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Alterations in oligodendrocyte proteins, calcium homeostasis and new potential markers in schizophrenia anterior temporal lobe are revealed by shotgun proteome analysis

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Abstract Global proteomic analysis of *post-mortem* anterior temporal lobe samples from schizophrenia patients and non-schizophrenia individuals was performed using stable isotope labeling and shotgun proteomics. Our analysis resulted in the identification of 479 proteins, 37 of which showed statistically significant differential expression. Pathways affected by differential protein expression include transport, signal transduction, energy pathways, cell growth and maintenance and protein metabolism. The collection of protein alterations identified here reinforces the importance of myelin/oligodendrocyte and calcium homeostasis in schizophrenia, and reveals a number of new potential markers that may contribute to the understanding of the pathogenesis of this complex disease.

Keywords Schizophrenia · Proteomics · Anterior temporal lobe · ICPL · Oligodendrocytes · Myelin · Calcium · Biomarker

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Introduction

Dysfunctions in synaptogenesis and neural plasticity (Mirnics et al. 2000; Hakak et al. 2001; Vawter et al. 2001; Aston et al. 2004; Arion et al. 2007), energy metabolism (Vawter et al. 2001; Middleton et al. 2002; Prabakaran et al. 2004), cytoskeleton assembly (Hakak et al. 2001; Vawter et al. 2001; Tkachev et al. 2003) and oligodendrocyte metabolism (Tkachev et al. 2003; Aston et al. 2004; Katsel et al. 2005a, b; Arion et al. 2007) have been previously reported in studies of global gene expression in different brain regions of schizophrenia (SCZ) patients. However, as post-transcriptional mechanisms may prevent mRNA fluctuations to be directly translated into protein differential expression, proteomic studies are a nice complement to studies of differential gene expression. Some proteomic studies were performed in brain regions such as the anterior cingulate cortex (Clark et al. 2006) and the corpus callosum (Sivagnanasundaram et al. 2007), showing that the proteome alterations on these related pathways have been confirmed.

Patterns of synchronization and desynchronization communicate between brain areas through specific neuronal activity (Singer 1999). In a complex disease such as

SCZ it is possible that all brain areas play a role in the etiology since they are all connected. However, there are some areas which seem to be more involved based on their exerted functions: the pre-frontal cortex because of its executive functions (Miller and Cohen 2001), the basolateral amygdala because of its involvement in affective behavior (Davis and Whalen 2001), and the anterior cingulate cortex because of its participation in cognitive and affective processes (Carter et al. 1997).

The temporal lobe concentrates important functions such as high-level auditory and visual processing, language, and transference from short- to long-term memory. All these functions can be compromised in SCZ in a process that apparently involves an imbalance of glutamate and gamma-aminobutyric acid (GABA) leading to dopaminergic dysfunctions (Deakin and Simpson 1997). A reduction of the temporal lobe size in SCZ has been shown by several studies with magnetic resonance imaging (MRI) (Bogerts 1993; Suddath et al. 1989). The left temporal pole gray matter was smaller and left-greater than right asymmetry was reduced in SCZ patients (Kasai et al. 2003; Antonova et al. 2005), however, results are not consistent (Turetsky et al. 2003). Interestingly, the volume of the left anterior temporal cortex was negatively correlated with hallucinations (Crespo-Facorro et al. 2004). In the polar temporal cortex of SCZ patients, deficits were reported in glutamate presynaptic components (Deakin and Simpson 1997); glutamate and GABA uptake sites were reduced on the left side (Deakin et al. 1989; Simpson et al. 1989) with no losses of post-synaptic glutamate receptors (Nishikawa et al. 1983). Deakin and Simpson (1997) have shown that degenerated glutamate terminals in the anterior temporal lobe originate in the frontal cortex with important implications for SCZ.

In the present work we performed a quantitative proteomic analysis of the left anterior temporal lobe (ATL) of SCZ and control samples using isotope-coded protein label (ICPL), a method for the accurate quantitative comparative analysis of protein regulation (Schmidt et al. 2005). ICPL is based on isotope labeling of free amino groups in intact proteins. After the modification, the heavy and light isotope labeled proteins are digested and analyzed by liquid chromatography (LC) followed by tandem mass spectrometry (MS/MS). Relative quantification of differential protein expression is based on the comparison of the peak intensities of the heavy- and light-labeled peptides from the mass spectra. The ICPL method is very reproducible and compatible with all known protein and peptide separation techniques, providing highly accurate quantification of regulated proteins.

The identification of proteins differentially expressed in the ATL of SCZ patients was performed by MS/MS followed by subsequent database searches. After their validation in a large set of samples and patients, these proteins can provide valuable information not only for a better understanding of the biological basis of the disease, but can also serve as biomarkers for disease monitoring or as targets for pharmaceutical applications.

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 anterior temporal lobe samples

Frozen tissue blocks from the left anterior temporal lobe tissue, Brodmann Area (BA) 38, were collected *post-mortem* from five SCZ patients and four controls free of psychiatric disorders. The left side was selected due to its importance in SCZ (DeLisi et al. 1989).

All brain samples were obtained from the brain bank of the Central Institute of Mental Health (Mannheim, Germany), dissected by an experienced neuropathologist and deep-frozen in liquid nitrogen-cooled isopentane immediately after dissection. Patient samples derived from in-patients of the Mental State Hospital Wiesloch, Germany, while controls were collected at the Institute of Neuropathology, Heidelberg University, and their clinical records were collected from their relatives and general practitioners. On average, the samples were collected 17.8 h (minimum: 4 h/maximum 28 h) after death. All cases were white and German with one female in each group. Detailed patient data are listed in Table 1. All SCZ patients had been long-term inpatients at the Mental State Hospital Wiesloch, Germany, and had been diagnosed antemortem by an experienced psychiatrist according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV) of the American Psychiatry Association for Schizophrenia (American PA 1994). For each patient the history of antipsychotic treatment was assessed by examining the medical charts and calculated in chlorpromazine equivalents (CPE) using the algorithm developed by Jahn and Mussgay (1989). All assessments and *post-mortem* evaluations and procedures had been approved by the Ethics Committee of the Faculty of Medicine of Heidelberg University, Germany.

All patients and controls underwent thorough neuropathologic characterization to rule out associated neurovascular or neurodegenerative disorders such as Alzheimer's disease and multi-infarct dementia (Braak and Braak 1991;

Table 1 Patient and control clinical data

Sample ID	Case	Age (years)	Gender	PMI (h)	pH values	Type of SCZ	Duration of disease (years)	Duration of medication (years)	atyptyp	CPE last dosis	CPE last 10 years	Cause of death	DSM IV	Age at onset	Last medication	Cigarettes	Alcohol	Hosp	ECT
01/00	SCZ	51	M	12	6.7	Residual, Chronic Paranoid episodes	28	25	2	450	1.8	Heart infarction	295.6	23	Clozapine 500 mg	30/day	No	17	No
35/00	SCZ	64	F	23	6.6	Residual, Chronic Paranoid episodes	41	40	2	54.5	4.6	Heart infarction	295.6	24	Zotepine 150 mg Olanzapine 10 mg	20/day	No	5	Yes
36/02	SCZ	73	M	20	6.9	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
83/01	SCZ	71	M	28	6.5	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
50/01	SCZ	81	M	4	6.8	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
43/01	Control	91	F	16	6.8							Cardio-pulmonary insuffiency				No	No		
57/02	Control	53	M	18	7.1							Heart infarction				No	No		
61/01	Control	66	M	16	6.9							Heart infarction				No	No		
72/02	Control	79	M	24	6.4							Heart infarction				No	No		

atyptyp duration of atypical treatment/duration of treatment with typical neuroleptics 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

Braak et al. 2006). The staging according to Braak was stage 2 or less for all subjects. Patients and normal comparison subjects had no history of alcohol or drug abuse, or severe physical illness.

Sample preparation

Fifty milligrams of human temporal lobe tissue from control and SCZ subjects were homogenized in 1.5 ml tubes using glass spheres in 200 µl 6 M guanidine and 0.1 M HEPES buffer. Protein concentrations were determined using the Bradford assay (Bradford 1976). Brain proteins were evaluated in pools containing 100 µg of protein. The SCZ pool was prepared by combining 20 µg protein from each of the five SCZ samples, whereas the control pool contained 25 µg of each of the four controls. Pooled samples were used in order to reduce individual variability not related to the SCZ phenotype (Weinkauf et al. 2006).

ICPL labeling

A total of 100 µg of total protein from SCZ or controls (5 mg/ml) were reduced, as specified by the ICPL kit protocol, for 30 min at 60°C. After cooling to 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 ten-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 allowed to proceed for 2 h at RT. Four ml 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. Resultant peptides were fractionated on Immobilized pH gradient strips (IPG 17 cm), pH 3.5–4.5. The strips were rehydrated 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 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). Chromatography fractions were collected onto Prespotted AnchorChip targets (Bruker Daltonics, Bremen, Germany) and provided with matrix thin layer preparations using a PROTEINEER-FC robot (Bruker Daltonics).

Mass spectrometry

Mass spectra from each target spot were acquired fully automatically using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). Measurements were performed with a nitrogen laser in positive reflector mode and a 20,000 V acceleration voltage. For mass spectrometry (MS) spectra 100 shots and for MS/MS spectra 1,000 shots were accumulated. WARP-LC 1.0 software was used for spectra acquisition and controlling the automatic selection of peptides for further MS/MS analysis. The ICPL-labeled peptides were selected for the MS/MS analysis based on their H/L ratio.

Identification of proteins

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 (16 December 2006) using an in-house version of MASCOT 2.1 (Matrix Science, London, UK). Parameter settings: *Homo sapiens* for organism, trypsin and Arg-C for enzymes, 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 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.

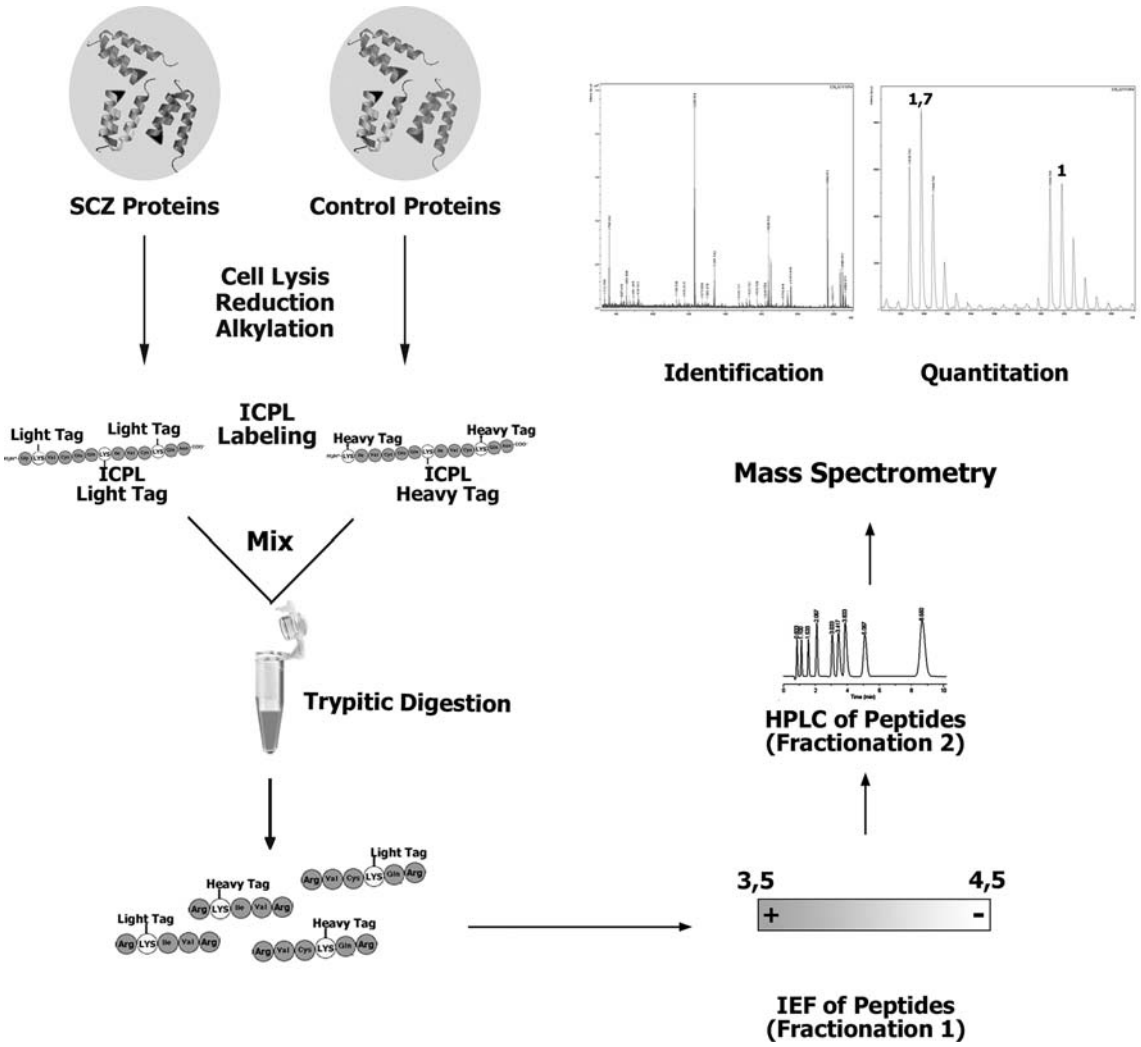

Fig. 1 Proteomic workflow: after cell lysis proteins are labeled with light or heavy ICPL reagents, combined and digested with trypsin. Tryptic peptides are fractionated by isoelectric focusing (IEF) on an IPG strip and then subjected to LC-MALDI mass spectrometry for identification and quantitation

1. BioTools software returns for each identified protein a MASCOT score value that is derived from the peptide hit scores. Only peptide scores greater than 38 were considered significant identifications.
2. The regulation status of a protein was defined by the ratio of the relative peptide signal intensities. We labeled SCZ proteins with heavy tag and control proteins with light tag. The proteins in both samples generated the same peptides after digestion, but peptides from SCZ samples had an approximately 6 Da per labeled amino group greater mass than peptides labeled with light tags (Fig. 2). In our analysis we considered a minimum 40% variation as significant regulation. Thus, proteins upregulated in SCZ should have ratios $\geq$1.4, whereas downregulated proteins should have ratios $\leq$0.6. Ratio values of 0.6–0.8 or 1.2–1.4 were considered borderline.
3. Protein identity and function were analyzed using the Human Protein Reference Database (HPRD) based on Gene Ontology criteria.

Results

Protein regulation in schizophrenic anterior temporal lobe

Shotgun mass spectrometry resulted in the analysis of 837 peptide sequences (Fig. 2a) and led to the identification of 479 proteins in ATL (Fig. 2b); 269 proteins (56.2%) were identified by unlabeled peptides and 210 (43.8%) by labeled peptides.

For relative quantification, the peptide data of the 210 labeled proteins were evaluated. No significant variations between SCZ and control samples and proteins identified

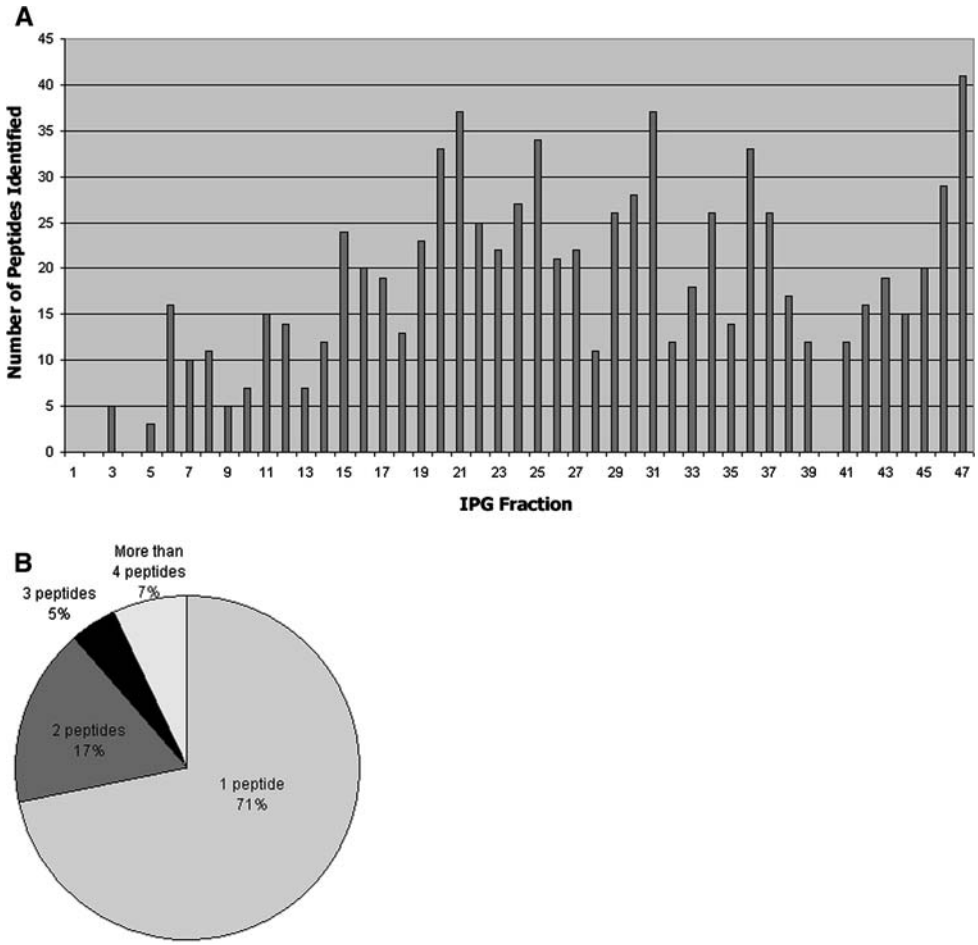

Fig. 2 Shotgun mass spectrometry results. **a** Number of peptides identified from each IPG fraction; **b** Number of peptides that identified a protein

by 1 peptide were represented for 173 proteins (82.4%). Of the remaining 37 proteins, 6 (16.2%) appeared to be upregulated and 31 (83.8%) downregulated. All proteins that appeared to be regulated in the ATL of SCZ patients, and could be unambiguously identified, are listed in Table 2.

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 (10/37), cell growth maintenance (8/37) and energy metabolism (8/37) pathways.

Discussion

ICPL methodology for proteomic analysis

For our analysis, we employed shotgun proteomics instead two-dimensional gel electrophoresis to overcome limitations such as the limited representation of small and large proteins as well as proteins with extreme isoelectric points (Gygi et al. 2000). In addition, low abundant proteins such as transcriptional factors can be better detected by using the shotgun strategy. As a result, this allowed us to investigate a greater fraction of the proteome and to more accurately measure differences in protein expression. The quantification of shotgun-generated data is improved by the use of stable isotope labeling of the proteins and allows a more precise comparison and quantification (Schmidt et al. 2005). This approach was applied to pooled control and SCZ samples, in order to identify proteins with altered regulation in the disease. Most of the regulated proteins identified (84.4%) were found to be reduced in SCZ ATL. The overall reduction in protein translation could be a hallmark of the hypotemporality described in SCZ (Catafau et al. 1994).

Shotgun proteomics confirms the regulation of previously identified SCZ-related proteins

Oligodendrocyte-myelin regulated proteins

The most important function of the oligodendroglia is the myelination and maintenance of myelin sheets in axons of

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

Biological process	Regulation in SCZ	Protein name	Gene name	Accession	H/L ratio	pI (th)	MW (th)	Chr loci	Id. pept	MASCOT score
Transport	↑	Plasma membrane calcium-transporting ATPase 4	PMCA-4	P23634	1.59	6.19	137920.16	1q25-q32	3	76.49
	↓	Hemoglobin subunit beta	HBB	P68871	0.57	6.81	15867.22	11p15.4-p15.5	2	44.1
Signal transduction	↑	Ezrin-radixin-moesin-binding phosphoprotein 50	EBP50	O14745	1.58	5.55	38737.24	17q25.2	2	50.76
Cell communication	↑	Prohibitin	PHB	Q6FHP5	1.57	5.57	29832.16	17q21	2	62.38
	↑	Phosphatidylethanolamine-binding protein 1	PEBP1	P30086	2.86	7.43	20925.59	12q24.23	3	44.07
	↓	14-3-3 protein eta	YWHAH	Q04917*	0.49	4.76	28087.53	22q12-q13	2	65.33
	↓	14-3-3 protein gamma	YWHAG	P61981	0.46	4.8	28171.4	7q11.23	4	112.84
	↓	14-3-3 protein zeta/delta	YWHAZ	P63104	0.52	4.73	27745.1	2p25.2-p25.1	4	58.22
	↓	Calcineurin or serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	PPP3CA or CALN	Q08209	0.60	5.58	58687.85	4q21-q24	3	64.34
	↓	Visinin-like protein 1	VILIP-1	P62763	0.51	5.01	22011.14	2p24.3	3	91.89
	↓	Signal-regulatory protein beta-1	SIRPB1	O00241	0.56	6.06	43255.18	20p13	4	118.36
	↓	Calmodulin	CALM	P62158	0.20	4.09	16706.39	14q24-q31	8	131.36
Metabolism; energy pathways	↑	Hexokinase brain form	HK1	P19367	1.84	6.44	102484.91	10q22	2	43.49
	↓	NADH-ubiquinone oxidoreductase 13 kDa-A subunit	NDUFS6	O75380	0.58	8.58	13711.61	5p15.33	3	92.52
	↓	NADH-ubiquinone oxidoreductase SGDH subunit	NDUFB5	O43674	0.48	9.62	21750.27	3q26.33	2	51.45
	↓	NADH-ubiquinone oxidoreductase 30 kDa subunit	NDUFS3	O75489	0.53	6.98	30241.53	11p11.11	4	81.08
	↓	NADH dehydrogenase (ubiquinone) flavoprotein 2	NDUFV2	Q6IB76	0.48	8.22	27349.47	18p11.31-p11.2	2	112.02
	↓	Carbonic anhydrase 2	CA2	P00918	0.50	6.86	29114.86	8q22	2	74.22
	↓	Fructose bisphosphate; aldolase C	ALDOC	Q6FH94	0.56	6.41	39455.87	17cen-q12	5	79.21
	↓	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase	CNP	P09543	0.51	9.17	47578.63	17q21	13	87.29
Cell growth and/or maintenance	↑	Tubulin, beta polypeptide	TUBB	Q9BVA1*	1.46	4.78	49953.06	*6p25*	3	83.45
	↓	Aggrecan core protein	AGC	P16112*	0.50	4.1	250193.19	15q26.1	2	56.55
	↓	Spectrin alpha chain; Fodrin	SPTAN1	Q13813	0.43	5.22	284539.36	9q33-q34	2	55.93
	↓	Glial fibrillary acidic protein, astrocyte	GFAP	P14136	0.59	5.42	49880.21	17q21	16	103.48
	↓	Neurofilament triplet M protein	NEFM	P07197*	0.46	4.9	102316.8	8p21	3	68.45
	↓	Hyaluronan and proteoglycan link protein 2	HAPLN2	Q5T3J1*	0.16	8.37	20827.82	1q23.1	3	82.95
	↓	Neurofilament triplet L protein	NEFL	P07196*	0.32	4.64	61385.41	8p21	2	70.24
	↓	Clathrin light chain B	CLTB	P09497*	0.50	4.57	25190.49	5q35	3	74.98

Table 2 continued

Biological process	Regulation in SCZ	Protein name	Gene name	Accession	H/L ratio	pI (th)	MW (th)	Chr loci	Id. pept	MASCOT score
Protein metabolism	↓	Ubiquitin-conjugating enzyme E2N	UBE2 N	P61088	0.32	6.13	17137.82	12q21.33	2	82.01
	↓	Eukaryotic initiation factor 4A-II	EIF4A2	Q14240	0.52	5.33	46402.27	3q28	2	46.03
	↓	60S ribosomal protein L13	RPL13	P26373	0.46	11.65	24130.28	16q24.3	2	62.73
	↓	40S ribosomal protein S10	RPS10	P46783*	0.53	10.15	18897.77	6p21.31	2	54.19
Nucleic acid metabolism	↓	Histone H3.1	HIST1H3E	P68431*	0.31	11.13	15272.89	6p21.3	2	58.35
	↓	Ribonuclease/angiogenin inhibitor 1	RNH1	P13489	0.59	4.71	49842.28	11p15.5	2	82.56
Immune response	↓	Myelin oligodendrocyte glycoprotein	MOG	Q5SSB7*	0.43	8.29	23235.99	6p22.1	3	88.54
Neurogenesis	↓	Myelin basic protein	MBP	Q65ZS4	0.39	11.35	22316.06	18q22-qter	7	78.43
Synaptic Transmission Oligodendrocyte metabolism	↓	Ermin (myelinating oligodendrocyte-specific protein)	ERMN	Q8TAM6	0.31	4.75	32782.95	2q24.1	2	61.42

Proteins marked with (*) are encoded by genes that map to genomic regions previously associated with schizophrenia. The standard deviation for all H/L ratios is ±0.00 and the accession numbers are from the Swiss-Prot database

pI (th) predicted protein isoeletric point; *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

the central nervous system (CNS). The diminution or malformation of the myelin sheath results in an increased ion leakage and a reduced propagation of nerve impulses. Besides, 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 (Du and Dreyfus 2006; Deng and Poretz 2003).

Whereas an analysis by magnetic transfer imaging and diffusion tensor imaging showed a disruption of white-matter integrity in SCZ patients (reviewed in Segal et al. 2007), several cDNA microarray studies (Hakak et al. 2001; Tkachev et al. 2003 Aston et al. 2004; Katsel et al. 2005a (2)) and individual gene expression analysis (Webster et al. 2005; Dracheva et al. 2006; McCullumsmith et al. 2007) revealed the alteration of a series of myelin-related genes in SCZ. A set of proteins encoded by these genes were also identified in our study, including 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP, downregulated here: −1.96x), glial fibrillary acidic protein (GFAP, downregulated here: −1.7x), and myelin oligodendrocyte glycoprotein (MOG, downregulated here: −2.32x).

Myelin Basic Protein (MBP, downregulated here: −2.56x), the major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the CNS, has a neuroprotective role in vivo (Moalem et al. 1999) and its expression is controlled by brain-derived neurotrophic factor (BDNF) (Hohlfeld et al. 2000) whose mRNA and protein were found regulated in SCZ patients' brain and serum (Angelucci et al. 2005; Chambers and Perrone-Bizzozero 2004). The myelinating oligodendrocyte-specific protein, Ermin (downregulated here: −3.23x) is a protein that seems to be exclusively expressed by oligodendrocytes. It regulates cytoskeletal rearrangements during myelinogenesis and is also important in the maintenance and stability of the myelin sheath in the adult brain (Brockschnieder et al. 2006).

The downregulation of the above mentioned proteins, revealed by an approach never seen over an independent sample set of a different brain area, strongly reinforces the importance of oligondendrocyte homeostasis in the pathogenesis of SCZ.

Regulation of Ca^{2+} homeostasis

The identification of an altered regulation of five proteins related to Ca^{2+} homeostasis and metabolism reinforces the concept of the centrality of Ca^{2+} in SCZ. Abnormal brain Ca^{2+} concentrations, probably mediated by an altered regulation of calmodulin (CALM, downregulated here: −5x), an intracellular Ca^{2+} sensor, and by the plasma membrane calcium-transporting ATPase 4 (PMCA-4, upregulated here: 1.59x), which is involved in the maintenance of Ca^{2+}

homeostasis in the cell (Strehler and Treiman 2004), may increase Ca^{2+}-dependent phospholipase A2 (PLA2) activity and account for the accelerated phospholipid turnover and reduced dopaminergic activity seen in the SCZ frontal lobe (Gattaz et al. 1990; Gattaz and Brunner 1996).

Ca^{+2} is considered a pivotal metabolite for the dopamine hypothesis in SCZ and plays a crucial role in the function of dopamine receptors D1 and D2 (Bergson et al. 2003). It should be noted that calcineurin, which appeared to be downregulated here (−1.67x) as well as in other SCZ studies (Hakak et al. 2001; Eastwood et al. 2005), has important neuronal functions (Malenka 1994; Liu et al. 1994) and is a regulator of dopaminergic (Greengard 2001) and glutamatergic (Zeng et al. 2001) neurotransmission, which are frequently compromised in SCZ (Seeman 1987; Carlsson et al. 2001). Dopaminergic hyperactivity in SCZ may result in altered *N*-methyl-D-aspartic acid (NMDA) receptor activation, which can lead to excitotoxicity and excess Ca^{2+} influxes through NMDA receptors (Lee et al. 1999).

The altered regulation of PMCA-4 and MBP support the findings of Fu et al. (2007) which show myelin degradation by cytosolic PLA2 induced by lysophosphatidylcholine (lyso-PtdCho) via Ca^{2+} influx into myelin. We submit that PMCA-4 upregulation promotes a higher Ca^{2+} influx, resulting in stimulation of Ca^{2+}-dependent PLA2 (Gattaz et al. 1990), which increases lyso-PrdCho (Pangerl et al. 1991) and myelin degradation.

Visinin-like protein 1 (VILIP-1, downregulated: −1.96x), previously related with SCZ (Bernstein et al. 2002) is a calcium sensor that has Ca^{2+}-dependent modulatory effects on signaling (Polymeropoulos et al. 1995) and has roles in neurotransmitter systems and in plasma membrane receptor expression and recycling. Spectrin alpha chain (also known as fodrin, downregulated: −2.32x) has proteolytic activity which is initiated by calcium-activated proteases. Both proteins were found to be regulated in ATL SCZ.

Energy metabolism

Transcriptome and proteome alterations in energy metabolism have been extensively described in patients with SCZ (Prabakaran et al. 2004; Ben-Shachar and Laifenfeld 2004; Bubber et al. 2004; Karry et al. 2004; (Clark et al. 2006; Martorell et al. 2006; Mehler-Wex et al. 2006) and our data have confirmed these findings. We detected a downregulation of several members of the NADH-ubiquinone oxidoreductase complex NDUFS3, NDUFS6, NDUFB5 and NDUFV2 (−1.89x, −1.72x, −2.08x and −2.08x, respectively). The connection of energy metabolism in neuronal plasticity and synapse (reviewed in Ben-Shachar and Laifenfeld 2004) as well as evidence for oxidative damage in SCZ brains (reviewed in Yao et al. 2001) and dopamine toxicity through mitochondrial complex I inhibition (Ben-Shachar et al. 2004) point to an important energetic component in SCZ.

Other regulated proteins

In our analysis we found a number of regulated proteins that have been previously reported to be altered in SCZ (Table 3, contains the references). These include hemoglobin subunit beta (−1.75x), which can compromise neuronal microcirculation (Nakashima et al. 1996) and result in attention deficits (Toichi et al. 2004) and Ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50, upregulated: 1.58), that controls the activity of Na+/H+ exchanger type 3, participates in recycling of membrane receptors (Heydorn et al. 2004) and transport proteins to the cell surface (Shenolikar and Weinman 2001); EBP50 mRNA was found downregulated with altered expression in peripheral blood lymphocytes of SCZ patients (Bowden et al. 2006). Moreover, we found the downregulation of three of the five known subunits of the 14-3-3 family of proteins (eta, zeta/delta and gamma) (−2.04x, −1.92x and −2.17x, respectively). All three 14-3-3 subunits, including the beta subunit, were also found to be downregulated in SCZ brains in a publication by Middleton et al. (2005). 14-3-3 proteins bind and regulate other proteins, modulate neurodevelopment, cell-division, signal transduction and gene transcription.

The neurofilaments M (NEFM, downregulated: −2.17x) and L (NEFL, downregulated: −3.13x) belong to a family of proteins recently named DRIP (dopamine receptor interacting protein) and have important functions in the dopamine receptor signal transduction pathway (Bergson et al. 2003); NEFL is directly associated with NMDA receptors (Ehlers et al. 1995). Recently, prohibitin (PHB, upregulated: 1.57x), that has many roles in apoptosis (Bruneel et al. 2005), maintenance of mitochondrial function and protection against senescence (Arnold and Langer 2002), was found downregulated in SCZ dorsolateral prefrontal cortex (Behan et al. 2008) differently of our results.

New potential SCZ markers

Next to proteins that had been previously associated with SCZ pathogenesis we also found 11 proteins that had not been described previously to be regulated in ATL-SCZ. Significant differences in expression were seen for these proteins and suggest their potential role in the disease.

Phosphatidylethanolamine-binding protein 1 (PEBP1, upregulated: 2.86), is a substrate of calpain (Chen et al.

Table 3 Proteins found regulated in our study of ATL-SCZ, which have previously been described in other reports as relevant in SCZ

Gene symbol	Product name	Type of analysis	Tissue	Described as regulated by
MOG	Myelin oligodendrocyte glycoprotein	1. Microarray and qPCR 2. Microarray 3. Microarray and qPCR	1. Pre-frontal cortex 2. Several brain region 3. Pre-frontal cortex	1. Tkachev et al. 2003 2. Katsel et al. 2005a 3. Arion et al. 2007
MBP	Myelin basic protein	1. Immunoassay 2. Microarray and qPCR	1. Anterior frontal cortex 2. Pre-frontal cortex	1. Honer et al. 1999 2. Tkachev et al. 2003
GFAP	Glial fibrillary acidic protein	1. Proteomics 2. In situ hybridization 3. Microarray and qPCR	1. Frontal cortex 2. Cingulate cortex 3. Pre-frontal cortex	1. Johnston-Wilson et al. 2000 2. Webster et al. 2005 3. Tkachev et al. 2003
CNP	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase	1. Microarray 2. Microarray and qPCR 3. Microarray 4. Microarray and proteomics 5. Microarray 6. Microarrays 7. qPCR	1. Cortical tissues 2. Pre-frontal cortex 3. Temporal gyrus 4. Pre-frontal cortex 5. Several brain region 6. Anterior cingulate cortex and hippocampus 7. Anterior cingulate cortex	1. Hakak et al. 2001 2. Tkachev et al. 2003 3. Aston et al. 2004 4. Prabakaran et al. 2004 5. Katsel et al. 2005a 6. Dracheva et al. 2006. 7. McCullumsmith et al. 2007
PPP3CA or CALN	Calcineurin	1. Microarray 2. Microarray	1. Cortical tissues 2. Hippocampus	1. Hakak et al. 2001 2. Eastwood et al. 2005
SPTAN1	Spectrin alpha chain (fodrin)	Western blot	Left superior temporal cortices	Kitamura et al. 1998
14-3-3 eta (YWHAH)	Protein kinase C inhibitor protein 1 (eta)	1. In situ hybridization 2. Microarray	1. Pre-frontal cortex 2. Cerebellum	1. Middleton et al. 2005 2. Vawter et al. 2001
14-3-3 zeta/delta (YWHAZ)	Protein kinase C inhibitor protein 1 (zeta/delta)	1. In situ hybridization 2. Proteomics	1. Pre-frontal cortex 2. Corpus callosum	1. Middleton et al. 2005 2. Sivagnanasundaram et al. 2007
14-3-3 gamma (YWHAG)	Protein kinase C inhibitor protein 1 (gamma)	1. In situ hybridization 2. Proteomics	1. Pre-frontal cortex 2. Corpus callosum	1. Middleton et al. 2005 2. Sivagnanasundaram et al. 2007
ALDOC	Fructose bisphosphate aldolase C	1. Proteomics 2. Microarray and proteomics 3. Proteomics 4. Immunoassay*	1. Anterior cingulate cortex 2. Pre-frontal cortex 3. Frontal cortex 4. Human CSF	1. Clark et al. 2006 2. Prabakaran et al. 2004 3. Johnston-Wilson et al. 2000 4. Willson et al. 1980
HIST1H3A	Histone H3.1	Immunoblotting, immunohistochemical, microarray and qPCR	Pre-frontal cortex	Akbarian et al. 2005
CA2	Carbonic anhydrase 2	1. Proteomics 2. Proteomics	1. Frontal cortex 2. Anterior cingulate cortex	1. Johnston-Wilson et al. 2000 2. Beasley et al. 2006
RPS10	40S ribosomal protein—family S10	Microarray	Pre-frontal cortex	Vawter et al. 2002
EBP50	Ezrin-radixin-moesin-binding phosphoprotein 50	Microarray	Peripheral blood lymphocytes	Bowden et al. 2006
TUBB	Tubulin beta polypeptide	1. In situ hybridization 2. Proteomics	1. Frontal cortex 2. Corpus callosum	1. Virgo et al. 1995 2. Sivagnanasundaram et al. 2007
NEFL	Neurofilament triplet L protein	Proteomics	Corpus callosum	Sivagnanasundaram et al. 2007
NEFM	Neurofilament triplet M protein	Proteomics	Corpus callosum	Sivagnanasundaram et al. 2007

2006), a Ca^{2+}-dependent protease implicated in synaptic chemistry and structure (Etienne and Baudry 1987) with functions in membrane biogenesis (Moore et al. 1996) which reinforces the importance of membrane phospholipid metabolism in SCZ. A recent publication on a PEBP1-knockout mouse has revealed a role of this protein in the control of emotions and complex behavior responses (Theroux et al. 2007). The loss of PEBP1 function has been implicated in AD and behavioral testing in mice revealed a learning deficit (George et al. 2006).

We also found the downregulation of the aggregan core protein (AGC, −2x), a proteoglycan that regulates neurite growth (Ruoslahti 1989) and contains a hyaluronic acid (HA) binding domain, which may associate this protein to the hyaluronan and proteoglycan link protein 2 (HAPLN2) that we found downregulated (−6.25x). HA is of critical importance for the formation of brain extracellular matrix, which maintains brain shape and correct functioning.

Validation of differentially expressed proteins

Whereas we had limitations to perform experiments to validate the potential markers identified by our shotgun approach, we could observe that most of the markers seen here have been previously identified or validated by other groups. Such recurrent genes/proteins (Table 3), gives us confidence that the repetitive findings are true disease identifiers, and that the new markers found here are potentially related to the disease.

Analysis of protein pools

Dramatic alterations in certain proteins of a single individual might 'contaminate' the pool, suggesting unreal alterations. However, we believe that the advantages of sample pooling may overcome the disadvantages.

The main advantage of sample-pooling is the possibility of reducing individual proteome variations not related to the disease, while highlighting the most consistent disease-related alterations. Other advantages include an important reduction in the amount of protein required from each sample, allowing experimental replicates and subsequent studies.

The sample pooling has been successfully used by different authors not only for proteomics (Jiang et al. 2003; Lehmensiek et al. 2007a, b), but also for gene expression analysis (Vawter et al. 2001; Katsel et al. 2005a). In this context, as presented here, the analysis of sample pools seems to be capable of indicating recurring alterations of some pathways, in a solid and consistent manner.

Confounding factors: the ATL-SCZ proteome and the effect of antipsychotics

At this point we do not know whether the alterations detected in our analysis are a cause or consequence of the disease. We also cannot rule out that these markers are caused by a combination of confounding factors such as age, smoking, duration of disease (Table 1), circadian rhythm variations, physical exercise, food intake, medication or agonal state, as all these features can potentially contribute to proteomic alterations. Moreover, for the particular samples used in this study, the electrophoretic profile of proteins from individual samples (cases and controls) suggests that the *post-mortem* intervals did not generate false proteome alterations in these particular analyses (data not shown). The brain samples used in this study were derived from SCZ patients taking varying doses of different neuroleptics (Table 1). Two of the five patients in our study were using haloperidol just before their death. Sugai et al. (2004), using cDNA arrays from cynomolgus monkeys and Narayan et al. (2007), using in situ hybridization in mice, showed that MBP can be modulated by haloperidol treatment. It was also reported that apolipoprotein A-I expression in plasma of SCZ medicated patients is altered (La et al. 2007). Malate dehydrogenase, peroxiredoxin 3, vacuolar ATP synthase subunit beta and mitogen-activated protein kinase kinase 1 were found regulated in the hippocampus of chlorpromazine/clozapine treated rats (La et al. 2006). However, a great number of the identified proteins found to be regulated in the present study have been reported to be associated with SCZ either through genetic linkage or transcriptomic regulation, which are processes largely independent of an exogenous drug effect.

As a consequence we do not know which proteins among the 37 described here are modulated by antipsychotic medication. For example, despite the Narayan et al. (2007) findings, we do not know whether the oligodendrocyte-related proteins found in this study are the consequence of antipsychotic use. In future studies the characterization of the proteome from naive tissues and animal studies will show whether the identified differences are SCZ pathogenesis-related or due to other confounding factors.

Conclusion

The comparison of protein profiles of ATL from SCZ and controls by shotgun proteomics enabled the identification of proteins differentially expressed in diseased samples. Whereas gene polymorphisms and gene expression alterations can play an important role in SCZ, the identification

of disease-related proteins, the true biological effectors, is critical for the understanding of the pathophysiology of the disease.

We do not know whether the identified proteins whose expression is regulated in SCZ are causative, a consequence of the disease or reflect medication effects. However, the identification of proteins that are part of pathways previously reported to be associated with the disease indicates some kind of involvement in the pathology of SCZ.

The observation that many of the markers identified in our analysis have been previously revealed by other groups using alternative approaches such as cDNA microarrays, reinforces their association with SCZ. Together with other studies, our findings suggest the involvement of oligodendrocytes and myelin dysfunction as well as calcium and energy imbalance in SCZ. A central concept of SCZ neurobiology is that its symptoms may arise from a malfunctioning communication between different brain areas, leading to a disruption of the circuitry that underlies behavior and perception. In this context, structural and functional abnormalities leading to brain dysfunction may comprise not only neurodevelopment and neurotransmitters, but also factors important for impulse propagation such as intracellular Ca^{2+} and axonal insulation.

Whereas it is tempting to speculate that a decomposition of the oligodendrocyte-axonic system may be responsible for some SCZ symptoms, the results should still be viewed with caution. It is not clear if the alterations seen here reflect oligodendrocyte dysfunction, a reduction in the number of oligodendrocytes in the samples or a combination of both, or the simple use of haloperidol by two out of our five patients.

The regulation of Ca^{2+} homeostasis-related proteins reinforces the importance of this pathway in SCZ. Ca^{2+} is of pivotal importance for dopamine receptor function (Bergson et al. 2003) and studies of genetic association have revealed calcium-activated potassium-channels as important players in SCZ (Chandy et al. 1998). Moreover, Ca^{2+} influences the activity of several enzymes related to neuronal membrane function, such as a group of phospholipases A2, which consistently have been shown to be altered in schizophrenia (Gattaz et al. 1987; Barbosa et al. 2007). Mitochondrial dysfunction in SCZ is also indicated by the downregulation of several proteins from mitochondrial complex I. In addition, we have identified many proteins that have not previously been associated with SCZ pathogenesis. These include PEBP1 which was recently identified as an important protein involved in complex behavior responses in the brain (Theroux et al. 2007).

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