on complex I enzyme activity, in freeze–thawed preparations of rat brain mitochondria.^{7,8} Maurer and Möller⁹ analyzed the activity of the mitochondrial respiratory chain enzyme complexes, which produce ATP via oxidative phosphorylation (OXPHOS), and found that antipsychotics may inhibit the mitochondrial complex I in human brain tissue.¹⁰ The mechanisms underlying the antipsychotic-associated neurotoxicity, and the toxic effects on mitochondria, have not yet been fully identified. Proteomics is a powerful tool for identifying protein expression alterations in disease tissue and has been successfully employed to study a variety of disorders, including SCZ.^{11–13} In this study, we used comparative proteomics to analyze expression changes of all mitochondrial proteins from the cerebral cortex and hippocampus of Sprague–Dawley (SD) rats in response to antipsychotic medication. The rats were divided into four groups, three of which were treated with CPZ, CLZ and QTP, respectively, and one control group. The goal of this study was to evaluate the effects of antipsychotic drugs on mitochondria function, and to provide further understanding of the curative effects and the side effects of antipsychotics.

Materials and Methods

Animals and Treatment. Male SD rats (6 weeks old, $n = 50$) weighing 200 ± 10 g on arrival were obtained from the Shanghai Laboratory Animal Co., Ltd. (SLAC, Shanghai, China). The rats were randomly divided into one of four groups: a CPZ-treatment group (11 rats), a CLZ-treatment group (12 rats), a QTP-treatment group (14 rats) and a control group (13 rats). Three or four rats were housed in one cage and kept on a 12/12 h light/dark cycle with food and water freely available. The animal room was air conditioned and the ambient temperature was maintained at 23.5 ± 3.5 °C and relative humidity at $55 \pm 15\%$. After 2 days of acclimatization, all rats were given their respective antipsychotics which were dissolved in 0.9% (w/v) saline and delivered by oral gavage using an injector with a metal ball tip, either CPZ (90 mg/(kg of body weight/day)),¹⁴ CLZ (45 mg/(kg/day))¹⁵ or QTP (50 mg/(kg/day)).¹⁶ Control animals were treated in the same way with 0.9% (w/v) saline. At the beginning of each week, all four groups were weighed in order to adjust the dose according to the weight gained the previous week. After a 34-day exposure to the antipsychotics, the rats were killed by cervical dislocation. This procedure was conducted in compliance with the Guide for the Care and Use of Laboratory Animals as approved by the local animal ethics committee.

Isolation of Mitochondria. Fresh mitochondria samples were collected from the cerebral cortex and hippocampus of the three treated groups and the control group. The cerebral cortex were rapidly removed, placed on ice, and divided into

three parts. Part I was placed in a freezing tube containing 0.8 mL of TRIzol reagent (Invitrogen, CA) and kept in liquid nitrogen to isolate total RNA; Part II was stored in a freezing tube and placed in liquid nitrogen to isolate all the proteins; Part III contained most of the cerebral cortex and hippocampus and these were examined immediately after collection. The hippocampus was divided into two parts, Part I and Part III. First, the tissues were washed three times using a mitochondrial isolation homogenization buffer (MIB) consisting of 320 mM sucrose, 1 mM EGTA, 1 mM EDTA, 0.23 mM PMSF, 10 mM Hepes-KOH (pH 7.4) and a correspondingly superfluous protease inhibitor cocktail set I (Merck-Calbiochem, Darmstadt, Germany). The tissue was then pooled (two random rats from the same group) and minced with scissors and gently homogenized with ultrasonic vibrations on ice. After diluting the homogenate to 5 mL with the MIB, large cellular debris and nuclei were eliminated by centrifuging for 3 min at 1500g, 4 °C, and the supernatant was transferred into a new centrifuge tube. The deposition was resuspended in 3 mL of MIB, after centrifuging for 3 min at 1500g, 4 °C and the supernatant was combined with that of the first step. The combined supernatant was centrifuged for 3 min at 1500g for 5 min, 4 °C. The supernatant was transferred into a new centrifuge tube and centrifuged for 10 min at 15 000g, 4 °C. The resultant deposit was resuspended in 2 mL of MIB and layered onto 1.5 mL of 7.5% (w/v) Ficoll-sucrose medium (7.5% Ficoll–70 mM sucrose, 210 mM mannitol, 1 mM EDTA-K₂, 0.23 mM PMSF and 5 mM Hepes-Tris, pH 7.4) on top of 1 mL of 10% (w/v) Ficoll–sucrose medium and centrifuged in a Beckman Optima MAX-E Ultracentrifuge (Beckman Coulter, CA) at 100 000g for 90 min. Mitochondrial pellets were enriched below the 10% Ficoll–sucrose medium. The pellets were suspended in 4.5 mL of MIB and then centrifuged for 5 min at 15 000g, 4 °C. The pellets were gently mixed and resuspended in 4.5 mL of fatty acid-free bovine serum albumin (BSA) containing MIB and centrifuged at 9800 rpm (revolutions per minute) for 10 min. The nonsynaptic purified mitochondrial pellets were then washed once in MIB alone, pelleted at 15 000g for 5 min (the mitochondrial pellets were evaluated by electron microscope) and resuspended in an appropriate volume of MIB and stored at –80 °C for subsequent analyses.¹⁷

Protein Extraction and 2D Electrophoresis. Total mitochondrial protein was prepared from each specimen. The specimen was centrifuged at 4 °C, 15 000g for 5 min. After removal of the supernatant, the deposit was suspended in 1 mL of sample buffer containing of 7 M Urea, 2 M Thiourea, 4% CHAPS, 65 mM 1,4-dithioerythritol, 40 mM Tris, 10 μL of 100 mM EDTA, 10 μL of 0.1 mM PMSF, and 10 μL of protease inhibitor cocktail set I (Merck-Calbiochem). The mitochondria pellets were gently homogenized with ultrasonic vibrations on ice until the sample buffer was limpid. After 1 h of incubation at room temperature, the sample was centrifuged at 12 °C, 14 000g for 1 h to remove insoluble material. The supernatant protein was added to 4 mL of sample buffer and treated for salt removal three times using the Amicon Ultra-4 (Millipore, Billerica, MA) at 25 °C, 4000g for 20 min. The protein content in the supernatant was determined by the Coomassie blue method. A total of 450 μL of sample buffer containing 1.6 mg of total protein was added to the 2.4 μL of IPG buffer (pH 3–10, GE Healthcare, Piscataway, NJ) and centrifuged at 4 °C, 14 000 rpm for 30 min. Supernatant was used for the first dimension. Hydration was carried out using PH 3–10 nonlinear Immobiline DryStrips (GE Healthcare) on an Ettan IPGphor apparatus

(GE Healthcare) for 14 h at 100 V/h, followed by 1 h at 500 V/h, 1 h at 1000 V/h, and 1 h at 4 000 V/h, and then focused for 12 h at 8 000 V/h until 96 000 V/h. After the isoelectric focusing, the Immobiline DryStrips were incubated for 15 min in a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and 60 mM DTT at room temperature. The strips were then alkylated for 15 min in a similar solution with DTT replaced by 100 mM iodoacetamide.¹⁸ The second-dimensional separation was performed in 12.5% SDS polyacrylamide gels. Gel was fixed in a 400 mL fix buffer containing 200 mL of methanol, 20 mL of Orthophosphoric acid and 180 mL of deionized H₂O. Protein spots were visualized using 0.1% Coomassie Blue G-250 staining overnight. Each gel was twice discolored for 2 h using 400 mL of 20% Ammonium sulfate.

Gel Image Analysis. Stained gels were scanned on a UMAX PowerLook III scanner (resolution 300 DPI, GE Healthcare), and the resulting TIFF images were analyzed using ImageMaster 2D Platinum software 5.0 (GE Healthcare). The automatic detection of the spot from the gel images included spot detection, modification, warping, background subtraction and matching. Spot volumes were normalized against the total volume of all the spots in the gel.¹⁸

Protein Identification using Mass Spectrometry. Protein spots of interest were excised manually from the Coomassie blue-stained gel and diced into small pieces ($\sim 4 \text{ mm}^3$) with a sheared tip and placed into a 96 hole board. The gel fragments were destained and dehydrated by washing twice for 30 min with 25 mM NH₄HCO₃ in 50% acetonitrile until shrunken and white. The destained gel particles were then dried for 19 min at 37 °C. Then, every dry gel particle was incubated in 10 μL of 25 mM NH₄HCO₃ with trypsin (GE Healthcare) for digestion for 3 h. Total peptides were extracted twice with 50% acetonitrile (ACN), 5% Trifluoroacetic acid (TFA) (Sigma-Aldrich, St Louis, MO), and then, the peptides supernatants were dried at 37 °C for 2 h. The dried peptides were added to a 3 μL buffer A (0.3% TFA, 50% ACN) and then thoroughly crushed with pipettes. The mix was then added to 3 μL of saturated α -Cyano-4-hydroxycinnamic acid (Fluka, Milwaukee, WI) and again crushed. A total of 0.3 μL of the mixture was then subject to mass spectrometric analysis (MALDI-ToF/MS Pro, GE Healthcare). The peptide fingerprinting was compared using the GE Healthcare database and a mascot database.

RNA Isolation and Q-RT-PCR. The cerebral cortex and hippocampus samples with added 0.8 mL of TRIzol reagent were removed from the liquid nitrogen and thawed on ice, then homogenized on a Mini-Bead Beater (Biospec, Bartlesville, OK). Total RNA of the sample was extracted using a TRIzol reagent recommended protocol, and quantities from 260/280 were measured to adjust the initial total RNA. The cDNA was synthesized by random hexamers using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). We measured the gene expression level of objective genes which code those proteins with the most significant changes using a Q-RT-PCR system on SyberGreen I (Molecular Probe, Inc.) and ABI PRISM 7900 (Applied Biosystems, Los Angeles, CA). Q-RT-PCR was performed using gene-specific primers designed by Primer Express 2.0. A comparative Ct method was employed for quantification according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used for normalization.¹⁹ The relative gene expression was evaluated using the comparative cycle threshold method.²⁰

Protein Isolation and Western Blotting. Total proteins were extracted from the cerebral cortex and the accessory of RNA

was extracted from the hippocampus using TRIzol reagent. We applied Western blotting to some of the differential expression proteins of ETC and Gfap, Cyb5b assessed by 2-D. Western blotting was performed with the following primary antibodies: mouse monoclonal anti-Gfap (2E1), sc-33673; Atp6v1b1/2 (F-6), sc-55544; Atp5b (3D5), sc-58618; Ndufs3 (17D95), sc-58393; goat polyclonal anti-Atp6v1a1 (C-16), sc-31462; Cyb5b (K-18), sc-48470 (Santa Cruz Biotechnology, CA) and using Polyacrylamide gradient (7.5–15%) SDS gels. A total of 50 μg of soluble protein was loaded per lane. Gels were blotted onto PVDF membranes and blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) overnight at 4 °C. After primary antibody (1:1000) incubation for 3 h at room temperature, membranes were washed three times using TBST for 5 min, and then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:8000) for 2 h at room temperature. Membranes were then washed using TBST for 5 min, three times. Blots were developed with 1 mL of Western blotting luminol reagent for 3 min (Santa Cruz-2048, Santa Cruz, CA) and visualized by autoradiography. HRP-conjugated monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was introduced as a reference gene. Western blots were digitized using Image Quant 5.2 (Molecular Dynamics).

Statistical Analysis. All the data were tested for normal distribution using the Kolmogorov-Smirnov Test before calculation of differences. Normally distributed data were analyzed using one-way ANOVA followed by a Post Hoc Dunnett Test for comparison with the control group.²¹ Data that was not distributed normally were analyzed using nonparametric Wilcoxon Mann-Whitney Test.²¹ P-values <0.05 between the treated groups and the control group were considered as statistically significant. After analyzing the data in line with the normal distribution, Pearson's correlation test was performed for the correlations between the expressions of all the differential expression of 2D proteins and the levels of the mRNA, respectively.²¹ The results of Western blotting were analyzed using the Independent-Samples T Test. SPSS version11.0 software was used for statistical analyses.

Results

Electron Microscope. Highly purified mitochondria were isolated by differential centrifugation. We used the transmission electron microscope (TEM) to check the quality of the isolated mitochondria and the results are shown (Figure 1).

Comparative Proteomics Analysis. A number of proteins whose levels were statistically significantly changed after the antipsychotic medication were identified by 2-D analysis. We compared global mitochondrial protein expression patterns of the three groups' of medicated rats and the control group rats. A total of 29 (6:6:10:7) 2-D gels (Figure 2) of the total mitochondrial proteins from the hippocampus, and 36 (11:12:6:7) 2-D gels of the total mitochondrial proteins from the cerebral cortex were evaluated. After the visualization processing, we obtained the TIFF images per gel. The volume of each protein spot was normalized against the total volume of all the spots in the gel. After statistical analysis, we obtained 14 differentially expressed proteins and P-value as shown in Table 1.

MALDI-TOF/MS, Bioinformatics and Pathway Analysis. We identified only the differentially expressed proteins using MALDI-TOF/MS. The 14 differentially expressed proteins were Ndufa10, Ndufv2, Ndufs3, Atp5b, Atp6v1b2, Atp6v1a1, calmodulin 1 (Calm1); complement component 1 Q subcomponent-

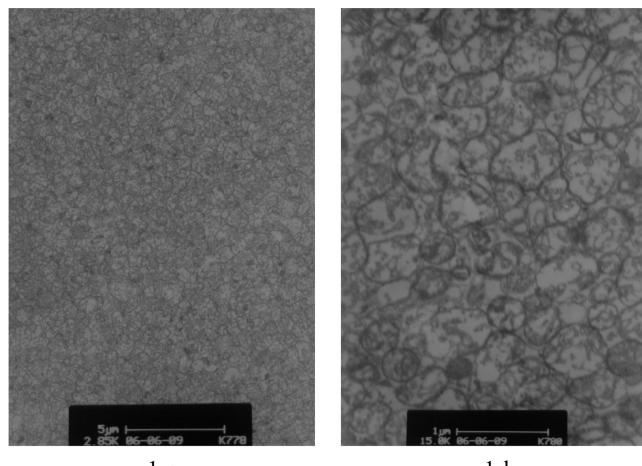


Figure 1. Mitochondria photos were taken by TEM. Purity of mitochondria: (a) for 2.85K and (b) for 15.0K magnifying power; using transmission electron microscopy (PHILIPS CM120). Mitochondria of rat cerebral cortex and hippocampus were processed for TEM using glutaraldehyde and osmic acid fixation and routine processing of dehydration, endosmosis, embedment, slice, uranyl acetate staining and TEM.

binding protein, mitochondrial (C1qbp); cytochrome b5, outer mitochondrial membrane (Cyb5b); glial fibrillary acidic protein (Gfap); mitogen-activated protein kinase-activated protein kinase 2 (Mapkapk2); phosphatidylethanolamine binding protein 1 (Pebp1); Tu translation elongation factor, mitochondrial (Tufm) and tyrosine 3-monooxygenase/tryptophan 5-monoox-

genase activation protein (Ywhah). Further pathways analysis was carried out using the DAVID online Gene Functional Classification Tool (<http://david.abcc.ncifcrf.gov/gene2gene.jsp>). The pathway analysis indicated that 6 proteins, Ndufa10, Ndufv2, Ndufs3, Atp5b, Atp6v1b2, and Atp6v1a1, were ETC members participating in OXPHOS. The top two pathways are OXPHOS of GOTERM and KEGG_PATHWAY. The *P*-values (Bonferroni or Benjamini) of these two significantly implicated pathways were reached, 0.00005 and 0.00025, respectively. To further study the relationship between the differentially expressed proteins, Pearson's correlation was performed between each drug treatment group and the control group using 2D data of mitochondrial protein from the cerebral cortex and hippocampus. However, the correlation between the differentially expressed proteins showed there were no stable relationships among them (data not shown).

Q-RT-PCR Primers and Results. The cDNA sequences of all selected genes were obtained from genome.ucsc.edu and www.ncbi.nlm.nih.gov. The primers of qPCR were designed by Primer Express 2.0 (Table 2). The results of mRNA show that transcription levels were inconsistent with protein expression levels. In the hippocampus, following 34 days of antipsychotic medication, there was a wide range of differentially expressed proteins, while their corresponding gene expression showed no statistically significant differences. In the cerebral cortex, the mRNA expression level of Cyb5b (CLZ vs control, *P*-value = 0.030) was up-regulated, and consistent with the trend of its protein expression (CLZ vs control, *P*-value = 0.001). The mRNA result of Cyb5b showed that the gene continued to exhibit up-regulated expression. Gfap was exactly the opposite. The mRNA

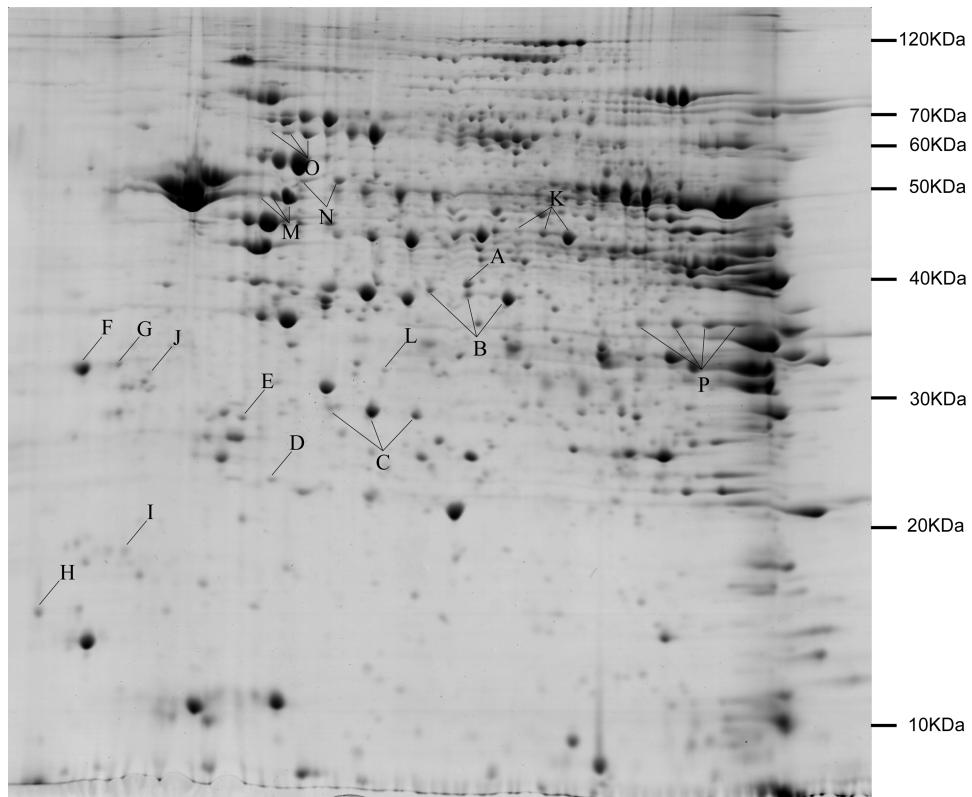


Figure 2. 2-D gels of total mitochondrial proteins. Representative 2-D gels of total mitochondrial proteins were visualized using Coomassie Blue G-250 staining. The letters of the protein spots in the gel correspond to the protein spots as follows: A, Ndufa10; B, Ndufa10; C, Ndufs3; D, Pebp1; E, Ndufv2; F, C1qbp; G, Ywhah; H, Calm1; I, Cyb5b; J, Atp5b; K, Tufm; L, Mapkapk2; M, Gfap; N, Atp6v1b2; O, Atp6v1a1; and P, Gapdh.

Table 1. Statistical Significantly Changed Proteins Identified by 2D Analysis^a

protein	%volume (means ± std.)					
	control		CPZ-treated (<i>P</i> -value)		CLZ-treated (<i>P</i> -value)	
	H	C	H	C	H	C
Ndufa0-A	0.176 ± 0.087	-	-	-	-	-
Ndufa0-B	0.284 ± 0.061	0.413 ± 0.034	-	-	-	-
Ndufs3	-	0.025 ± 0.003	-	0.369 ± 0.028 (0.018)	-	-
Pebp1	0.027 ± 0.003	0.025 ± 0.012	-	-	0.013 ± 0.003 (0.000)	0.036 ± 0.005 (0.005)
Nduf2	0.029 ± 0.012	-	0.316 ± 0.080 (0.018)	-	-	0.054 ± 0.014 (0.000)
C1qbp	0.406 ± 0.030	-	0.030 ± 0.005	-	-	-
Ywhah	-	0.045 ± 0.004	-	0.032 ± 0.003 (0.000)	-	-
Calm1	0.042 ± 0.009	0.009 ± 0.004	-	0.018 ± 0.002 (0.005)	0.027 ± 0.003 (0.000)	0.064 ± 0.013 (0.012)
Cyb5b	0.028 ± 0.004	0.020 ± 0.003	-	0.016 ± 0.004 (0.020)	0.014 ± 0.002 (0.008)	0.020 ± 0.003 (0.027)
Atp5b	0.023 ± 0.007	-	0.616 ± 0.069	0.048 ± 0.069 (0.049)	0.651 ± 0.126 (0.047)	0.016 ± 0.002 (0.031)
Tufm	0.479 ± 0.069	-	-	-	-	-
Mapkapk2	0.019 ± 0.009	-	-	-	0.009 ± 0.002 (0.035)	-
Gfap	0.536 ± 0.130	0.784 ± 0.047	-	-	0.375 ± 0.121 (0.031)	0.521 ± 0.105 (0.005)
Atp6v1b2	0.184 ± 0.050	0.184 ± 0.035	0.115 ± 0.038 (0.009)	0.138 ± 0.028 (0.038)	0.092 ± 0.012 (0.000)	0.123 ± 0.044 (0.005)
Atp6v1a1	0.265 ± 0.037	0.189 ± 0.023	-	-	0.190 ± 0.014 (0.006)	0.131 ± 0.024 (0.001)

^a Quantitative image analysis revealed a total of 14 proteins that changed their intensities significantly (*P*-value <0.05) on the basis of one-way ANOVA followed by a post-hoc Dunnett Test. Data results are Means ± Std.

expression was up-regulated in the CLZ treatment group (CLZ vs control, *P*-value = 0.000), but its protein expression was down-regulated (CLZ vs control, *P*-value = 0.005). This paradox could be caused by the body demonstrating a metabolic compensation for self-protection after chronic treatment. The differential expression of Mapkapk2-cerebral cortex (CPZ vs control, *P*-value = 0.046) and Ndufv2-cerebral cortex (CLZ vs control, *P*-value = 0.013; QTP vs control, *P*-value = 0.003) was only shown at the mRNA expression level not that of protein expression level. The results of mRNA quantization are shown Figure 3. To confirm the correlations of the mRNA expression levels of the genes from each group and the control group, Pearson's correlation test was used. Correlation analysis showed an irregular relationship between mRNA expression levels of the various genes (data not shown).

Western Blotting. Total hippocampus proteins suffered serious degradation when extracted from the accessory of RNA using TRIzol reagent. We therefore, used Western blotting only for total proteins of the cerebral cortex. Of the total proteins of the cerebral cortex identified by 2D gels after antipsychotic medication, the abnormally expressed mitochondrial proteins selected for confirmation by Western blotting were Gfap, Atp6v1b2, Atp5b, Ndufs3, Atp6v1a1 and Cyb5b. Some statistically significant differences in the levels of protein expression were confirmed, including Atp6v1b2, Atp5b and Ndufs3. Western blotting showed protein expression levels of Atp6v1b2 to be down-regulated in the CPZ (*P*-value = 0.040) group and the CLZ (*P*-value = 0.025) group. Similarly, the protein expression of Atp6v1b2 revealed by 2D was significantly decreased in the CPZ (*P*-value = 0.038) group and in the CLZ (*P*-value = 0.005) group. Western blotting showed a significantly reduction of Atp5b expression in the CLZ (*P*-value = 0.048) group. 2D showed similarly decreased levels of Atp5b in the CLZ group. 2D showed Ndufs3 to be significantly decreased in the CPZ (*P*-value = 0.018) group, while Western blotting showed a similar reduction in the CPZ (*P*-value = 0.006) group. We did not obtain any band of Atp6v1a1 or Cyb5b using the same protocol with other antibodies. Using Ponceau S staining, we found that proteins had transferred to the PVDF membranes. We therefore speculated that a possible cause of the failure might be a problem with polyclonal antibodies. The results of Western blotting are shown as Figure 4.

Discussion

The results of this study demonstrate a distinctly different expression of mitochondrial proteomics, especially in the OXPHOS pathway, following exposure to antipsychotic drugs. These drugs have been reported to disturb mitochondrial function by inhibiting complex I activity directly, both *in vitro* and *in vivo* in rodents and in humans.^{7,9,22–25} Antipsychotics, typical as well as atypical, interact with the dopamine pathway. Strong experimental evidence has accumulated over the past decade implicating dopamine in mitochondrial respiration in general and in complex I function in particular.²⁶ It has been reported that, both *in vivo* and in isolated mitochondria, dopamine inhibits complex I activity, electron transport, adenosine triphosphate (ATP) production, mitochondrial respiration, and mitochondrial membrane potential.^{27–31} These interactions between dopamine, one of the most prominent candidates in the etiology and in antipsychotic treatment of SCZ, and mitochondrial complex I are also evident in SCZ.²⁶ Because cerebral metabolism is highly dependent on the energy supply of mitochondria, mitochondrial dysfunction is often the

Table 2. The Primers of the Q-RT-PCR

gene	forward primer	reverse primer
Gapdh	ACCACCAACTGCTTAGCCCC	CATGGACTGTGGTCATGAGCC
1. Atp5b	ACCACCAAGAAGGGCTCAT	GCATCCAATGGCAAAGG
2. Atp6v1a1	AAAGCTGCCAGCCAACCA	ACAGCCAAAGCACCTGGAA
3. Atp6v1b2	TGAGAACATGTCCTGGTCATC	TCTTCCCTGGCAGCTGAAAC
4. Cyb5b	GTCTACGATATCACCCGCTTCCT	GCACCAGCTTGTTCAGTAGAA
5. Gfap	GGAGGTGGAGAGGGACAATCT	GGTTGGTTCATCTGGAGCTT
6. Mapkapk2	GGTGCTCGGGATCTCGA	TGGGACAGTCCTGCAGCAT
7. Ndufs3	GGCTTCGAGGGACATCCCTT	CCGCTTCACCTCATCGTCAT
8. Ndufv2	CAGAATGGGTGGCTGCCTAT	ACTCTCATTGGAGGCAGTTGTAAA

first to affect brain function. Brain injury can lead to abnormal brain discharge; it is consistent with the symptoms of patients with SCZ and is associated with impaired cerebral energy metabolism, developmental aberrations, abnormal neurotransmission, and neuronal connectivity and may account for the multifactorial manifestation pattern in SCZ.²⁶

The most important result of the pathway analysis in our study using DAVID online tool is that antipsychotic drugs treatment affects the normal function of OXPHOS. Of 6 ETC members proteins, 4 had down-regulated expression, and only Ndufv2 (QTP vs control) showed up-regulated expression. Interestingly, an Ndufa10 isoform (A in Figure 2) was significantly decreased in hippocampus mitochondria of QTP group; however, the total expression level of Ndufa10 has not changed because other three isoforms expression of Ndufa10 (B in Figure 2) was up-regulated. The phenomenon is likely to be caused by the dissimilar post-translational modifications of differential isoforms, changes in the activity of Ndufa10. The down-regulated expression of Ndufs3, Atp5b, Atp6v1b2 and Atp6v1a1 will inhibit the efficiency of ETC, but the up-regulated

expression of Ndufv2 produces a similarly result. Three subunits, NDUFV1, NDUFV2 and NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NADH-coenzyme Q reductase) (NDUFS1) form complex I with a stoichiometry of 1 mol of each subunit for 1 mol of complex I.³² Surprisingly, any deviation from this ratio could lead to abnormal complex I activity. Therefore, the up-regulation of Ndufv2 will disturb the balance of the ratio among them, thereby inhibiting the efficiency of complex I.

Complex I is the rate-limiting step of the ETC, and if the efficiency of decline is affected, then ETC efficiency will be affected. SCZ-specific abnormalities have been observed at the level of transcription and translation of complex I subunits in postmortem brain specimens. Some alterations in complexes I–IV of the ETC have been found in multiple brain regions of SCZ patients in several independent investigations.^{11,33,34} Karry et al.²⁶ found that both mRNA and protein levels of the NDUFV2 and NDUFV1 subunits of complex I were significantly decreased in the prefrontal cortex, but increased in the ventral parietooccipital cortices of SCZ patients compared with normal

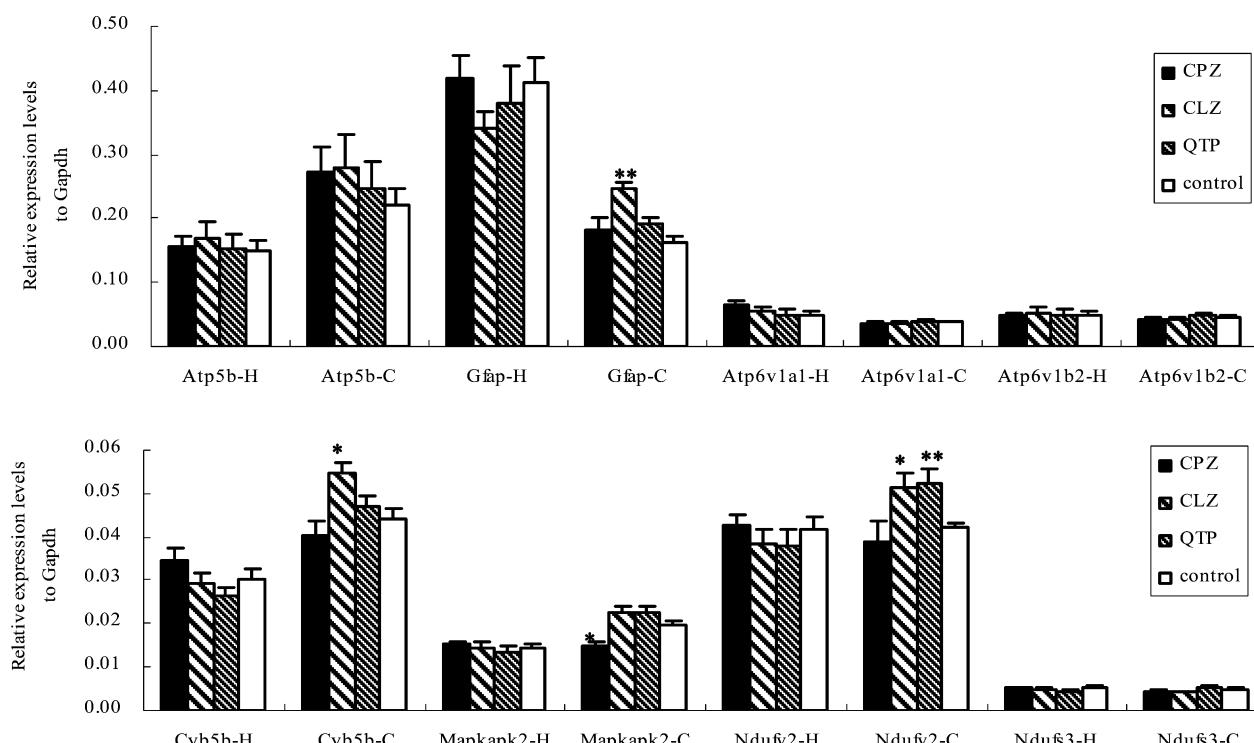


Figure 3. Gene expression was analyzed by Q-RT-PCR. Data analysis results are Means \pm Std. The significant difference between treated subjects and controls was analyzed by Dunnett Test as * $p < 0.05$, ** $p < 0.01$ vs control. Gfap-C (CLZ vs control, P -value = 0.000); Cyb5b-C (CLZ vs control, P -value = 0.030); Mapkapk2-C (CPZ vs control, P -value = 0.046); Ndufv2-C (CLZ vs control, P -value = 0.013; QTP vs control, P -value = 0.003). H, hippocampus; C, cerebral cortex.

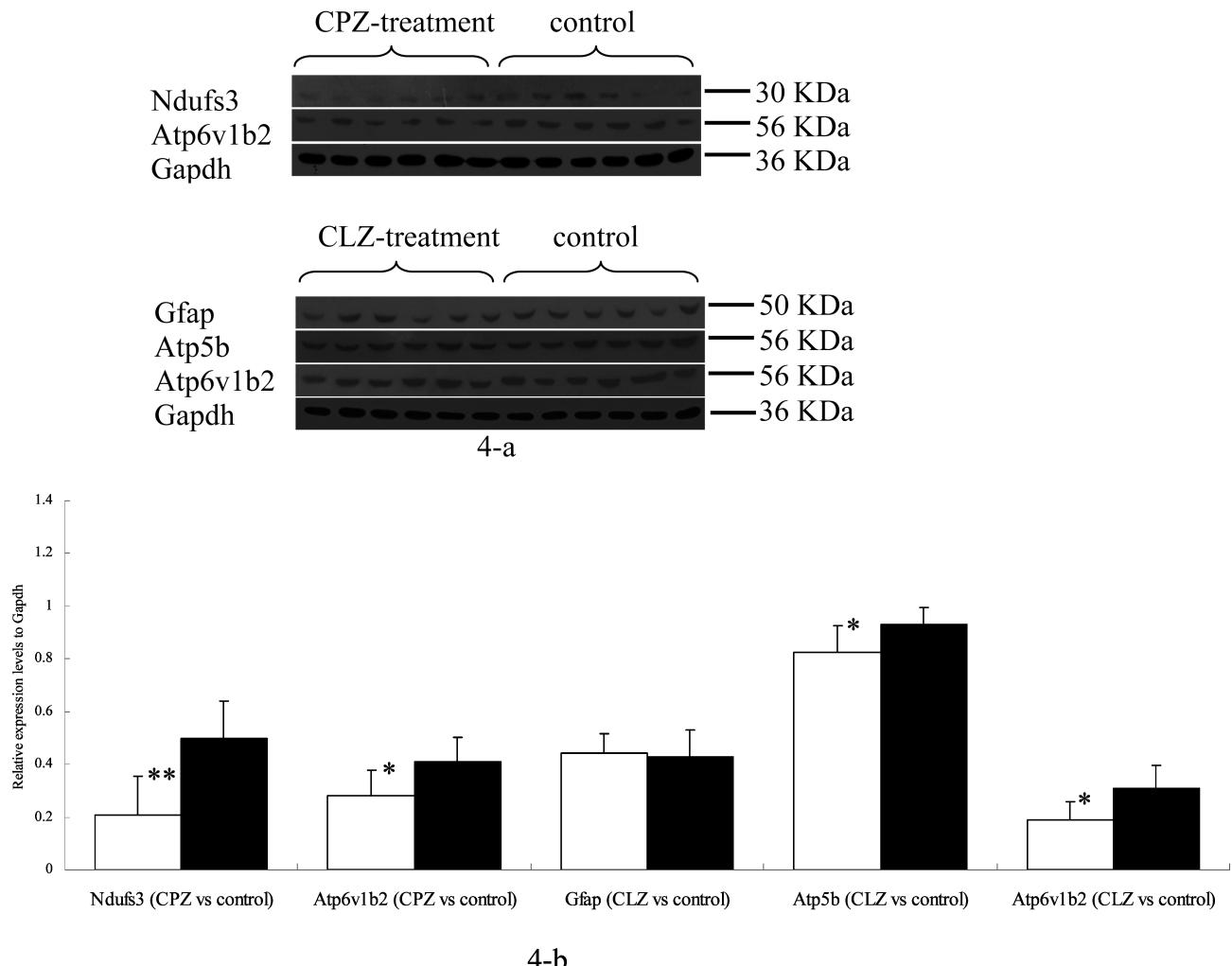


Figure 4. The levels of protein expression were analyzed using Western blotting (a). Results are means \pm Std. The significant changes between antipsychotic treated groups and controls were measured by Independent-samples T-Test. * $p < 0.05$, ** $p < 0.01$ vs control. Ndufs3, P -value = 0.006 (CPZ vs control); Atp5b, P -value = 0.048 (CLZ vs control); Atp6v1b2, P -value = 0.040 (CPZ vs control) and 0.025 (CLZ vs control), respectively (b).

control subjects. Significantly reduced activity of complexes I–III has been found in the frontal and temporal cortex as well as the basal ganglia of psychotics.^{35,36} Similarly, mitochondrial gene expression has been shown to indicate increased cytochrome oxidase subunit II in the postmortem frontal cortex tissue of schizophrenic patients.³⁷ Focusing on the mitochondrial OXPHOS in postmortem brains has revealed region-specific alterations in the activities of NADH-cytochrome *c* reductase (complexes I–III), succinate dehydrogenase (complex II) and cytochrome *c* oxidase (complex IV).³⁸

In our study, antipsychotic drugs not only inhibited the complex I, but also by inhibiting the subunits of ATP synthase (complex V) Atp5b, Atp6v1b2 and Atp6v1a1, achieved inhibition of ATP synthesis. Defects in mitochondrial function and inability to maintain cellular ATP levels have been suggested as a possible cause for the slow neurodegeneration and aberrations associated with neuronal diseases such as SCZ, Parkinson's and Alzheimer's diseases.³⁹ Morphological and functional abnormalities of mitochondria have been found in the brains of SCZ patients.³³ Autopsy evidence shows that the brain tissues of SCZ patients indicate reductions in the number and density of mitochondria in the frontal cortex, striatum, and substantia nigra.^{40,41} An electron micrograph study has also

reported a decreased number of mitochondria per axon in the striatum of medication-free SCZ patients.^{40,42}

Alterations in mitochondria-related genes/proteins have been observed in postmortem schizophrenic brains.⁴³ In a study by Prabakaran et al.,¹¹ several pathways were found to be significantly up-regulated, while OXPHOS, energy pathways, RNA metabolism, vesicle transport, protein transport, carbohydrate biosynthesis, lipid biosynthesis and glycolysis were among the most significantly down-regulated in SCZ postmortem brain tissues. The results of a cDNA microarray of the 71 major metabolic pathways in frontal cortex samples of psychotic and normal subjects further support complex I as significant in SCZ. The SCZ-specific reduction in complex I subunits in the prefrontal cortex is consistent with one of SCZ's most prominent deficits, thus further supporting the hypothesis of mitochondrial dysfunction in this disorder.⁴⁴

In our study, we also found that other mitochondria-related proteins, Calm1, C1qbp, Cyb5b, Gfap, Mapkapk2, Pebp1, Tufm and Ywhah were differentially altered in the antipsychotic drugs treatment groups as compared with the controls. The functions of Calm1 include roles in growth and the cell cycle as well as in signal transduction and the synthesis and release of neurotransmitters. CALM1 has been found differentially expressed

in the temporal lobe of SCZ patients.⁴⁵ C1QBP may contribute directly to thrombosis, inflammation, and endovascular infections.⁴⁶ CYB5B participates in electron transport and directs movement of substances (such as macromolecules, small molecules, ions) into, out of, within or between membranes. GFAP has also been reported differentially expressed in the dorsolateral prefrontal cortex¹² and the temporal lobe of SCZ patients.⁴⁵ The abnormal mRNA expression of Gfap was also reported.⁴⁷ A significant increase was observed in mRNA levels of Gfap in the cerebral cortex following CLZ treatment (*P*-value = 0.000). Mapkapk2 has been shown to be involved in many cellular processes including stress and inflammatory responses, nuclear export, gene expression regulation and cell proliferation. A mouse model study showed that MAPKAPK2 may be involved in the pathology of Alzheimer's disease.⁴⁸ PEBP1 may be involved in the function of the presynaptic cholinergic neurons of the central nervous system, and has been found differentially expressed in the temporal lobe in patients with SCZ by Martins-de-Souza et al.⁴⁵ Tufm is a GTPase which promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.⁴⁹ The Ywhah gene contains a 7 bp repeat sequence in its 5' UTR, and changes in the number of this repeat have been associated with early onset schizophrenia. YWHAH has been found differentially expressed in the temporal lobe of SCZ patients.⁴⁵

Animal models of SCZ will contribute to a deeper understanding of the pathogenesis of the disease and the mechanism of antipsychotic medications. Paulson et al.⁵⁰ observed that the cytochrome c oxidase gene expression in the cerebral cortex was up-regulated in two groups of MK-801-treated rats. A cDNA microarray had shown only the altered proteins on the 2-D gel in these rats, the four proteins Atp6v1b2, gamma-enolase, hsp70 and hsp60. In fact, the literature does offer a good explanation of these discrepancies between mRNA and corresponding protein levels, namely, that the abundance of a protein at a given mRNA expression level may vary 30-fold.^{50,51} Another possible explanation of this phenomenon is that timing of mRNA and protein expression is not synchronized. Interestingly, in the process of protein identification, we discovered that some different isoforms (Ndufa10, Gfap) arose at multiple positions on the gel, possibly due to some post-translational modifications, such as glycosylation and phosphorylation.

In conclusion, our results indicate that some components of mitochondria, especially ETC components, have been changed following antipsychotic medication. Our study may help to provide further understanding of the curative effects and side effects of antipsychotics and encourage new directions in SCZ research. However, our experimental design has some limitations, which will require modifications in future experiments. On the one hand, neither the hippocampus nor the cerebral cortex is a homogeneous structure and has physiological differences in each subarea. Since, in our experiments, the hippocampus and the cerebral cortex were central to the focus of the research, some important aspects may have been missed. On the other hand, it should be stressed that proteomic techniques cannot resolve every problem, in particular when dealing with proteins of infrequent occurrence, or which are exceptionally small or exceptionally large or with proteins that are highly hydrophobic.¹³ In addition, we did not confirm all the differential expression proteins with the Western blotting. There is no doubt that more research is needed to clarify both basic research and clinical studies of SCZ.

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