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Proteomic Analysis Identifies Dysfunction in Cellular Transport, Energy, and Protein Metabolism in Different Brain Regions of Atypical Frontotemporal Lobar Degeneration

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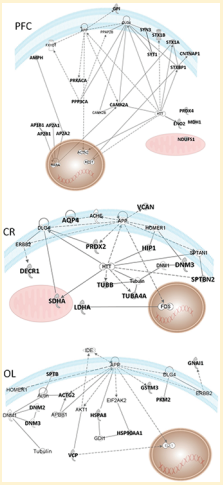
Supporting Information

ABSTRACT: Frontotemporal lobar degeneration (FTLD) is an umbrella term for a heterogeneous group of young-onset dementias of uncertain prevalence and incidence worldwide. Atypical cases of FTLD with fused in sarcoma inclusions (aFTLD-U) have been described recently, but their molecular characterization is still due. Using shotgun mass spectrometry, we identified a total of 107 differentially expressed proteins in the prefrontal cortex, cerebellum and occipital lobe from aFTLD-U patients compared to controls. These proteins are involved in a range of biological pathways such as cellular transport in the prefrontal cortex, energy metabolism in the cerebellum, and protein metabolism in the occipital lobe. In addition, they were validated by selective reaction monitoring (SRM). Comparison of the aFTLD-U proteomic findings with similar studies of Alzheimer's disease and schizophrenia led to identification of proteins that may be related to dementias and psychoses, respectively. Further studies of aFTLD-U and other FTLD subtypes are warranted, although this will require intensive biobanking efforts.



KEYWORDS: *aFTLD, cerebellum, occipital lobe, prefrontal cortex, proteomics*

INTRODUCTION

Frontotemporal lobar degeneration (FTLD) is a general term of young-onset heterogeneous dementias, including progressive nonfluent aphasia, semantic dementia, and behavioral variant frontotemporal dementia (bvFTD).[1] FTLD commonly presents with bilateral and asymmetrical atrophy of the frontal and anterior temporal lobes, as well as progressive decline in behavior and language.[2] The different forms of FTLD present with distinct genetic and neurohistological backgrounds and distinct symptomatology.[3] Because of this heterogeneity, the true prevalence and incidence of FTLD worldwide has still not been determined, although some data has been recently published on the disease prevalence in the U.S.A.[4] and in an isolated Italian province.[5] Nevertheless, FTLD appears to be the second most common form of young-onset dementia after Alzheimer's disease (AD) and fourth most common delayed-onset dementia.[6]

The bvFTD subtype can be characterized by three types of histopathology. The first of these shows TAU-inclusions (FTLD-TAU). The second contains ubiquitin-positive inclusions, described originally as FTLD-U[7] and classified later as FTLD-TDP considering that the ubiquitinated protein is mostly TDP-43.[8] The final type is characterized by Fused in Sarcoma (FUS)-positive inclusions (FTLD-FUS), negative for TDP-43.[9]

Atypical cases of FTLD-FUS (aFTLD-U) have also been reported.[10] aFTLD-U is likely to have an early onset (35−40 years old),[10,11] and patients are usually first diagnosed as having bvFTD. Considering the rareness of this particular subtype, prevalence and incidence have not yet been determined. The symptoms of aFTLD-U include decreased social conduct,

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disinhibition, inappropriate sexual behavior, aggression, and overeating. In addition, aFTLD-U patients often present psychotic symptoms, such as hallucinations or delusions.[11,12]

Understanding the biochemical basis of the different types of FTLD through molecular phenotyping may lead to better classification of the various subtypes, with relevance to the underlying pathology. In turn, this increased understanding could lead to more specific and effective treatments. For example, molecular fingerprints of the different FTLD types can be assessed by analyzing global protein expression in a large-scale manner by using proteomic methodologies.[13] However, only a small number of such efforts have been performed.

Here, we have carried out a label-free shotgun mass spectrometry analysis (LC-MS^E), of *post-mortem* prefrontal cortex (PFC), cerebellum (CR), and occipital lobe (OL) tissues from aFTLD-U patients. Ten protein candidates were further analyzed by selected reaction monitoring (SRM) for a technical validation of the obtained results. Our objectives are to reveal proteins and biochemical pathways that may be related to aFTLD-U for a later comparison with other types of FTLD, which could provide a basis for more suitable and directed treatments. Also, considering the degenerative aspect of FTLD and the presence of psychosis as feature of aFTLD-U patients, it would be of interest to compare the molecular fingerprints of aFTLD-U with those of AD and schizophrenia.[14,15] Our results represent a pilot study considering the prevalence of FTLD conditions in general, but they are limited by the rare availability of the aFTLD-U subtype of samples in brain repositories. Therefore, future studies will require increased availability of these tissues by ongoing brain banking programs.

MATERIAL AND METHODS

Clinical Samples

Post-mortem samples from PFC (Brodmann area 9), CR, and OL (Brodmann area 17) from 5 aFLTD patients and 7 controls were obtained from BrainNet Europe (Munich, Germany) and the Manchester Brain Bank (U.K.) (Supporting Information Table 1). All brains had been obtained at autopsy through appropriate consenting procedures with Local Ethical Committee approval. In addition, all procedures for processing human tissues were approved by the ethics committee at the University of Cambridge.

Sample Preparation

Brain tissue samples (20−30 mg) were macerated individually using the Sample Grinding Kit (GE Healthcare; Little Chalfont, Bucks, U.K.) in 150 uL of 7 M urea, 2 M thiourea, 4% CHAPS, 2% ASB-14, and 70 mM DTT as described previously.[16] Samples were centrifuged for 10 min at 16000×*g* and the supernatants collected. Protein concentrations were determined using the Bradford dye-binding assay (Sigma, Poole, Dorsett, U.K.).

Proteome Purification and Digestion

Each sample (15 μg protein) was diluted separately in SDS-PAGE sample loading buffer [2% w/v SDS, 100 mM Tris (pH 6.8), 10% glycerol, 100 mM DTT and 0.001% w/v bromophenol blue]. Samples were incubated for 5 min at 95 °C prior to electrophoresis on NuPAGE 4−12% bis-tris polyacrylamide gels (Invitrogen; Paisley, UK). Protein bands were visualized using Coomassie blue staining. Each lane containing stained protein bands was sliced in the horizontal direction to produce 2 sections, which were subjected separately to trypsin digestion in situ as described previously.[17] Both resulting peptide mixtures were combined and lyophilized. We used SDS-PAGE here as a means of sample clean up and for compatibility with MS analyses.

Nano-High-Performance Liquid Chromatography−Mass Spectrometry Analyses

Lyophilized peptides were dissolved in 0.1% formic acid and 0.5 μg injected in duplicate into a nano-Ultra Performance Liquid Chromatography instrument containing a BEH-130 C18 column (75 um X 200 mm) run at a flow rate of 0.3 uL/min. This system was connected online to a quadrupole-time-of-flight (Q-TOF) Premier Mass Spectrometer (Waters Corporation; Manchester, U.K.). The HPLC buffers were (A) 0.1% aqueous formic acid and (B) acetonitrile with 0.1% formic acid. The following 140 min minutes gradient was applied to the nano-HPLC system: 97/3% (A/B) to 70/30% in 90 min; 70/30% to 10/90% in 25 min; 10/90% to 3/97% in 5 min; 10 min at 3/97%; and 3/97% to 97/3% in 1 min. Eluted peptides were measured in MS^E mode (data independent analysis) using the ion accounting algorithm[18] for data processing. Analysis of the resulting chromatograms/mass spectra and database searching were performed using the ProteinLynx Global Server (PLGS) v.2.4 (Waters Corp.). The resulting data were searched against the SwissProt human database (version 57.4) as well as a randomized database to exclude false positives. The maximum false identification rate was set to 4% and peptides had to be detected in >70% of samples to ensure biological reproducibility. The criteria for protein identifications were set at a minimum of 3 ion fragments per peptide, 7 ion fragments per protein, and 2 peptides per protein. Modifications considered were carbamidomethylation of cysteines and oxidation of methionine.

Quantitative protein expression and statistical analyses were performed with the Rosetta Elucidator system, version 3.3.0.1.SP3.19 (Rosetta Inpharmatics; Seattle, WA, U.S.A.), using data processed by PLGS. Accounting for the possibility that the generated data were not normally distributed, Wilcoxon signed-rank tests were used to determine significant differences between the groups under comparison ($p < 0.05$).

Validation Experiments by Selected Reaction Monitoring

To validate our findings, quantitative differences in the levels of proteins were determined by SRM. At least 2 peptides and 2 SRM transitions of protein candidates were selected for SRM according to their identification during the discovery phase (Table 2). We have also considered whether those peptides were proteotypic.[19] Protein and peptide samples (0.5 μg) were prepared and injected in duplicate into the same LC as described above, although this was coupled to a Xevo triple-quadrupole mass spectrometer (Waters). For peptide fractionation, the following gradient of 48 min was applied: 97/3% (A/B) to 60/40%B in 30 min; 60/40% to 15/85% in 2 min; 5 min at 15/85%; and 15/85% to 97/3% in 1 min. Eluted peptides were measured in SRM mode using an electrospray voltage of 22 kV and a cone voltage of 35 V. All SRM functions had a 5 min window of the observed retention time and the scan time was automatically determined with a minimum of 20 ms. The collision energy for each transition was optimized using Skyline software[20] based on the equation: $CE = 0.034 \times m/z + 3.314$. Skyline was also used to optimize SRM methods and to generate quantitative data. Considering the sample size, differences in protein expression were analyzed by nonparametric Mann−Whitney tests.

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Classification of differentially expressed proteins

Differentially expressed proteins in each brain region were classified according to their biological processes, molecular function and cellular localization using the Human Protein Reference Database (http://www.hprd.org). For interpretation of the functional significance of differentially expressed proteins, and for determination of cellular localizations, the associated SwissProt accession numbers were uploaded into the Ingenuity Pathways Knowledgebase (IPKB) (www.ingenuity.com). These were analyzed to identify potential interactions between these proteins and other proteins in the IPKB.

RESULTS

Proteomic Analyses

The current LC-MS^E method has been established previously for brain tissue analyses.[21] Application of this method here allowed the identification of 14,971 peptides, comprising 1,209 proteins in the PFC. In the case of the CR proteome, 13,784 peptides were detected, which translated to 1,089 proteins. In addition, 14,774 peptides were detected in the OL, which was equivalent to 1,076 proteins. Across all 3 brain regions, 486 were identified in common, and 547, 466, and 459 proteins were identified as unique in the PFC, CR, and OL, respectively (Supporting Information Figure 1). These differences might be related to functions particularly associated to each of the brain regions analyzed. Proteins identified by only one peptide or that did not have a minimum of 3 ion fragments per peptide and 7 ion fragments per protein were not considered in the quantitative analyses. Thus, 402, 330, and 391 proteins were analyzed for PFC, CR, and OL, respectively.

Differentially Expressed Proteins

Fifty-seven proteins were found to be differentially expressed in the PFC of aFTLD-U (Table 1a). These proteins belonged to nine biological categories, and transport of molecules was the most highly represented category (Figure 1a). In addition, 19% of the differentially expressed proteins in PFC did not belong to a known biological class. Most of the differentially expressed proteins in the PFC were cytosolic (56%) and 21% were membrane proteins (Figure 1b).

In the CR analyses, we found 31 differentially expressed proteins (Table 1b). Most of these (36%) were involved in energy metabolism and 26% in cell growth and maintenance (Figure 1a). Interestingly, 23% of the differentially expressed proteins were localized to the nucleus, which was higher than seen in the other brain regions analyzed (Figure 1b).

We also found 24 differentially expressed proteins in the OL (Table 1c). The most significant differences were found for proteins involved in protein metabolism (29%) (Figure 1a). This contrasted with the other brain regions which showed fewer changes in proteins related to this function. In addition, 21% of the differentially expressed proteins were localized to the membrane.

None of the proteins were found to be differentially expressed in all three brain regions. However, dynamin 2 (DNM2) levels were increased in PFC and OL. Interestingly, DNM2 gene has been associated previously to a specific type of AD.[22]

Protein TANC2 (TANC2) and immunoglobulin superfamily, member 8 (IGSF8) levels were decreased and increased respectively in both PFC and CR. TANC2 interacts with PSD-95 and is suggested to increase dendritic spines density as well as excitatory synapses. TANC2 deficiency can be lethal.[23] Additionally, IGSF8 is involved in neurite outgrowth regulation and neural network maintenance.[24,25]

Putative elongation factor 1-alpha-like 3 (EEF1A1P5) was decreased in both OL and CR. No reports have described the involvement of this putative form so far, but elongation factor 1-alpha has been associated with motor neuron degeneration.[26] Thus, EEF1A1P5 might provide leads about the degenerative aspect of aFTLD-U to be further investigated.

Experimental Validation

Nine protein candidates from the LC-MS^E experiments were chosen for validation by SRM mass spectrometry based in the most represented biochemical pathways for each brain region as described in Figure 1. From the PFC the proteins syntaxin binding protein 1 (STXB1 − p = 0.0356) and syntaxin 1B (STX1B − p = 0.0332) confirmed using Mann−Whitney test the findings of the large-scale study while syntaxin 1A (STX1A − p = 0.1399) did not. Peroxiredoxin-2 (PRDX2 − p = 0.0085), glutamate dehydrogenase 1 (GLUD1 − p = 0.0156) and L-lactate dehydrogenase A chain (LDHA − p = 0.0177) were confirmed in the CR but not succinate dehydrogenase (SDHA − p = 0.1193). Finally, putative heat shock protein HSP 90-alpha A5 (HSP90AA5 − p = 0.0328) was confirmed in the OL, but Heat shock 70 kDa protein 1 (HSPA1A) showed only borderline significant (p = 0.0786). All the nonsignificant findings at least showed the same directional change as found in the large scale profiling study. We analyzed a maximum of seven, and in some cases, only two peptides per protein by SRM (Table 2). Several peptide candidates were discarded since they were not proteotypic[19] and quantotypic.[27]

Systems Biology Analyses

The 3 analyzed proteomes were subjected to a systems biology analyses using IPKB as described in the methods to identify the most over-represented pathways, which may be altered in the pathogenesis of aFTLD-U (Figure 3). In addition, some of the differentially expressed proteins have been associated previously with other brain disorders including AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and schizophrenia (Table 3).

DISCUSSION

aFTLD-U has been recently described[10] and studies of this condition are ongoing. Disorders such as FTLD require a molecular phenotyping and characterization, especially due to the symptom heterogeneity and histological variations. In contrast to the extensive histopathological characterization of FTLD,[28] there have been only minor efforts in large-scale, and nonhypothesis driven analyses of FTLD variants using transcriptomic and proteomic approaches. Recently, a miRNA array profiling study was performed using samples from FTLD-TDP patients, which resulted in identification of 20 miRNAs potentially related to mutations in the progranulin (PGRN) gene.[29] Schweitzer and colleagues identified 48 differentially expressed proteins in the frontal cortex from patients with frontotemporal dementia and Parkinsonism linked to chromosome 17, using a two-dimensional gel electrophoresis (2-DE) profiling approach.[30] In addition, a more detailed proteome analysis has been performed by identification of protein phosphorylation differences in FTLD-TDP.[31] However, no proteomic studies of aFTLD-U have been carried out to date.

The most striking finding of this study was the presence of distinct molecular alterations in the three brain regions of aFTLD-U subjects (Figure 1). The most prominent changes

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Table 1. Differentially Expressed Proteins in aFTLD-U Compared to Controls[a]

A: Prefrontal Cortex								
biological process	gene	protein description	MW	cell. local.	FC	ID Pep	*p*-value	SC (%)
transport	AP1B1	adaptor-related protein complex 1, beta 1 subunit	104636.67	cytoplasm	1.20	28	0.0173	45
	AP2A1	adaptor-related protein complex 2, alpha 1 subunit	107545.89	cytoplasm	1.35	39	0.0303	32
	AP2A2	adaptor-related protein complex 2, alpha 2 subunit	103960.42	cytoplasm	1.15	46	0.0173	47
	AP2B1	adaptor-related protein complex 2, beta 1 subunit	104552.57	cytoplasm	1.31	44	0.0043	19
	ATP6 V1H	ATPase, H+ transporting, lysosomal 50/57 kDa, V1 subunit H	55883.07	cytoplasm	1.18	23	0.0303	78
	SLC25A13	solute carrier family 25, member 13 (citrin)	74175.58	cytoplasm	−1.21	3	0.0173	23
	SLC3A2	solute carrier family 3, member 2	67994.01	membrane	−1.46	15	0.0303	56
	STX1A	syntaxin 1A (brain)	33023.43	cytoplasm	1.27	21	0.0303	21
	STX1B	syntaxin 1B	33244.69	membrane	1.48	35	0.0043	54
	STXBP1[b,c]	syntaxin binding protein 1	67568.71	cytoplasm	1.36	113	0.0173	34
	SYN3	synapsin III	63302.66	membrane	1.25	5	0.0303	21
cell communication and signaling	AMPH	amphiphysin	76256.85	membrane	1.44	20	0.0043	18
	CAMK2A[c]	calcium/calmodulin-dependent protein kinase II alpha	54087.71	cytoplasm	1.27	54	0.0303	31
	CNTNAP1	contactin associated protein 1	154252.2	membrane	1.64	5	0.0087	44
	DNM2	dynamin 2	98064.31	membrane	1.23	56	0.0173	57
	PPP3CA[c]	protein phosphatase 3, catalytic subunit, alpha isozyme	58687.85	cytoplasm	1.30	27	0.0303	70
	PPP3CC	protein phosphatase 3, catalytic subunit, gamma isozyme	58129.38	cytoplasm	1.13	10	0.0303	83
	PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	40458.48	cytoplasm	1.51	2	0.0043	74
	SIRPB1[c]	signal-regulatory protein beta 1	40304.52	membrane	1.34	10	0.0303	38
	STIP1	stress-induced-phosphoprotein 1	62639.26	cytoplasm	−2.55	7	0.0303	54
	SYT1[b]	synaptotagmin I	47573.11	cytoplasm	1.33	33	0.0303	45
cell growth and maintenance	EVPL	envoplakin	231604.11	membrane	−1.20	10	0.0173	36
	EZR	ezrin	69281.61	membrane	−1.31	8	0.0087	32
	IMMT	inner membrane protein, mitochondrial	83677.91	cytoplasm	1.37	12	0.0303	25
	MYH15	myosin, heavy chain 15	224618.97	unknown	1.43	3	0.0087	60
	TUBA4B	tubulin, alpha 4b (pseudogene)	27551.24	cytoplasm	1.21	19	0.0173	66
	TUBB1[c]	tubulin, beta 1	50326.92	cytoplasm	1.17	39	0.0303	35
	TUBB2A	tubulin, beta 2A	49906.97	cytoplasm	1.20	23	0.0303	26
	TUBB2C[b]	tubulin, beta 2C	49831.01	cytoplasm	1.17	22	0.0303	44
energy metabolism	ENO2[b,c]	enolase 2 (gamma, neuronal)	47137.39	cytoplasm	1.32	62	0.0173	55
	GLUD2	glutamate dehydrogenase 2	56052.79	cytoplasm	−1.20	40	0.0173	41
	GPI	glucose-6-phosphate isomerase	63015.93	extracellular	1.25	75	0.0173	42
	HPRT1	hypoxanthine phosphoribosyltransferase 1	24448.19	cytoplasm	1.25	9	0.0303	33
	MDH1[b]	malate dehydrogenase 1, NAD (soluble)	36294.93	cytoplasm	1.19	41	0.0173	33
	NDUFS1[c]	NADH dehydrogenase (ubiquinone) Fe−S protein 1	76975.37	cytoplasm	−1.23	43	0.0173	22
	PHGDH	phosphoglycerate dehydrogenase	56519.31	cytoplasm	−1.79	14	0.0173	59
	PRDX4	peroxiredoxin 4	26572.09	cytoplasm	−1.25	8	0.0087	34
immune response	C4B	complement component 4B (Chido blood group)	71678.89	extracellular	−1.23	4	0.0303	42
	CADM3	cell adhesion molecule 3	40957.97	membrane	1.55	5	0.0087	29
	IGSF8	immunoglobulin superfamily, member 8	62173.57	membrane	1.22	38	0.0303	28
reg nucleic acid	DDX10	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	100887.95	nucleus	1.65	2	0.0087	24
	HIST1H2BL	histone cluster 1, H2bl	13821.02	nucleus	1.35	3	0.0303	26
	TDRD1	tudor domain containing 1	132023.65	cytoplasm	1.36	3	0.0173	28
regulation of cell cycle	SEPT2[c]	septin 2	41487.47	cytoplasm	−1.30	21	0.0303	42
	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	50140.86	cytoplasm	−1.19	25	0.0087	57
protein Metabolism	TCP1	t-complex 1	60343.58	cytoplasm	−1.14	3	0.0173	65
unknown		putative tubulin beta chain-like protein	41775.06	unknown	1.20	22	0.0303	24
	BEST2	bestrophin 2	57138.97	membrane	1.92	2	0.0087	22
	GOT1L1	glutamic-oxaloacetic transaminase 1-like 1	47305.01	unknown	−3.41	2	0.0173	34
	MAST4	microt assoc serine/threonine kinase family member 4	284377.65	unknown	1.56	2	0.0173	30
	NCDN	neurochondrin	78864.26	cytoplasm	1.19	19	0.0303	26
	NEGR1	neuronal growth regulator 1	31426.38	extracellular	1.55	3	0.0087	21

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Table 1. continued

A: Prefrontal Cortex								
biological process	gene	protein description	MW	cell. local.	FC	ID Pep	*p*-value	SC (%)
	NME2P1	nonmetastatic cells 2 protein	15529.06	unknown	−1.19	5	0.0303	34
	NOC3L	nucleolar complex associated 3 homologue (S. cerevisiae)	92547.76	nucleus	1.66	2	0.0173	57
	PIK3R6	phosphoinositide-3-kinase, regulatory subunit 6	84258.05	cytoplasm	−2.56	3	0.0303	19
	PRRT2	proline-rich transmembrane protein 2	34944.91	unknown	1.62	4	0.0303	22
	TANC2	Protein TANC2	219649.61	nucleus	−1.56	8	0.0087	38
B: Cerebellum								
biological process	gene	protein description	MW	cell. local.	FC	ID Pep	*p*-value	SC (%)
energy metabolism	AHCYL2	putative adenosylhomocysteinase 3	66721.13	unknown	1.79	5	0.0177	32
	CA1[c]	carbonic anhydrase 1	28739.02	cytoplasm	1.76	12	0.0177	21
	CBR3	carbonyl reductase NADPH 3	30719.06	cytoplasm	1.38	14	0.0051	31
	DECR1	2,4-dienoyl-CoA reductase, mitochondrial	32149.96	cytoplasm	1.44	4	0.0303	35
	GLS	glutaminase kidney isoform mitochondrial	71560.71	cytoplasm	1.25	13	0.0051	27
	GLUD1[b]	glutamate dehydrogenase 1 mitochondrial	56008.68	cytoplasm	1.10	39	0.0303	24
	LDHA[c]	L-lactate dehydrogenase A chain	36557.53	cytoplasm	1.18	45	0.0303	28
	PGD[c]	6-phosphogluconate dehydrogenase	53008.79	cytoplasm	1.19	7	0.0480	27
	PRDX2[b]	peroxiredoxin-2	21760.73	cytoplasm	1.13	30	0.0303	25
	PSAT1	phosphoserine aminotransferase	40422.68	cytoplasm	−1.16	2	0.0480	65
	SDHA	succinate dehydrogenase	68012.04	cytoplasm	1.34	10	0.0101	70
cell growth and maintenance	GFAP[bc]	glial fibrillary acidic protein	49880.21	cytoplasm	−1.48	149	0.0480	26
	PRPH	peripherin	53650.89	membrane	−1.47	4	0.0101	23
	RAN	GTP-binding nuclear protein Ran	24291.91	nucleus	1.27	4	0.0480	20
	SPTBN2[c]	spectrin beta chain, brain 2	271324.87	cytoplasm	−1.14	60	0.0303	22
	TUBA4A	tubulin alpha-4A chain	49924.4	cytoplasm	−1.13	23	0.0303	29
	TUBB[c]	tubulin beta chain	49670.82	cytoplasm	−1.11	29	0.0480	20
	VCAN	versican core protein	370516.18	extracellular	−1.15	89	0.0303	24
reg. of nucleic acid	HIST1H2BM	histone H2B type 1 M	13858.08	nucleus	1.68	4	0.0177	21
	HNRPDL	heterog. nucl. ribonucleoprotein D like	46437.54	nucleus	−1.33	3	0.0177	18
	INTS6	integrator complex subunit 6	100390.07	nucleus	−1.10	3	0.0480	18
	NME2	nucleoside diphosphate kinase B	17166.84	nucleus	1.67	3	0.0303	24
	TCFL5	transcription factor-like 5 protein	52697.08	nucleus	−1.19	2	0.0101	15
cell communication and signaling	CALB2	calretinin short CR	31539.87	cytoplasm	1.20	16	0.0480	19
	SH3GL1	endophilin A2	41489.95	cytoplasm	1.21	13	0.0480	42
cell death	HIP1	Huntingtin-interacting protein 1	116221.15	cytoplasm	−1.34	2	0.0480	49
immune response	IGSF8	immunoglobulin superfamily member 8	62173.57	membrane	1.23	29	0.0303	37
reg. of cell cycle	EEF1A1P5	putative elongation factor 1-alpha-like 3	50185.02	unknown	−1.18	11	0.0177	32
transport	AQP4	aquaporin 4	34829.69	membrane	−1.50	3	0.0480	21
vesicle transport	CLTCL1	clathrin heavy chain 2	187029.91	membrane	1.12	84	0.0303	27
unknown	TANC2	protein TANC2	219649.61	nucleus	−1.11	22	0.0177	28
C: Occipital Lobe								
biological process	gene	protein description	MW	cell. local.	FC	ID Pep	*p*-value	SC (%)
protein metabolism	EIF4A2[c]	eukaryotic initiation factor 4A-II	46402.27	cytoplasm	−1.17	13	0.0101	41
	HSP90AA1[b,c]	heat shock protein HSP 90-alpha	84528.52	cytoplasm	−1.13	70	0.0177	52
	HSP90AA2	putative heat shock protein HSP 90-alpha A2	39364.8	unknown	−1.17	14	0.0303	23
	HSP90AA5P	putative heat shock protein HSP 90-alpha A5	38738.01	unknown	−1.16	14	0.0101	31
	HSPA12B	heat shock 70 kDa protein 12B	75687.56	unknown	1.12	5	0.0480	47
	HSPA1A[bc]	heat shock 70 kDa protein 1	69921.04	cytoplasm	−1.15	59	0.0101	20
	HSPB1	heat shock protein beta 1	22782.52	cytoplasm	−1.54	2	0.0303	39
cell communication and signaling	ARF5	ADP-ribosylation factor 5	20398.46	cytoplasm	1.13	14	0.0303	29
	CSRP1	cysteine and glycine rich protein 1	20436.2	nucleus	−1.35	3	0.0480	31
	DNM2	dynamin 2	98064.31	plasma membrane	1.36	59	0.0177	26
	GBF1	golgi-specific BFA-resistant GEF 1	206445.89	cytoplasm	−1.14	7	0.0303	31

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Table 1. continued

C: Occipital Lobe								
biological process	gene	protein description	MW	cell. local.	FC	ID Pep	*p*-value	SC (%)
	GNAI1[b]	guanine nucl bind prot G i alpha 1 subunit	40229.89	plasma membrane	1.10	23	0.0480	41
energy metabolism	AKR1B1	aldose reductase short AR	35722.21	cytoplasm	−1.25	6	0.0101	28
	CBR1	carbonyl reductase	30243.75	cytoplasm	−1.10	34	0.0480	42
	GSTM3[b]	glutathione S transferase Mu 3	26428.4	cytoplasm	1.83	4	0.0101	71
	VCP	transitional endoplasmic reticulum ATPase	89190.61	cytoplasm	−1.11	39	0.0480	20
cell growth and enance	PCDHA4	protocadherin alpha 4	99092.67	plasma membrane	−1.74	2	0.0480	23
	SPTB	spectrin beta chain	246336.92	plasma membrane	−1.17	37	0.0303	19
immune response	SCRN1	secernin 1	46382.05	cytoplasm	−1.11	16	0.0480	50
reg. of nucleic acid	CAND1	Cullin-associated and NEDD protein 1	136244.53	cytoplasm	−1.12	14	0.0480	20
reg.of cell cycle	EEF1A1P5	putative elongation factor 1-alpha-like 3	50185.02	unknown	−1.10	16	0.0025	22
unknown	APOA1BP	apolipoprotein A I binding protein	29128.43	extracellular space	−1.16	3	0.0025	45
	ERLIN2	erlin 2	37839.54	plasma membrane	−1.11	2	0.0303	29
	WDR96	WD repeat-containing protein C10orf79	191983.67	unknown	−1.14	2	0.0177	37

[a]FC = fold change, ID Pep = number of identified peptides; SC (%) = sequence coverage in percentage. [b]Proteins previously found in proteomic studies of AD. [c]Proteins previously found in proteomic studies schizophrenia

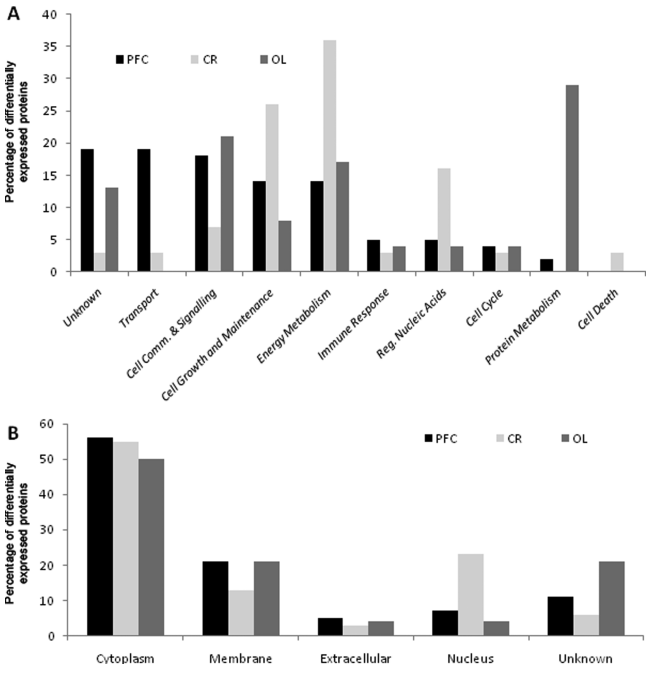

Figure 1. (A) Biological processes and (B) cellular sublocalization of the aFTLD differential proteomes from prefrontal cortex (PFC), cerebellum (CR), and occipital lobe (OL).

involved protein transport in the PFC, energy metabolism in the CR and protein metabolism in the OL. Common pathways were also altered in the 3 brain regions, such as effects on the immune response, cell cycle, cell growth and maintenance, and cell communication and signaling. In terms of the cellular localization of the changes, similar cellular compartments were affected in all three brain regions apart from the over representation of changes in nuclear proteins in the CR.

It should be noted that the present study has some limitations. For example, the results were derived from analysis of a small group of samples, and no replication was possible due to the lack of availability of a separate sample set. In addition, the protein alterations described here may have been potentially confounded by factors such as post-mortem interval, age, gender, or medications used, although none of these factors appeared to have influenced the outcome here. Nevertheless, this still represents a study on the central nervous system effects in aFTLD-U subjects and should lay the groundwork for future studies on this neurodegenerative condition.

In the PFC, changes in the cellular transport and communication pathway were indicated by changes in syntaxin subunits (STX1A, STX1B, and STXBP1) and clathrin-associated adaptor proteins (AP1B1, AP2A1, AP2A2, AP2B1), consistent with effects on synaptic connectivity. In addition, the PFC showed changes in the calcium-related proteins calcium/calmodulin-dependent protein kinase II alpha (CAMK2A) and protein phosphatase 3, catalytic subunit, alpha isozyme (PPP3CA). In the CR, the molecular changes regarding the cellular structure and cytoskeleton assembly pathway included subunits of tubulin (TUBB and TUBA4A), dynamin (DNM3), and spectrin (SPTBN2). In the OL, the alterations regarding protein metabolism included heat shock proteins (HSPA8, HSP90AA1), as well as detoxification of endogenous compounds and xenobiotics through the differential expression of glutathione S transferase Mu 3 (GSTM3).

All pathway analyses using the IPKB software implicated changes in the amyloid beta A4 (APP) protein in all 3 brain regions. This was seen by the linking of this protein as a branch-point of the IPKB-determined biochemical networks (Figure 3), suggesting a central role of this protein in aFTLD-U. APP plays various roles in brain tissue including neurite and axon growth, cell mobility, and transcription regulation and is most known for its central involvement in AD.[32] Although APP mutations have not been observed in FTLD,[33] there have been reports of a decreased concentration of APP in the CSF of patients with FTLD.[34,35] The mass spectrometry method employed here did not detect APP and so other targeted

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Table 2. Protein and Peptides Evaluated by SRM

protein	peptide sequence	parent ion and charge	daughter ions	brain region
STX1A	IEYNVEHAVDYVER	579.2810+++	851.4258+	PFC
			780.3886+	
			681.3202+	
STX1A	SIEQSIEQEEGLNR	816.3972++	1087.5378+	PFC
			974.4538+	
			845.4112+	
STX1B	LSEDVEQVK	523.7719++	846.4203+	PFC
			717.3777+	
STX1B	LAIFTDDIK	518.2897++	851.4509+	PFC
			738.3668+	
			591.2984+	
STXB1	AIVPILLDANVSTYDK	866.4800++	1448.7631+	PFC
			1125.5422+	
			1012.4582+	
STXB1	EVLLDEDDDLWIALR	605.6439+++	886.5145+	PFC
			658.4035+	
STXB1	VLVVDQLSMR	580.3288++	848.4295+	PFC
			749.3610+	
STXB1	WEVLIGSTHILTPQK	861.4829++	1081.6000+	PFC
			372.2241+	
STXB1	WEVLIGSTHILTPQK	574.6577+++	836.4989+	PFC
			699.4400+	
LDHA	FIIPNVVK	465.2946++	782.5135+	CR
			669.4294+	
			556.3453+	
LDHA	LNLVQR	371.7321++	629.3729+	CR
			515.3300	
			402.2459+	
LDHA	LVIITAGAR	457.2951++	588.3464+	CR
			475.2623+	
LDHA	VHPVSTMIK	506.2864++	775.4382+	CR
			678.3855+	
PRDX2	GLFIIDGK	431.7553++	692.3978+	CR
			545.3293+	
PRDX2	LSEDYGVLK	512.2715++	823.4196+	CR
			694.3770+	
			579.3501+	
PRDX2	SVDEALR	395.2087++	603.3097+	CR
			488.2827+	
PRDX2	TDEGIAYR	462.7247++	708.3675+	CR
			579.3249+	
			522.3035+	
SDHA	GFHFTVDGNK	561.2724++	917.4476+	CR
			780.3886+	
SDHA	NTVVATGGYGR	547.7831++	780.3999+	CR
			681.3315+	
			610.2944+	
GLUD1	ALASLMTYK	499.2730++	742.3804+	CR
			542.2643+	
GLUD1	AYVNAIEK	454.2478++	574.3195+	CR
			460.2766+	
GLUD1	FTMELAK	420.2202++	591.3171+	CR
			460.2766+	
GLUD1	GFIGPGIDVPAPDMSTGER	958.4646++	1159.5412+	CR
			1060.4728+	
			963.4200+	
			892.3829+	
GLUD1	HGGTIPIVPTAEFQDR	579.9688+++	963.4530+	CR
			866.4003+	
			765.3526+	
GLUD1	HYSEAVADR	524.2463++	747.3632+	CR

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Table 2. continued

protein	peptide sequence	parent ion and charge	daughter ions	brain region
			660.3311+	
GLUD1	YSTDVSVDEVK	621.2984++	890.4466+	CR
			775.4196+	
			676.3512+	
HSPA1A	SAVEDEGLK	474.2376++	789.3989+	OL
			690.3305+	
HSPA1A	NQVALNPQNTVFDAK	829.9285++	1133.5586+	OL
			1019.5156+	
HSP90AA5P	TKPIWTR	451.2663++	672.3828+	OL
			462.2459+	
HSP90AA5P	HGLEVIYMIELIDK	558.3041+++	861.4750+	OL
			730.4345+	
yeast enolase	AADALLLK	407.7553++	672.4291+	normalization
			557.4021+	
			486.3650+	
yeast enolase	SIVPSGASTGVHEALEMR	614.3122+++	885.4247+	normalization
			748.3658+	
			619.3232+	
yeast enolase	NVNDVIAPAFVK	643.8588++	745.4607+	normalization
			561.3395+	
yeast enolase	VNQIGTLSESIK	644.8590++	834.4567+	normalization
			777.4353+	
			676.3876+	

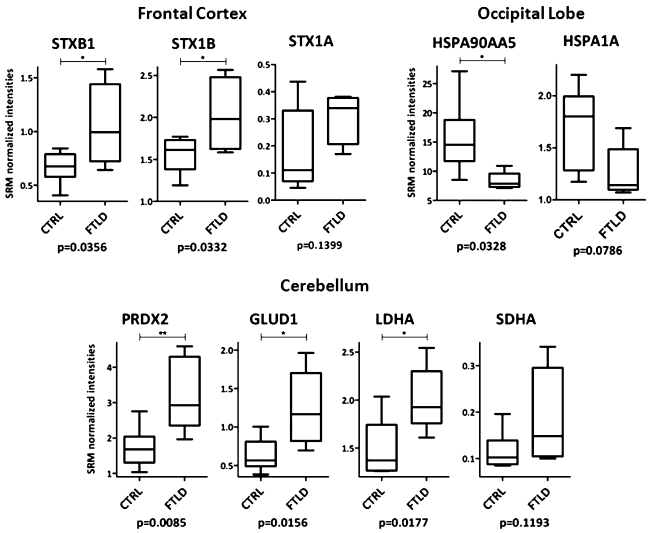

Figure 2. Validation by SRM of the differentially expressed proteins in all the 3 brain regions analyzed. Sample groups were analyzed by Mann−Whitney test. Differences with $p < 0.05$ were considered statistically significant.

methods will be required to establish a role of this protein in aFTLD-U. In the case of the PFC and CR networks, the major branch-point identified was the huntingtin (HTT) protein. This protein plays a role in microtubular transport and vesicle function and is most notable for its involvement in Huntington disease (HD).[36] HD and FTLD show similarities in the molecular composition of the inclusion bodies, as both are TDP-43 positive.[37] An HD-like phenotype has been suggested to be part of the clinical spectrum of the bv-FTD phenotype.[38] However, this may be distinct from cases of aFTLD-U, which are characterized by TDP-43 negative inclusions. The systems biology analysis also identified molecular similarities of aFTLD-U with other brain disorders (Table 3). We compared our proteome profiling to that found in brains from AD patients.[15] Five proteins found differentially expressed in the PFC of aFTLD-U were previously revealed in different brain regions of AD [syntaxin binding protein 1 (STXBP1), tubulin, beta 2C (TUBB2C), synaptotagmin I (SYT1), enolase 2 (ENO2) and malate dehydrogenase 1 (MDH1)]. Three proteins from the CR of aFTLD-U patients [glial fibrillary acidic protein (GFAP), glutamate dehydrogenase 1 mitochondrial (GLUD1) and peroxiredoxin-2 (PRDX2)] have been identified previously in AD studies. Five proteins in the OL analyses have also been identified in AD brains [metabolic-related proteins glutathione S transferase Mu 3 (GSTM3), pyruvate kinase isozymes M1/M2 (PKM2), guanine nucl bind prot G i alpha 1 subunit (GNAI1), HSPA1A and HSP90AA1]. These similarities might represent overlaps in pathogenesis but could also reflect downstream events shared by both conditions as a result of neurodegeneration.

We also compared current proteomic findings with previous studies of schizophrenia, considering the fact that aFTLD-U patients may present similar psychotic symptoms.[11,12] Alterations in tubulin-related proteins was one finding in common, with changes in tubulin subunits reported in Wernicke's area in schizophrenia patients, as well as in the PFC in patients with schizophrenia and bipolar disorder.[39,40] We also found changes in adapter-related proteins in PFC. These proteins, along with several other components, govern clathrin-mediated endocytosis, and this mechanism has been implicated in the pathophysiology of schizophrenia and bipolar disorders.[41] Other proteins highlighted in Table 1 have also been described previously in proteome studies of schizophrenia.[14,42] These proteins might be associated with psychotic events, and should be investigated further.

This study also identified molecular similarities and differences in aFTLD-U brain tissues compared with those from other FLTD variants. Alterations in alpha tubulin, neuronal enolase and peroxiredoxin 1 were found in a previous proteomic

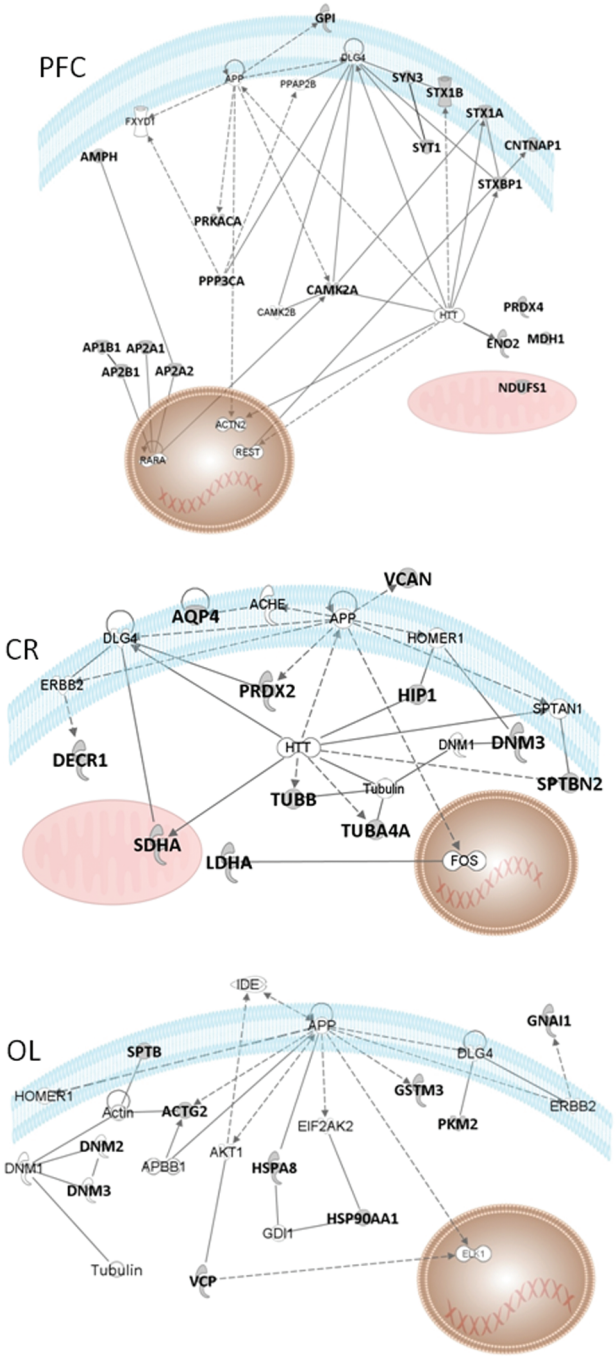

Figure 3. Network of proteins interactions among the differentially expressed proteins from prefrontal cortex (PFC), cerebellum (CR), and occipital lobe (OL) according to systems biology analyses by Ingenuity Pathways Knowledgebase.

study of brains from patients with frontotemporal dementia.[30] We also identified changes in neuronal enolase (ENO2), a subunit of alpha tubulin (TUBA4B) and a member of peroxiredoxin family (PRDX4) (Table 1). However, most of the changes that we identified in the PFC were related to transport molecules, although Schweitzer and colleagues[30] found more changes in energy metabolism proteins. This is probably due to the different proteomic methodologies used. Shotgun analyses as performed here are more likely to detect a greater fraction of membrane proteins. On the other hand, 2-DE analyses as performed by Schweitzer and colleagues are more likely to detect differences in soluble proteins such as energy metabolism enzymes, and this demonstrates the potential complementary nature of these approaches.[13]

Table 3. Involvement of the Differentially Expressed Proteins Found in aFTLD-U in Other Brain Disorders

functions annotation	*p*-value	molecules	molecules
	frontal cortex		
Schizophrenia[43,44]	5.31×10^{-5}	AMPH, HPRT1, MDH1, PPP3CC, STX1A, SYN3	6
Parkinson's disease[45,46]	6.47×10^{-3}	EEF1A1, ENO2, MDH1	3
Bipolar disorder[44,47]	4.34×10^{-2}	CAMK2A, SYN3	2
	cerebellum		
Progressive motor neuropathy[46,48,49]	3.59×10^{-7}	AQP4, GFAP, HNRPDL, LDHA, PRPH, RAN	6
Parkinson's disease[46]	6.71×10^{-5}	AQP4, HNRPDL, LDHA, RAN	4
Amyotrophic lateral sclerosis[48−50]	9.30×10^{-4}	GFAP, PRPH	2
Alzheimer's disease[51,52]	4.48×10^{-2}	GFAP, TUBB	2
	occipital lobe		
Alzheimer's disease[53−55]	4.28×10^{-2}	ACTA2, CSRP1, EIF4A2, HSPB1	4
Parkinson's disease[46,56]	1.94×10^{-2}	EIF4A2, HSPB1	2

This is the first investigation of aFTLD-U of its type. The study employed the use of rare samples, and it was therefore not possible to replicate the findings in a follow up series, although they have been validated using the SRM method. Nonetheless, the results are encouraging and suggest that it might be possible to classify subtypes of FTLD based on brain molecular profiles. The other intriguing finding was the effects on different molecules and molecular pathways in distinct brain regions. This suggests that, like other neurological conditions, FTLD may be manifested as a result of altered communication between multiple brain regions. Further studies in this area may lead to a better understanding of the molecular changes of FTLD and will drive studies toward the development of biomarker tools for better classification of these subjects which could be used in the development of novel treatment strategies.

■ ASSOCIATED CONTENT

Supporting Information

Additional table and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

¶In Memoriam.

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