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Proteomic studies on the white matter of human brain ☆

Stefano Pozzi Mucelli [a], Federico Odreman [a], Marlen Lujardo Gonzales [a], Ernestina Gerardi [b], Giorgio Stanta [a], Alessandro Vindigni [a,*]

[a] *International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy*
[b] *Department of Clinical, Morphological, and Technological Sciences, University of Trieste, Cattinara Hospital, University of Trieste, Strada di Fiume, 447, 34149 Trieste, Italy*

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Abstract

Limited information on the protein expression profiles of the different components of mammalian brain is available to date. In the present study, proteomic analysis was performed on 32 white matter samples obtained from 8 different regions of brains of four *post mortem* cases. Proteins were separated by 2D gel electrophoresis and identified by mass spectrometry. Most of the protein spots (98%) are reproducibly present in all the samples analyzed. A total of 64 different proteins were identified and divided into seven functional groups. These include metabolic proteins (33%), structural proteins (9%), proteins involved in signal transduction (9%), blood proteins (8%), stress related proteins (23%), and proteins involved in the ubiquitin mediated proteolysis (6%). This protein database obtained from the white matter of human brain contributes to deepen our knowledge on the molecular mechanisms that control several pathologies affecting this key component of the brain.

Keywords: Proteomics; Human brain; Brain white matter; 2D electrophoresis; Mass spectrometry

1. Introduction

The brain is the most complex organ of our Central Nervous System (CNS) and is mainly composed of neurons, blood vessels, and gial cells [1]. A neuron has one cell body, containing the nucleus and surrounding cytoplasm (perikaryon), one axon, and one or more dendrites, which extend from the cell body [1]. The axon splits into several branches. Each of them ends in so called terminal buttons. Those terminal buttons exchange signals over the synaptic gap via neurotransmitters to dendrites of a neighboring dendritic tree belonging to another neuron. Glial cells include ependymal cells, oligodendrocytes, astrocytes, and microgliocytes [2]. They are more numerous than neurons, and unlike neurons, do not generate electrical signals. The gray matter of the CNS contains most of neuron cell bodies, while the white matter is mainly formed by gial cells and the axons of the neurons. The white matter plays a crucial role in the physiology of the brain [3]. In fact, it provides nutrition and mechanical support for the neurons, protects the entire CNS against chemical injures and pathogens, and it is also involved in the transmission of the nervous impulse. The white matter is the target for various diseases [4]. For example, brain gliomas are neoplastic diseases, which account for about 45% of all primary brain tumors [5], while the demyelinating disorders are degenerative diseases that cause a progressive loss of the glial support and an indirect damage of the axons [6–10]. A through description of the proteins that characterize the white matter of human brains is essential for a better understanding of the molecular processes that regulate the functioning of this key component of our CNS.

Proteomics is a rapidly emerging field of study dedicated to the characterization of proteins from cell or tissue extracts. Using proteomic approaches, protein databases for several human tissues have been developed, including heart, liver, brain, kidney, red blood cell, breast cancer, plasma, lymphoma, lung cancer, thyroarytenoid muscle, and cell lines for Jurkat T-cells, colon carcinoma and glioblastoma [5,11–20]. In addition, comparative proteomics has been widely utilized for the identification of protein markers that can allow the distinction of pathological versus healthy tissues or that are selectively present in mutant versus wild type cells [12,14,21–26]. In the present

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* Corresponding author. Tel.: +39 040 3757326; fax: +39 040 226555.
E-mail address: vindigni@icgeb.org (A. Vindigni).

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work, a proteomic approach has been used to analyze the protein expression profiles of the white matter of human brain. White matter samples from frontal, parietal, temporal, and occipital lobes of both brain hemispheres were compared using 16 cm × 18 cm two-dimensional (2D) polyacrylamide gels. The majority of protein spots was conserved among the different samples and information on the identity of several of these proteins was obtained by mass spectrometry analysis. The results presented here provide an initial reference map and database of proteins that are present in the white matter of human brain.

2. Experimental

2.1. Materials

3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), IPG buffer, immobiline DryStrips, and cover fluid were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Dithiothreitol (DTT), urea, thiourea, TrisHCl, iodoacetamide, glycine, agarose, sodium thiosulphate, potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA), and sodium dodecyl sulphate (SDS) were from Sigma (St. Louis, MO, USA). Acrylamide, Bradford reagent and broad range molecular mass markers were from Bio-Rad (Munich, Germany). Silver nitrate, glycerol, *N*,*N*,*N*,*N*-tetramethyl-ethylenediamine (TEMED), ammonium persulfate, acetonitrile, trifluoroacetic acid (TFA), and ammonium bicarbonate were bought from Fluka (Buchs, Switzerland). Methanol, ethanol, and acetic acid were from Merck (Darmstadt, Germany). Protease inhibitors cocktail was from Roche (Mannheim, Germany) and sequencing grade trypsin was from Promega (Madison, WI, USA).

2.2. Sample preparation

Human white matter samples were obtained from *post mortem* cases of four patients died for diseases not connected to the CNS. The deceased patients had no history, symptoms or signs of any psychiatric or neurological condition and the cause of death was unrelated to any dysfunction of the central or peripheral nervous system. The samples were obtained between 24 and 48 h from death. From each patient, eight tissue samples, from frontal, parietal, temporal, and occipital lobes of both hemispheres were taken, frozen in liquid nitrogen and stored at −80 °C until processed. Tissues were homogenized in 700 μl of lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.5 mM EDTA, and 1× protease inhibitors cocktail. Successively, the suspension was sonicated for 30 s to facilitate protein solubilization and incubated for 1 h at room temperature. The samples were then centrifuged at 16,000 × *g* for 30 min. The protein concentration in the supernatant was estimated by SDS-PAGE gels stained with coomassie brilliant blue R-250 and with the DC Protein assay from Bio-Rad (a kit compatible with detergents). BSA was used as a standard. Samples were either directly used for 2-DE analysis or stored at −80 °C. Both commercial kits and prefractionation methods are available for the selective removal of highly concentrated and contaminant proteins, in particular blood proteins [27]. In this study we decided not to apply any of these methods in order to give a complete view of the proteomic profiling of the white matter. The amount of blood proteins was, however, low enough to not compromise the quality of the gels.

2.3. 2D electrophoresis

Immobilized pH gradient (IPG) strips (pH 3–10NL and pH 4–7) of 13 cm were rehydrated passively overnight in a strip holder with 250 μl of rehydration buffer containing 8 M urea, 2% CHAPS, 1% DTT, 0.6% v/v IPG Buffer pH 3–10NL, and ∼250 μg of protein extract. Isoelectrofocusing was carried out under the following conditions: 500 V for 1 h, 1000 V for 1 h, and finally 8000 V hold for 6 h. The strips were kept at 200 V until loaded on the second dimension. Before starting the second dimension the strips were equilibrated in 6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris pH 8.8, and 1% DTT for 15 min. Afterwards, they were briefly rinsed in double distilled water (ddwater) and equilibrated in 6 M urea, 30% glycerol, 2% CHAPS, 50 mM Tris pH 8.8, and 2.5% iodoacetamide for an additional 15 min. The second dimension was carried out using 12% polyacrylamide gels and a current of 30 mA per gel.

2.4. Protein visualization

Proteins were visualized using the modified silver staining protocol developed by Mortz et al. [28]. Briefly, after the second dimension, the gels were fixed overnight at room temperature in 50% methanol, 12% acetic acid, and 0.05% formalin. The day after, the gels were washed three times in 35% ethanol for 20 min, and then sensitized in a 0.02% solution of $Na_2S_2O_3$ for 2 min. Next, the gels were washed three times in ddwater for 5 min and placed into a solution containing 0.2% silver nitrate and 0.076% formalin for 30 min. Successively, the gels were washed twice with ddwater for 1 min and developed in a solution containing 6% Na_2CO_3, 0.05% formalin, and 0.0004% $Na_2S_2O_3$. Finally, the gels were placed in a solution containing 50% methanol, 12% acetic acid for 5 min to stop the reaction. The stained gels were stored in 1% acetic acid.

2.5. Computer analysis of 2D gels

The silver-stained gels were scanned with a VersaDoc imaging system (Model 3000 from Bio-Rad). The data were analyzed using the PDQuest software from Bio-Rad. Five spot were selected as landmarks in the 32 gels series, and after the automated matching of the detected spots, the results were manually inspected.

2.6. Protein digestion

Selected spots were excised manually from the preparative gel with a scalpel. Individual gel samples were placed in 1.5 ml

microcentrifuge tubes and washed twice for 10 min with ddwater. To remove silver, samples were placed in a 1:1 mix of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until the gel pieces turned clear. This solution was then discarded and each gel sample was washed with ddwater for 15 min. Successively, 300 μl of 100 mM of ammonium bicarbonate were added to the solution, and the gel pieces were incubated for additional 15 min. After discarding the solution, the bands were washed with 100 mM of ammonium bicarbonate/acetonitrile (50:50, v/v) for 15 min. This solution was removed and gel samples were crushed with a Teflon stick, after which 100 μl of acetonitrile were added for dehydration. After 5 min, the acetonitrile was removed and the bands were dried in a Speed Vac for 5 min. Samples were resuspended in 50 μl of 10 mM DTT in 100 mM ammonium bicarbonate and incubated for 1 h at 56 °C. After this time, DTT was removed and samples were placed in 50 μl of 50 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 30 min in the dark. The solution was removed and gel samples were washed with 300 μl of 100 mM ammonium bicarbonate for 15 min. Samples were then placed in 300 μl of 20 mM ammonium bicarbonate/acetonitrile (50:50, v/v) for 15 min. The previous solution was replaced with 100 μl of acetonitrile and samples were left at room temperature for 5 min. The samples were then dried in a Speed Vac for 10 min and resuspended with 5 μl of trypsin (0.1 μg/μl) in 100 mM ammonium bicarbonate pH 8.0. After 10 min, 100 μl of 100 mM ammonium bicarbonate were added and the digestion was carried out overnight at 37 °C. The supernatant was collected in a second microcentrifuge tube. The gel pieces were washed once with 100 μl ddwater and twice with 100 μl of 60% acetonitrile/1% TFA. The washes were pooled and added to the previously collected supernatant. The volume of the solution was reduced to 5–10 μl in a Speed Vac and the samples were stored at 4 °C until analyzed with the mass spectrometer.

2.7. Mass spectrometry analysis

Preparative gels were stained using the silver staining protocol described above. Spots of interest were excised from the gels and digested with sequencing grade trypsin. LC-ESI-MS/MS was performed on a LCQ Deca ion trap (ThermoFinnigan, San Jose, CA, USA) coupled with a LC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a Surveyor micro HPLC pump (ThermoQuest-Finningan). For each experiment, 20 μl of sample in 0.1% formic acid was injected on a C8 reverse-phase column (1 mm × 150 mm, particle size 5 μm, pore size 100 Å) from Thermo-Hypersil. Peptides were eluted from the column using a linear gradient of acetonitrile (from 0 to 85% CH_3CN in 70 min) in the presence of 0.1% formic acid at 25 °C. When smaller amounts of material were available, 5 μl of sample were loaded onto a C18 PicoFrit column (0.075 mm × 50 mm, particle size 5 μm, pore size 300 Å) from New Objective and eluted with a CH_3CN gradient (from 0 to 80% CH_3CN) using a flow rate of ∼200 nl/min at 25 °C. Helium was used as collision gas and the collision energy was set at 35% of the maximum. Data analysis was performed either using TurboSequest 2.0 or MASCOT 2.0. The peptide tolerance was ±1.2 Da, while the MS/MS tolerance was ±0.5 Da. For MASCOT analysis, the spectra were converted to DTA files and regrouped using in-house software. The combined Swiss-Prot and TrEMBL database was searched without species restriction.

3. Results

Protein extracts from white matter samples of four patients deceased for reasons not connected to defects in the CNS were analyzed by 16 cm × 18 cm 2D gel electrophoresis. For first dimension analysis, we initially utilized broad (pH 3–10NL) and narrow (pH 4–7) pH range IPG strips. Moreover, the experiments with the broad range IPG strips were performed using

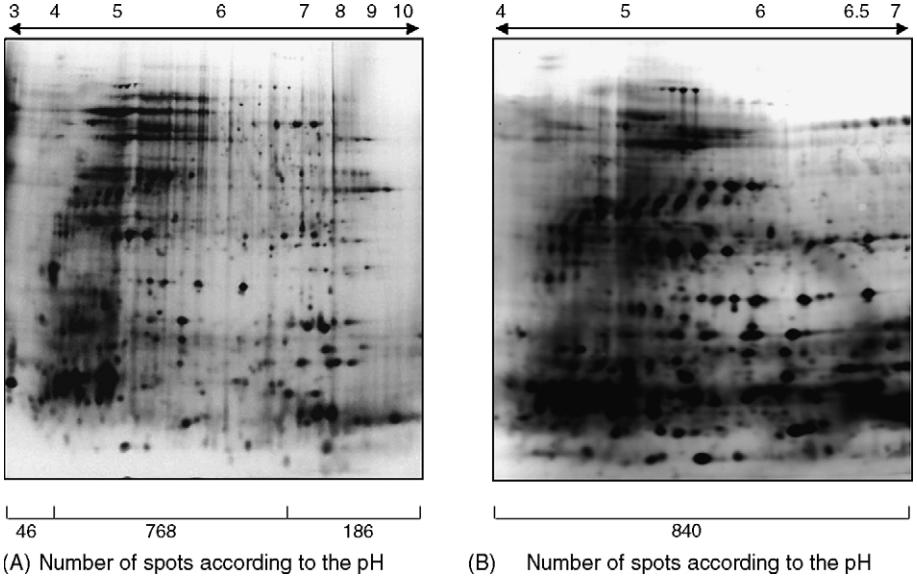

Fig. 1. Representative 2-DE maps of proteins of the white matter separated on a 3–10 non-linear gradient (A) and on a 4–7 gradient (B). Each gel was loaded with 250 μg of proteins and silver stained. The pH range is outlined at the top of the gel, while the number of spots detected in the analyzed ranges is shown at the bottom.

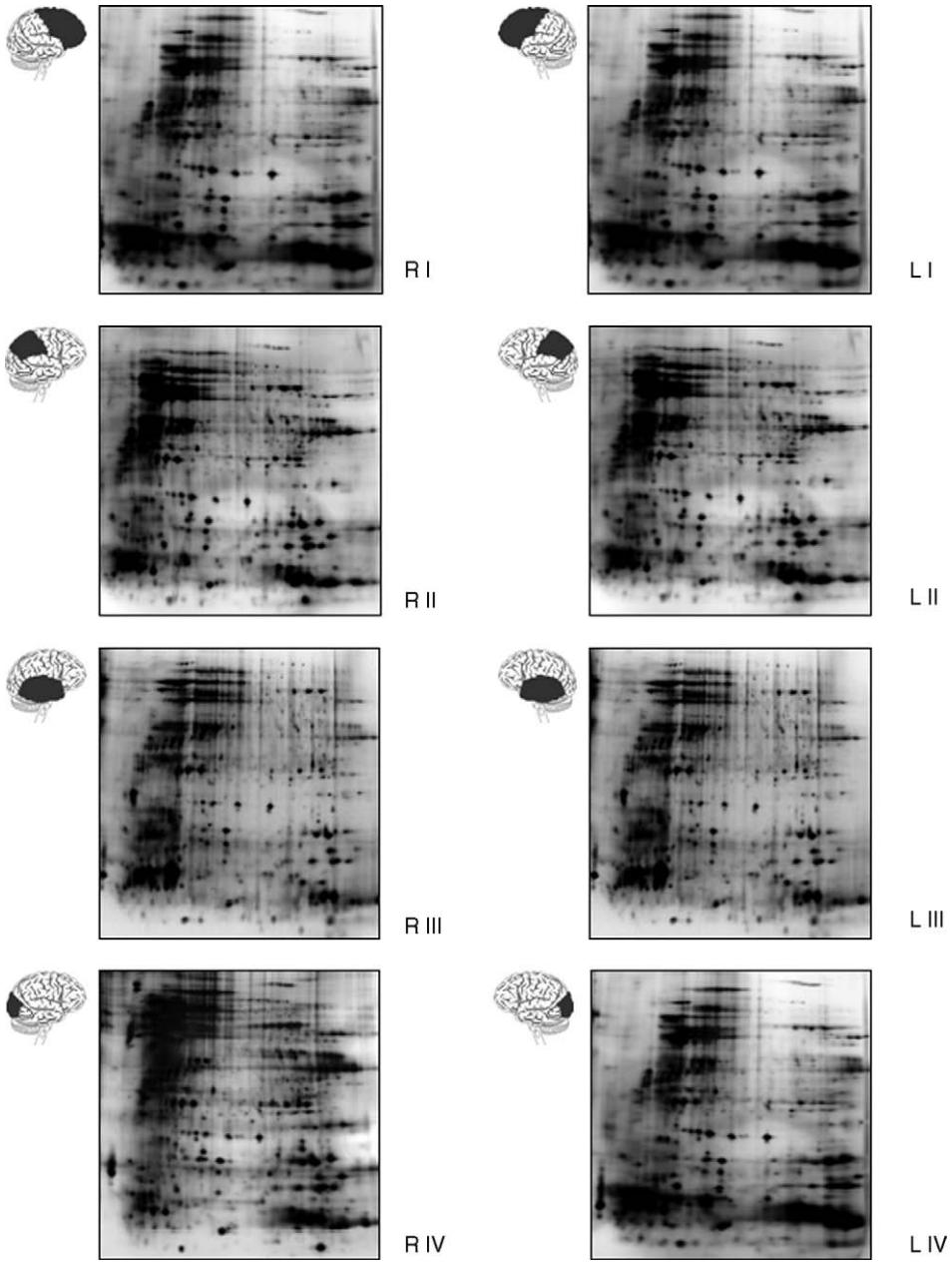

Fig. 2. Representative 2-DE patterns from the eight different lobes of the brain analyzed, as indicated in the picture on the top left corner of each gel image. (I) Frontal lobe, (II) parietal lobe, (III) temporal lobe, (IV) occipital lobe (R, right hemisphere, L, left hemisphere).

Table 1
Distribution of detected protein spots according MW and pH

MW	pH							Total number of spots
	3–4	4–5	5–6	6–7	7–8	8–9	9–10	
>70 kDa	8	16	54	11	2	0	0	91
70–60 kDa	4	9	24	6	0	0	0	43
60–45 kDa	9	26	52	34	17	9	10	157
45–20 kDa	21	64	159	98	49	25	16	432
20–10 kDa	4	49	69	44	25	14	15	220
<10 kDa	0	8	13	12	1	3	0	37
Total number of spots	46	172	371	205	94	51	41	980

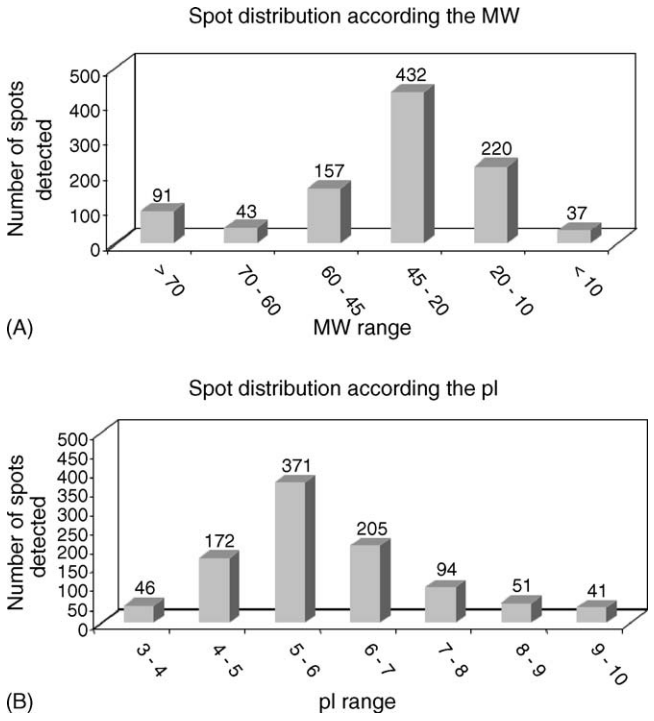

Fig. 3. Schematic representation of protein spots distribution according the molecular weight (A) and the isoelectric point (B), as reported in Table 1.

both the 3–10 linear gradient (L) and the 3–10 non-linear gradient (NL) strips. On the other hand, the use of 3–10 linear gradient strips caused a loss of resolution in the 5–7 region compared to the non linear gradient strips, without giving reasonable advantages in the other pH regions (data not shown). Thus, the 3–10 L strips were not utilized further in the present study. For the second dimension, the use of 12% polyacrylamide gels allowed us to detect proteins with molecular masses ranging from 15 to 150 kDa. Typical 2D gel images of a white matter sample obtained with the broad range pH strips (pH 3–10NL) and the narrow range strips (pH 4–7) are shown in Fig. 1. Following computational analysis, we detected approximately 1000 protein spots on gels (16 cm × 18 cm) obtained with the broad range strips using a silver-stained based protocol optimized for sensitivity and compatible with mass spectrometry analysis [28]. Most of the protein spots were distributed in a molecular mass range from 20 to 120 kDa and approximately 23% of these spots lied outside the 4–7 region of the gel. Fewer protein spots (∼840) were detected using the IPG strips with pH range 4–7 (Fig. 1). Thus, we decided to perform all further experiments with the 3–10NL broad range pH strips.

A total of 32 white matter samples coming from four *post mortem* cases were analyzed with this technique. Eight white matter samples, respectively from frontal, parietal, temporal, and occipital lobes of both brain hemispheres, were taken from each patient. The 2D electrophoresis profiles and relative spot

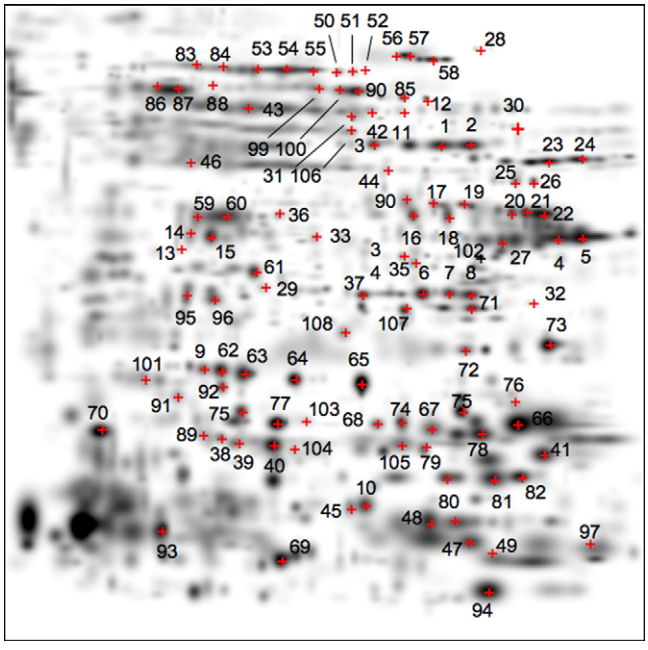

Fig. 4. Master gel obtained with the PDQuest software overlapping the results of all the 32 electrophoretic runs. The master gel contains the 980 common spots. The crosses indicate the spots that have been identified with LC-ESI-MS/MS, and the numbers refer to Table 2.

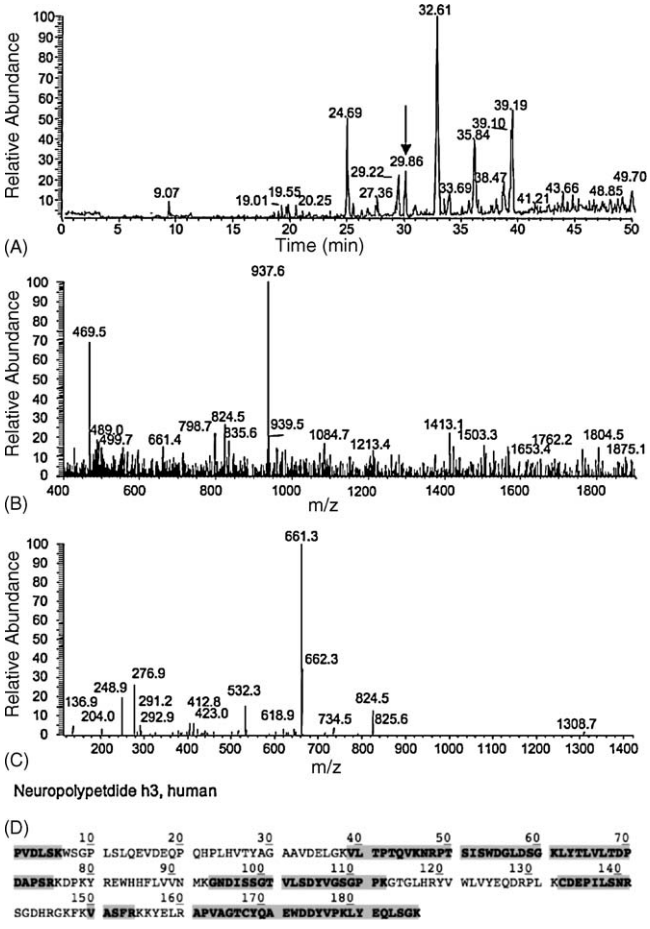

Fig. 5. (A) Representative elution of a tryptic digest from the C18 chromatographic column used in the LC-ESI-MS/MS identification of protein spots from human white matter sample. The arrow indicates the retention time of the peptide for which the MS (B) and MS/MS spectra (C) are also shown. The two major peaks of the MS spectrum correspond to the *n* and 2*n* charged states of the peptide. (D) Sequence of the corresponding protein (Neuropolypeptide H3). The peptides identified by mass spectrometry are highlighted in bold.

Table 2
Proteins present in all white matter samples identified by mass spectrometry

Number	Protein	Swiss-Prot or NCBI entry	Theoretical M_r (kDa)/p*I*	Function
Metabolism				
1–3	Carbonic anhydrase IX	Q16790	47.4/7.01	Reversible hydration of carbon dioxide. May be involved in the control of cell proliferation and transformation
4, 5a, 27a	Glyceraldehyde-3-phosphate dehydrogenase	P00354	36.2/8.26	Homotetramer involved in the second phase of glycoslysis
5b	Mitocondrial malate dehydrogenase precursor	P40926	35.9/8.92	Oxidative decarboxylation of pyruvate and TCA cycle
6–8	Triosephosphate isomerase	P60174	26.5/6.51	Plays an important role in several metabolic pathways
9	Apolipoprotein A–I	P02647	30.7/5.56	Participates in the reverse transport of cholesterol
10	Fatty acid binding protein 5	O15540	14.7/5.4	B-FABP could be involved in the transport of a so far unknown hydrophobic ligand with potential morphogenic activity during CNS development
11, 12	3-Phosphoglycerate dehydrogenase	O43175	57/6.29	Involved in the serine biosynthesis
13–15	Pyruvate dehydrogenase E1 component beta subunit, mitochondrial [Precursor]	P11177	39.2/6.20	Catalyzes the overall conversion of pyruvate to acetyl-CoA and CO(2)
16–19	Fructose aldolase C	P09972	39.3/6.46	Involved in the glycolysis pathway
20–22	Fructose aldolase A	P04075	39.7/8.39	Involved in the glycolysis pathway
23, 24	Phosphoglycerate kinase 1	P00558	44.9/8.30	Glycolytic enzyme that might also act as a polymerase alpha cofactor protein
25, 26	Acetyl-CoA acetyltransferase, mitochondrial	P24752	45.5/9.07	Plays a major role in ketone body metabolism
28	Aconitase 2	Q99798	86.1/7.36	Aconitase is involved in the turnover of citrate within the TCA cycle
29	L-Lactate dehydrogenase B chain	P07195	36.5/5.72	Belongs to the LDH family; involved in the final step of the anaerobic glycolysis pathway
30	Glutamate dehydrogenase 1, mitochondrial [Precursor]	P00367	61.3/7.66	Belongs to the Glu/Leu/Phe/Val dehydrogenases family; catalyses the reaction of L-glutamate metabolism
31	Alpha enolase	P06733	47/7.01	Involved in the glycolysis pathway
32	3-Hydroxyacyl-CoA dehydrogenase type II	Q99714	27/7.66	Binds intracellular amyloid and it may contribute to the neuronal dysfunction associated with Alzheimer disease
33	Carbonic anhydrase II	P00921	29/6.63	Catalyses the reversible hydration of carbon dioxide
34	Electron-transfer flavoprotein α-subunit	P13804	33/6.95	Specific electron acceptor for several dehydrogenases
35	Aldose reductase	P15121	36/6.56	Catalyzes the NADPH-dependent reduction of a wide variety of carbonyl-containing compounds to their corresponding alcohols
36	Glycerol-3-phosphate dehydrogenase	P21695	38/5.81	Converts NAD + in NADH
Structural proteins				
37	Actin, cytoplasmic	P02570	41.7/5.29	Are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells
38–40	Stathmin (oncoprotein 18)	P16949	17.1/5.77	Involved in the regulation of the microtubule (MT) filament system by destabilizing microtubules
41	Cofilin, non-muscle isoform	P23528	19.0/8.2	Controls reversibly actin polymerization and depolymerization in a pH-sensitive manner
42, 43a	Alpha-tubulin	P05209	50/5.02	Is the major constituent of microtubules
44	Glial Fibrillary Acidic Protein (GFAP)	P14136	49.8/5.42	During the development of the central nervous system, distinguishes astrocytes from other glial cells
45	Profilin II	P35080	15/6.78	Binds to actin and affects the structure of the cytoskeleton

Table 2 (*Continued*)

Number	Protein	Swiss-Prot or NCBI entry	Theoretical M_r (kDa)/p*I*	Function
Blood proteins				
46	Fibrinogen fragment D	2781208	38.0/5.84	Fibrinogen has a double function: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation
47, 48	Hemoglobin, beta chain	P02023	16/6.8	Involved in oxygen transport
49	Hemoglobin, alpha chain	P01922	15/8.7	Involved in oxygen transport
50–55	Human Serum Albumin	P02768	71/5.93	Its main function is the regulation of the colloidal osmotic pressure of blood
56–58	Serotransferrin [Precursor]	P02787	79.2/6.81	It is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization
Proteins involved in signal transduction				
59, 60	NG,NG-dimethylarginine dimethylaminohydrolase 1	O94760	31.4/5.53	Has a role in nitric oxide generation
61	Dimethylarginine dimethyilaminohydrolase 2	O95865	29.9/5.66	Has a role in nitric oxide generation
62–65	DJ-1	Q99497	20/6.33	Protect against polyglutamine toxicity
66, 67a, 68	Neuropolypeptide h3	P30086	21/7.4	Involved in the function of the presynaptic cholinergic neurons of the central nervous system
69	Trascription factor BTF3	P20290	12/9.7	General transcription factor
70	Adenylate cyclase	P00936	24/5.43	Cellular communication and signal transduction
Proteins involved in stress responses				
71	Protein-L-isoaspartate(D-aspartate) *O*-methyltransferase	P22061	25.2/6.23	Catalyzes the methyl esterification of L-isoaspartyl and D-aspartyl
72	Manganese superoxide dismutase	Q7Z7M4	24.9/8.35	Destroys radicals which are normally produced within the cells
73–76	Peroxiredoxin 1	Q06830	22.1/8.27	Involved in redox regulation of the cell
77	Superoxide dismutase Cu/Zn	P00441	15.8/5.7	Destroys radicals normally produced within the cells and which are toxic to biological systems
67b, 78, 79a	Alpha-B-crystallin	P02511	20.0/6.8	Member of the small heat shock protein family. Acts as molecular chaperones and possess autokinase activity
80–82	Cyclophillin A	P05092	18.0/7.8	Accelerates the folding of proteins; catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides
83, 84	Heat Shock Protein 70	P08107	70/5.97	In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides
85	Stress induced phosphoprotein 1	P31948	63/6.40	Mediates the association of the molecular chaperones HSC70 and HSP90
86, 87	Heat Shock 60 kDa Protein	P10809	61/5.70	Implicated in mitochondrial protein import and macromolecular assembly
88	T-complex protein 1, epsilon subunit	P48643	60/5.45	Molecular chaperone; assist the folding of proteins upon ATP hydrolysis
43b	Protein disulfide isomerase	P07237	57/5.98	PDI, the beta subunit of prolyl 4-hydroxylase, and the cellular thyroid hormone binding protein are identical
89	Peptidyl-prolyl isomerase-like 1 protein 3 isoform PPIL3a	NP_115861	18.85/6.51	catalyze the cis-trans isomerization of peptidylprolyl imide bonds in oligopeptides
90	Aflatoxin B1 aldehyde reductase 1 AFB1 dialcohol	O43488	39/6.52	Can reduce the dialdehyde protein-binding form of aflatoxin B1 to the nonbinding
91	Glutathione *S*-transferase P	P09211	23.2/5.44	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles

Table 2 (*Continued*)

Number	Protein	Swiss-Prot or NCBI entry	Theoretical M_r (kDa)/p*I*	Function
92	Heat shock 27 kDa protein	P04792	23/5.98	Involved in stress resistance and actin organization
Proteins involved in the ubiquitin-mediated proteolysis				
93	Ubiquitin A-52 residue ribosomal fusion protein	NP_003324.1	16.1/9.95	This ribosomal protein is synthesized as a C-terminal extension protein (CEP) of ubiquitin
94	Ubiquitin	P62988	8.5/6.5	Involved in the ATP-dependent selective degradation of cellular proteins, the maintenance of chromatin structure, the regulation of gene expression, the stress response, and ribosome biogenesis
95, 96	Ubiquitin carboxyl-terminal hydrolase isozyme 1	P09936	24.8/5.43	Ubiquitin-protein hydrolase involved both in the processing of ubiquitin precursors and of ubiquinated proteins
Other proteins				
97	Macrofage migration inhibitory factor	P14174	12/8.2	The expression of MIF at sites of inflammation suggest a role for the mediator in regulating the function of macrophage in host defense
98–100	Dihydropirimidinase-like 2	Q86U75	62.7/5.95	Involved in signal transduction and cell communication
101	QRWT5810	Q6UXS0	15.6/6.17	Interacting selectively with any mono-, di-or trisaccharide
103	Nucleoside diphosphate kinase A	P15531	17.3/5.83	Major role in the synthesis of nucleoside triphosphates other than ATP
80b, 104, 105	Myelin Basic Protein	P02686-6	17.3/11.14	The classic group of MBP isoforms (isoform 4-isoform 14) are with PLP the most abundant protein components of the myelin membrane in the CNS
106	Heterogeneous nuclear ribonucleoprotein H'	P55795	50/5.89	Nucleic acid synthesis and processing
107	Poly(rC)-binding protein 1	Q15365	38/6.66	Single-stranded nucleic acid binding protein that binds preferentially to oligo dC
108	Pirin	O00625	32/6.42	Nucleic acid synthesis and processing

intensities obtained for most of the samples were perfectly reproducible when performed in duplicate or triplicate. The 2D gels obtained from the eight different regions of the brain are shown in Fig. 2. Most of the protein spots were reproducibly present in all the 2D gels analyzed, with a distribution summarized in Table 1 and Fig. 3. In particular, the digitalized images of all the 32 gels were analyzed and overlapped using the PDQuest software from Bio-Rad, in order to identify the spots common to all the electrophoretic runs. The average coefficient of variation in spot intensity calculated on the five landmark spots used for the computational analysis was 5.35%. The results are summarized by the software in a master gel (Fig. 4), a virtual gel in which only common spots are visualized. Thus, we found that 980 spots are consistently present in all the analyzed samples, with differences confined to low intensity spots.

Proteins were then identified using an ion trap mass spectrometer equipped with a micro-high pressure liquid chromatography system and a nano-electrospray source (see Section 2). Fig. 5 shows a representative chromatogram of a tryptic digest of a protein from the spot no. 66 of the 2D gel reported in Fig. 4. The peptide eluting from the column were subjected to MS/MS analysis in order to achieve an unambiguous identification of the protein. A total of 111 spots were selected for mass spectrometric analysis. Information on the identity of the protein was obtained for 108 out of the 111 spots and a total of 64 different proteins were identified (Table 2). In a few cases, the same protein was detected in multiple spots suggesting the presence of post-translational and/or proteolytic modifications that affect the p*I* and/or the molecular mass of the selected protein. Identified proteins were classified into seven functional classes: (1) proteins associated with cell metabolism (33%); (2) structural proteins (9%); (3) blood proteins (8%); (4) proteins involved in signal transduction (9%); (5) proteins involved in stress responses (23%); (6) proteins involved in the ubiquitin-mediated proteolysis (5%);

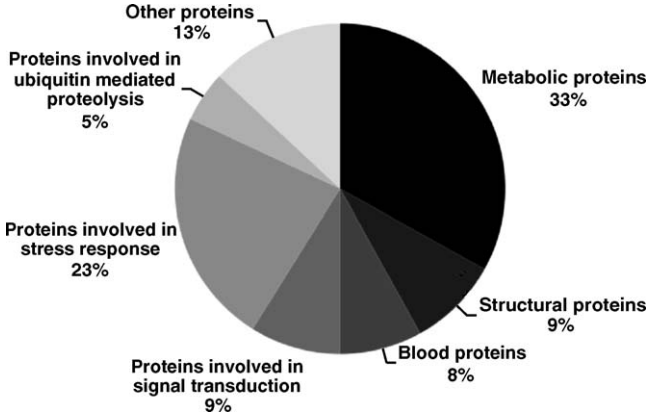

Fig. 6. Distribution of the identified proteins according to their general function.

(7) other proteins (13%) (Fig. 6). Membrane proteins and proteins that are highly hydrophobic can hardly be identified using this approach, since they tend to precipitate when placed in a pH near to their p*I*. Moreover, they are poorly soluble in the solutions used for sample preparation prior 2D gel electrophoresis [29,30]. Based on these results, a preliminary 2D database for the white matter of the human brain was developed (Table 2).

4. Conclusions

The white matter is a fundamental tissue for the physiology of the brain. It is composed by a relatively heterogeneous cell population called glia (or neuroglia) and by the axons of the neurons, whose nuclei are located in the grey matter. This tissue is affected by both neoplastic and non-neoplastic diseases. Human brain gliomas are heterogeneous neoplasms originating from glial cells and represent the most common type of brain tumors. Given the extensive heterogeneity of these tumors, an accurate distinction between the malignant high-grade and the indolent low-grade gliomas on the basis of their microscopic appearance can be difficult, thus affecting prognosis estimation and therapeutic decisions [31]. Proteomic studies performed by us and other groups allowed the identification of differentially expressed proteins that could eventually serve as novel molecular markers for this class of tumors [5,26,32,33]. Among the non-neoplastic diseases that affect the white matter, demyelinating disorders, such as multiple sclerosis, are degenerative disorders whose aetiology has still to be thoroughly investigated [34]. Thus, a detailed description of the normal protein expression pattern of the white matter of human brain might provide essential information for a better understanding of the molecular mechanisms altered in brain gliomas and in degenerative diseases affecting our central nervous system [14,23,35].

A number of studies have been previously performed to describe the protein expression profiles of the different components of a human brain [36,37]. Langen et al. utilized a combination of 2D gel electrophoresis and MALDI mass spectrometry analysis to characterize normal human CNS and identified approximately 180 parietal cortex proteins [38]. Successively, detergent solubilized samples from normal frontal cortex and cerebrospinal fluid (CSF) were fractionated by preparative liquid phase 2D-electrophoresis prior mass spectrometry analysis allowing the identification of novel proteins not previously described as expressed in the brain [39]. A protein database of normal fetal brain describing approximately 1700 proteins from 437 genes was also constructed using a proteomic approach by Fountoulakis et al. [40]. Moreover, proteins up-or down-regulated in several brain related disorders, such as multiple sclerosis, Alzheimer disease, and Down's syndrome, have been identified by several groups using proteomic technologies. These studies paved the way for the development of novel molecular bio-markers and provided novel information on the molecular processes associated with these brain disorders [41–43]. While much of information is available on proteins present in the CSF and in the cortex of the CNS, little has been done so far to characterize the protein expression profile of the human white matter. In particular, Newcombe et al. analysed human white matter samples from normal and multiple sclerosis post-mortem brains to identify proteins which undergo disease-related changes [27]. On the other hand, the number of proteins identified in this study was very limited (<20). In a second study, protein extracts from human white matter samples from uncomplicated alcoholics and alcoholics with hepatic cirrhosis were compared to obtain specific information on changes in protein expression levels affected by the alcohol-related disorders and a total of 18 proteins were identified by MALDI-TOF mass spectrometry [44]. Notwithstanding the information that already emerged from these studies, much further investigation is still required to reach a more comprehensive knowledge of the proteins that are expressed in the white matter of human brain.

The proteomic study presented here provides the first description of the protein expression profile of the white matter of human brain. The protein expression profiles of eight white matter samples taken from frontal, parietal, temporal, and occipital lobes of both brain hemispheres were compared for the first time by 2D gel electrophoresis. The vast majority (98%) of protein spots are reproducibly present in all the samples analyzed. The best results are obtained using a non-linear 3–10 pH gradient, which gives a comprehensive view of the proteins expressed in the white matter with a good resolution in the median region of pH.

Mass spectrometry analysis was performed on 111 excised spots and a total of 64 different proteins were identified. In five cases two proteins were found within a single spot, while in 25 cases multiple spots contained the same protein that migrated with different molecular masses and/or p*I*. Multiple spots are commonly due to the presence of post-translational modifications and/or enzymatic cleavage of the protein. For example, spots 50–55 all match to human serum albumin (HSA) and spots 62–65 all match to DJ-1, a protein known to carry a high number of post translational modifications [45,46]. The largest protein identified is aconitase 2 (86.1 kDa), while the smallest is ubiquitin (8.5 kDa). The identified proteins were divided in seven functional groups. A great number of them (33%) are enzymes associated with cell metabolism, including triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate dehydrogenase. Structural proteins (9%) include actin, and the glial fibrillary acidic protein (GFAP), a marker used for the identification of glial derivation of intracranial tumours [47,48]. The presence of blood proteins could be easily explained by the presence of blood vessels in the white matter, but could be also partially due to an incomplete washing of the tissues before the freezing. A fourth group of identified proteins is related to signal transduction. Among these proteins, particularly interesting is the presence of DJ-1, an oncogene of the Ras pathway [49] that has been found associated to early onset of the Parkinson disease [50–53]. The neuropolypeptide h3 is another brain specific protein, involved in the functioning of the presynaptic fiber and responsible for the inhibition of some members of the Raf pathway [54]. The large number of stress response proteins (23%) may be due to oxidative stress following the death of the patient. In fact, some of these proteins were already described by Lescuyer et al. [55] as possible markers of neurodegenerative processes in

the cerebrospinal fluid of deceased patients. However, a large amount of stress induced proteins has been also reported in various proteomic profiling studies of several other human healthy tissues [56]. Thus, we cannot rule out the possibility that stress related proteins are ubiquitously present in the white matter. In particular, one of them, alpha-B-crystallin, has been found implicated in multiple sclerosis, the most important among the demyelinating diseases [9]. Three proteins (5%) belong to the ubiquitin-mediated proteolysis pathway and are involved in the degradation of the proteasome. On the other hand, their presence might be again related to the autoptic origin of the samples. Among the other proteins, it is interesting to remark the presence of various spots pertaining to the 17.3 kDa splicing variant of the Myelin Basic Protein, a protein involved in remyelination of the damaged neuron. These different spots migrate to the same molecular mass, but have different p*I* suggesting that they are due to post-translational modifications of the Myelin Basic Protein. The remaining proteins represent a group whose function in the brain needs to be further investigated. For example, macrophage migration inhibitory factor is an intracellular cytokine involved in the endotoxic stress [57], the nucleoside diphosphate kinase A is a protein involved in the synthesis of nucleoside triphosphates implicated in Alzheimer Disease and Down Syndrome [58], and dihydropirimidinase-like 2 is a protein that seems to be related to the aging process in mammals [59].

Some of the identified proteins were not, to our knowledge, previously described as expressed in human brain. For example, QRWT5810 is probably a secreted or membrane protein, whose function has been poorly characterized to date [60]. Poly(rC)-binding protein 1 is single strand DNA binding protein involved in the regulation of the translation of genes involved in the folate metabolism [61], while Pirin is another nuclear protein that has been recently described as potentially involved in tumor metastasis [62]. Among the other proteins that we have identified, some were previously found by other groups as expressed in the brain or as differentially regulated in brain disorders, but their specific presence in the white matter was never described before. For example, carbonic anhydrase IX is a protein involved in the maintenance of the intracellular pH, normally expressed at low level in the brain and up-regulated in brain tumors [63], while its isoform II has recently been described as a potential target of oxidative stress induced by Alzheimer disease [64]. The macrophage migration inhibitory factor is likely to be involved in the regulation of the macrophage activity in the immune response and it has recently been described also as a potential regulator of neuronal activity [65]. Among the other examples, 3-phosphoglycerate dehydrogenase is a protein which deficiency is associated to a neurometabolic disorder of the L-serine biosynthesis; 3-hydroxyacyl-CoA dehydrogenase type II is also implicated in Alzheimer disease, as it seems to mediate the neuronal dysfunction associated to this pathology by binding to the intracellular amyloid-beta [66]; DJ-1 is implicated in a early onset of Parkinson disease, while the transcription factor BTF3 is a apoptotic inhibitor that we had previously discovered among the proteins overexpressed in high grade gliomas [26].

In summary, our study offers a preliminary description of proteins that characterize the white matter of human brain and provide essential information that deepen our understanding on the molecular mechanisms that control brain function. Moreover, our database can be utilized in comparative proteomic studies to identify novel proteins whose expression levels might be modified in pathologies affecting the white matter. Similar proteomic studies on other regions of human brain are greatly needed to increase our knowledge regarding the molecular processes that regulate the main component of our central nervous system.

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