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Identification of protein biomarkers for schizophrenia and bipolar disorder in the postmortem prefrontal cortex using SELDI-TOF-MS ProteinChip profiling combined with MALDI-TOF-PSD-MS analysis

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This paper describes the high-throughput proteomic analysis of the dorsolateral prefrontal cortex (DLPFC) from schizophrenia (SCHIZ), bipolar (BD), and normal control cohorts from the Harvard Brain Tissue Resource Center performed using ProteinChip technology based on the surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS). The resultant profiles were utilized in classification-tree algorithms for selection of protein biomarker peaks contributing maximally to the differentiation between the examined diagnostic cohorts. Twenty-four such protein biomarker peaks were identified. All of them had lower levels in the SCHIZ cohort as compared to the BD cohort. Also, 21 of these peaks were down-regulated in the SCHIZ cohort vs. the control cohort, and 7 peaks were up-regulated in the BD cohort vs. the control cohort. The proteins constituting these biomarker peaks were recognized via matrix-assisted laser desorption time of flight/postsource decay mass spectrometry (MALDI-TOF-PSD-MS). These proteins represent a wide range of functional groups involved in cell metabolism, signaling cascades, regulation of gene transcription, protein and RNA chaperoning, and other aspects of cellular homeostasis. Finally, after statistical evaluation suggesting that the selected protein biomarkers are not significantly impacted by epidemiological/tissue storage parameters (although, influence of antipsychotic and mood stabilizing drugs could not be fully excluded), the ProteinChip-based profiling was engaged again to demonstrate that the detected SCHIZ-associated changes in the levels of our protein biomarkers could also be seen in DLPFC samples from the brain collection of the Mount Sinai Medical School/Bronx Veteran Affairs Medical Center. This study demonstrates the usefulness of ProteinChip-based SELDI-TOF protein profiling in gaining insight into the molecular pathology of SCHIZ and BD as it points to changes in protein levels characterizing these diseases.

Keywords: Humans; Dorsolateral prefrontal cerebral cortex; Mental disorders; Proteomics; Classification-tree algorithms

Introduction

It is now quite apparent that complex psychiatric diseases, such as schizophrenia (SCHIZ) and bipolar disorder (BD), cannot be traced to alterations in just a few specific molecules, but rather reflect changes in large sets of diverse molecular factors in the brains of the affected individuals (Corsico and McGuffin, 2001; Lidow, 2003). This realization of the complexity of molecular changes underlying major psychiatric diseases, together with the availability of sequences for the full human genome (reviewed in: Rogers, 2003) and development of DNA microarrays (Mirnics and Pevsner, 2004; Ryan et al., 2004), has resulted in an explosion of high-throughput genomic studies in brain tissue from SCHIZ and BD patients (Aston et al., 2004; Chung et al., 2003; Hakak et al., 2001; Iwamoto et al., 2005a,b; Middleton et al., 2002, 2005; Mirnics et al., 2000, 2001; Pongrac et al., 2004; Tkachev et al., 2004; Vawter et al., 2001, 2002). The latter studies identified multiple genes, the levels of which may be affected in SCHIZ and/or BD, which have greatly enhanced our appreciation of the very broad scope of molecular changes associated with these two diseases. Furthermore, they demonstrated that these changes likely involve not just molecules engaged in supporting structural features and activities specific for neural cells, but also molecules essential for such basic aspects of cell functionality as energy production, waste removal, survival in adverse environmental conditions, etc. It is well known, however, that mRNA expression levels do not necessarily translate into a proportional protein abundance (Greenbaum et al., 2003); yet, presently there are very few studies employing high-throughput protein profiling of brain tissue from SCHIZ and/or BD patients (Johnston-Wilson et al., 2000).

This paper describes a high-throughput proteomic analysis of the dorsolateral prefrontal cortex (DLPFC) from the postmortem SCHIZ, BD, and normal control cases from the Harvard Brain Tissue Resource Center (HBTRC). The analysis employed Ciphergen ProteinChip technology that is based on the surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS; Issaq et al., 2002; Weinberger et al., 2002). The resultant profiles were then utilized in classification-tree-based

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computer algorithms (Webb, 2002) for selection of protein biomarker peaks, which among the detected peaks, contributed maximally to the diagnostic differentiation between SCHIZ, BD, and control cohorts (Knable et al., 2002). The proteins constituting these biomarker peaks were identified via matrix-assisted laser desorption time of flight/postsource decay mass spectrometry (MALDI-TOF-PSD-MS; Lieber, 2002). Finally, after statistical evaluation of possible impacts of the epidemiological/tissue storage parameters of the originally examined cases on the selected protein biomarkers, the ProteinChip-based profiling was employed again to verify that the detected changes in the levels of these biomarker proteins could also be seen in postmortem DLPFC samples from the brain collection of the Mount Sinai Medical School/Bronx Veteran Affairs Medical Center (MSMS/BVAMC). The DLPFC was selected for this study as a well-recognized site of psychopathology in both SCHIZ and BD (Manoach, 2003; Selemon and Rajkowska, 2003). While, for obvious reasons, the protein biomarkers found in this study cannot be used in diagnosis, they nevertheless are helpful in evaluating the scope of the prefrontal cortical molecular pathology of SCHIZ and BD.

Materials and methods

Tissue samples

The DLPFC samples of SCHIZ, BD, and control cases for identification of protein biomarkers, were provided to us by the HBTRC (Belmont, MA). Originally, we received samples from 20 cases per cohort. However, visual examination of the protein profiles from 3 cases in the SCHIZ cohort revealed patterns very different from those produced by the remaining 17 cases of this cohort. Although sent to us within the SCHIZ cohort, these 3 cases were actually diagnosed with schizoaffective disorder (SCHD), rather than SCHIZ proper. While the observed dissimilarities in the overall protein profiles between samples from SCHIZ and SCHD are potentially interesting, the low number of the latter samples made it impossible to conduct a meaningful protein biomarker analysis of these samples. Therefore, SCHD samples were not subjected to this analysis and are not included in the present paper. Nevertheless, these and all the rest of the ProteinChip profiles generated in our study from NBTRC-derived cases will soon be available via the web site of this center (http://national_databank.mclean.harvard.edu/brainbank/index.jsp) where they will be linked to individual case histories and the DNA microarray data obtained from the same cases. The removal of 3 SCHD cases from the study left 17 SCHIZ cases (along with 20 BD and 20 normal cases), which is well within the range used in recent high-throughput profiling of psychiatric cases (Iwamoto et al., 2005a,b; Middleton et al., 2005); this number of cases is also sufficient for the selection of protein biomarker peaks via the employed classification-tree algorithms (Webb, 2002). The medical history, and tissue preservation epidemiological and data (the latter derived from case histories supplemented, where appropriate, by toxicology assays) for individual cases from HBTRC are available at http://national_databank.mclean.harvard.edu/brainbank/index.jsp. Table 1 contains the summary of these data for the cases used in our analysis. Among the 17 SCHIZ cases, 8 belonged to paranoid and 9 to undifferentiated types. Also, 16 of these cases had an onset of SCHIZ between 20 and 30 years of age, with only 1 case having an onset of the illness at 45 years of age. For 14 cases, the duration of illness was more than 20 years, while, for 3 cases, the duration of illness was close to 10 years. All BD cases were classified as type 1. Sixteen of these cases had the onset of BD between 20 and 30 years of age, and 4 cases had the onset of the illness when they were more than 40 years of age. Seventeen cases had BD for more than 20 years; 3 cases had BD for 15–10 years.

The verification of general validity of the protein biomarkers identified in the cases from the HBTRC was performed using DLPFC samples from six control and six SCHIZ cases from the MSMS/BVAMC (New York, NY). These cases were selected to match the age range of the cohorts used to identify the protein biomarkers; these matching criteria also determined the number of MSMS/BVAMC cases included in our study. A relatively low number of samples is acceptable since they are employed for protein biomarker verification rather than original identification (Seibert et al., 2004). The demographic and preservation characteristics of individual cases from the MSMS/BVAMC are published in Table 1 in Bai et al. (2004). In the latter table, the control cases used are #12, #25, #27, #28, #29, and #30; the SCHIZ cases used are #35, #36, #42, #49, #72, and #75. Table 1 of the present paper contains the summary of the epidemiological and storage data for these cases. All selected SCHIZ cases in this study belonged to the undifferentiated type and had the illness for more than 20 years, with the onset between 20 and 30 years of age.

While due to space limitations, the epidemiological information in Table 1 includes only summaries, rather than detailed descriptions, of the causes of death it must be stressed that all cases in both HBTRC and MSMS/BVAMC collections died without coma, anoxia, or renal failure, which are known to produce significant deterioration of the molecular organization of brain tissue (Buesa et al., 2004; Webb, 2002). The diagnosis for all the cases used in this study was done by the research clinicians supervising the aforementioned brain collections based on the complete medical charts. In addition, postmortem examinations conducted by the collection's pathologists confirmed that all cases were free of any discernable neuropathological lesions. In all cases, the DLPFC area 46 was identified according to the criteria of Rajkowska and Goldman-Rakic (1995); the gray matter was carefully separated from the underlying white matter and snap-frozen in liquid nitrogen. The tissue samples were stored at −80°C, without thawing until the time of analysis.

Three ~1.0 g pieces of the DLPFC (areas 46/9, identified as above) for purification of the biomarker proteins were provided to us by the HBTRC from normal cases included in the original collection. These pieces were snap-frozen in liquid nitrogen and stored at −80°C as the samples used for protein profiling.

Sample preparation for protein profiling

For each protein profiling, 10 mg tissue per sample was homogenized for 30 s by sonication with a Sonic Dismembrator (Fisher, Hampton, NH) in 500 μl of deionized water containing 0.1 M phenyl methyl sulfonyl fluoride (PMSF). To remove cellular debris, the resultant homogenate was centrifuged for 30 sec at 80 × *g* on a Scientific Micro-centrifuge (Fisher, Pittsburgh, PA). The supernatant was collected and the total protein concentration was determined using CBQCA Protein Quantitation kit (Molecular Probes, Eugene, OR) with fluorescence measured on a SpectraMAXGeminiXS spectrofluorometer (Molecular Devices, Sunny-

Table 1
Summary of demographic, tissue preservation, and treatment characteristics of the diagnostic cohorts from the Harvard Brain Tissue Resource Center and Mount Sinai Medical School/Bronx Veteran Administration Medical Center

	#	PMI (h)	pH	Age (years)	Race	Sex	Cause of death	Side	Substance abuse						Treatment		
									Smok.	Opiates (cases)	Benzodiazepines (cases)	Psychostimulants (cases)	Cannabinoids (cases)	Alc. (cases)	Antipsychotic (cases)	Mood stabilizer (cases)	Antidepressants (cases)
Harvard brain tissue resource center collection																	
Control	20	19.5 ± 5.8	6.5 ± 0.2	63.7 ± 26.2	15 W 3 mixed	11 F 9 M	7 Card. 4 Resp. 5 Canc. 4 Other	9 L 11 R	8 S 10 NS 2 U	7	2	0	0	0	0	0	0
BD	20	22.5 ± 9.0	6.5 ± 0.2	66.0 ± 17.8	17 W 3 mixed	9 F 11 M	7 Card. 7 Resp. 3 Canc. 3 Other	11 L 9 R	9 S 9 NS 2 U	6	0	2	1	4	12	17	3
SCHIZ	17	21.6 ± 5.8	6.4 ± 0.3	62.8 ± 17.6	16 W 1 mixed	8 F 9 M	6 Card 5 Resp. 4 Canc. 2 Other	7 L 10 R	7 S 8 NS 2 U	4	2	0	0	2	14	1	2
Significant		$P = 0.810$ One-way ANOVA	$P = 0.600$	$P = 0.856$	$P = 0.632$	$P = 0.856$	$P = 0.210$ χ^2 test	$P = 0.266$	$P = 0.321$	$P = 0.476$							
Mount Sinai Medical School/Bronx Veteran Administration Medical Center Brain Collection																	
Control	6	13.4 ± 5.0	6.3 ± 0.2	65.5 ± 2.6	6 W	1 F 5 M	3 Card. 3 Resp.	6 L	0 cases	2	0	0	0	0	0	0	0
SCHIZ	6	18.1 ± 5.6	6.3 ± 0.3	63.3 ± 3.6	6 W	2 F 4 M	2 Card. 4 Resp.	6 L	0 cases	2	0	0	0	0	6	0	0
Significant.		$P = 0.405$ Two-tailed t test	$P = 0.999$	$P = 0.243$		$P = 0.411$ χ^2 test	$P = 0.328$										

This summary is based on the case-by-case data available at http://national_databank.mclean.harvard.edu/brainbank/index.jsp for the former collection and Bai et al. (2004) for the latter collection. W—white; mixed—mixed race; F—female; M—male; L—left hemisphere; R—right hemisphere. Smok.—smoking; Alc.—alcohol abuse; Card.—death due to cardiac disease; Resp.—death due to respiratory disease; Canc.—death due to cancer; Other—causes of death ascribed to no more than one case per diagnostic cohort; S—smokers; NS—non-smokers; U—unknown.

vale, CA). Also, the quality of the samples in relation to possible proteolysis (Fountoulakis et al., 2001) was verified using protein mass spectra of each sample generated with Protein 200 Plus LabChip on a 2100 Bioanalyzer (Agilent, Palo Alto, CA). The mass spectrum of each sample was divided into 10 kDa intervals, and the area under the curve for each interval was calculated as a percentage of the area under the curve of the entire spectrum. The intersample CV of the area under the curve for all intervals was <8%, which we considered acceptable for our study.

ProteinChip SELDI-TOF-MS analysis

Three ProteinChip arrays were selected for protein profiling in this study as yielding the best resolution of proteins from our samples based on the preliminary testing with ProteinChip System Starter Kit-4 (Ciphergen, Fremont, CA): weak cation exchange, CM10; strong anion exchange, Q10; and immobilized metal affinity capture-copper, IMAC30-Cu (Ciphergen, Fremont, CA). In addition, non-selective normal phase NP20 arrays (Ciphergen, Fremont, CA) were used exclusively in the verification of specificity and purity of biomarker proteins during their purification for MALDI-TOF-PSD-MS-based identification. All arrays were processed using a C503-006 96-well Bioprocessor (Ciphergen, Fremont, CA), which holds 12 8-spot arrays. The array chips were prepared by washing in the binding buffers: 100 mM sodium acetate (pH 4.0) for CM10; 10 mM Tris (pH 9.0) for Q10, 100 mM sodium phosphate/500 mM sodium chloride (pH 7.0) for IMAX30, and deionized water for NP20. IMAX30 also required activation with 100 mM cupric sulfate followed by neutralization with 100 mM sodium acetate (pH 4.0) prior to washing. The incubation step was conducted at room temperature for 30 min with vigorous shaking. For the protein profiling of the DLPFC samples, 6 'experimental' spots on each array were loaded with 150 μl of mixtures containing 50 μl of sample supernatant and 100 μl of binding buffer. To select the concentrations of the total sample protein to be loaded per 'experimental spot', we examined the linearity of the assays on the CM10, Q10, and IMAX30-Cu chips. For this purpose, a mixture of all control samples (from both collections used in this study) was profiled on each type of ProteinChip at serial dilutions from 2000 to 50 μg/ml followed by analysis of the intensity of 20 peaks in the MW range from 10 to 70 kDa (a resultant protein concentration-intensity chart for 3 such peaks, with MW 15,199 Da, 20,950 Da, and 31,126 Da, on IMAC30-Cu arrays is shown in Fig. 1). Based on these data, we selected the total protein concentration of 800 μg/ml for all three chips. In addition, each array included two 'quality control' (QC) spots. One QC spot (QC1) was loaded with 150 μl of the chip-appropriate binding buffer containing two protein standards: 3 μg of recombinant sperm whale myoglobin (MW, 17.0 kDa; cat. # 70035, Sigma, St. Louis. MO) and 6 μg of bovine serum albumin (MW, 66.6 kDa; cat. # A9306, Sigma, St. Louis, MO). In our preliminary studies, we found that these two proteins bind to all ProteinChip types used in this study. The loading amounts of these proteins were chosen to generate clear peaks within the linear concentration–signal range based on the serial-dilution analysis similar to the one described above for the total sample protein. Another QC spot (QC2) was loaded with 2 μl of reconstituted All-in-1 Protein Standard II (containing 7 proteins with the molecular weights (MW) 7034 Da, 12,230 Da, 16,951 Da, 29,023 Da, 46,671 Da, 66,433 Da, and 147,300 Da; Ciphergen, Fremont, CA) diluted 1:4 with a solution of saturated sinapinic acid (Ciphergen, Fremont, CA) in 50% acetonitrile (ACN) containing 0.5% trifluoroacetic acid (TFA).

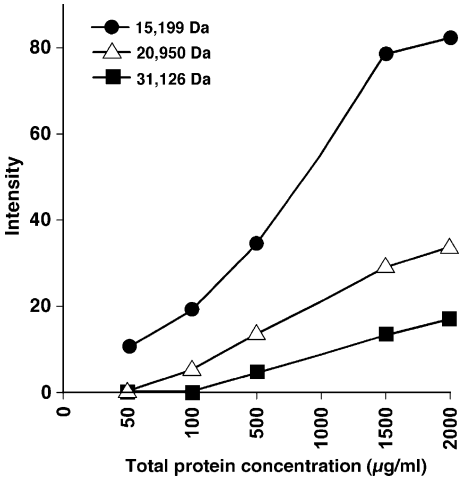

Fig. 1. Relationship of the signal intensity for three peaks with MW 15,199 Da, 20,950 Da, and 31,126 Da to the total DLPFC protein concentration applied to IMAC30-Cu ProteinChips. Note close to linear relationship in 500–1500 μg/ml total protein concentration range.

The DLPFC samples and standards were loaded on each array in random order. Every sample was loaded in duplicates per array on 2 separate arrays of each type. For NP20 arrays used in the process of protein biomarker purification, sample supernatants in the sample/binding buffer mixtures loaded on the 'experimental spots' were substituted with equal volume of various protein aliquots requiring analysis of specificity and/or purity. Upon completion of the incubation, all array spots (with the exception of the QC1 spot) were washed several times, and the arrays were air-dried. To facilitate desorption and ionization of the retained proteins, 1 μl saturated sinapinic acid prepared as described above was applied twice to all array spots (once again, with the exception of the QC1 spot); the spots were allowed to air-dry after each application.

The time of flight spectra from each array was generated using a ProteinChip Reader (Ciphergen, Fremont, CA) by averaging 130 laser shots collected in a positive mode at laser intensity of 245. The detector was set at the sensitivity—8, optimization range—10,000–40,000 Da, and high mass—70,000 Da. The mass accuracy for each type of ProteinChip was calibrated externally using All-in-1 Protein Standard II (Ciphergen, Fremont, CA). For the arrays employed in the DLPFC protein profiling, the interchip performance reproducibility was determined by the examination of the normalized peak intensities produced by myoglobin and albumin in QC1 spots from all chips of a given type. The intra-array variability was assessed by comparing the normalized values of all protein peaks in the 10–70 kDa range between the duplicates of control samples in an array. The calculated interarray CV values were <12% and intra-array CV values were <9% for all array of a given type used in the protein profiling. This suggests a reasonable reproducibility of the measurements (Nomura et al., 2004; Qu et al., 2002). Also, outside the myoglobin and albumin peaks, none of the QC1 spots revealed signal elevations above the normal ionic noise levels, indicating the absence of background interference with the data collection. Examples of protein profiles obtained in this study are shown in Fig. 2.

Peak detection was performed with ProteinChip Software 3.2.1 (Ciphergen, Fremont, CA) The peak calibration for each chip was performed based on the peaks generated by All-in-1 Protein Standard II from the QC1 spot of that chip. Baseline subtraction

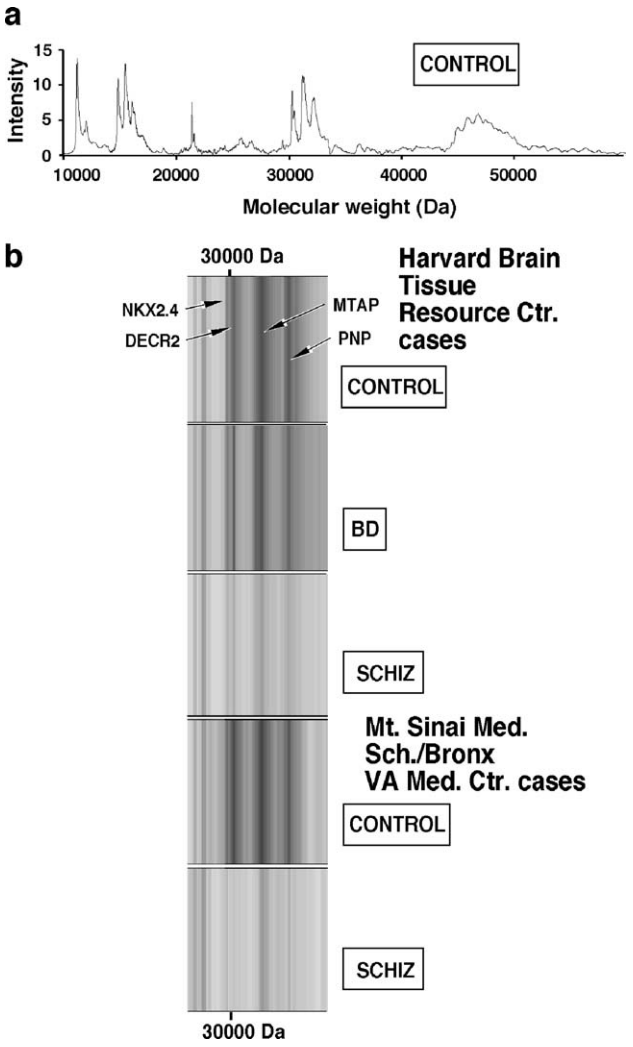

Fig. 2. Examples of protein spectra generated in this study. A typical IMAC30-Cu spectrum (in the chart format) from a normal control case covering the entire range of molecular weights examined in this study (a). A typical example of comparative regional IMAX30-Cu spectra (in densitomatic format) obtained from control, BD, and SCHIZ DLPFC samples from the Harvard Brain Tissue Resource Center and control and SCHIZ DLPFC samples from the Mount Sinai Medical School/Bronx Veteran Administration Medical Center (b). The matching bands containing four biomarker protein peaks are pointed by arrows.

and normalization of peak intensities were performed in batches of ProteinChip arrays of the same type based on the total ion current. The preliminary selection of the peaks with potential interdiagnostic differences to be included in the protein biomarker identification analysis was accomplished with the Biomarker Wizard module of the software at the settings: signal/noise ratio for the first pass—5; minimum peak threshold—30%; cluster mass window—2%; signal/noise ratio for the second pass—2. The peaks expressed by <30% of the cases in the collections were not considered.

Biomarker protein selection

The normality of the intensity distributions of the peaks selected by the Biomarker Wizard was examined by Kolmogorov–Smirnov analyses for each diagnostic cohort (SPSS 13, SPSS Inc., Chicago, IL). In all cases, P values >0.05 suggested a Gaussian distribution. This justified the use of one-way ANOVAs as the appropriate statistical tests for the comparison of the intensities of the protein peaks between the cohorts. These ANOVAs were performed using parallel ANOVA paradigm [with the Benjamini and Hochberg's false discovery rate (FDR) correction procedure for FDR of 0.05] followed by Tukey's post hoc test. The peaks showing statistically significant differences between diagnostic cohorts were further processed with two classification-tree computer algorithms: (a) Classification and Regression Tree (CART; Breiman et al., 1984) implemented in the Biomarker Pattern Software 5.0 (Ciphergen, Fremont, CA) and (b) Random Forest (Breiman, 2001) available as a part of the R-project package at http://www.r-project.org.

The first algorithm was run under the following rules: tree type—classification; method—Gini; root nodes—3; splitting—maximum reduction of cost in the descendant nodes; pruning to prevent overfitting—the minimal tree size is determined by the cost of branch removal. The importance score for each peak as a biomarker was calculated based on the improvement attributable to this peak as a surrogate to the primary split. For each peak, the values of the improvements from all nodes were totaled and scaled relative to the best performing peak, which was designated as having importance score of 100.

For the second algorithm, all the tested peaks were used at each branching point based on the Rftune function analysis demonstrating that this results in the minimal classification error. Each tree was built using ~66% of cases, with the remaining cases acting as 'out of the bag' source. This created 500 trees 'voting' for inclusion of individual cases in a diagnostic group. The measure of biomarker peak importance of each peak was calculated as an average reduction of the accuracy of the diagnostic group assignment produced by randomly permuting the intensity of this peak and running the new values down the trees.

Biomarker protein purification and digestion

The biomarker proteins were purified from homogenates of the designated human DLPFC from the HBTRC prepared by sonication and centrifugation as described earlier. The initial step of homogenate fractionation was performed employing CM-Ceramic HyperD F, Q-Ceramic HyperD F, and IMAC HyperCel separation spin columns (Ciphergen, Fremont, CA). The absorbents in these columns match those on the surface of CM10, Q10, and IMAC30-Cu ProteinChip arrays, respectively. These columns were used in a Scientific Micro-centrifuge (Fisher, Pittsburgh, PA) and spun at 80×g for 30 s for each solution removal. After being washed in column-appropriate binding buffers (and IMAC HyperCel column activation by 100 mM cupric sulfate), the columns were incubated for 40 min with 1.5 ml of the same buffers diluted 3:1 with the tissue homogenate containing 2000 μg/ml total protein. The incubation was followed by two washes in the binding buffer. In the cases of CM-Ceramic HyperD F columns, the bound proteins were eluted with a series of sodium sulfate gradients (100–500 mM); for Q-Ceramic HyperD F columns, the elution was performed with a series of sodium chloride gradients (100–1000 mM); and for IMAC HyperCel columns, the elution gradients were based on concentrations of imidazole (100–500 mM). The elution aliquots (5–6 per column) were collected, desalted using Protein Desalting Spin Columns (Pierce, Rockford, IL), and examined with the column-corresponding ProteinChip arrays to determine the fractions containing the peaks of interest. The fractions containing such peaks were mixed 1:1 with Laemmli

buffer (Sigma, St Louis, MO). After 1 h of incubation, these mixtures were loaded onto ready made 4–20% gradient Criterion gels (Bio-Rad, Hercules, CA) and run for 1 h at 200 V. The gels were stained with Gelcode Blue Stain (Pierce, Rockford, IL) and the bands with the molecular weight corresponding to those of the biomarker peaks were punched out. To check the purity and specificity of the collected proteins, punched out pieces from one line on each gel were subjected to protein extraction with ProtoPlus130 kit (Q-BIOgene, Irvine, CA). The extracted proteins were dried on a SpeedVac (Savant, Irvine, CA), reconstituted in deionized water to the concentration 100 μg/ml (measured with CBQCA Protein Quantitation kit; Molecular Probes, Eugene, OR), and subjected to SELDI-TOF-based protein MW verification using non-selective NP20 ProteinChip arrays. The gel punches parallel to those failing to display a protein of interest or containing complex protein mixtures were discarded. The punches selected for further analysis were digested overnight using Trypsin Profile IGD Kit (Sigma, St. Louis, MO). The duration of the digestion was chosen to generate peptides with MW <3500 Da. The digests were acidified by TFA added to achieve the concentration of 0.5% and purified with ZipTip C18 pipette tips (Millipore, Billerica, MA). Each ZipTip tip was activated with 100% ACN and equilibrated with 0.1% TFA. Each digest was aspirated and dispensed 10 times. The tip was washed with aqueous 0.1% TFA and the bound peptides were eluted with 5 μl 50% ACN/0.1% TFA.

Protein identification using MALDI-TOF-PSD-MS analysis

The ZipTip protein eluants were mixed 1:1 with MALDI Matrix solution (50% ACN/0.05% TFA/α-cyano-4-hydroxycinnamic acid at saturation) and spotted in duplicates on AnchorChip Plates (Bruker Daltonics, Billerica, MA) and processed for spectrometric analyses on an Autoflex Mass Spectrometer (Bruker Daltonics, Billerica, MA) operating in reflector mode for positive ion detection. The masses were calibrated externally using ProteoMass Peptide MALDI-MS Calibration kit (Sigma, St. Louis, MO). The linear spectra data and spectra after MALDI-MS-PSD for several peaks in each trypsin digestion were collected with FlexControl 1.1 software and analyzed with FlexAnalysis 2.0 software (both from Bruker Daltonics, Billerica, MA). The identification of proteins from peptide mass fingerprinting data was done using the following programs: PeptIdent program (http://us.expasy.org/tools/peptident.html) accessing the UniProt (Swiss-Prot/TrEMBL) database; Protein Prospector 3.4. MS Fit and MS Tag tools (University of California, San Francisco, CA) accessing the NCBInr database; and Mascot (Matrix Science, Boston, MA) accessing the NCBI and MSDB databases. The protein search in the databases was performed at the following search parameters: molecular mass range ±10%; mono-isotopic peptide mass; mass tolerance ±1.0 Da; matching at least four peptides; two missed cleavages allowed; cysteine modified by acrylamide; fragment mass tolerance ±2.0 Da; and maximum 25% unmatched ions.

Statistics employed for evaluation of influences of tissue preservation, epidemiological, and treatment parameters on protein biomarkers in the cases from the HBTRC and verification of changes in the levels of these biomarkers using cases from MSMS/BVAMC

The initial statistical evaluation of tissue preservation and epidemiological parameters in the cases from the HBTRC included one-way ANOVA comparisons between control, BD, and SCHIZ cohorts for the continuous variables, such as postmortem intervals (PMI), tissue pH, and age at the time of death. For the categorical variables, such comparison was performed using χ^2 tests. The latter comparisons, included: race, sex, causes of death (collapsed into the categories of cardiac diseases, respiratory diseases, cancer, and other causes seen in no more than one case per cohort; Table 1), brain side, smoking, and opiate abuse. Alcohol, benzodiazepines, psychostimulants, cannabinoids, and antidepressants were used by too few cases to warrant statistical analysis (Table 1). In both ANOVA and χ^2 tests (SPSS 13, SPSS Inc., Chicago, IL), significance was determined at FDR of 0.05 (Benjamini et al., 2001). Possible influences of the continuous epidemiological/tissue preservation variables on each protein biomarker were further examined by Pearsons's least squares correlation analyses. The presence of correlation was inferred at $r > 0.5$ combined with $P < 0.05$ (Norman and Streiner, 1998). The influence of the bimodal categorical variables (with the exception of those noted earlier as unwarranting statistical evaluation) on each biomarker protein was examined using parallel two-tailed Student's t tests or parallel ANOVAs implemented in GeneSpring GX software (Agilent, Palo Alto, CA) with the Benjamini and Hochberg's FDR correction procedure with FDR of 0.05.

Only 3 BD cases were free of mood stabilizers and 3 SCHIZ cases were free of antipsychotics at the time of death (for > 3 years in all cases based on treatment record). Such small numbers are insufficient to perform proper statistical comparisons between medicated and unmedicated cases in these two cohorts. In our previous studies (Bai et al., 2004; Koh et al., 2003a,b), the effects of these medications were inferred from the examinations of drug-treated monkeys. Unfortunately, this approach is impossible to implement in the present study since monkey protein databases lack information about the majority of our biomarker proteins. Consequently, we cannot identify with any certainty either SELDI-TOF-MS peaks or MALDI-TOF-MS peptide sequences for the monkey versions of these proteins. To provide at least some appreciation of the possible role of medications in regulating the levels of our protein biomarkers, we plotted the overall distribution of the intensities of the ProteinChip-generated peaks for each of these proteins in both disease and control cohorts. Then, the intensities of the corresponding peaks in the antipsychotic-free SCHIZ cases and mood stabilizer-free BD cases were marked within the distributions of the respective cohorts. The influence of a disease on a protein biomarker was considered as likely exceeding that of the corresponding medication when, compared to the mean peak intensity for this protein of the diseased cohort, its peak intensities in the medication-free cases fell in the direction away from the control distribution or within 1 SD toward the control distribution. For an additional evaluation of the role of antipsychotics, we took advantage of the fact that, based on the medical records, the treatment of 12 BD cases included these drugs, while the rest of BD cases received no antipsychotics for at least 10 years prior to death (Table 1). Therefore, we compared the intensities of the protein biomarker peaks between the antipsychotic-treated and untreated cases from the BD cohort. These comparisons were conducted using parallel two-tailed Student's t tests, with FDR of 0.05 as described above (GeneSpring GX software, Agilent, Palo Alto, CA).

In addition, since the SCHIZ cohort from the HBTRC used for the protein profiling included tissue from both paranoid and undifferentiated patients, we employed parallel two-tailed Stu-

dent's t tests with the FDR of 0.05 (GeneSpring GX software, Agilent, Palo Alto, CA) for comparison of the protein biomarker levels between the cases with these two types of the disease. No intertype comparison was required for BD cases since all belonged to the type 1 of this disease. Also, since the great majority of SCHIZ and BD cases had the onset of their illness between 20 and 30 years of age and by the time of death had suffered from it for at least 20 years, no statistical analysis was used for the evaluation of either the onset of the disease or its duration on the levels of the protein biomarkers identified in this study. We were prepared to conduct ANCOVAs for our protein biomarkers by diagnosis with all the demographic and storage parameters showing significant effects in the above-mentioned tests as covariates. However, none of the tests revealed significant effects warranting such ANCOVAs.

The cases from the MSMS/BVAMC included only control and SCHIZ cohorts (Table 1). The comparability of PMI, tissue pH, and age between these two cohorts was checked with two-tailed Student's t tests, while the comparability of these cohorts in respect to sex and cause of death compositions was checked with χ^2 test (SPSS 13, SPSS Inc. Chicago IL) with the application of FDR of 0.05 (Benjamini et al., 2001). None of the cases had a history of substance abuse, except 2 smokers in each cohort. In addition, all SCHIZ cases were of the same type with similar time of the onset and length of duration. Finally, all SCHIZ cases were on antipsychotic drugs at the time of death.

The examination of the differences in the selected protein biomarkers between the control and SCHIZ cohorts from the MSMS/BVAMC was performed using parallel two-tailed Student's t tests with FDR of 0.05 as described above (GeneSpring GX software, Agilent, Palo Alto, CA).

Results

Overview of the protein profiles and protein biomarker identification in the DLPFC of control, BD, and SCHIZ cohorts from the Harvard Brain Tissue Resource Center

The ProteinChip software-based analysis of the CM10, Q10, and IMAC30-Cu profiles of the DLPFC samples from the Harvard Brain Research Tissue Resource Center detected ~200 protein peaks for each type of these Protein Chips. Out of these peaks, the Biomarker Wizard algorithm selected 53 peaks for further examination as having potentially different intensities between at least 2 diagnostic cohorts. All the peaks were detectable in all three diagnostic cohorts examined in this study. The majority of the selected peaks had unique MW. The exceptions were 3 peaks from CM10 arrays and 3 peaks from IMAC30-Cu arrays with closely matching MWs. For these peaks, further analysis was performed using the data from IMAC30-Cu arrays. The application of one-way ANOVAs confirmed the significant interdiagnostic differences for 46 non-overlapping peaks (P values < the assigned FDR levels). When compared to their intensities in the control cohort, 10 of the latter peaks were up-regulated and 23 peaks down-regulated in SCHIZ cohort while 13 peaks were up-regulated and 11 peaks were down-regulated in BD cohorts (P values < 0.05 in Tukey's post-tests followed ANOVAs with P values < the assigned FDR levels). Among the aforementioned 46 peaks, the Biomarker Pattern and Random Forest classification algorithms identified 24 peaks as diagnosis biomarkers with the importance levels above 0. Although being ranked differently on the order of importance, the same 24 peaks were selected by both classification algorithms (Fig. 3). The identification of the proteins constituting the biomarkers was based on the linear spectra of 4–11 peptides/protein digest

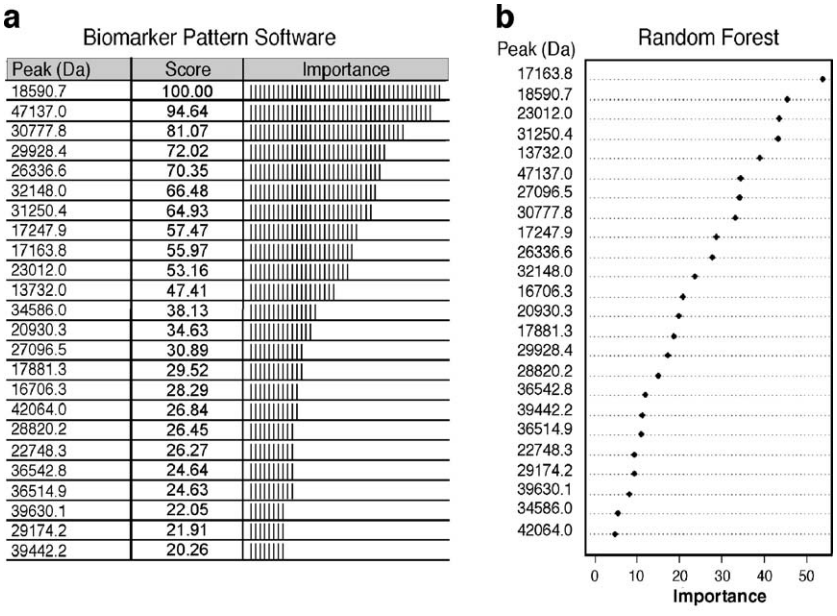

Fig. 3. Protein peaks (identified by their molecular weight) with diagnostic biomarker importance above 0 selected by Biomarker Pattern (a) and Random Forest (b) classification algorithms. In the Biomarker Pattern software, the importance score for each peak as a biomarker was calculated based on the improvement attributable to this peak as a surrogate to the primary split of the classification tree. For each peak, the values of the improvements from all nodes were totaled and scaled relative to the best performing peak, which was designated as having an importance score of 100. In the Random Forest paradigm, the measure of biomarker importance of each peak was calculated as an average reduction on the accuracy of the diagnostic group assignment produced by randomly premuting the intensity of this peak and running the new values down the classification trees. Note that, although being ranked differently on the order of importance, the same 24 peaks were selected by both algorithms.

with the ion-PSD spectra confirmation for 2 of these peptides (for the number of peptides used to identify each biomarker protein, see Table 2). The identities of the protein biomarkers (along with the common abbreviations of their names and the number of peptides used for their identification) are presented in Table 2.

Levels of protein biomarkers in the diagnostic cohorts from the Harvard Brain Tissue Resource Center

Among the peaks representing the 24 biomarker proteins identified in this study, 21 peaks displayed significantly different intensities in the profiles of the DLPFC samples from the SCHIZ cohort as compared to their intensities in the profiles of the corresponding samples from the control cohort (Fig. 4). Specifically, the peaks representing LFA-3, CALM1, MAGOH, MAXb, RGS11, UCTH-L1, CBF2, Hsp60s2, PGAM1, ε14-3-3, NKX2.4, DECR2, MTAP, PNP, BYSL, ANKRD 12, HSPC 124β, ALDOC, SMPD1, GS, and NSE were down-regulated by 25–65% (Fig. 4). The peaks of the remaining 3 biomarker proteins (CYCL-A, MBP-18.5, and DKK2) failed to exhibit significant differences in intensity between SCHIZ and control cohorts (Fig. 4).

The significant differences between BD and control cohorts were detected for the intensities of the peaks representing 7 biomarker proteins (Fig. 4). All these peaks (MBP-18.5, DKK2, CBF2, DECR2, BYSL, ANKRD 12, and ALDOC) were up-regulated (by 30–45%). The other biomarker proteins (LFA-3, CALM1, MAGOH, MAXb, CYCL-A, RGS11, UCTH-L1, Hsp60s2, PGAM1, ε14-3-3, NKX2.4, MTAP, PNP, HSPC 124β, SMPD1, GS, and NSE) showed no differences between the BD and control cohorts (Fig. 4). The peaks for all 24 biomarker proteins displayed much lower intensities (by 25–65%) in the SCHIZ cohort as compared to those in the BD cohort (Fig. 4). This includes, CYCL-A, the only biomarker protein with statistically significant differences confined to SCHIZ vs. BD cohorts.

Examination of possible influences of tissue storage, epidemiological, and treatment parameters on the biomarker protein levels

The control, SCHIZ, and BD cohorts used for the identification of biomarker proteins were statistically similar in respect to PMI, tissue pH, age, race, sex, cause of death, side of the brain, smoking, and opiate abuse (Table 1). The lack of significant influence of PMI, tissue pH, and age on the levels of the biomarker proteins was further demonstrated by the results of the analysis of possible correlation between the values of these parameters and the protein biomarker peak intensities in the cases from all three cohorts (for all correlations, r was within ±0.25 with $P > 0.05$; for typical examples see Fig. 5). Similarly, the absence of significant influence on the protein biomarkers by race, sex, brain side, cause of death, smoking, and opiate abuse was demonstrated by P values > the assigned FDR levels in all the t tests and ANOVAs in which biomarker intensities were grouped by these parameters (for typical examples see Fig. 5). As mentioned earlier, too few cases in each cohort abused substances other than nicotine (smoking) or opiates, or were treated with antidepressants to affect our results (Table 1).

In the present study, only 3 antipsychotic-free cases and 3 mood stabilizing medication-free cases were present in the entire SCHIZ and BD cohorts, respectively. This forced us to focus our analysis of the role of the disease-specific treatment in determining the levels of the protein biomarkers in these two cohorts to the examination of the position of the aforementioned antipsychotic and mood stabilizer-free cases in relation to the overall distribution of cases in the corresponding diagnostic cohorts. In the 3 antipsychotic-free SCHIZ cases, the peak intensities of all 21 protein biomarkers affected in SCHIZ cohort, when compared to their mean cohort values, fall either in the direction away from the distribution of the control cohort or within 1 SD in the direction of the latter distribution. Similar positioning within the intensity distributions of the 7 protein biomarkers altered in BD cohort was also observed for the corresponding peaks generated by the 3 mood stabilizer-free cases (not shown). According to the criteria chosen for this analysis, these observations could be viewed as indicating that the influence of the disease on the protein biomarkers may exceed that of medications. In addition, the t test comparison of the peak intensities for all protein biomarkers between the antipsychotic-treated ($n = 12$) and untreated ($n = 8$) cases from the BD cohort revealed no significant differences (for all tests, $P >$ the assigned FDR levels). A typical example of the latter comparisons is shown in Fig. 5.

The earlier-noted similarity in the time of the onset and duration of the illness for most cases either within SCHIZ or within BD cohorts, precluded us from the examination of the influence of these parameters on the levels of our protein biomarkers. Also, all the BD cases belonged to the type 1. Therefore, no inter-BD type comparison for the protein biomarkers was conducted. Finally, while SCHIZ cohort contained both paranoid and undifferentiated cases, the t tests failed to detect significant differences between these two SCHIZ types for any of the protein biomarkers (for all tests, $P >$ the assigned FDR levels).

Verification of changes in the levels of the protein biomarkers in SCHIZ cases from MSMS/BVAMC

To verify the general validity of the selected protein biomarkers, the intensities of peaks representing all 24 biomarker proteins identified in this study were examined in the control and SCHIZ cohorts from the second tissue source, the Mount Sinai Medical School/Bronx Veteran Affairs Medical Center. These two cohorts were statistically similar in respect to PMI, tissue pH, age, gender, and substance abuse (Table 1). Our examinations revealed differences matching those found in our primary SCHIZ and control cohorts from the HBTRC. The SCHIZ cohort displayed significantly lower intensities (by 25–65%; t tests with P values < the assigned FDR levels) of peaks for all protein biomarkers, except CYCL-A, MBP-18.5, and DKK2. The latter 3 protein biomarkers had statistically similar peak intensities in both SCHIZ and control cohorts (t tests with P values > the assigned FDR levels; Fig. 6).

Discussion

Limitations of analysis

SELDI-TOF-MS provides a highly sensitive analysis of proteins deposited on Protein Chip (Seibert et al., 2004). Nevertheless, the ProteinChip-based methodology is not designed either for complete comprehensive profiling of proteins (Freeman and Hemby, 2004; Rocken et al., 2004) or allows a preselection of proteins for analysis on a defined functional basis. The presence of specific peaks on a chip depends on a complex combination of the

Table 2
The identity of the protein biomarkers identified in this study (with the number of peptides in trypsin digests used to establish this identity in MALDI-TOF-PSD-MS analysis), the common abbreviations of their names used in this paper, their major functional activities, and their presence in neurons and glia

MW (Da)	Number of peptides from: linear spectrum MALDI-TOF/ion-PSD spectra	Name	Abbreviation	Function							Expressing cells	
				Cell metabolism	Glycolysis	Lipid metabolism	Signal transcription	Transcription factor/cofactor	Chaperone	Involved in response to stressful environment	Glia	Neuron
13732.0	6/2	Lymphocyte function-associated antigen 3	LFA-3							Yes	Yes	U
16706.3	4/2	Calmodulin-1	CALM1				Yes				Yes	Yes
17163.8	4/2	Human mago-nashi homolog	MAGOH						RNA chaperon		Yes	Yes
17247.9	4/2	Myc-associated factor X isoform b	MAXb					Yes		Yes	Yes	Yes
17881.3	8/2	Cyclophilin A	CYCL-A				Yes		Yes	Yes	Yes	Yes
18590.7	9/3	Myelin basic protein 18590	MBP-18.5								Yes	No
20930.3	4/2	Dickkopf homolog 2	DKK2				Yes				Yes	Yes
22748.3	4/2	Regulator of G-protein signaling 11	RGS11				Yes				Yes	Yes
23012.0	4/2	Ubiquitin carboxyterminal hydrolase L1	UCTH-L1	Yes						Yes	Yes	Yes
26336.6	6/2	CCAAT-box-binding transcription factor 2	CBF2								U	U
27096.5	5/2	Heat shock protein 60 short isoform 2	Hsp60s2						Yes	Yes	Yes	Yes
28820.2	4/2	Phospho-glycerate mutase 1	PGAM1	Yes	Yes						Yes	Yes
29174.2	4/2	14-3-3 ε isoform	ε14-3-3				Yes		Yes	Yes	Yes	Yes
29928.4	5/2	Human NK2 homeobox transcription factor homolog 4	NKX2.4					Yes			U	U
30777.8	5/2	Peroxisomal 2,4-dienoyl- CoA reductase 2	DECR2	Yes		Yes				Yes	Yes	Yes
31250.4	4/2	5′-methyl-thioadenosine phosphatase	MTAP	Yes							Yes	Yes
32148.0	4/2	Purine nucleoside phosphatase	PNP	Yes			Yes			Yes	Yes	Yes
34586.0	Bystin	BYSL								Yes	Yes	No
36514.9	4/2	Ankyrin repeat domain protein 12	ANKRD12					Yes			Yes	Yes
36542.8	4/2	Mitochondrial inorganic pyrophosphatase β subunit	HSPC124β	Yes						Yes	Yes	Yes
39442.2	6/2	Aldolase C	ALDOC	Yes	Yes						Yes	Yes
39630.1	4/2	Acid sphingomyelinase isoform 1	SMPD1	Yes		Yes	Yes			Yes	Yes	Yes
42064.0	4/2	Glutamine synthetase	GS	Yes	Yes		Yes				Yes	No
47137.0	11/2	Enolase 2	NSE	Yes	Yes						No	Yes

U—unknown.

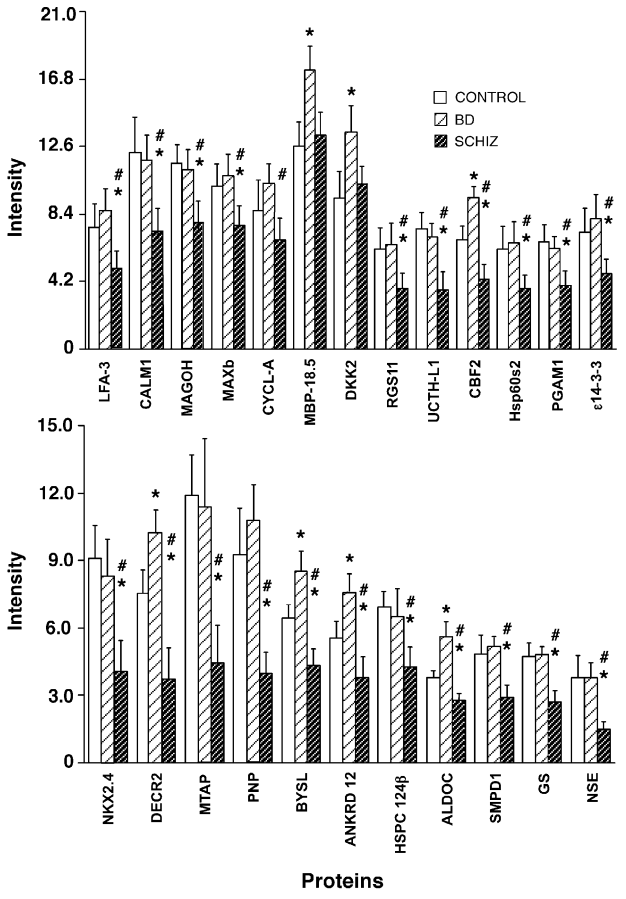

Fig. 4. The levels of the biomarker proteins identified in this study in control (n = 20), BD (n = 20), and SCHIZ (n = 17) cohorts from the Harvard Brain Tissue Resource Center. Each bar represents the mean of the peak intensity for a given biomarker obtained from the ProteinChip SELDI-TOF-MS analysis ± SD. The significant differences in biomarker levels from the BD and SCHIZ cohorts as compared to those from the control cohort are marked by asterisks ($P < 0.05$ in Tukey's post-tests followed ANOVAs with $P <$ the assigned FDR levels). The significant differences in the biomarker levels from SCHIZ cohort as compared to those from the BD cohort are marked by the number sign ($P < 0.05$ in Tukey's post-tests followed ANOVAs with P values < the assigned FDR levels). Note that, compared to the control cohort, the SCHIZ cohort shows down-regulation of 21 biomarkers, while BD cohort shows up-regulation of 7 biomarkers. There are significant differences between SCHIZ and BD cohorts for all 24 biomarkers.

binding property of the array surface, composition of the binding buffer, and chosen binding conditions making prediction of the appearance of a particular peak in advance difficult (Chapman, 2002; Fung and Enderwick, 2002, James, 2002), which precludes specific hypothesis testing. Instead, ProteinChip-based profiling is best suited for identifying protein biomarkers of various abnormal conditions in a tissue (Nomura et al., 2004; Ye et al., 2003; Zhu et al., 2004). These protein biomarkers, however, provide an insight into the etiology of a disease by pointing to changes in the protein levels and pathway alterations characterizing this disease (Johnston-Wilson et al., 2001).

In this study, use of detergent-free water in tissue homogenization (for consistent protein extraction) further limited biomarker selection by favoring hydrophilic proteins. Also, our examinations were narrowed to the optimally resolved 10–70 kDa protein range. These restrictions likely contributed to our ability to detect only a relatively small number of peaks showing significant differences in intensities between the diagnostic cohorts. Finally, due to cohort composition, our data could be directly applied only to paranoid and undifferentiated SCHIZ and BD type 1.

Recently, similar to that for DNA microarrays (Bammler et al., 2005; Irizarry et al., 2005; Wang et al., 2005), concerns have been raised regarding inter- and intralaboratory reproducibility of Protein Chip arrays (Baggerly et al., 2004; Diamandis, 2004). The issue of interlaboratory variability in the ProteinChip analysis is beyond the scope of this paper and, as in the case of DNA microarrays, requires standardization of array processing and analysis universally accepted by the field. The intralaboratory consistency of the results has shown to be achievable by careful use of uniform procedures and standards (Semmes et al., 2005). In the present study, the consistency of the data was assured by the use of molecular weight and quantitative QC standards on each chip, uniformity of protein preparation and loading, and examinations of protein peak levels within the linear range of the assay.

Protein biomarkers

The present study has identified 24 proteins in the DLPFC as having importance for differentiating between diagnostic cohorts. None of the aforementioned proteins were unique to a particular diagnostic group—the interdiagnostic differences were in their levels. All biomarker proteins had significantly lower levels in SCHIZ than BD. The majority of these protein biomarkers were down-regulated in SCHIZ relative to normal cases. Only a few protein biomarkers displayed differences between control and BD (all up-regulated). We do not know whether these differences in protein biomarker regulation between SCHIZ and BD cohorts reflect the selection of proteins from which the biomarkers were chosen or some more fundamental aspects of the protein organization of the DLPFC in SCHIZ and BD. We only like to emphasize that the above-noted down-regulation of SCHIZ protein biomarkers and up-regulation of BD protein biomarkers could not be simply ascribed to some technical artifact in chip processing since the original pool of proteins subjected to biomarker-identifying classification-three algorithms included both up-regulated and down-regulated proteins for both cohorts.

It is impossible to account for all non-diagnostic parameters that might influence the biomarker protein levels. Nevertheless, HBTRC's original case selection, combined with our statistical analysis, suggests a low probability that the observed differences between the diagnostic cohorts reflect intercohort dissimilarity in such major preservation/epidemiological variables as cause of death, PMI, tissue pH, age, race, sex, side of the brain, antidepressant treatment, and substance abuse. Similarly, the choice of the protein biomarkers was unlikely to be significantly influenced by a potential variability in the proportion of the underlying white matter in the DLPFC samples. The latter would skew the biomarkers toward major glial (particularly oligodendroglial) proteins. However, only three proteins with the known glia-specific expression (MBP-18.5, BYSL, and GS; Boggs, 2002; Sheng et al., 2004; Tsacopoulos and Magistretti, 1996) are present among the biomarkers, with MBP18.5 being affected only in BD and GS being affected only in SCHIZ. An important confirmation that the differences in protein levels leading to biomarker selection in this study may truly represent the diagnostic divisions comes from the discovery that similar

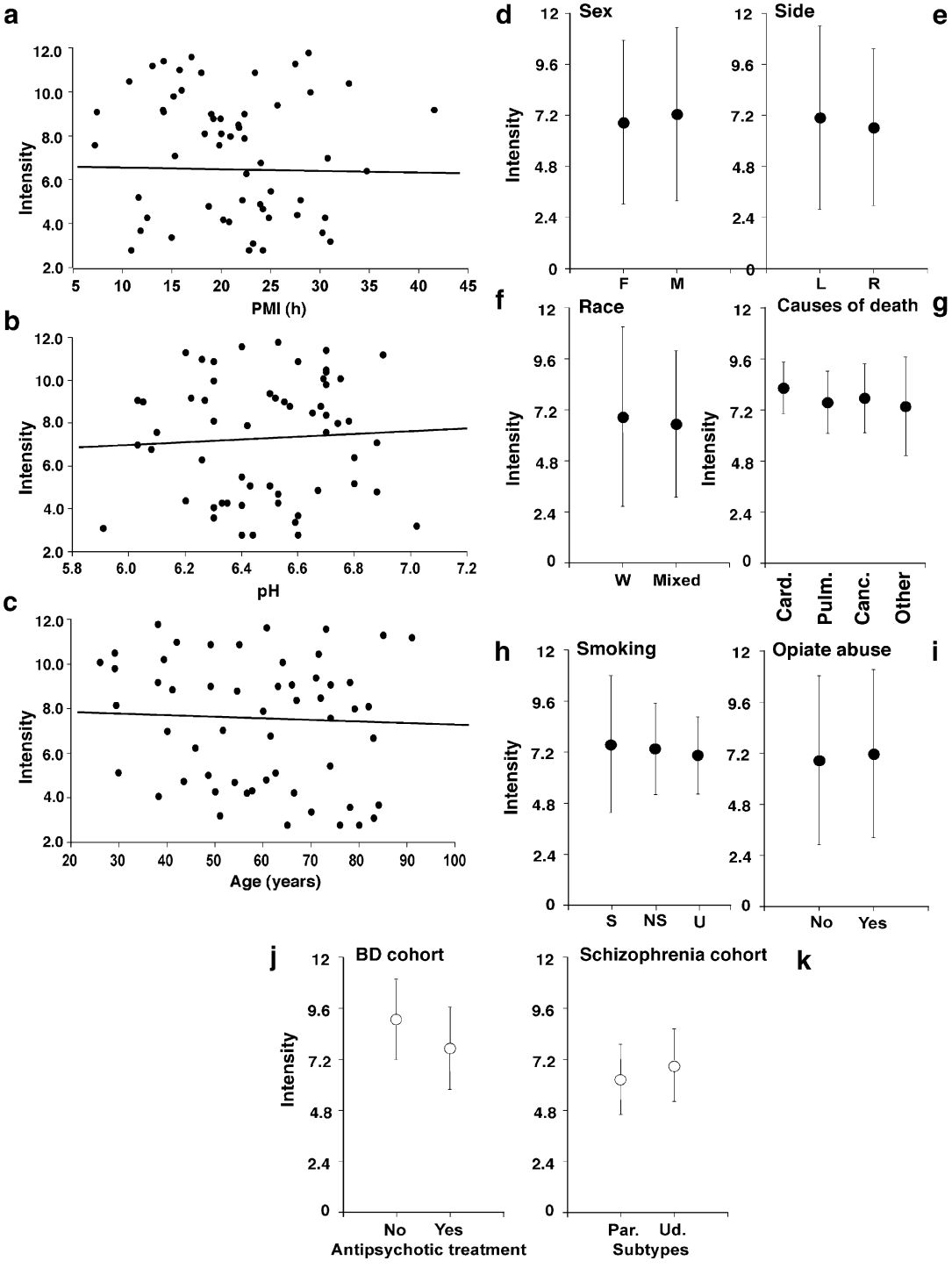

Fig. 5. Typical relationship of the biomarker peak intensities in the cases from the Harvard Brain Tissue Collection to the tissue preservation and epidemiologic parameters, antipsychotic treatment in BD cohort, and types of SCHIZ in the cohort of cases with these disease. (a–c) Plots of correlation analyses of the intensities of LFA-3 peaks in the profiles of the DLPFC samples used in this study (n = 57) with PMI (a), tissue pH (b), and the age of death (c) for the corresponding cases. r correlation coefficient; P significance of r. These correlations are not significant because for all three of them $r <\pm 0.5$ with $P > 0.05$. (d–i) Plots of the intensities of LFA-3 peaks as functions of sex (d), side of the brain (e), race (f), cause of death, (g), smoking (h), and opiate abuse (i). In these plots, the data are presented as mean values ± SD (for n see Table 1); P—significance from t tests (d–f,i) and ANOVAs (g, h). These tests show no significant differences because they generated P values > the assigned FDR levels. (j) Plot of the intensities of LFA-3 peaks in the BD cohort as a function of antipsychotic treatment (for n see Table 1). (k) Plot of the intensities of LFA-3 peaks in the SCHIZ cohort as a function of paranoid (n = 8) and undifferentiated (n = 9) subtypes. The data in the latter two plot are presented and analyzed as described for the plots (d–f,i). For the statistical analysis of the data sets in both plots, P values > the assigned FDR levels indicate no significant differences. S—smokers; NS—non-smokers; U—unknown; Card—death due to cardiac disease Resp.—death due to respiratory diseases; Canc.—death due to cancer; Other—other causes.

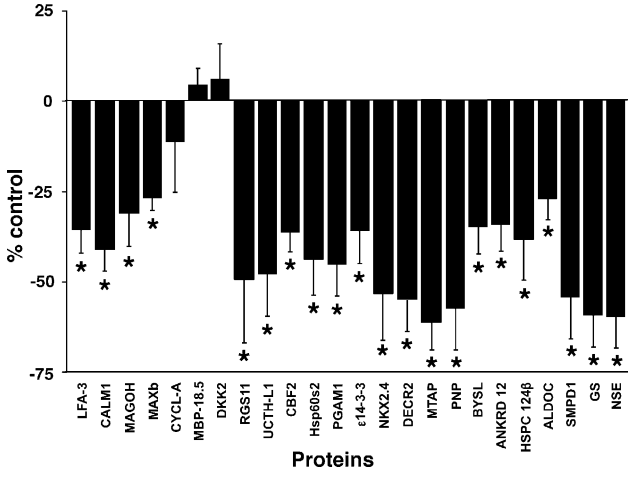

Fig. 6. Verification of the SCHIZ-associated changes in the biomarker protein levels using DLPFC samples from control (n = 6) and SCHIZ (n = 6) cases from the Mount Sinai Medical School/Bronx Veteran Administration Medical Center Brain Collection. Each bar represents the mean percent change ± SD in specific biomarker levels in the SCHIZ cohort as compared to those in the control cohort. The differences reaching statistical significance are marked by asterisks (P value < the assigned FDR levels). Note that SCHIZ cohort from this collection showed significantly reduced levels of the same 21 biomarkers that were down-regulated in the collection of the Harvard Brain Tissue Resource Center (Fig. 4).

alterations in all protein biomarkers were observed in the SCHIZ cohort from our second tissue source, the MSMS/BVAMC.

The influence of antipsychotics and mood stabilizers on the protein biomarker levels in SCHIZ and BD cohorts, respectively, however, could not easily be excluded because in our brain collections only 3 SCHIZ were antipsychotic-free and 3 BD cases were mood stabilizer-free. Some indication that BD diagnosis might have exerted larger influence on protein biomarker selection than mood stabilizers could be derived from our observation that no protein biomarker showing alterations in BD was expressed by the mood stabilizer-free BD cases at levels that fall father than 1 SD from this cohort's mean in the direction of the control cohort. Likewise, a preeminence of SCHIZ diagnosis vs. antipsychotic treatment may be suggested by the biomarkers altered in the SCHIZ cohort being expressed in the antipsychotic-free SCHIZ cases at levels that fall in a similar manner within the later cohort. For antipsychotics, a possible lack of influence on the protein biomarkers is further supported by the absence of differences in biomarker levels between antipsychotic-treated and untreated BD cases.

While the present data cannot fully exclude the effect of psychiatric treatment on the levels of our biomarker proteins, the ProteinChip profiling described in this study still represents an important novel step in the molecular analysis of psychiatric diseases since it demonstrates the capacity of such profiling to identify proteins differently expressed in normal tissue vs. tissue from patients with specific mental disorders. We hope that, in the nearest future, the non-human primate protein databases would expand significantly to allow a meaningful application of SELDI-TOF-MS analysis for addressing the effects of drugs on the protein levels in monkey tissue. While awaiting these advances, we are presently employing Western blots to examine the effects of antipsychotic and mood stabilizers in rhesus monkeys on the biomarkers for which antibodies are available. The resultant data will be published separately.

Protein biomarkes altered in SCHIZ

The levels of all but three protein biomarkers were affected in SCHIZ. They represent a range of functional pathways and emphasize the complex multifactorial nature of this disease. The majority of these protein biomarkers are involved in cell metabolism (Table 2). They include: UCTH-L1 (salvaging of ubiquitin; Wilkinson, 1997), PGAM1, ALDOC, and NSE (glycolysis; Fothergill-Gilmore and Watson, 1990; Gerlt et al., 2005; Thompson et al., 1982); DECR2 (unsaturated fatty acid degradation; Osmundsen et al., 1991); MTAP (salvaging of adenine and methionine in polyamine pathway; Subhi et al., 2003); PNP (adenine and guanine recovery in purine turnover; Ealick, 2002); HSPC 124β (mitochondrial energy generation; Mansurova, 1989), SMPD1 (sphingomyelin degradation in lipid metabolism; Sawai and Hannun, 1999), and GS (glutamate turnover; Tsacopoulos and Magistretti, 1996). These proteins were down-regulated in SCHIZ supporting the view (Iwamoto et al., 2005b; Vawter et al., 2004) that SCHIZ is associated with significant intracellular metabolic abnormalities in the DLPFC. Moreover, among these metabolic protein biomarkers, three are major glycolytic enzymes, ALDOC, PGAM1, and NSE (Fothergill-Gilmore and Watson, 1990; Gerlt et al., 2005; Thompson et al., 1982), which suggests that the DLPFC in SCHIZ suffers from a major glycolytic deficit. This deficit may represent a basis for a reduction in glucose metabolism in the DLPFC reported in positron emission tomographic studies (Buchsbaum and Hazlett, 1998). Down-regulation of GS protein and mRNAs encoding UCTH-L1 and NSE have previously been demonstrated in the DLPFC of SCHIZ patients (Burbaeva et al., 2003; Iwamoto et al., 2005b; Vawter et al., 2001). It also must be noted that, in contrast to our findings, an increase in ALDOC levels was reported in the Brodmann's frontal area 10 of SCHIZ and BD patients (Johnston-Wilson et al., 2000). Unfortunately, the latter studies failed to examine the 9/46 cortical region sampled in this study, while we did not extended our analysis to the area 10. Consequently, it remains unresolved whether the aforementioned discrepancy in ALDOC-related findings reflects regional differences or has some other origin.

Many protein biomarkers down-regulated in SCHIZ are involved in intracellular signaling (Table 2): CALM1 (Ca^{+2} cascade; Means, 2003), RGS11 (Gi/Go signaling: Hooks et al., 2003); ε14-3-3 (multiple signaling cascades; Mackintosh, 2004), PNP (purine nucleoside signaling cascades; Barsotti et al., 2002), and SMPD1 (synaptic lipid signaling; Bazan, 2003). Abnormal intracellular signaling is proposed within multiple hypotheses of SCHIZ (Lidow, 2003). Several earlier studies examined CALM, RGS, and 14-3-3 proteins in SCHIZ. No changes in CALM protein levels, and even up-regulation in CALM gene expression, were reported in the DLPFC of SCHIZ patients (Vargas and Guidotti, 1980; Hakak et al., 2001). The apparent disagreement of these reports with our data may reflect the measurement of only free cytosolic CALM1 in this study. Thus, our findings could represent the redistribution of CALM between the cytosolic and membranous cellular compartments. Alternatively, since earlier studies did not distinguish between CALM isoforms, it is possible that they missed SCHIZ-associated CALM1-specific alterations. Previous investigations involving RGS and 14-3-3 identified SCHIZ-associated changes in mRNAs encoding subtypes of these proteins

different from those registered as biomarkers in this study (Mirnics et al., 2001; Middleton et al., 2005). Consequently, our findings expand the list of RGS and 14-3-3 subtypes affected in SCHIZ.

An important finding is that several protein biomarkers represent transcription factors and chaperones (Table 2), categories of proteins that have not yet attracted wide attention as possible sites of abnormality in SCHIZ (Bates et al., 1996; LaMantia, 1999). These include: MAXb (cofactor of Myc and Mad transcription factors; Luscher, 2001), NKX2.4 (homeodomain transcription factor; Small et al., 2000), ANKRD 12 (cofactor for multiple transcription factors including P53, 4B1, PML, P16; Kojic et al., 2004), Hsp60s2 (mitochondrial chaperone; Martin, 1997), and MAGOH (RNA chaperone; Le Hir et al., 2001).

Interestingly, multiple protein biomarkers down-regulated in SCHIZ are involved in cellular and/or neural tissue responses to adverse environmental conditions such as oxidative stress, hypoxia, inflammation, etc. (Table 2; Aloisi et al., 1992; Bazan, 2003; Ciccarelli et al., 2001; Conway de Macario and Macario, 2003; Harada et al., 2004; Mansurova, 1989; McGahan et al., 1998; Satoh et al., 2004; Sheng et al., 2004; Sawai and Hannun, 1999). It is tempting to suggest that a core mechanistic deficit leading to SCHIZ is the inability of brain cells to master adequate molecular responses to environmental challenges. Finally, two SCHIZ-affected protein biomarkers (CALM1 and ε14-3-3) activate the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (Perez et al., 2002; Berg et al., 2003), while another protein biomarker (DECR2) influences the presynaptic pool of dopaminergic vesicles (Logan, 2004). Therefore, these protein biomarker changes may contribute to the hypodopaminergia characteristic of the DLPFC in SCHIZ (Dworkin and Opler, 1992; Knable and Weinberger, 1997).

Three protein biomarkers affected in SCHIZ did not belong to any of the above-listed functional groups. Among them, LFA-3 is involved in cellular adhesion (Aloisi et al., 1992), and the roles of CBF2 and BYSL in the brain are unknown (Senderek et al., 2003; Sheng et al., 2004; Table 2).

Protein biomarkers altered in BD

Only seven protein biomarkers had altered levels in BD; all were up-regulated, and two were affected exclusively in BD: DKK2 and MB-18.5. DKK2 is a member of Wnt cascade (Table 2; Krupnik et al., 1999). While BD-associated alterations in DKK2 were not previously reported, abnormal Wnt signaling in BD has been proposed (Gould and Manji, 2002). MBP-18.5 is one of the major human MBP isoforms involved in myelin formation by oligodendrocytes (Table 2; Krupnik et al., 1999). The increase in the MBP-18.5 levels specific to BD is unexpected, considering that a down-regulation of MBP gene expression was reported for both BD and SCHIZ (Tkachev et al., 2004). This discrepancy likely reflects differences in the subtypes of MBP examined in the present vs. earlier analysis. For instance, SCHIZ-associated down-regulation was detected for the mRNA containing exon 2 (Tkachev et al., 2004), which is not included in MBP-18. 5 (Boggs, 2002).

The remaining BD-affected proteins (CBF2, DECR2, BYSL, ANKARD 12, and ALDOC) were also altered in SCHIZ, with their diversity (Table 2) suggesting that BD, as SCHIZ, is associated with complex multifactorial molecular changes involving altered gene transcription, cell signaling, lipid and glucose metabolism, and other intracellular processes (Kojic et al., 2004; Osmundsen et al., 1991; Senderek et al., 2003; Sheng et al., 2004; Thompson et al., 1982). However, since the levels of these proteins changed in the direction opposite to that in SCHIZ, the results should be different. For example, the observed BD-associated up-regulation of the glycolytic enzyme, ALDOC, supports the recent proposition (Dager et al., 2004) that, while, as noted above, a faulty glycolytic machinery may play a significant role in the deficient glucose metabolism in the DLPFC in SCHIZ, the reported reduction in glucose metabolism in the DLPFC in BD may not involve its glycolytic component.

One protein biomarker, cyclophilin A, did not display changes in either SCHIZ or BD as compared to controls, but showed significant differences in its levels between SCHIZ and BD cohorts (BD > SCHIZ; Table 2). This protein plays an important role in cerebral cortical plasticity (Arckens et al., 2003). Therefore, differences in cyclophilin A levels between SCHIZ and BD may indicate potentially different capacity for plasticity by the DLPFC in these two diseases.

As the first study employing SELDI-TOF-MS technique for high-throughput protein profiling in postmortem brain tissue, the present report demonstrates the power of this analysis to reveal molecular pathology of SCHIZ and BD by focusing our attention on change in the levels of specific proteins characterizing these diseases. It also shows the need for further evaluation of this technique in relation to psychiatry. We hope that this paper will serve as impetus for the utilization of ProteinChip profiling in molecular psychiatry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbd.2006.02.002.

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