Prefrontal cortex shotgun proteome analysis reveals altered calcium homeostasis and immune system imbalance in schizophrenia

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ORIGINAL PAPER

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Prefrontal cortex shotgun proteome analysis reveals altered calcium homeostasis and immune system imbalance in schizophrenia

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Abstract Schizophrenia is a complex disease, likely to be caused by a combination of serial alterations in a number of genes and environmental factors. The dorsolateral prefrontal cortex (Brodmann's Area 46) is involved in schizophrenia and executes high-level functions such as working memory, differentiation of conflicting thoughts, determination of right and wrong concepts and attitudes, correct social behavior and personality expression. Global proteomic analysis of *post-mortem* dorsolateral prefrontal cortex samples from schizophrenia patients and non-schizophrenic individuals was performed using stable isotope labeling and shotgun proteomics. The analysis resulted in the identification of 1,261 proteins, 84 of which showed statistically significant differential expression, reinforcing previous data supporting the involvement of the immune system, calcium homeostasis, cytoskeleton assembly, and energy metabolism in schizophrenia. In addition a number of new potential markers were found that may contribute to the understanding of the pathogenesis of this complex disease.

Key words schizophrenia · proteomics · shotgun · prefrontal cortex · biomarkers

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Introduction

Previous studies of global gene expression in different brain regions of schizophrenia (SCZ) patients revealed dysfunctions in synaptogenesis and neural plasticity, energy metabolism, cytoskeleton assembly and oligodendrocyte metabolism [8, 10, 41, 50, 67, 69, 92, 93]. Since gene fluctuations are not always directly correlated with a differential protein expression, gene expression studies are nicely complemented by proteomics. A few proteome studies were performed in distinct brain regions such as anterior cingulate cortex and the corpus callosum [13, 30, 73, 75, 78, 86].

The prefrontal cortex (PFC) is the anterior region of frontal lobes located above the motor and premotor areas, and it is the neocortical region that is most elaborated in primates in order to provide a diverse and flexible repertoire of behaviors. Divided into dorsolateral, orbitofrontal, and medial areas, PFC functions are neurologically denoted as "Executive Functions". These functions include differentiation of conflicting thoughts, determination of good and bad perspectives in accordance with determined actions,

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moderating correct social behavior, future consequences of current activities, as well as working memory. An important function influenced by PFC is personality expression. Basically, the activities of this region are the organization of thoughts and actions according to internal aims (cognitive control) [61, 68]. Dysfunction of the dorsolateral prefrontal cortex (DLPFC) has been implicated in the pathophysiology of SCZ [12, 98]. SCZ hallmarks such as differential eye and hand movements probably are dysfunctions in frontal cortical circuits [80].

In the current work, a shotgun proteomic analysis of the DLPFC of SCZ and control samples was performed and a quantitative analysis was done using Isotope-Coded Protein Label (ICPL), a method for the accurate quantitative comparative analysis of protein regulation [82], aiming the identification of proteins differentially expressed.

Defining a set of proteins that are consistently altered in this disease and define specific pathways altered in SCZ will be valuable not only for a better understanding of the biological basis of the disease, but also for drug development efforts as well as the determination of potential protein markers to the diagnosis.

Materials and methods

Materials

All chemicals and solvents were from Bio-Rad (Hercules, CA, USA) and of the highest purity available. The ICPL kit was from Serva Electrophoresis (Heidelberg, Germany) and Prespotted AnchorChips were obtained from Bruker Daltonics (Bremen, Germany).

Human dorsolateral prefrontal cortex samples

Post-mortem brain samples from the DLPFC tissue (BA46) were collected from 9 schizophrenia patients and 7 controls, who were free from psychiatry disorders, somatic diseases or brain tumors and were never treated with antidepressant or antipsychotic medications. Brain samples were dissected by an experienced neuropathologist (on average 24.3 h after death) and deep-frozen immediately after collection.

All samples were obtained from the brain bank of the Central Institute of Mental Health (Mannheim, Germany). Controls were collected at the Institute of Neuropathology, Heidelberg University, and their clinical records were collected from their relatives and general practitioners. Patient samples derived from in-patients of the Mental State Hospital Wiesloch, Germany. All cases and controls were German whites. All SCZ patients have been long-term inpatients at the Mental State Hospital Wiesloch, Germany, and the diagnosis of schizophrenia was made *ante mortem* by an experienced psychiatrist according to the DSM IV criteria [7]. For each patient the antipsychotic treatment history was assessed by examining the medical charts and calculated in chlorpromazine equivalents (CPE), through the algorithm developed by Jahn and Mussgay [45]. All patients and controls underwent neuropathologic characterization to rule out associated neurovascular or neurodegenerative disorders. The classification according to Braak was stage II or less for all subjects [19, 20]. Patients and controls had no history of alcohol, drug abuse, or severe physical illness. All assessment and *post mortem* evaluations and procedures were previously approved by the ethics committee of the Faculty of Medicine of Heidelberg University, Germany. Detailed patient information are given in Table 1.

Sample preparation

Fifty milligrams of human DLPFC (gray matter) were individually homogenized in 1.5 ml tubes with glass spheres in 200 µl of 6 M Guanidine·HCl and 0.1 M HEPES buffer. Samples were centrifuged for 10 min at 14,000 rpm and quantified [22] to prepare equimolar pools. To achieve a similar final quantity of protein (100 µg), control pools were made with 14.3 µg of protein from each of the seven samples, whereas SCZ pools were made with 11.1 µg of protein from each of the nine samples.

ICPL labeling

One hundred micrograms of total protein from SCZ or controls (5 mg/ml) were reduced for 30 min at 60°C, as specified by the ICPL kit protocol. After cooling to room temperature (RT), free thiol groups were alkylated in the dark with 1 ml of 0.4 M iodoacetamide for 30 min at RT. Excess iodoacetamide was quenched by adding 1 ml of 0.5 M *N*-acetylcysteine. For protein labeling, a 10-fold molar excess (based on free amino groups) of light tag for the control sample and heavy tag for the SCZ samples were added to the proteins and the reactions were allowed to proceed for 2 h at RT. Four mililiter of 1.5 M hydroxylamine were added to each sample to inactivate the remaining Nic-NHS reagents, and equal aliquots of both samples were combined. Esters, which are also formed during the labeling procedure, were hydrolyzed by raising the pH to 11–12 for 20 min.

Digestion of labeled proteins and fractionation of peptides by isoelectric focusing

Protein samples were digested in 200 mM NH_4HCO_3, pH 8.3, with 1 mg/ml trypsin at a ratio 1:50 (P:E) at 37° C for 4 h. Resulting peptides were fractionated on Immobilized pH gradient strips (IPG 17 cm), pH 3.5–4.5. The strips were rehydratated for 12 h and run for 8 h with a constant voltage of 10,000 V. The strip was manually cut in 47 pieces and the peptides were extracted with 1% formic acid.

Fractionation of peptides by nano high performance liquid chromatography

Each of the 47 peptide samples from isoelectric focusing was further fractionated on a micro-LC-System (HP1100 Agilent Technologies, Waldbronn, Germany) using an RP-C-18 monolithic column (200 µm id. × 5 cm, Dionex, Sunnyvale, CA) with a flow rate of 4 µl/min and a 40 min gradient from 10 to 100% of solvent B (ACN; 0.1% TFA). Each isoelectric focusing eluate was chromatographed and fractions were collected onto Prespotted AnchorChip targets (Bruker Daltonics) using a PROTEINEER-FC robot (Bruker Daltonics).

Mass spectrometry

Mass spectra from each target spot were acquired using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in fully automatic mode. Measurements were performed with a nitrogen laser in positive reflector mode and a 20,000 V acceleration voltage. Hundred shots and 1,000 shots were accumulated, respectively, for MS spectra and for MS/MS spectra. WARP-LC 1.0 software was used for spectra acquisition and to control the automatic selection of peptides for further MS/MS analysis. The ICPL–labeled peptides were selected for MS/MS analysis based on their H/L ratio.

Table 1 Patient and control clinical data

Sample ID	Case	Age (years)	Gender	PMI (hours)	Type of SCZ	Duration of disease (years)	Duration of medication (years)	atyptyp	CPE last dosis	CPElifetime	Cause of death	DSM IV	Age at onset	Last medication	Cigarettes	Alcohol	Hosp	ECT
13/00	SCZ	64	F	11	Residual, Chronic Paranoid episodes	48	45	3	1,536	7.7	Pulmonary insufficiency	295.6	16	Clozapine 500 mg, haloperidol 40 mg, ciatyl 40 mg	0	No	21	Yes
36/02	SCZ	73	M	20	Residual, chronic Paranoid episodes	43	40	1	507.4	1.7	Heart infarction	295.6	30	Perphenazine 32 mg, promethazine 150 mg	30/day	No	33	No
39/02	SCZ	43	M	18	Residual, chronic paranoid episodes	22	20	2	464	2.6	Heart infarction	295.6	20	Zuclopethixol 40 mg, valproate 1,200 mg, tiapride 300 mg	0	No	13	No
39/03	SCZ	77	F	32	Residual, chronic Paranoid episodes	49	48	2	2,555	8.3	Lung emboly	295.6	28	Clozapine 400 mg, benperidol 25 mg, chlorprothixen 150 mg	0	No	48	Yes
43/03	SCZ	76	F	17	Residual, chronic Paranoid episodes	49	47	1	300	4.9	Cardio-pulmonary insufficiency	295.6	27	Perazine 300 mg	0	No	30	Yes
46/00	SCZ	63	F	31	Residual, chronic paranoid episodes	40	30	3	75	1.8	Heart infarction	295.6	24	Olanzapine 15 mg	30/day	No	30	Yes
50/01	SCZ	81	M	4	Residual, chronic paranoid episodes	62	50	1	92.8	1.4	Cor pulmonale, heart insufficiency	295.6	19	Haloperidol 4 mg, prothypendyl 80 mg	20/day	No	48	No
75/02	SCZ	92	F	37	Residual, chronic paranoid episodes	51	48	1	100	3.4	Pancreas-carcinoma	295.6	41	Prothipendyl 160 mg, perazine 100 mg	0	No	51	No
83/01	SCZ	71	M	28	Residual, chronic paranoid episodes	40	35	1	782.4	10	Heart infarction	295.6	30	Haloperidol 32 mg, pipamperone 40 mg	40/day	No	12	No
02/02	Control	41	M	7							Heart infarction				0	No		
43/01	Control	91	F	16							Cardio-pulmonary insuffiency				0	No		
50/02	Control	69	F	96							Lung emboly				0	No		
51/02	Control	57	M	24							Heart infarction				0	No		
57/02	Control	53	M	18							Heart infarction				0	No		
59/02	Control	63	M	13							Heart infarction				0	No		
61/01	Control	66	M	16							Heart infarction				0	No		

atyptyp duration of atypical treatment/duration of treatment with typical neuroleptis during lifetime, *CPE* medication calculated in chlorpromazine equivalents (mg), *CPE last 10 years* the sum of medications during the last 10 years in kg, *Hosp* hospitalization time in years, *ECT* electroconvulsive therapy

Protein identification

Acquired MS and MS/MS spectra were automatically sent as combined peak lists by the WARP-LC 1.0 to Biotools software 3.0 (Bruker Daltonics) and searched against the NCBI database (Dec.16th, 2006) using an in-house version of MASCOT 2.1 (Matrix Science, London, UK). The parameter settings were as follows: *Homo sapiens* for organism, trypsin and Lys-C for enzymes (considering 1 missed cleavage), carbamidomethylation as fixed modification and oxidized methionine and heavy and light ICPL labels of lysines and N-terminal protein as variable modifications.

ICPL quantitative analysis

The determination of the ratios of isotope-labeled peptide pairs (heavy and light) was performed by the WARP-LC 1.0 Protein Browser (Bruker Daltonics), comparing the relative heavy and light cluster signal intensities. The identified heavy and light peptide-pair sequences containing up to four labeled lysines with a mass difference of 6.0204 Da per labeled amino group were obtained by BioTools 3.0. The workflow of protein shotgun mass spectrometry and ICPL-quantitation of differentially expressed proteins is shown in Fig. 1.

Determination of regulated proteins in SCZ samples

Three parameters were applied to determine the putative regulated proteins:

1) BioTools software provides for each identified protein a MASCOT score value that is derived from the peptide hit scores. Significant identifications were considered only for peptide scores greater than 38.
2) We only considered proteins identified with two or more peptides. Proteins identified by a single peptide were considered provisional.
3) The regulation status of a protein was defined by the ratio of the relative peptide signal intensities. SCZ proteins were labeled with heavy tags and control proteins with light tags. The proteins in both samples generated the same peptides after digestion, but peptides from SCZ samples had an approximately 6 Da greater mass per labeled amino group than peptides labeled with light tags. In the analysis, a minimum of 40% variation was considered as significant regulation. Thus, proteins considered upregulated in SCZ had ratios ≥1.4, whereas downregulated proteins had ratios ≤0.6. Ratio values between 0.6–0.8 and 1.2–1.4 were considered borderline.

Results

Protein regulation in schizophrenia dorsolateral prefrontal cortex

Shotgun mass spectrometry allowed the analysis of 2,541 peptide sequences (Fig. 2a), leading to the identification of 1,261 proteins in DLPFC (Fig. 2b); 634 proteins (50.28%) were identified by unlabeled peptides and 627 (49.72%) by labeled peptides.

For relative quantitation, peptide data for 627 proteins were evaluated. No significant variations between SCZ and control samples were observed for 433 proteins (69.1%). Of the remaining 194 proteins, 110 (56.7%) were discarded due to low identification scores or because it were identified by only one peptide ("one hit wonders"), whereas 77 (39.7%) appeared to be upregulated and 7 (3.6%) downregulated, yielding a total of 84 confirmed regulated proteins. All proteins that showed regulated expression in the DLPFC of SCZ patients could be unambiguously identified and are listed in Table 2.

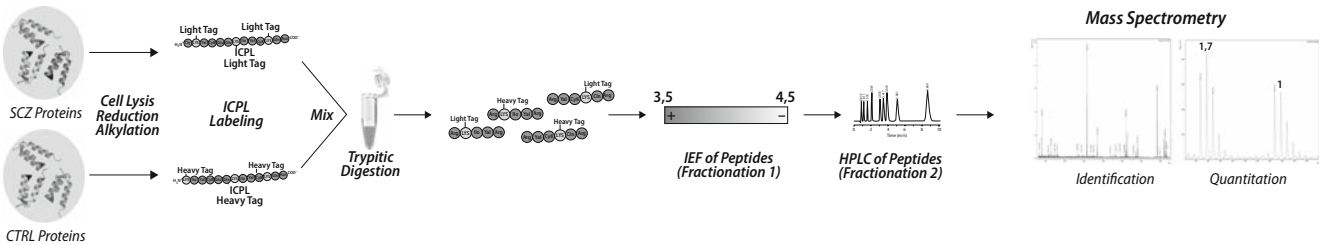

Fig. 1 Shotgun sequencing workflow: Cells were disrupted and proteins are labeled with light or heavy ICPL reagents, combined and digested with trypsin. Tryptic peptides were fractionated by isoelectric focusing (IEF) on an IPG strip and then subjected to LC-MALDI mass spectrometry for identification and ICPL quantitation

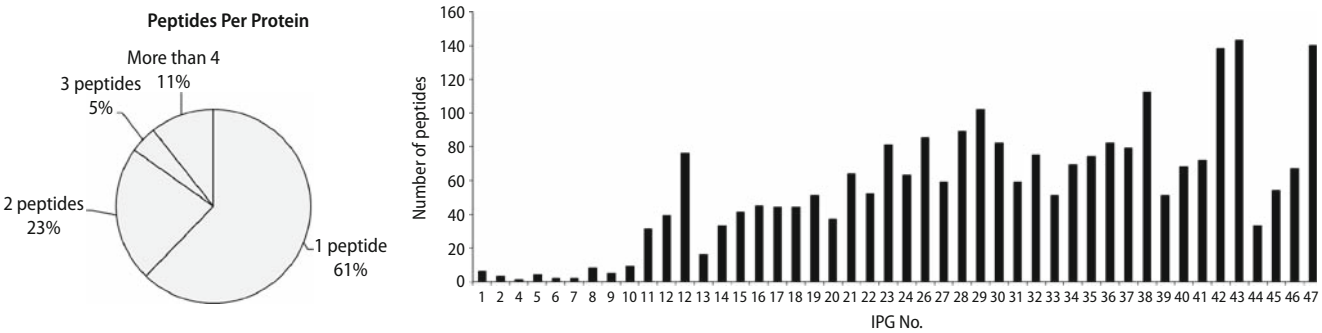

Fig. 2 Shotgun mass spectrometry results. **a** Number of peptides identified from each IPG fraction; **b** Number of peptides that identified a protein

Table 2 Proteins regulated in schizophrenia brains, classified according to their biological function

Biological process	Reg. in SCZ	H/L ratio	Gene name	Protein name (HPRD)	MW (th)	Chr loci	Id. pept	MASCOT score	Previously described in SCZ (*= proteome and # = gene expression)
Signal transduction/Cell communication, **protein modification	↓	0.39	SIRPA	SIRP alpha 1	54,967	20p13	2	125.11	
	↑	1.42	PEBP1	Phosphatidylethanolamine binding protein (Raf kinase inhibitor protein)	21,057	12q24.23	19	51.19	Clark et al. [30]*
	↑	1.43	SORBS1	Sorbin and SH3 domain containing 1	142,496	10q23.3-q24.1	2	42.14	
	↑	1.46	PPP1R9B	Protein phosphatase 1, regulatory subunit 9B	89,451	17q21.33	2	56.27	
	↑	1.49	GSPT1	G1 to S phase transition 1	55,756	16p13.1	2	65.64	
	↑	1.52	CTNND2	Delta catenin	132,656	5p15.2	2	58.55	
	↑	1.52	OPA1	Dynamin like 120 kDa protein, mitochondrial	117,770	3q28-q29	4	73.1	Clark et al. [30]*; Pennington et al. [75]*
	↑	1.53	SH3GL2	SH3 containing GRB2 like protein 2	39,962	9p22	5	65.91	Prabakaran et al. [78]*
	↑	1.55	SLC9A3R1	Solute carrier family 9, isoform A3, regulatory factor 1	38,868	17q25.1	3	38.88	
	↑	1.60	HOMER1	Homer neuronal immediate early gene	40,277	5q14.2	2	73.93	
	↑	1.61	SIRPB1	Signal regulatory protein beta 1	43,254	20p13	3	106.8	
	↑	1.65	PRKCG	Protein kinase C, gamma	78,448	19q13.4	3	61.91	Vawter et al. [93] (Lymp) #;
	↑	1.98	CSNK2A1	**Casein kinase II, alpha 1	45,143	20p13	2	43.43	
	↑	2.00	CHP	Calcium binding protein P22	22,456	15q13.3	2	51.92	
	↑	2.20	STRN	Striatin calmodulin binding protein	86,132	2p22-p21	2	44.18	
Cell growth/maintenance	↓	0.53	MYO1D	Myosin ID	116,202	17q11-q12	2	76.37	
	↑	1.40	PKP4	Plakophilin 4	131,868	2q23-q31	2	39.5	
	↑	1.41	EPB41L3	Band 4.1 like protein 3	120,677	18p11.32	7	84.97	
	↑	1.42	DES	Desmin	53,536	2q35	2	75.79	
	↑	1.46	EPB41L1	Neuron type nonerythroid protein 4.1	98,503	20q11.2-q12	2	59.57	
	↑	1.52	TRIM3	Brain expressed RING finger	80,829	11p15.5	2	78.65	
	↑	1.52	MAP6	Microtubule associated protein 6	86,505	11q13.3	3	41.03	
	↑	1.52	MRLC2	Myosin regulatory light chain MRLC2	19,779	18p11.31	2	65.91	
	↑	1.53	MAP2	Microtubule associated protein 2	202,758	2q34-q35	7	94.94	
	↑	1.58	MAP1A	Microtubule associated protein 1A	291,964	15q13-qter	5	50.55	
	↑	1.58	TUBB2B	Tubulin beta polypeptide paralog	49,953	6p25	7	98.31	Virgo et al. [95]#; Beasley et al., [13]*; Sivagnanasundaram et al. [86]*
	↑	1.62	LMNB2	Lamin B2	67,689	19p13.3	4	47.66	
	↑	1.64	KIF21A	Kinesin family member 21A	185,510	12q12	2	47.63	Hakak et al.[41]#
	↑	1.67	VIM	Vimentin	53,652	10p13	2	74.17	
	↑	1.70	NSFL1C	NSFL1 (p97) cofactor (p47)	40,573	20p13	2	44.69	Prabakaran et al. [78]*
Metabolism/energy pathways	↓	0.50	CNP	2′,3′ cyclic nucleotide,3′-phosphodiesterase	45,099	17q21	18	93.81	Hakak et al. [41]#; Tkachev et al. [92]#; Aston et al. [10]#; Prabakaran et al. [78]*; Katsel et al. [50]#; Dracheva et al. [33]#; McCullumsmith et al. [65]
	↑	1.40	ADH5	Alcohol dehydrogenase 5, chi polypeptide	39,724	4q21-q25	2	57.78	
	↑	1.41	GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	36,054	12p13	25	87.78	Prabakaran et al. [78]*
	↑	1.42	STUB1	STIP1 homologous and U box containing protein 1	34,856	16p13.3	2	86.17	
	↑	1.44	PRDX2	Peroxiredoxin 2	21,892	19p13.2	2	90.83	
	↑	1.53	HK1	Hexokinase 1	102,738	10q22	2	62.27	Prabakaran et al. [78]*
	↑	1.55	UQCRC1	Cytochrome bc1	52,646	3p21.3	1	94.36	Prabakaran et al. [78]*
	↑	1.56	NDUFV2	NADH ubiquinone oxidoreductase flavoprotein 2	27,363	18p11.31-p11.2	2	76.63	
	↑	1.68	PFKM	Phosphofructokinase	85,182	12q13.3	2	52.28	

Table 2 continued

Biological process	Reg. in SCZ	H/L ratio	Gene name	Protein name (HPRD)	MW (th)	Chr loci	Id. pept	MASCOT score	Previously described in SCZ (*= proteome and # = gene expression)
	↑	1.71	CKB	Creatine kinase brain type	42,644	14q32	5	86.64	Prabakaran et al. [78]*; Clark et al. [30]*; Beasley et al. [13]*; Sivagnanasundaram et al. [86]*
	↑	1.78	PPT1	Palmitoyl protein thioesterase 1	34,193	1p32	2	41.11	
	↑	2.47	AK1	Adenylate kinase 1	21,635	9q34.1	3	50.9	
	↑	3.69	PRDX6	Peroxiredoxin 6	25,035	1q25.1	4	55.98	
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism *also belongs to this class	↑	1.42	HNRPU	Heterogeneous nuclear ribonucleoprotein U	90,584	1q44	5	45.76	
	↑	1.44	SFRS7	Splicing factor, arginine/serine-rich 7,35 kDa	27,366	2p22.1	2	94.2	
	↑	1.45	NONO	Non pou domain containing octamer binding protein	54,231	Xq13.1	2	71.75	
	↑	1.49	FUBP1	Far upstream element binding protein	68,604	1p31.1	2	47.36	
	↑	1.53	SFRS6	Splicing factor, arginine/serine-rich 6	39,587	20q12-q13.1	3	43.85	
	↑	1.56	HNRPK	Heterogeneous nuclear ribonucleoprotein K	51,028	9q21.32-q21.33	3	83.54	
	↑	1.69	PurA	Purine rich element binding protein A	34,911	5q31	2	54.29	
	↑	1.83	NUCKS1	Nuclear ubiquitous casein kinase and cyclin dependent kinase substrate	27,296	1q32.1	2	14,4.54	
	↑	1.90	HP1BP3	HP1-BP74	61,207	1p36.12	2	51.2	
	↑	1.92	BSN	Zinc finger protein 231	416,464	3p21.31	2	59.78	
	↑	1.96	HNRPA0	Heterogeneous nuclear ribonucleoprotein A0	30,841	5q31	2	62.9	
Protein metabolism	↓	0.54	HSPA5	BIP	72,333	9q33-q34.1	2	81.99	Prabakaran et al. [78]*; Clark et al. [30]*; Sivagnanasundaram et al. [86]*
	↑	1.41	CKAP1	Cytoskeleton associated protein 1	27,325	19q13.11-q13.12	4	66.94	
	↑	1.46	SERPINA3	Alpha 1 antichymotrypsin	47,651	14q32.1	2	41.1	Arion et al. [8]#
	↑	1.46	HSPA1A	Heat shock 70 KD protein 1A	70,038	6p21.3	10	94.04	Prabakaran et al. [78]*; Clark et al. [30]*; Arion et al. [8]#
	↑	1.49	FARSLA	Phenylalanyl-tRNA synthetase alpha chain	57,563	19p13.2	2	44.11	
	↑	1.50	DNAJC6	DnaJ (Hsp40) homolog subfamily C member 6	99,996	1pter-q31.3	2	76.4	Arion et al. [8]#
	↑	1.53	CCT3	Chaperonin containing T complex polypeptide 1, subunit 1	60,534	1q23	2	93.78	
	↑	1.61	UBE2 N	Ubiquitin conjugating enzyme E2N	17,138	12q22	3	97.82	
Transport	↓	0.54	HBB	Hemoglobin beta chain	15,998	11p15.5	7	56.38	
	↑	1.47	ATCAY	Caytaxin	42,120	19p13.3	2	53.87	
	↑	1.50	VAPB	VAMP associated protein B	27,228	20q13.33	5	59.09	
	↑	1.51	APOE	Apolipoprotein E	36,154	19q13.2	2	43.33	
	↑	1.66	ATP6V0D1	ATPase H+ transporting lysosomal 38 KD V0 subunit D, isoform 1	40,329	16q22	2	89.36	
	↑	1.72	RLBP1	Retinaldehyde binding protein 1	36,474	15q26	2	51	
Immune response	↓	0.31	MOG	Myelin oligodendrocyte glycoprotein	28,647	6p22.1	1	97.74	Tkachev et al. [92]#; Katsel et al. [50]#; Arion et al. [8]#
	↓	0.57	IGSF4B	Immunoglobulin superfamily member 4B	47,021	1q21.2-q22	2	66.04	
	↑	1.69	KIR3DL2	NK associated transcript 4	50,216	19q13.4	2	109.11	
Cytoskeletal anchoring	↑	1.68	PLEC1	Plectin 1	531,789	8q24	2	46.48	
Osmoregulation/hormone metabolism	↑	1.56	CRYM	Crystallin mu	33,775	16p13.11-p12.3	2	106.46	Hakak et al. [41]#; Middleton et al. [67]#; Vawter et al. [93] (Lymp)#; Sivagnanasundaram et al. [86]*, Arion et al. [8]#

Table 2 continued

Biological process	Reg. in SCZ	H/L ratio	Gene name	Protein name (HPRD)	MW (th)	Chr loci	Id. pept	MASCOT score	Previously described in SCZ (*= proteome and # = gene expression)
DNA repair	↑	1.89	APEX2	DNA-(apurinic or apyrimidinic site) lyase 2	57,400	Xp11.22	2	56.43	
Biological_process unknown	↑	2.92	CGI–38	CGI-38 brain specific protein	18,985	16q22.1	2	94.3	
	↑	1.41	SEPT4	Septin 4	55,098	17q22-q23	2	53.71	
	↑	1.42	TTC9C	Hypothetical protein MGC29649	20,013	11q12.3	2	41.67	
	↑	1.43	RTN4	Reticulon 4	12,031	2p16.3	5	40.83	
	↑	1.45	L1CAM	L1 cell adhesion molecule	140,002	Xq28	2	71.6	
	↑	1.49	LANCL2	LanC lantibiotic synthetase component C like 2	50,854	7q31.1-q31.33	2	48.03	
	↑	1.51	KIAA1189	(Ermin (myelinating oligodendrocyte-specific protein))	34,301	2q24.1	2	78.22	
	↑	1.54	RBMXL1	Similar to RNA binding motif protein X linked	42,141	1p22.2	2	74.47	
	↑	1.59	EFHD2	EF hand domain family member D2	26,697	1p36.21	5	53.89	
	↑	1.88	WDR1	WD repeat protein 1	66,193	4p16.1	2	55.84	

MW (th) predicted protein molecular weight, *Chr Loci* gene locus, *Id. Pept* number of peptides that identified the protein, *MASCOT score* identified peptide database search score; other researches which found the differential regulation of the same gene or protein)

*Previously described in reports of SCZ proteome; #Previously described in reports of SCZ gene expression; **CSNK2A1 belongs to Protein Modification Biological Process

Functional classification of regulated proteins

The regulated proteins were divided in functional classes according to the human protein reference database (HPRD—http://www.hprd.org) and are shown in Table 2. Most of them belong to cell communication and signal transduction (15/84), cell growth maintenance (15/84) and energy metabolism (13/84) pathways.

Discussion

Shotgun and ICPL methodologies for proteomic analysis

We employed in our analysis a shotgun methodology consisted in two methods of peptide fractionation, aiming to reveal the differential expression of the widest proteome possible, including mainly the low-expressed proteins, which are normally not detected in conventional proteome analysis.

The quantification of shotgun-generated data was improved using ICPL, a stable isotope labeling which allows a more precise proteome comparison and quantification [82]. This kind of analysis is quite superior in sensibility and accuracy if compared with the known post-eletrophoretic staining methodologies.

The differentially expressed proteins in schizophrenia

Our findings support previous reports on altered protein expression in SCZ, and suggest new targets which may be relevant for the pathobiology of the disease.

Schizophrenia as an immune system disease

The first time immunological effects were implicated in SCZ was in 1845 by Esquirol, who described SCZ as an epidemic outbreak of psychotic disorders. Many years later a new concept emerged, now known as 'psychoneuroimmunology', which is defined as the investigation of influences of the immune system on the central nervous system (CNS) and its consequences on behavior and mental illness. Previously-observed alterations in the concentrations of immunotransmitters, cytokines such as interleukin-2, -4, -6 and -10, interferon-gamma and tumor necrosis factor-alpha strongly suggested that the immune system plays a role in disease pathology reviewed in [70, 88]. The differential regulation of immune system related-proteins presented in this study reinforces this concept.

Signal-regulatory protein alpha (SIRPA—down-regulated: 2.56x) and Signal regulatory protein beta 1 (SIRPB1—upregulated: 1.61x) are transmembrane

glycoprotein receptors, involved in the negative regulation of receptor tyrosine kinase-coupled signaling processes and belong to the immunoglobulin superfamily. SIRPA interacts with Janus kinase 2 (JAK2) and Olanzapine treatment (used by 1 of 9 patients—Table 1) demonstrates activation of the JAK-STAT signaling cascade, increasing the phosphorylation of both proteins [85]. SIRPA regulates neutrophil transmigration [62], B lymphocyte recruitment [102] and destruction of host cells in autoimmune diseases [74] through CD47 binding. SIRPB1 regulates neutrophil transepithelial migration and plays an important role in the response to inflammatory stimuli [63]. Peroxiredoxin 2 (PRDX2—upregulated: 1.44x), which contributes to CD8(+) T-cell activity [66], due to its antioxidant role [81], may represent a link with oxidative stress previously described in SCZ [79]. Plectin 1 (PLEC1—upregulated: 1.68x) is a regulator of T lymphocyte cytoarchitecture [24].

Cytoskeleton assembly

Cytoskeleton proteins display a tissue-specific pattern of expression and their altered expression can directly influence key cellular processes, including symmetrical shape, structural polarity, neuritogenesis, and neurotransmission, that are essential for the physiology of the neuron reviewed in [15].

Abnormalities in cytoarchitectural pattern were previously described in brain tissues of SCZ patients [9]. The differential regulation of proteins such as Vimentin (VIM—upregulated: 1.67x), Lamin B2 (LMNB2—upregulated: 1.62x), Desmin (DES—upregulated: 1.42x), Plectin 1 (PLEC1—upregulated: 1.68x), Tubulin beta polypeptide (TUBB2B—upregulated: 1.58x), reinforces these observed abnormalities. In addition, Cytoskeleton associated protein 1 (CKAP1—upregulated: 1.41x), that facilitates the dimerization of alpha- and beta-tubulin through nitric oxide signaling, and Kinesin family member 21A (KIF21A—upregulated: 1.64x),) which interacts with disrupted-in-schizophrenia 1 (DISC1), are also likely causal factors of SCZ. Moreover, we found the upregulation of 3 MAP (Microtubule-associated protein) proteins (MAP1A, MAP2 and MAP6—upregulated: 1.58x, 1.53 and 1.53) that are involved in microtubule assembly, an essential step in neurogenesis. Proteins of the MAP family were previously described to be involved in SCZ and have been suggested as potential therapeutic targets [15].

TAU, APOE, Alzheimer's disease and SCZ-altered proteins

TAU is a neuronal microtubule-binding protein and its inclusions are the main feature of several neurodegenerative diseases, including Alzheimer's disease (AD). TAU ubiquitination and hyperphosphorylation [48] generate filamentous polymers that lead to neuronal apoptosis [46]. STIP1 homologous (STUB1—upregulated: 1.42x) and Ubiquitin conjugating enzyme E2N (UBE2N—upregulated: 1.61x—gene was found downregulated in SCZ DLPFC [94]) participate in the ubiquitination of hyperphosphorylated TAU leading to filamentous polymers [84]. Moreover, STIP1 interacts with the chaperone heat shock 70 KDa protein 1A (HSPA1A—upregulated: 1.46x), whose gene was recently described to be upregulated in SCZ DLPFC [8].

Apolipoprotein E (APOE—upregulated: 1.51x) and Serpin peptidase inhibitor (SERPINA3—upregulated: 1.46x) are proteins involved in lipid metabolism and are present in AD amyloid deposits [29, 90]. The interaction of APOE with TAU has been reported numerous times [14]. Moreover, Peroxiredoxin 2 (PRDX2—upregulated: 1.44x) have previously been described to be involved in AD and to be differentially regulated in SCZ DLPFC.

Crystallin (CRYM—downregulated 1.56x) that modulates cytoskeleton assembly, was found regulated in astrocytes associated with senile plaques and cerebral amyloid angiopathy in AD patients [99] and in gene expression and proteome analysis [8, 41, 86].

Ca^{2+} homeostasis

Ca^{2+} and neurotransmission Ca^{2+} is considered to be a pivotal metabolite for the dopamine hypothesis in SCZ [17]. In addition, the identification of the altered expression of many Ca^{2+}-related proteins corroborates the concept of Ca^{2+} altered homeostasis in SCZ.

Intracellular calcium levels control the dopamine receptor function [17] and the maintenance of neurotransmitter exocytosis during stimulation [23, 28]. These processes are controlled by Neuronal protein 4.1 (EPB41L1—upregulated: 1.46x), which stabilizes dopamine receptors at the neuronal plasma membrane [18] and also by Endophilin A1 (SH3GL2—upregulated: 1.53x), which is essential for the formation of synaptic vesicles from the plasma membrane. Phosphatidylethanolamine-binding protein 1 (PEBP1—upregulated: 1.42x), is a substrate of calpain [27], a Ca^{2+}-dependent protease that has been implicated in processes that produce persistent changes in synaptic chemistry and structure [36].

Calcium plays a critical role in signaling glutamatergic synapses. The regulation of Presynaptic cytomatrix protein (BSN—upregulated: 1.92x) suggests a glutamatergic dysfunction. In BSN-null mice an inactivation of a significant fraction of glutamatergic synapses [6] occurs. Moreover, Homer homolog 1 (HOMER1—upregulated: 1.6x), a protein previously associated with SCZ [72], regulates the metabotropic glutamate receptor function [91]. In addition, Haloperidol treatment induced the increased expression of

HOMER1 in different brain region in rats [76]. This is important in light of the fact that 3 out of 9 patients in the present study had been treated with Haloperidol. Finally, Myosin regulatory light chain (MRLC2—upregulated: 1.52x) is an enzyme activated by calcium.

■ **Calmodulin and calcineurin related proteins** Calmodulin, the most prominent calcium-modulated protein, is involved in the control of many biochemical processes and binds several proteins such as Striatin (STRN—upregulated: 2.2x), Casein kinase II, alpha 1 (CSNK2A1—upregulated: 1.98x), Myosin ID (MYO1D—downregulated: 1.88x), and Casein kinase II, alpha 1 (CSNK2A1—upregulated: 1.98x—found downregulated in frontal cortex of SCZ [4]. This protein has been implicated in several cellular functions including cellular growth control, proliferation and apoptosis [1].

Calcineurin is a regulator of dopaminergic [38] and glutamatergic [103] neurotransmission, which is vital for normal cognitive and behavioral functioning and is frequently compromised in SCZ [25, 57, 83]. Calcineurin homologous protein (CHP—upregulated: 2x), which interacts with Solute carrier family 9, isoform A3 (SLC9A3—upregulated: 1.55x), inhibits calcineurin activity, most likely compromising regulation of neurotransmission.

■ **Ca^{2+} and actin-related proteins** Cell adhesion is mediated by Cadherins, which are Ca^{2+} dependent, and Catenins, which bind actin [51, 64]. We found in SCZ DLPFC the regulation of Catenin 4 (PKP4—upregulated: 1.4x) and Delta catenin (CTNND2—upregulated: 1.52x) proteins, which regulate cadherin function and interaction with actin [43], and Band 4.1 like protein 3 (EPB41L3—upregulated: 1,41x), a protein that acts in intercellular junctions by binding actin [101].

Spinophilin (PPP1R9B—upregulated: 1.46x), a dendritic protein that regulates glutamate receptor activity, has an actin-binding domain, necessary for targeting spinophilin to dendrites [39] and interacts with D1 and D2 dopamine receptors [87] which are directly controlled by Ca^{2+}. Despite a study implicating PPP1R9B in SCZ hippocampal dendritic pathology [56], the administration of clozapine and haloperidol (antipsychotics used by our patients) could regulate the PPP1R9B concentrations [31]. Moreover, PPP1R9B may contribute to the dysfunction of the dopamine system in DLPFC SCZ, since altered levels of dopamine receptor-binding proteins may lead to a dopamine imbalance [11, 52].

Mitochondrial metabolism

Mitochondrial hypoplasia, imbalance of the oxidative phosphorylation system, and differentially expressed genes and proteins related to mitochondria are believed to be involved in SCZ mitochondrial dysfunction [16, 30, 78]. This leads to alterations in ATP production, generation of reactive oxygen species and alterations in intracellular calcium concentrations. These processes combined result in an altered synaptic state, compromising the plasticity, neuronal polarity and synaptogenesis reviewed in [16].

Our data show the differential regulation of mitochondrial proteins such as Dynamin-like 120 kDa protein (OPA1—upregulated: 1.52x), that participate in mitochondrial biogenesis and stabilization of mitochondrial membrane integrity [5]; Cytochrome bc1 (UQCRC1—upregulated: 1.55x), that was previously implicated in SCZ [49]; and NADH ubiquinone oxidoreductase flavoprotein 2 (NDUFV2—upregulated: 1.56x) that participates in nervous system development [42].

Energy metabolism

Glucose metabolism involves different interconnected pathways that have been identified as involved in the pathogenesis of SCZ [21, 53]. The relationship between glucose regulation and SCZ is significant since glucose administration decreases deficits in verbal declarative memory [37] and atypical antipsychotic medications generate hyperglycemia [40, 77]. In addition, alterations in basal glucose metabolism in DLPFC could generate SCZ hypofrontality [100].

We found several regulated enzymes involved in glucose metabolism. These include Glycolysis enzymes such as Hexokinase 1 (HK1—upregulated: 1.53x), Phosphofructokinase (PFKM—upregulated: 1.68x), and Glyceraldehyde 3 phosphate dehydrogenase (GAPDH—upregulated: 1.41x). We also found regulated proteins involved in lipid metabolism. Peroxiredoxin (PRDX6—upregulated: 3.69x) is a bifunctional enzyme that, despite its redox regulation role in the cell, acts as a Ca^{2+}-independent phospholipase A2 for cellular phospholipid turnover [26]. Rats treated with chlorpromazine and clozapine showed altered PRDX levels in the hippocampus [54]. The upregulation of a redox regulation enzyme could be a hallmark of cellular oxidative stress previously described in SCZ [78]. Palmitoyl protein thioesterase 1 (PPT1—upregulated: 1.78x) is a key enzyme for fatty acid synthesis that participates in many biochemical pathways, including brain and neuronal development [44, 60] and sphingolipid catabolic processes [3]. PPT1 is present in axons and metabolizes synaptic vesicles [2]. Alcohol dehydrogenase 5, chi polypeptide (ADH5—upregulated: 1.40x) metabolizes, among many other substrates, lipid peroxidation products [35]. Apolipoprotein E (APOE—upregulated: 1.51x) and Alpha 1 antichymotrypsin (SERPINA3—upregulated: 1.46x) are essential for lipid metabolism.

Our study revealed differential regulation of myelin oligodendrocyte glycoprotein (MOG—downregulated: 3.23x), 2′,3′ cyclic nucleotide, 3′-phosphodiesterase (CNP—downregulated: 2.00x) and ermin—ERM-like protein (ERMN—upregulated: 1.51x), corroborating several cDNA microarray study data [10, 41, 50, 92] and other gene expression analyses [33, 65, 97]. All these studies revealed an alteration of a series of myelin-related genes in SCZ. The myelination and maintenance of myelin sheets in axons of the CNS is the most important function of the oligodendroglia, and the diminution or malformation of the myelin sheath results in an increased ion leakage and a reduced propagation of nerve impulses. Moreover, other functions such as trophic signaling to nearby neurons, synthesis of growth factors, neuronal survival and development, neurotransmission and synaptic function are executed by oligodendrocytes [32, 34].

One hit wonders

We discard from our list of differentially expressed proteins the ones that were identified by only one peptide. However, we sustained in Table 2 the proteins UQCRC1 and MOG, even identified by one peptide, since there were proteins previously identified by our group and other groups as differentially expressed besides the potential their role previously described in SCZ.

Analysis of protein pools

For a number of reasons we compared protein pools instead of individual protein extracts in the present study. While we are aware that more dramatic alterations in certain proteins of a single individual might 'contaminate' the pool, suggesting unreal alterations, we believe that the advantages of sample pooling may overcome its disadvantages. Foremost, sample-pooling reduces the influence of individual proteome variations (not related to the disease) while highlighting the most consistent (disease-related) alterations. An additional advantage is the reduction of the amount of protein required from each sample, allowing experimental replicates and subsequent studies. This approach has been successfully used by several groups not only for proteomics [47, 58, 59], but also for gene expression analysis [50, 93] and genotyping [96]. By using comparative genome hybridization for the analysis of copy number variations, the authors showed that neither specific DNA variations nor specific genes were consistently associated with SCZ. Rather, their results showed that the disease is likely to be caused by rare alterations which disrupt pathways related to neural regulation and development. As shown in the present report, the analysis of sample pools is capable of implicating recurring pathway alterations in a consistent manner.

Confounding factors

We cannot confirm that all protein alterations detected in our analyses are directly associated with SCZ. If associated with SCZ, we also cannot confirm that their regulation is causative for the disease process, or rather a consequence of age, gender, diet and/or medications taken by the patients. All these variables can directly influence the proteome. Thus, validation experiments have to be done.

As is evident from Table 1, all SCZ samples used in this study came from patients that have taken different antipsychotic drugs. This is a limitation of the current and many additional studies. Three out of nine patients from our study were taking haloperidol at death. Sugai et al. [89], using cDNA arrays from cynomolgus monkeys and Narayan et al. [71], using in situ hybridization analysis of mice, showed that myelin basic protein (MBP) is modulated as a result of haloperidol treatment. An altered expression of Apolipoprotein A-I in plasma of SCZ medicated patients was also found [55], whereas Malate dehydrogenase, Peroxiredoxin 3, Vacuolar ATP synthase subunit beta and Mitogen-activated protein kinase kinase 1 were found to be regulated in the hippocampus of chlorpromazine/clozapine treated rats [54]. However, a considerable number of proteins identified here have not been reported to be associated with any of the drugs used by the patients and many have been associated with SCZ processes largely independent of an exogenous drug effect, such as genetic linkage studies. We expected that the pooling strategy adopted here would contribute to dilute protein alterations driven by haloperidol in 1/3 of our patients, but we cannot exclude that some of the alterations seen here could be drug-related, rather that SCZ-related.

Whereas the study of brain samples derived from psychotropic drug-naïve patients [79] and animal models are of utmost importance, the vast majority of samples available worldwide are derived from treated patients. As the samples used in the studies were derived from patients under distinct therapeutic regimens, the recurrent identification of the same targets might implicate certain genes and proteins in the pathobiology of the disease. Thus, we believe that the findings described here will reinforce the interest in certain pathways, and moreover suggest new protein candidates, which can be further investigated in future studies.

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