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NMDA Receptor Hypofunction Induces Dysfunctions of Energy Metabolism And Semaphorin Signaling in Rats: A Synaptic Proteome Study

Kejun Zhou[1,2], Yifeng Yang[1,2], Linghan Gao[1,2], Guang He[1,2], Weidong Li[1,2], Kefu Tang[1,2], Baohu Ji[1,2], Ming Zhang[1,2], Yang Li[1,2], Jinglei Yang[1,2], Liya Sun[1,2], Zhao Zhang[1,2], Hui Zhu[1,2], Lin He[1,2,3], and Chunling Wan[1,2,*]

[1]Bio-X Center, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders (Ministry of Education), Shanghai Jiao Tong University, 1954 Huashan Road, Shanghai 200030, China; [2]Institutes for Nutritional Sciences, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, Shanghai, China; [3]Institutes of Biomedical Sciences, Fudan University, Shanghai, China

*To whom correspondence should be addressed; tel: 00-86-21-62932779, fax: 00-86-21-62822491, e-mail: clwan@sjtu.edu.cn

There is considerable evidence to suggest that aberrations of synapse connectivity contribute to the pathophysiology of schizophrenia and that *N*-methyl-D-aspartate (NMDA) receptor–mediated glutamate transmission is especially important. Administration of MK-801 ([+]-5-methyl-10, 11-dihydro-5H-dibenzo-[a, d]-cycloheptene-5, 10-iminehydrogenmaleate) induces hypofunction of NMDA receptors in rats, which are widely used as a model for schizophrenia. We investigated synaptosomal proteome expression profiling of the cerebral cortex of MK-801–treated Sprague-Dawley rats using the 2-dimensional difference gel electrophoresis method, and 49 differentially expression proteins were successfully identified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight mass spectrometry. We carried out a literature search for further confirmation of subsynaptic locations and to explore the relevance to the diseases of differentially expressed proteins. Ingenuity Pathways Analysis (IPA) was used to further examine the underlying relationship between the changed proteins. The network encompassing "cell morphology, cell-to-cell signaling and interaction, nervous system development and function" was found to be significantly altered in the MK-801–treated rats. "Energy metabolism" and "semaphorin signaling in neurons" are the most significant IPA canonical pathways to be affected by MK-801 treatment. Using western blots, we confirmed the differential expression of Camk2a, Crmp2, Crmp5, Dnm1, and Ndufs3 in both synaptosome proteins and total proteins in the cerebral cortex of the rats. Our study identified the change and/or response of the central nervous transmission system under the stress of NMDA hypofunction, underlining the importance of the synaptic function in schizophrenia.

Key words: synaptosome/proteomics/NMDA/MK-801/schizophrenia

Introduction

Schizophrenia is a complex and severe brain disorder that affects approximately 1% of the world's population. There is considerable evidence showing that abnormalities of synapse connectivity contribute to the pathophysiology of the disease.[1–4]

The *N*-methyl-D-aspartate (NMDA) receptor mediates glutamate transmission and plays a key role in plastic processes in the nervous system.[5,6] NMDA receptor hypofunction has long been implicated in schizophrenia.[7,8] NMDA receptor antagonists exacerbate preexisting symptoms in schizophrenia patients and produce positive and negative symptoms as well as characteristic cognitive deficits similar to those seen in schizophrenia in rats and healthy human volunteers.[9,10] Impairment in phosphorylation of the NR1 subunit (NMDA receptor) at serine (S) 897 leads to behavioral deficits in social interaction and sensor motor gating that are associated with psychiatric disorders.[11] Postmortem brain proteomic studies have revealed synaptic dysfunction of the dorsolateral prefrontal cortex in schizophrenia cases.[12] MK-801 ([+]-5-methyl-10, 11-dihydro-5H-dibenzo-[a, d]-cycloheptene-5, 10-iminehydrogenmaleate) is a specific NMDA receptor antagonist that induces NMDA receptor hypofunction and schizophrenia-like symptoms in rodents.[13,14] Rats treated with MK-801 are widely used as pharmacological animal models of schizophrenia. Paulson et al studied the cerebral cortex and thalamic protein expression profiles of MK-801–treated rats[15–17] but did not focus on the synapse that is an essential structure affecting neuronal functions.

To investigate this possible aspect of schizophrenia, we systematically analyzed the synaptic proteome in subchronic MK-801–treated rats using the 2-dimensional difference gel electrophoresis (2D-DIGE) method. Ingenuity

Pathways Analysis (IPA; http://www.ingenuity.com) was used to scrutinize the underlying molecular interaction between the altered proteins and thus to obtain further insight into the integrated cellular response to MK-801 treatment.

Methods

Animals and Treatment

Sprague-Dawley rats (n = 36, male, 220–250 g) were obtained from the Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). The rats were randomly divided into 3 groups with 12 rats in each cohort: a control group, a 7-day treatment group, and a 21-day treatment group. All the rats were injected subcutaneously with physiological saline 3.5 ml/kg (0.9% wt/vol NaCl [aqueous]) or 0.7 mg/kg MK-801 (Research Biochemicals, Natick, Massachusetts) (saline as vehicle) for 21 days. The control group was given saline for 21 days, the 7-day treatment group was given saline for 14 days and then MK-801 for 7 days, and the 21-day treatment group was given MK-801 for 21 days. All the rats were weighed before injection to adjust the dose of MK-801 or saline. They were kept in a 12:12-hour light/dark cycle with food and water available ad libitum. On day 22, approximately 24 hours after the final injection, the rats were killed by cervical dislocation. The brain was immediately removed and put in an ice-chilled petri dish. All the procedures were conducted in compliance with the Guide for the Care and Use of Laboratory Animals as approved by the local animal ethics committee.

Isolation of Synaptosome

Synaptosome fractionations were prepared following the protocols of Booth and Clark[18] with minor modification. All the procedures were performed at 4°C. The entire cerebral cortex was immediately dissected from the whole brain and homogenized in buffer A (5mM (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 320mM sucrose, pH 7.4, with protease inhibitor cocktail set I [Merck-Calbiochem, Darmstadt, Germany]). Half of the homogenate was used for the isolation of synaptosome, and the other half was stored at −80°C for western blot analysis. To remove large cellular debris and nuclei, the homogenate was centrifuged twice for 10 minutes at 1000g. The supernatants were combined and centrifuged for 10 minutes at 17 000g. The resulting deposit which was mainly composed of synaptosome and mitochondria was resuspended in 3 ml buffer A and laid carefully onto a Ficoll gradient consisting of 1 ml of 7.5% Ficoll on top and 1 ml of 12% Ficoll at the bottom. After centrifuging at 100 000g (Beckman Optima™ MAX-E Ultracentrifuge; Beckman Coulter, Fullerton, California) for 30 minutes, the synaptosome was enriched at the 7.5%/12% Ficoll interface. The synaptosomes were recovered by aspiration and resuspended in 4 ml buffer A. After centrifuging at 17 000g for 20 minutes, the pellet was stored at −80°C. The purity of cerebral cortex synaptosomes was checked by western blotting.

Protein Extraction and 2D-DIGE Analyses

Total synaptosome proteins were prepared as follows: 1 ml of sample buffer (7 M urea, 2 M thiourea, 4% (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 30mM Tris, pH 8.5, protease inhibitor cocktail set I [Merck-Calbiochem, Darmstadt, Germany]) was added to each of the specimen. The synaptosomes were gently homogenized with ultrasonic vibration on ice until the sample buffer was transparent. After 1 hour of incubation at room temperature, the samples were centrifuged at 14 000g, 25°C for 20 minutes to remove insoluble materials. Each of the supernatants was transferred to the Amicon Ultra-4 centrifugal filter unit (Millipore, Billerica, Massachusetts) for desalting. The protein concentration was measured using the Bradford method and adjusted to 5 mg/ml using sample buffer.

Ten control samples, ten 7 day–treated samples, and ten 21 day–treated samples, labeled with Cy3 or Cy5, were subjected to DIGE analysis. The internal standard (a pool of equal amounts from all samples) was labeled with Cy2. Proteins labeled with Cy2, Cy3, and Cy5 were mixed (supplementary table 1) and subjected to subsequent 2D-DIGE analyses. The protein mixtures were adjusted to 450 μl and separated by isoelectric focusing (IEF) using 24 cm nonlinear immobiline Drystrips, pH 3-10 (GE Healthcare, Uppsala, Sweden). Rehydration was carried out at 100 V for 14 hours and IEF at 500 V for 1 hour, 1000 V for 1 hour, 4000 V for 1 hour, and then focused at 8000 V up to 96 000 Vh. After IEF, the immobilized pH gradient strips were first equilibrated for 15 minutes using the equilibration buffer (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, and 50mM Tris, pH 8.8) containing 2% dithiothreitol and then equilibrated for 15 minutes with equilibration buffer containing 2.5% iodoacetamide. Strips were then transferred onto vertical 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and sealed with 0.5% low–melting point agarose. Electrophoresis was run for 5–6 hours at 12 W per gel at 15°C. After electrophoresis, gels were first rinsed with Milli-Q water and then scanned on a Typhoon TRIO$^+$ imager (GE Healthcare, Piscataway, New Jersey) with 100 μm resolution and appropriate photomultiplier tube voltages.

Spot detection, matching, and quantification were performed using DeCyder software (V6.0; GE Healthcare, Piscataway, New Jersey). Gel images were processed with 10 000 expected spots, and spots with areas smaller than 300 and slopes greater than 1.5, mostly dust artifacts, were excluded from further analysis. Protein spots with P values $< .05$ (1-way ANOVA) were matched to the silver-stained gels and excised for identification using

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight (MALDI-TOF/TOF) mass spectrometry following trypsin digestion.

Protein Identification by MALDI-TOF/TOF Tandem Mass Spectrometry

Differentially expressed protein spots were excised from the silver-stained gels and plated into a 96-well microtiter plate. Excised spots were destained by a mixture of 15mM potassium ferricyanide and 50mM sodium thiosulfate (1:1) for 20 minutes at room temperature. After being washed twice with deionized water, the spots were dehydrated with 100% acetonitrile. The dried pieces of gel were then incubated in an ice-cold digestion solution (trypsin 12.5 ng/μl and 20mM NH_4HCO_3) for 20 minutes and then transferred into a 37°C incubator for digestion overnight. The digested peptides were extracted using extraction solution (0.1% trifluoroacetic acid and

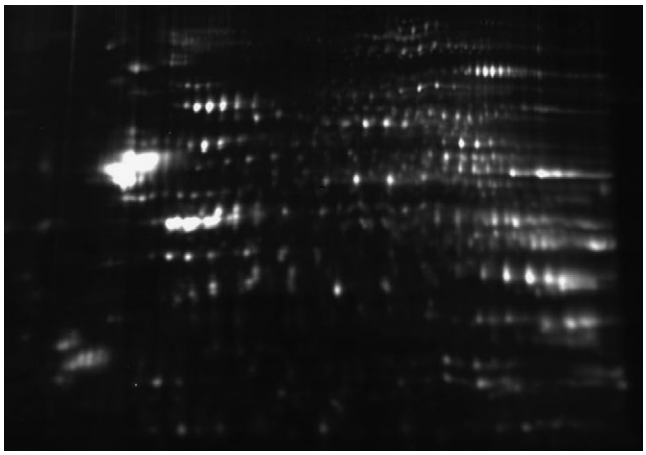

Fig. 2. The Representative Difference Gel Electrophoresis of Rat Synaptosome Samples. One hundred fifty micrograms protein mixture of 50 μg Cy3-labeled, 50 μg Cy5-labeled, and 50 μg Cy2-labeled synaptosome proteins were separated on a 24-cm immobilized pH gradient strip (pH 3–10, nonlinear) as the first dimension and 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as the second dimension.

the isolation results, we used western blot analysis with antibodies specific to Synaptophysin (Syp), GluR1, Syntaxin1, and Tpi1 for cerebral cortex homogenate and synaptosomes (figure 1). Cerebral cortex homogenate and synaptosomes samples were randomly selected from the control group. Synaptophysin is a synaptic vesicle (SV) protein, and GluR1 is a postsynaptic density (PSD) protein, and both proteins were strongly enriched after fraction. Syntaxin1 is ubiquitously distributed on subcellular membranes, which showed no differences between homogenate and synaptosome fractions. Tpi1 was greatly decreased after fraction implying a high degree of purity in the cerebral cortex synaptosome samples.[21]

Differential Expression of Synaptosome Proteins Induced by MK-801

Cerebral cortex synaptosome samples from ten 21-day MK-801–treated rats, ten 7-day MK-801–treated rats, and 10 normal control rats were analyzed using 2D-DIGE (figure 2). The 2D-DIGE results were analyzed on Decyder 6.0 software. Significantly altered spots ($P < .05$) identified by 1-way ANOVA were picked from the silver-stained gels (figure 3) for protein identification. Forty-nine significantly altered proteins were identified (table 1). Among them 32 were differentially expressed proteins of the 7-day MK-801–treated group and 24 were differentially expressed proteins of the 21-day MK-801–treated group. Thirteen proteins (Dnm1, Hsph1, Dlst, Pcmt1, Crmp5, Lonp1, Ndufs3, Eno2, Camk2a, Ap2b1, Uqcrc1, LOC729708, and Sucla2) were altered in both treatment groups. Five proteins (Phgdh, Napa, Cndp2, Gnao1, and Ckb) were differentially expressed between the 7-day MK-801 treatment group and the 21-day MK-801 treatment group.

A comprehensive literature search was undertaken to obtain the subsynaptic location information of these

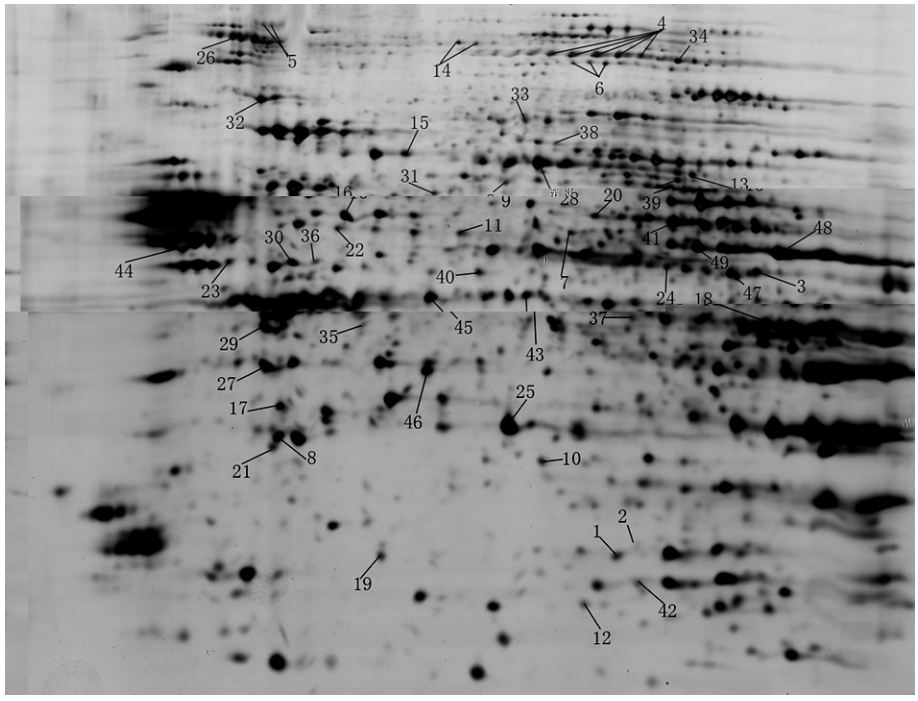

Fig. 3. The Silver-Stained Gel of Rat Synaptosome Samples for MS Identification. Proteins (400 μg) were separated on a 24-cm immobilized pH gradient strip (pH 3–10 nonlinear) as the first dimension and 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as the second dimension.

Table 1. Synaptosome Proteins Altered by Subchronic MK-801 Treatment

	Accession no. NCBI	Gene Name	Mascot Score	Theoretical Molecular Weight (Da)/PI	7-Day Group		21-Day Group		ANOVA	Presynaptic Proteins	Postsynaptic Proteins	Synaptic Complex
					FC	*P* Value	FC	*P* value				
1	gi|114326546	Pgam1	119	28813.9/6.67	−1.36	.00001	1.03	.674	0.00002	√[22]	√[21]	
2	gi|157824168	Hdhd3	89	27776.2/6.5	1.17	.000031	1.04	.2	0.00011			
3	gi|55741424	Ndufv1	248	50698.6/8.37	−1.21	.000034	−1.06	.151	0.00017	√[22]		
4	gi|18093102	Dnm1	351	95867.2/6.32	1.2	.000097	1.18	.00022	0.00017	√[22–25]	√[21]	SV[25]
5	gi|58865372	Hsph1	102	96357.4/5.4	1.12	.0006	1.11	.00666	0.00064		√[21]	
6	gi|158187544	Pygb	442	96676.5/6.31	1.14	.018	1	.91	0.001	√[25]	√[21]	SV[25]
7	gi|201066380	Fscn1	239	54456.9/6.29	−1.03	.24	−1.09	.0022	0.0012		√[21], PSD-95–associated[26]	
8	gi|205829956	Napb	275	33448.5/5.32	1.07	.033	−1.05	.114	0.0013	√[23,25]	√[21]	SV[25]
9	gi|25742568	Crmp4	99	61928/6.04	1.18	.00042	1.07	.137	0.00178	√[23]		
10	gi|8393962	Pitpna	278	31887.1/5.97	1.1	.0031	−1.01	.772	0.00223			
11	gi|195927000	Dlst	113	48868.4/8.9	−1.26	.0028	−1.2	.0084	0.00226		√[21]	
12	gi|56961640	Pcmt1	136	24625.7/7.14	1.14	.00055	1.09	.0368	0.00252			
13	gi|6714522	Crmp5	85	61356.1/6.5	1.16	.0026	1.09	.035	0.00486		√[21]	
14	gi|19173766	Lonp1	204	105726.2/6.17	−1.18	.005	−1.12	.0026	0.0052			
15	gi|78365255	Dlat	180	67123.4/8.76	−1.14	.0096	−1.04	.334	0.0065	√[22,25]	√[21]	SV[25], MASC[21], NRC[27]
16	gi|17105370	Atp6v1b2	245	56514.9/5.57	1.14	.006	1.04	.362	0.007	√[22–25]	√[21]	SV[25]
17	gi|16758840	Crym	212	33533.1/5.34	1.11	.022	1.01	.585	0.0071		PSD-95–associated[26]	
18	gi|60678254	Ckmt1	111	46932.2/8.58	−1.09	.075	−1.14	1.0×10^{-5}	0.0073	√[22,25]	√[21]	SV[25]
19	gi|157817227	Ndufs3	205	28028.2/6.31	−1.07	.039	−1.15	.00046	0.0079	√[22,24]	√[21]	
20	gi|13928850	Phgdh	92	56457.2/6.28	1.12	.11	−1.05	.401	0.0081			
21	gi|18034791	Napa	120	33171.2/5.3	1.07	.071	−1.06	.169	0.0083	√[22,23]	√[21]	
22	gi|58219062	Cndp2	193	52659.6/5.43	1.06	.12	−1.06	.188	0.009			
23	gi|26023949	Eno2	173	47110.9/5.03	1.2	.0078	1.14	.0112	0.0098	√[23]	√[21]	
24	gi|6978593	Camk2a	78	54080.6/6.61	−1.09	.042	−1.13	.0084	0.011	√[22–25]	√[21], PSD-95-associated[26]	SV[25], MASC[21], NRC[27]
25	gi|15100179	Mdh1	178	36460.1/6.15	1.1	.014	1.02	.694	0.0119	√[23]		
26	gi|4557469	Ap2b1	208	104486/5.22	1.11	.012	1.06	.0459	0.0128	√[22–25]	√[21]	SV[25], MASC[21]
27	gi|8394152	Gnao1	337	40042.9/5.34	1.07	.11	−1.05	.203	0.0129	√[22,23,25]	√[21], PSD-95-associated[26]	SV[25], MASC[21]
28	gi|40254595	Crmp2	328	62238.6/5.95	1.1	.041	1.08	.057	0.0135	√[22–24]	√[21]	MASC[21]
29	gi|160406738	Sh3gl2	218	39874/5.26	1.11	.035	1	.968	0.0136	√[22,24]		
30	gi|51948476	Uqcrc1	148	52815.4/5.57	−1.11	.011	−1.09	.0067	0.0067		√[21]	
31	gi|157819689	Sept8	287	49825.2/5.68	1.12	016	−1.02	.693	0.0032	√[24]	√[21]	
32	gi|53850628	Ndufs1	546	79361.6/5.65	−1.09	.18	−1.15	.0011	0.0156		√[21]	
33	gi|6980978	Gpd2	236	80921.3/6.18	1.02	.75	−1.12	.0158	0.0168		√[21]	
34	gi|8393296	Eef2	226	95222.9/6.41	1.09	.011	1.01	.759	0.017	√[22,24]	√[21]	
35	gi|203476	Ckb	242	40598.3/5.32	−1.06	.29	1.09	.196	0.0176	√[22,24,25]	√[21]	SV[25]
36	gi|148747414	Gda	286	50868.7/5.48	−1.13	.034	−1.03	.579	0.0179		PSD-95–associated[26]	
37	gi|38181948	Glul	268	42241.3/6.47	1.18	.0056	1.03	.736	0.0179	√[23]	√[21], PSD-95–associated[26]	MASC[21]
38	gi|18426858	Sdha	251	71569.7/6.75	−1.01	.82	−1.1	.0108	0.0188		PSD-95–associated[26]	
39	gi|158186661	Crmp3	191	61940.6/6.51	1.05	.28	−1.08	.029	0.0198	√[25]	√[21]	SV[25]
40	gi|40254781	Gdi2	286	50504.7/5.93	1.09	.047	−1.03	.544	0.02			
41	gi|40786469	Dld	64	54004.1/7.96	−1.1	.082	−1.11	.0079	0.0232		√[21]	
42	gi|109505678	LOC729708	84	26919.8/6.45	1.09	.016	1.1	.01	0.0251			
43	gi|22122615	Actr1b	111	42254.7/5.98	1.06	.012	1.01	.638	0.0251			

Table 1. Continued

	Accession no. NCBI	Gene Name	Mascot Score	Theoretical Molecular Weight (Da)/PI	7-Day Group		21-Day Group		ANOVA	Presynaptic Proteins	Postsynaptic Proteins	Synaptic Complex
					FC	*P* Value	FC	*P* value				
44	gi|1374715	Atp5b	267	51170.6/4.920	−1.14	.05	1.02	.645	0.0281	√[22,23]	√[21], PSD-95–associated[26]	
45	gi|158749584	Sucla2	160	50274.1/7.57	−1.12	.024	−1.1	.0076	0.0287		√[21], PSD-95–associated[26]	
46	gi|16758446	Idh3a	64	39588/6.47	−1.08	.052	−1.1	.0003	0.0301	√[22]	√[21]	
47	gi|109468279	Mettl8	67	52417.4/8.94	−1.14	.066	−1.19	.0009	0.0303			
48	gi|149015801	Rbmx	76	27913.1/10.03	−1.1	.1	−1.14	.00243	0.046			
49	gi|6980956	Glud1	210	61377.3/8.05	−1.08	.342	−1.17	.00021	0.032	√[22,25]	√[21]	SV[25]

Note: NCBI, National Center for Biotechnology Information; FC, fold change; PSD-95, postsynaptic density-95; SV, synaptic vesicle; NRC, NMDA receptor complex; MASC, combines proteins found in both NRC and membrane-associated guanylate kinase (MAGUK) complex.[21]

differentially expressed proteins (table 1). Twenty-six proteins are involved in presynaptic terminals and 31 proteins are involved in postsynaptic terminals. Nine proteins are associated with PSD-95, which is one of the most abundant scaffold proteins at excitatory brain synapses. Twelve proteins are components of SV. Dlat, Camk2a, Ap2b1, Gnao1, Crmp2, and Glul participate in membrane-associated guanylated kinase-associated signalling complex (MASC) while MASC combines proteins involved in NMDA receptor complex and membrane-associated guanylate kinase (MAGUK) complex.[21] Among the 6 MASC proteins, Dlat and Camk2a are components of NMDA receptor complex.[27]

We also carried out a literature search to explore the relevance of the 49 differentially expressed proteins to neurological and psychiatric diseases. Eighteen genes/proteins were identified as being implicated in neurological and psychiatric diseases: 7 in schizophrenia, 6 in Alzheimer's disease, and 5 in major depression and other diseases (table 2). Twenty-five of the altered proteins have been found differentially expressed in the postmortem brains of schizophrenia patients (table 2).

IPA Pathways and Network Function Analysis

To better understand the biological function involved in the differentially expressed proteins and protein interactions between these proteins, the altered proteins in the 7-day and 21-day treatment groups were subjected to IPA analysis, respectively.

The most significant network in both the 7-day and the 21-day MK-801–treated groups was "cell morphology, cell-to-cell signaling and interaction, nervous system development, and function," with scores of 21 and 28, respectively (table 3a). The second most significant network in the 7-day MK-801–treated group was "cellular function and maintenance, lipid metabolism, neurological disease," with a score of 7. Figure 4A shows the merged figure of the top 2 significant networks in the 7-day MK-801–treated group, which incorporates 15 of the 32 proteins eligible for analysis (Sh3gl2, Gnao1, Eno2, Dlst, Lonp1, Ap2b1, Dlat, Camk2a, Atp5b, Pgam1, Dnm1, Dpysl2 [Crmp2], Hsph1, Uqcrc1, and Ndufs3). Only 2 of them, Lonp1 and sh3gl2, have not been found in postsynaptic terminals. Figure 4B shows the figure for the most significant network in the 21-day MK-801–treated group. It includes 12 proteins (Uqcrc1, Dnm1, Camk2a, Lonp1, Eno2, Dlst, Ap2b1, Hsph1, Ndufs1, Idh3a, Ndufs3, and Sdha) of the 24 differential expressed proteins identified. Within this protein-protein interaction network, only Lonp1 has not so far been detected in any postsynaptic terminals. Furthermore, all the kinases (CDK5, GSK3β, CAMK2A, and CAMK2B in figure 4A; GSK3β, CAMK2A, and CAMK2B in figure 4B) and receptors (GRIN2A and GRIN2B) participating in the function networks are involved in the postsynaptic phosphoproteome network.[55] The subcellular location of the proteins in the network supports the contention that

Table 2. Genes Association and Expression Related with Neurological And Psychiatric Diseases

Gene Name	Related with Neurological and Psychiatric Diseases	Postmortem Proteomic Research of Schizophrenia
Pgam1		Wernicke's area,[28] ACC white[29]
Ndufv1	Schizophrenia, bipolar disorder, major depression[30]	
Dnm1	Exercise-induced collapse[31]	ACC,[32] DLPFC[12,33,34]
Pygb		ACC white[29]
Fscn1		DLPFC white[33]
Crmp4		DLPFC[12]
Dlst	Alzheimer's disease[35]	
Pcmt1		DLPFC,[34] DLPFC white[36]
Crmp5		DLPFC white,[36] DLPFC[33]
Atp6v1b2	Major depressive disorder[37]	ACC white[29]
Crym	Alzheimer's disease[38]	DLPFC[34,39]
Ckmt1		ACC[32]
Ndufs3	Leigh syndrome[40]	
Phgdh		DLPFC[33]
Eno2	Schizophrenia[41]	DLPFC,[12] Wernicke's area[28]
Camk2a	Alzheimer's disease[42]; severe depression[43]	
Mdh1	Schizophrenia[44]	DLPFC,[33,34] DLPFC white[33]
Gnao1	Schizophrenia[44]	
Crmp2	Schizophrenia[45]	DLPFC,[33,34] Wernicke's area,[28] layer 2 IC,[46] ACC,[32,47] ACC white[29]
Sh3gl2		DLPFC[33,39]
Uqcrc1		DLPFC white,[33] DLPFC[33,34,39]
Ndufs1	Schizophrenia, bipolar disorder, major depression[30]; Leigh syndrome[48]	DLPFC,[33] DLPFC white[33]
Gpd2	Nonsyndromic mental retardation[49]	
Eef2	Alzheimer's disease[50]	
Ckb	Parkinson's disease[51]	Wernicke's area,[28] ACC,[32,47] DLPFC[33,39]
Gda		DLPFC,[12] layer 2 IC[46]
Glul	Alzheimer's disease, major depressive disorder[52]	DLPFC[33]
Sdha	Schizophrenia[53]	ACC white[29]
Crmp3		DLPFC[33]
Dld	Alzheimer's disease[54]	Wernicke's area[28]
Atp5b		Layer 2 IC[46]
Idh3a		ACC[32]

Note: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; IC, insular cortex.

the biological progress of "cell morphology, cell-to-cell signaling and interaction, nervous system development, and function" happens in post synapses.

The top 4 canonical pathways of the 2 treatments are shown in table 3b. These were "citrate cycle, glycolysis/gluconeogenesis, oxidative phosphorylation, and semaphorin signaling in neurons" in the 7-day MK-801 treatment group and "citrate cycle, mitochondrial dysfunction, oxidative phosphorylation, and glycolysis/gluconeogenesis" in the 21-day MK-801 treatment group. Semaphorin signaling is important in axon growth and guidance and regulates the morphology of synapses.[56] The treatment of MK-801 may significantly disturb the signaling of semaphorin.

Western Blot Validation of Selected Proteins

Based on the IPA analysis, we chose 5 proteins for the western blot assay. Altered proteins common to the 7-day and 21-day MK-801 treatment groups were given priority. Camk2a, Dnm1, and Ndufs3 were chosen because of their presence in the networks (figure 4). Camk2a is a key node in the networks, and Ndufs3 is a core subunit of the mitochondrial membrane respiratory chain complex I. Crmp2 and Crmp5 are components of semaphorin signaling in neurons. We confirmed the expression of the 5 proteins both in cerebral cortex synaptosome samples and in total cerebral cortex protein samples (figure 5). Camk2a was greatly downregulated

Table 3. Ingenuity Pathway Analysis of Differentially Expressed Proteins. (a) Top Networks and Associated Functions Involved in the Effect of MK-801. (b) Top Canonical Signaling Pathways Involved in the Effect of MK-801

(a) Top Networks

	Associated network functions	Score
21-Day treatment	Cell morphology, cell-to-cell signaling and interaction, nervous system development and function	28
	Carbohydrate metabolism	2
	Endocrine system disorders, hematological disease, metabolic disease	2
	Cancer, tumor morphology, cell death	2
7-Day treatment	Cell morphology, cell-to-cell signaling and interaction, nervous system development and function	21
	Cellular function and maintenance, lipid metabolism, neurological disease	7
	Carbohydrate metabolism	2
	Cancer, cell cycle, cell morphology	2
	Amino acid metabolism, molecular transport, small molecular biochemistry	2

(b) Top Canonical Pathways

7-Day treatment			21-Day treatment		
	P value	Ratio		*P* value	Ratio
Citrate cycle	2.56×10^{-5}	3/31 (0.097)	Citrate cycle	2.84×10^{-10}	6/31 (0.194)
Glycolysis/ gluconeogenesis	2.74×10^{-5}	4/100 (0.04)	Mitochondrial dysfunction	2.07×10^{-8}	6/132 (0.045)
Oxidative phosphorylation	1.97×10^{-4}	4/151 (0.026)	Oxidative phosphorylation	3.76×10^{-5}	4/151 (0.026)
Semaphorin signaling in neurons	2.09×10^{-4}	3/55 (0.055)	Glycolysis/gluconeogenesis	2.1×10^{-4}	3/100 (0.03)

after the MK-801 treatment both in the synaptosomal fraction and in the cerebral cortex. In the 2 MK-801–treated groups, western blotting confirmed that Dnm1 was upregulated in both synaptosome proteins and total proteins of the cerebral cortex. The upregulation of Crmp5 in synaptosome proteins (in both the 7-day and the 21-day MK-801 treatment groups) was replicated by western blot analysis, whereas this protein showed no change in total cerebral cortex proteins after MK-801 injection. Ndufs3 was decreased in synaptosomal proteins while increased in total proteins of the cerebral cortex. Western blot revealed that Crmp2 was increased in the synaptosome proteins of both treatment groups (figure 5) but was decreased (fold change = 0.68, $P = .0052$) in the total proteins from the cerebral cortex of the 21-day MK-801–treated rats. This reversal of change patterns for Crmp2 and Ndufs3 in synaptosomes and total cerebral cortex after MK-801 treatments may be caused by the intracellular redistribution of these proteins. Apart from the increasing trend of Crmp2 in the 21-day treatment group, all the alterations observed in the 2D-DIGE were confirmed by western blot. 2D-DIGE analysis found that Crmp2 was significantly increased ($P = .041$) in the synaptosomal proteins of the 7-day MK-801–treated rats but revealed only trend ($P = .057$) elevation in the synaptosomal proteins of the 21-day MK-801–treated group.

Discussion

The present study is the first synaptosomal proteomic research on MK-801–treated rats. Using 2D-DIGE, we compared synaptosome protein profiles in 21-day MK-801–treated rats, 7-day MK-801–treated rats, and a control group of rats and identified 49 differentially expressed proteins. Among these altered proteins, 32 were altered after 7 days of MK-801 treatment, and 24 were altered after 21 days of MK-801 treatment. The subsynaptic locations and diseases relevance of the altered proteins were confirmed by literature search.

IPA was carried out to determine the underlying relationship between the differentially expressed proteins. The most significantly disturbed network in both the 7-day MK-801 treatment and the 21-day MK-801 treatment groups was "cell morphology, cell-to-cell signaling and interaction, nervous system development, and function." Given that MK-801 is a specific NMDA receptor antagonist that induces hypofunction of glutamate transmission, it was not unexpected to find GRIN2A and GRIN2B (both are NMDA receptors) in the network. CAMK2A and HTT were 2 key nodes in the MK-801 treatment–related network. CAMK2 is an important protein kinase, which plays a key role in long-term potentiation and neurotransmitter release. The phosphorylation status of NMDA receptors regulates its interaction with CAMK2.[57] It has been reported that increased Camk2 activity enhances the spatial cognitive function in SAMP6 mice.[58] Camk2a is 1 of the 4 subunits of Camk2, and deficiency of Camk2a results in abnormal fear response and aggressive behavior in mutant mice.[59] The downregulation of Camk2a in the synapse of MK-801–treated rats may result in impaired cognitive and emotional function. HTT is a key protein

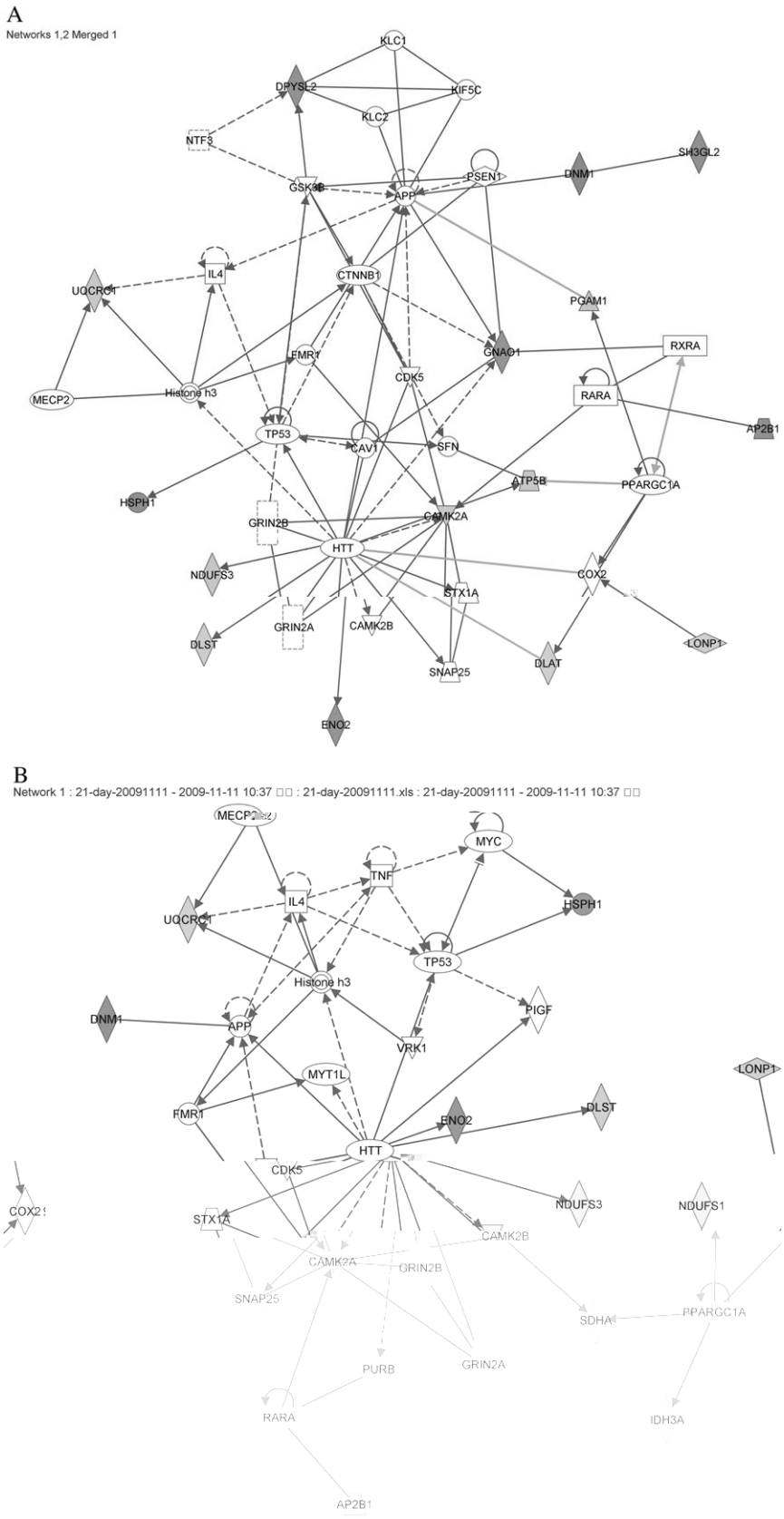

Fig. 4. Top Networks Associated With MK-801 Treatment Identified by Ingenuity Pathway Analysis. Protein symbols with red were upregulated while green were downregulated. Hub proteins not significantly altered in this study are shown as clear. (A) The merged network of 2 top networks associated with 7-day MK-801 treatment. (B) The top network associated with 21-day MK-801 treatment.

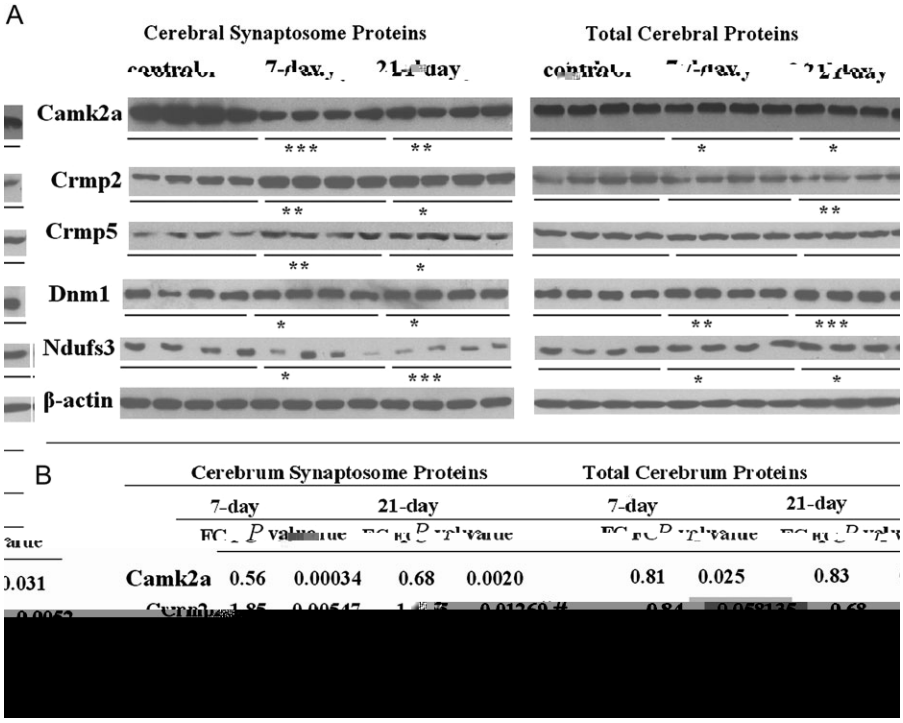

Fig. 5. Western Blot Analysis Confirming Results of 2-Dimensional Difference Gel Electrophoresis. (A) Western blot results of Camk2a, Crmp2, Crmp5, Dnm1, and Ndufs3 in cerebral synaptosome proteins and total cerebral proteins of the control, 7-day MK-801–treated, and 21-day MK-801–treated groups. (B) The fold changes and *P* values of western blot results. FC, fold change. #, not consistent with 2-dimensional difference gel electrophoresis result. *$P < .05$; **$P < .01$; ***$P < .001$.

involved in Huntington's disease. Inhibition of the extra function of NMDA receptors reduces cytotoxicity induced by mutant HTT.[60] Normal HTT is essential for nervous system functions. Deduced normal HTT content results in abnormal brain development[61] and reduced brain-derived neurotrophic factor vesicular transport.[62] Overexpression of wild-type HTT protects neurons against NMDA receptor–mediated apoptotic neurodegeneration.[63] The relationship between HTT and schizophrenia has not so far been the subject of published research and is worth investigating.

A number of mitochondrial proteins (NDUFS1, NDUFS3, SDHA, IDH3A, and UQCRC1 in the 21-day treated group and NDUFS3, UQCRC1, ATP5B, and DLAT in the 7-day treated group) featured in the MK-801 treatment–related network. IPA analysis also pointed to energy metabolism (''citrate circle, glycolysis/gluconeogenesis, mitochondrial dysfunction, and oxidative phosphorylation'') as top canonical pathways. Paulson et al studied the impact of MK-801 on cerebral cortex protein expression in rats.[15] Among the 10 differentially expressed proteins in Paulson's research, 4 ATP synthase beta subunit (Atp5b), pyruvate dehydrogenase lipoamide, and mitochondrial hsp70 and hsp60) were mitochondrial function related. This suggests a dysfunction of mitochondria in MK-801–treated rats and is consistent with our findings. Atp5b was the common differentially expressed protein in Paulson's research and our own. Atp5b combines with the other subunits of mitochondrial ATP synthase to form a complex that catalyzes ATP formation during oxidative phosphorylation. A number of other studies have reported that mitochondrial dysfunction plays an important role in schizophrenia and some other psychiatric disorders.[64,65] Aberrations in energy metabolism may potentially lead to neurodevelopment deficits and failure to maintain synaptic connection in mature nervous systems.[66] A desirable goal for new antipsychotic drugs would be to optimize energy metabolism.[66]

Top canonical pathway analysis of the 7-day MK-801–treated rats indicated enrichment of semaphorin signaling, another important pathway. Eastwood et al[67] found that semaphorin 3A was increased in the cerebellum in schizophrenia patients and the semaphorin receptor PLXNA2 has been identified as a susceptibility gene for the disease.[68] In our study, we found that 4 semaphorin signaling–related proteins (Crmp2, Crmp3, and Crmp5 in the 7-day MK-801–treated group; Crmp2, Crmp4, and Crmp5 in the 21-day MK-801–treated group) were altered in the MK-801–treated rats. The differential expressions of Crmp2 and Crmp5 were confirmed by western blot assay. CRMP2 has been found to be upregulated in the anterior cingulate cortex[47] while downregulated in layer 2 of the insular cortex[46] of

schizophrenia patients. The variation in Crmp2 expression in different brain regions may contribute to the occurrence of schizophrenia. Detailed research on semaphorin signaling in different brain regions of schizophrenia patients will be valuable.

There are some other interesting protein components in the network. Dynamin 1 (Dnm1) is a GTPase, which is essential for SV fission. It was altered both in the 7-day and in the 21-day treatment groups. This protein is increased in the dorsolateral prefrontal cortex[33] and anterior cingulate cortex[32] of schizophrenia patients. Changes in the level of Dynamin 1 are induced by beta-amyloid and are correlated with memory impairment in vivo.[69] Peroxisome proliferator-activated receptor gamma coactivator 1-alpha is a transcriptional coactivator for steroid receptors and nuclear receptors, which increases the activity of peroxisome proliferator-activated receptor gamma and thyroid hormone and regulates some key mitochondrial genes. Retinoic acid receptor, alpha, serves as a receptor for retinoic acid. Substantial evidence suggests that retinoid dysfunction is involved in the pathogenesis of schizophrenia.[70,71] Retinoid and its agonists have been proposed as candidates in schizophrenia treatment.[72–74]

In summary, the present synaptosome proteomic study on subchronic MK-801–treated rats detected a series of proteins that have also been highlighted in postmortem brain research in schizophrenia patients. The IPA pathway analysis of these proteins revealed a comprehensive network disturbance and dysfunction of energy metabolism and semaphorin signaling induced by NMDA receptor hypofunction. These findings should help in the understanding of the molecular mechanism of schizophrenia and offer some hits in the search for potential antipsychotic drug targets. Given the variety of aberrations in different brain areas of rodents and humans, more detailed and brain area–specific proteomic research into subchronic MK-801–treated rats is needed.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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