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Identification of proteomic signatures associated with depression and psychotic depression in post-mortem brains from major depression patients

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Major depressive disorder (MDD) is a leading cause of disability worldwide and results tragically in the loss of almost one million lives in Western societies every year. This is due to poor understanding of the disease pathophysiology and lack of empirical medical tests for accurate diagnosis or for guiding antidepressant treatment strategies. Here, we have used shotgun proteomics in the analysis of *post-mortem* dorsolateral prefrontal cortex brain tissue from 24 MDD patients and 12 matched controls. Brain proteomes were pre-fractionated by gel electrophoresis and further analyzed by shotgun data-independent label-free liquid chromatography-mass spectrometry. This led to identification of distinct proteome fingerprints between MDD and control subjects. Some of these differences were validated by Western blot or selected reaction monitoring mass spectrometry. This included proteins associated with energy metabolism and synaptic function and we also found changes in the histidine triad nucleotide-binding protein 1 (HINT1), which has been implicated recently in regulation of mood and behavior. We also found differential proteome profiles in MDD with ($n = 11$) and without ($n = 12$) psychosis. Interestingly, the psychosis fingerprint showed a marked overlap to changes seen in the brain proteome of schizophrenia patients. These findings suggest that it may be possible to contribute to the disease understanding by distinguishing different subtypes of MDD based on distinct brain proteomic profiles.
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Introduction

Major depressive disorder (MDD) is a serious psychiatric condition affecting approximately 10% of the world population with a lifetime prevalence of 17%.[1] The effects of MDD are wide-ranging, including a negative impact on families, work and relationships, and has been associated with debilitating co-morbidities such as general ill health, substance abuse and anxiety disorders. Together, these factors contribute to an enormous significant financial burden on the healthcare services.[2] In addition, MDD subjects account for 60% of suicides in the United States.[3] Although some molecular aspects of MDD have been identified, such as hypothalamic–pituitary–adrenal axis dysfunction,[4] effects on memory[5] and volume reduction of certain brain regions such as hippocampus[6] and prefrontal cortex,[7] the underlying pathophysiology of this disorder has only been partially elucidated. The association between inflammation and MDD has been supported by the fact that treatment of hepatitis C and certain cancer patients with interferon-alpha,[8,9] frequently induces depressive symptoms as side-effects. In addition, MDD is also associated with auto-immune diseases[10] and metabolic disorders,[11] and several studies have shown that the efficacy of antidepressants may be partly attributable to their anti-inflammatory properties.[12] As a consequence, currently available antidepressant medications often show only medium efficacy and can have serious side-effects.[13] Therefore, it is important to increase our understanding of the physiological factors underlying this condition before more effective medications can be developed.

Studies of MDD are complicated by the fact that it is a systemic, multifactorial disorder. Current hypotheses suggest that MDD most likely arises from complex interactions between genetic predisposition,[14] disturbance of key molecular pathways including neurotransmitter systems and synaptic plasticity,[4] along with the impact of environmental factors such as stressful life events.[15,16] Therefore, an increased understanding of this disorder is likely to be gained by application of molecular profiling analyses of relevant brain regions using approaches such as transcriptomics,[17,18] lipidomics[19] and proteomics.[20] Of these platforms, proteomics may be the most appropriate for studies of psychiatric conditions considering that it is better suited for capturing the dynamic nature of perturbed biological systems.[20,21]

MDD patients can present with a great variety of symptoms including low mood, low self-esteem, loss of interest or pleasure in normally enjoyable activities and melancholia. MDD patients may also present with severe psychotic

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symptoms, including delusions and hallucinations. Similar symptoms are often observed, for example, in schizophrenia albeit with subtle differences. Therefore, the identification of symptom-related potential biomarker candidates[22] could be useful in combination with the existing clinical assessment for more accurate classification of MDD patients and also to provide a biochemical comprehension of the molecular processes involved. These could be useful not only for potential diagnostic purposes but also to provide a molecular means of distinguishing patients to facilitate much-needed personalized medicine strategies.

With this in mind, we have carried out shotgun proteome analyses as represented in Supplementary Material 1 on *post-mortem* dorsolateral prefrontal cortex (DLPFC) tissue from MDD patients. Features such as volume reduction[7] and reduced activation[23] have previously suggested that effects on this brain region are associated with MDD.[24] We used label-free liquid chromatography mass spectrometry in data-independent mode (LC-MS^E) to analyze DLPFC tissue from 24 MDD patients and 12 control subjects. The DLPFC is known to be involved in executive and intellectual functions, integration of sensory and mnemonic information and in working memory.[25] Additionally, we have re-evaluated the data considering those MDD patients who presented with ($n=11$) or without ($n=12$) psychotic symptoms, in an attempt to identify a MDD psychosis-specific signature.

Materials and methods

Clinical samples. DLPFC tissues (Brodmann area 9) were collected *post-mortem* and frozen from MDD patients ($n=24$), either with psychosis ($n=11$) or without psychosis ($n=12$) plus matched control subjects ($n=12$) by the Stanley Medical Research Institute (Bethesda, MD, USA). One of the MDD patients could not be defined as psychotic or not. The diagnosis of MDD had been given *ante-mortem*. Consent was obtained by questionnaire-based telephone interview and signed by the interviewer and a witness. The Institutional Review Board at the Uniformed Services University of Health Sciences determined that the procedure was exempt from federal and state regulations governing human research, as specimens were obtained from cadavers and anonymized with respect to personal information. General patient information is given in Table 1 and detailed information in Supplementary Material 2.

Sample and proteome preparation. Tissue samples (20 mg) were homogenized individually in 100 μl of 7 M urea, 2 M thiourea, 4% CHAPS, 2% ASB-14 and 70 mM DTT, as described previously,[26] using the Sample Grinding Kit (GE Healthcare, Little Chalfont, Bucks, UK). Samples were centrifuged for 10 min at 16 000 × *g*, the supernatants were collected and protein concentrations were determined using the Bradford dye-binding assay (Sigma, Poole, Dorset, UK). For enhancing proteome coverage, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) pre-fractionation was used for separation of proteins according to apparent molecular size. Extracted protein samples (15 μg) were diluted 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 heated for 5 min at 95 °C before 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 four sections corresponding to different apparent molecular weight ranges. Each gel section was then subjected to trypsin digestion *in situ* as described previously,[27] and resulting peptide mixtures were lyophilized.

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 nanoUltra-Performance Liquid Chromatography instrument containing a BEH-130 C18 column (75 μm × 200 mm) at a flow rate of 0.3 μl min^{-1} connected online to a Q-TOF Premier Mass Spectrometer (Waters Corporation, Manchester, UK). The following gradient was applied over 140 min comprised of solvent A (0.1% aqueous formic acid) and solvent B (acetonitrile with 0.1% formic acid): 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%; returning to the initial condition in 1 min. Eluted peptides were measured in MS^E mode (data-independent

Table 1 Demographic information for the brain tissue samples used in the study

	Control	*All MDD*	*MDD-P*	*MDD-NP*	*Significance t-test for MDD x CTRL (P-value)*	*Significance ANOVA for MDD-P x CTRL x MDD-NP (P-value)*
Sample size	12	24	12	12		
Age	47 ± 12	42 ± 11	40 ± 11	43 ± 10	0.38	0.30
Postmortem interval	25.3 ± 10.6	29.7 ± 12.4	33.1 ± 11	23.6 ± 6.7	0.44	0.12
Refrigerator interval	7.4 ± 5.4	7.9 ± 6.3	7.2 ± 3.9	8 ± 8.2	0.80	0.94
Brain pH	6.6 ± 0.2	6.7 ± 0.2	6.6 ± 0.2	6.7 ± 0.1	0.44	0.12
Gender (male/female)	8/4	13/11	5/6	7/5	0.57	Fisher's exact test 0.41 (MDD-P x CTRL) 0.99 (MDD-NP x CTRL) 0.68 (MDD-P x MDD-NP)

Abbreviations: ANOVA, analysis of variance; CTRL, control; MDD, major depressive disorder.
Values are mean ± s.d. MDD patients have been considered as one group (All MDD) or separately according to the presence of psychosis (MDD-P and MDD-NP).

analysis) using the ion accounting algorithm[28] 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.). Firstly, raw data were processed and chromatograms aligned in time. Aligned peaks were extracted and abundance measurements obtained by integration of time, mass/charge (m/z) and intensity volumes, with normalization to the total ion current. Data were searched against the SwissProt human database (version 57.4; http://www.uniprot.org/) and also against 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 three ion fragments per peptide, seven ion fragments per protein and one peptide per protein. However, we only considered for differential expression analyses proteins identified by at least two peptides. Modifications considered were carbamidomethylation of cysteines and oxidation of methionine.

Quantitative protein expression and statistical analyses were performed using the Rosetta Elucidator system v.3.3.0.1.SP3.19 (Rosetta Inpharmatics, Seattle, WA, USA) and processed data from PLGS analysis. We established a fold change cutoff of ± 1.15 based on the following facts: (1) coefficient of variation calculated for all identified proteins was 0.18 ± 0.3 (mean $\pm$ s.d.); (2) label-free proteomics has been shown to underestimate fold changes,[29] which is supported by the fact that a protein with a fold change of 1.13 was validated by Western blot analysis revealing a 1.47-fold change.

Statistical analyses. Differences in protein expression between MDD patients and controls were accessed using Wilcoxon signed-rank test, as the data were not assumed to be normally distributed. Only differences with a $P<0.05$ were considered significant. False discovery rate (FDR) was calculated according to Benjamini and Hochberg.[30] No adjustments were made for multiple comparisons in order to not exclude possible true positives.[31] This approach will lead to fewer errors of interpretation as proteomic data are not necessarily random but can be physiologically interdependent observations. Nevertheless, a FDR threshold of approximately 0.4 and a fold change cutoff of 15% for the shotgun proteome analyses was established.

Although groups are matched for demographic variables (Table 1), the influence of gender, age, alcohol abuse, smoking, *post-mortem* interval and refrigeration interval on the data were accessed by using principal component analysis (PCA) as previously described.[32] The PCA results are presented in Supplementary Material 3. Differentially expressed proteins were not found to be correlated to demographics variables.

Selected reaction monitoring. Quantitative differences in the levels of histidine triad nucleotide-binding protein 1 (HINT1) and synaptosomal-associated protein 25 (SNAP25) were validated using whole tissue lysates by selected reaction monitoring (SRM) mass spectrometry. Three SRM transitions of the HINT1 peptides 'IIFEDDR', 'HISQISVAEDDDESLLGHLMIVGK' and 'MVVNEGSDGGQ SVYHVHLHVLGGR' were analyzed, as well as three SRM transitions of the SNAP25 peptides 'NELEEMQR', 'AWGNNQDGVVASQPAR' and 'IEEGMDQINK'. Peptides were selected based on identification in the LC-MS^E dataset with a high spectral quality, and if those peptides were proteotypic, which means an experimentally observable peptide that uniquely identifies a specific protein or protein isoforms.[33]

Samples (0.2 μg) were prepared exactly like for LC-MS^E analyses. Whole digested lysates were injected in duplicate into an identical LC system, as above, coupled to a Xevo triple-quadrupole mass spectrometer (Waters). For separation of peptides, the following 48 min gradient 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%; returning to the initial condition 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 2 min window of the predicted retention time and the scan time was 20 milliseconds. The collision energy for each transition was optimized using Skyline software[34] based on the equation: $CE = 0.034*m/z + 3.314$. Acquired data were processed using TargetLynx (Waters). Differences in protein levels between MDD and controls were determined using Student's *t*-tests considering $P<0.05$ as significant.

Western blot. Quantitative differences in the levels of amphiphysin (AMPH) and growth-associated protein 43 (GAP43) were assessed for validation of LC-MS^E findings by Western blot analyses due to their involvement in synapses. Total tissue lysates from MDD and controls were arranged in randomized order such that each of the groups were represented on both gels. For each sample, 20 μg total protein was electrophoresed using pre-cast Novex 10–20% Tricine polyacrylamide gels (Invitrogen) at 125 V for 60 min, followed by semi-dry transfer to Immobilon-FL polyvinyldiphenyl fluoride membranes (Millipore, Watford, UK). The membranes were incubated in a 1:1 mixture of Odyssey blocking buffer (Li-COR Biosciences, Cambridge, UK) and phosphate-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies (both from Abcam, Cambridge, UK): anti-amphiphysin (1:2000) and anti-GAP-43 (1/5000). Membranes were washed in Tris-buffered saline containing EDTA for 30 min, in phosphate-buffered saline for 2×5 min and then for 1 h at room temperature with the appropriate IR dye-conjugated secondary antibodies (1:7500 for secondary rabbit antibody and 1:15000 for secondary mouse antibody Li-COR Biosciences) in blocking buffer. Immunoreactive protein bands were visualized using the Odyssey Infra-red imaging system (Li-COR Biosciences) and the integrated intensities of the bands measured. Values outlying by more than two standard deviations from the mean were excluded from the analysis. Differences in protein levels between MDD and controls were determined using Student's *t*-tests considering $P<0.05$ as significant.

ATP assay. In order to validate the differential expression of several oxidative phosphorylation protein subunits, ATP

levels were measured using the ATP Assay Quantification Kit according the manufacturers' instructions (BioVision, San Francisco, CA, USA). Differences in ATP levels per 5 μg of brain tissue between MDD and controls were determined using Student's *t*-tests considering $P<0.05$ as significant.

Classification of differentially expressed proteins. Differentially expressed proteins in MDD DLPFC were classified according to their biological pathways and subcellular localization using the Human Protein Reference Database (http://www.hprd.org) and KEGG database (www.genome.jp/kegg). For interpretation of the functional significance of differentially expressed proteins, the associated SwissProt accession numbers were uploaded into the Ingenuity Pathways Knowledgebase (IPKB) (www.ingenuity.com) and these were analyzed to identify potential interactions between these proteins and other proteins in the IPKB.

Results

Shotgun proteomics results. The shotgun LC-MS^E strategy for protein profiling of *post-mortem* DLPFC samples from MDD ($n=24$) and control subjects ($n=12$) is outlined in Supplementary Material 1. This resulted in identification of 18 422 unique peptides in the 36 analyzed samples, which led to identification of 1422 non-redundant proteins. From these, 526 proteins were identified with a minimum of two distinct peptides and detected at least in >75% of samples. Overall, 35 of these were discarded, as they were keratin and trypsin contaminations. Therefore, 491 proteins were considered for differential analyses. These proteins, their peptides and mass spectra data are deposited at the PRIDE database[35] (http://www.ebi.ac.uk/pride; accession number: 15310). There were no significant differences between MDD patients and controls with regards to gender, age, post-mortem and refrigeration interval and brain pH (Table 1). In addition, PCA of the data showed no overt effects of any of these parameters (Supplementary Material 3).

Proteome differences between MDD and control subjects. Comparing all MDD patient samples to controls, significant differences ($P<0.05$; Wilcoxon test) in protein levels were observed for 39 proteins with a fold change cutoff of ±1.15 and a FDR of approximately 0.4 although most were approximately 0.350 (Table 2). No adjustment for FDR was used[31] as described in material and methods section. AMPH is the only exception for the fold change cutoff as it has been validated by Western Blot. Two of these proteins were decreased and 37 increased in MDD patients compared with controls. These proteins were classified according to their biological processes and cellular localization. The major pathways that are associated with MDD were metabolic/energy pathways (32%) and transport of molecules (22%) (Supplementary Material 4A). The majority of the differentially expressed proteins (72%) were identified as cytoplasmic, and 13% were membrane-associated proteins (Supplementary Material 4B). We also identified several proteins such as profilin 1 and 2 (PFN1 and PFN2), SNAP25 and gamma aminobutyric acid receptor-associated protein like 2 (GABARAPL2) that are involved in synaptic transmission (Figure 1). Also, some of these proteins have been previously associated with other neurological or psychiatric conditions such as Huntington's disease (27%), schizophrenia (18%) and Alzheimer's disease (3%) (Supplementary Material 5).

Proteome differences in MDD patients with and without psychosis. Shotgun LC-MS^E analysis of MDD patients with psychosis (MDD-P) and without psychosis (MDD-NP) showed significant differences ($P<0.05$; Wilcoxon test) in proteomic profiles with an FDR of approximately 0.4. Proteomic comparison of MDD-NP patients ($n=12$) with control subjects ($n=12$) led to identification of 18 altered proteins. However, comparison of MDD-P patients ($n=11$) with controls resulted in identification of 36 altered proteins and direct comparison of the MDD-P and MDD-NP proteomes resulted in identification of 30 significantly different proteins. All of the differentially expressed proteins associated with the categories above are listed in Table 3 and classified according to their biological processes and cellular localization (Figure 2).

The overlapping proteins across these different comparisons are shown in Figure 3. Five proteins (cystatin C (CST3), fatty acid-binding protein (FABP3), rho-related GTP-binding protein (RHOC), gamma synuclein (SNCG) and peptidylprolyl isomerase A (PPIA)) were changed in common between the MDD-P and MDD-NP patients relative to controls (Figure 3a). A total of 13 proteins were altered specifically in the MDD-NP subjects, including the HINT1 and the mitochondrial ATP synthase subunits b and e (ATP5F1 and ATP5I). Overall, 31 proteins were altered specifically in the MDD-P patients including the synaptic vesicle-related proteins AMPH and SNAP25. Direct comparison of the MDD-P and MDD-NP proteomes resulted in identification of 25 altered proteins, which were not found in either of the MDD-P vs control or MDD-NP vs control comparisons (Figure 3b). These proteins included ADP-ribosylation factor 1 and 3 (ARF1 and ARF3), synaptotagmin 2 (SYT2) and stress-induced phosphoprotein 1 (STIP1). Moreover, there was no overlap of this signature with that found in the total MDD vs control comparison.

Comparison of these datasets with those obtained from previous proteomic studies of *post-mortem* brain tissue from schizophrenia patients[36–38] showed a marked overlap between the MDD-P and schizophrenia proteomes (Figure 3a). Also, the psychosis-specific fingerprint obtained by direct comparison of MDD-P and MDD-NP subjects showed a significant overlap with the schizophrenia fingerprint (Figure 3b). This included proteins such as SYT2, septin 2 (SEPT2), syntaxin-binding protein 1 (STXBP1) and calreticulin (CALR).

Validation experiments. Considering the impairment of synaptic transmission associated with MDD, as observed here and previously,[1,4] we carried out technical validation of three such differentially expressed candidate proteins identified by the shotgun LC-MS^E molecular profiling method. Using Western blot, we analyzed the proteins AMPH and GAP43 in whole tissue lysates as well as with SRM-MS to validate the differential expression of SNAP25.

Table 2 Differentially expressed proteins in all post mortem brains from MDD patients compared with controls

Biological processes	*Gene name*	*Protein name*	*FC*	*IP*	P-*value*	q-*value*
Metabolism/energy	ACOT13	Acyl coenzyme A thioesterase	1.62	2	0.0445	0.3665
	ACYP2	Acylphosphatase 2	1.34	3	0.0107	0.3248
	COX4I1	Cytochrome c oxidase subunit 4 isoform 1 mitochondrial	1.24	5	0.0311	0.3248
	COX5B	Cytochrome c oxidase subunit 5B mitochondrial	1.44	6	0.0210	0.3665
	COX7A2	Cytochrome c oxidase polypeptide 7A2 mitochondrial	1.31	2	0.0325	0.4368
	CYCS	Cytochrome c	1.51	13	0.0311	0.3248
	NDUFA13	NADH dehydrogenase Ubiquinone 1-alpha subcomplex subunit 13	1.43	7	0.0248	0.3248
	NDUFA2	NADH dehydrogenase Ubiquinone 1-alpha subcomplex subunit 2	1.39	3	0.0349	0.3665
	NDUFA6	NADH dehydrogenase Ubiquinone 1 alpha subcomplex subunit 6	1.62	5	0.0025	0.2412
	NDUFS5	NADH dehydrogenase Ubiquinone iron-sulphur protein 5	1.57	2	0.0393	0.3665
	UQCRFSL1	Cytochrome b c1 complex subunit	1.38	2	0.0433	0.3665
Transport/energy	ATP5I	ATP synthase subunit e mitochondrial	1.37	4	0.0410	0.3248
	ATP5 L	ATP synthase subunit g mitochondrial short ATPase	1.34	4	0.0370	0.3248
Transport	APOE	Apolipoprotein E	1.17	4	0.0279	0.3356
	FABP3	Fatty acid-binding protein heart	1.62	7	0.0219	0.3248
	FABP7	Fatty acid-binding protein brain	1.35	4	0.0444	0.3665
	FXYD6	FXYD domain containing ion transport regulator 6	1.76	4	0.0008	0.3665
	GABARAPL2	Gamma aminobutyric acid receptor-associated protein-like 2	1.44	3	0.0318	0.3665
	HRSP12	Ribonuclease UK114	1.25	7	0.0270	0.3248
	SNAP25	Synaptosomal-associated protein 25	−1.15	26	0.0070	0.1247
Cell communication and signalling	AMPH[a]	Amphiphysin	1.13	41	0.0379	0.1347
	DPYSL2	Dihydropyrimidinase-related protein 2	1.17	10	0.0027	0.3248
	DPYSL3	Dihydropyrimidinase-related protein 3	1.16	2	0.0124	0.3665
	FKBP2	FK506-binding protein 2	1.30	3	0.0072	0.3665
	GAP43	Neuromodulin; growth-associated protein 43	1.20	27	0.0302	0.1247
	HINT1	Histidine triad nucleotide-binding protein 1	1.59	4	0.0038	0.2412
	RHOC	Rho-related GTP-binding protein	1.49	2	0.0095	0.3665
Cell growth and maintenance	MAP1LC3A	Microtubule-associated proteins 1A 1B light chain 3A	1.30	5	0.0455	0.3248
	PFN1	Profilin-1	1.42	6	0.0259	0.3248
	PFN2	Profilin 2	1.46	7	0.0032	0.3248
	TUBA4B	Putative tubulin-like protein alpha-4B	1.19	6	0.0238	0.3248
Protein metabolism	CST3	Cystatin C	1.68	3	0.0284	0.3356
	CSTB	Cystatin-B	1.29	2	0.0208	0.3665
	SNCA	Alpha synuclein	1.51	5	0.0382	0.3248
	SNCG	Gamma synuclein	1.38	11	0.0074	0.3248
Regulation of nucleic acid metabolism	HIST4H4	Histone H4	1.44	5	0.0265	0.3248
	SATB2	DNA-binding protein SATB2	1.35	3	0.0314	0.3248
	SSBP1	ss DNA-binding protein mitochondrial short Mt SSB	1.56	2	0.0397	0.4368
Unknown	CISD1	CDGSH iron sulfur domain-containing protein 1	−1.34	8	0.0140	0.3248

Abbreviations: FC, fold change; IP, number of identified peptides; MDD, major depressive disorder.
P-values for Wilcoxon test. [a]AMPH is an exception for the fold change cutoff because it has been validated by Western blot.

Considering the suggested role of histidine triad nucleotide-binding protein-1 (HINT1) in MDD,[39,40] we also carried out SRM-MS experiments for this protein. Apart from GAP43, differential expression of all of these proteins was validated (Figure 4). Of note, AMPH was only found to be significant in the MDD-NP versus MDD-P comparison with a fold change of 1.13 based on MS profiling and Western blot analysis revealed that this was increased by 1.47 fold in all MDD patients. This lends support to our choice of a cutoff of 1.15-fold in the LC-MS^E screening phase and also suggests that the quantification of label-free proteomics data may underestimate fold changes, as demonstrated previously.[29]

The results also suggested that the oxidative phosphorylation pathway was dysregulated in MDD through the differential expression of several subunits of the mitochondrial complexes (Supplementary Material 6). Therefore, we carried out a functional validation experiment of whole tissue lysates to test this possibility by measuring the levels of ATP in the DLPFC samples. This analysis showed that lower levels of ATP were observed in MDD tissues compared with controls, although this was not related to psychotic status (Figure 4).

Discussion

This is the first shotgun proteomics study of brain tissue samples from MDD patients. We employed an LC-MS^E method in combination with gel-based protein fractionation to increase proteome coverage, including the identification of membrane proteins,[26] which are mediators of signal transduction and potential therapeutic targets. We also applied LC-SRM-MS for the first time in proteome studies of psychiatric disorders. This method was used here as a means of validating differences in the levels of identified proteins with greater level of sensitivity and specificity compared to the conventional LC-MS^E profiling. The multiplex nature of SRM allows the accurate quantification of tens of proteins in a single experimental run allowing its use in both pre-clinical and clinical trials as well as in clinical pipelines for detection of

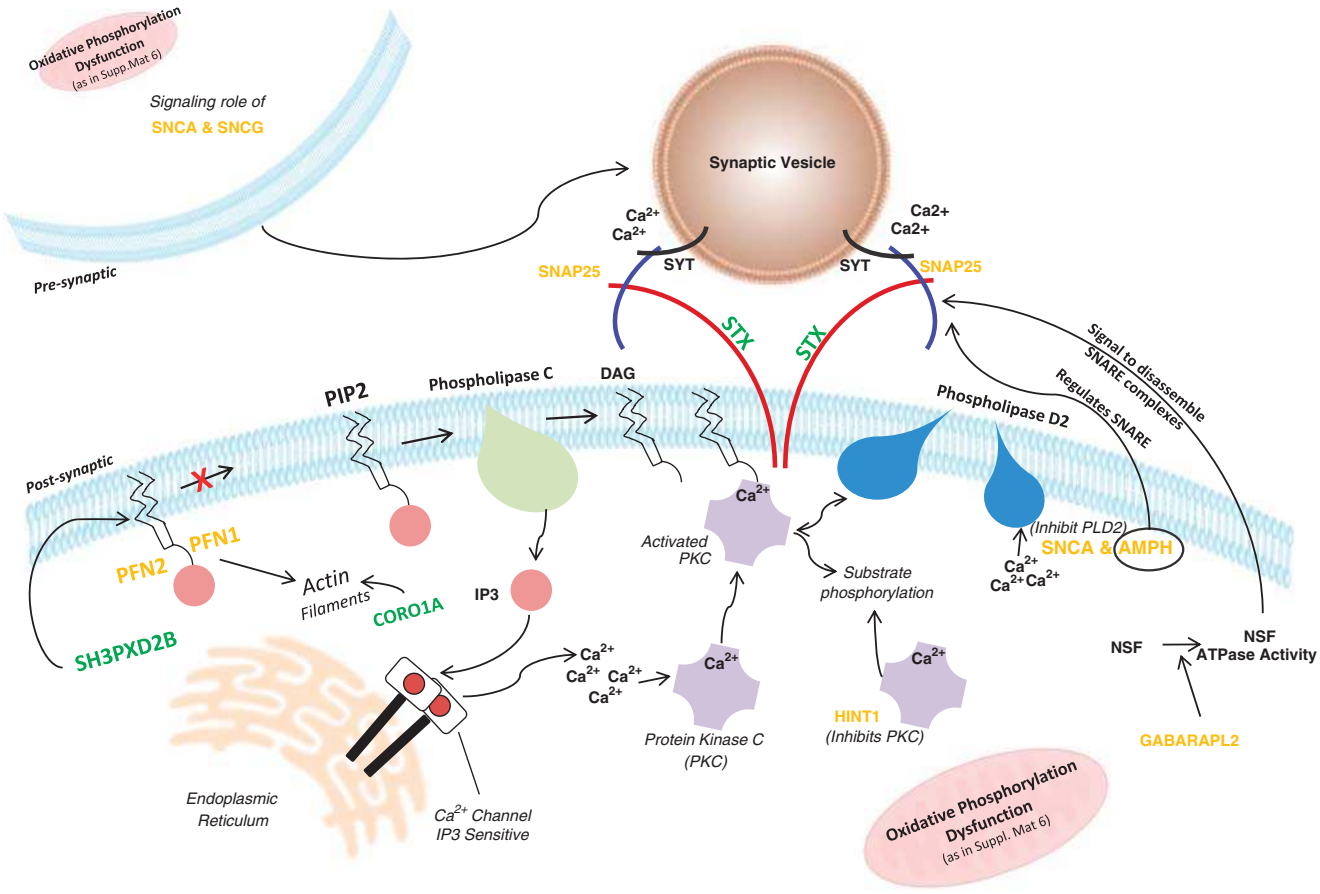

Figure 1 Proteins associated with synaptic dysfunction in major depressive disorder brains. Proteins found differentially expressed between major depressive disorder patients *versus* controls presented in Table 2 are indicated in yellow. In green, proteins found differentially expressed which were further eliminated by the fold change cutoff established.

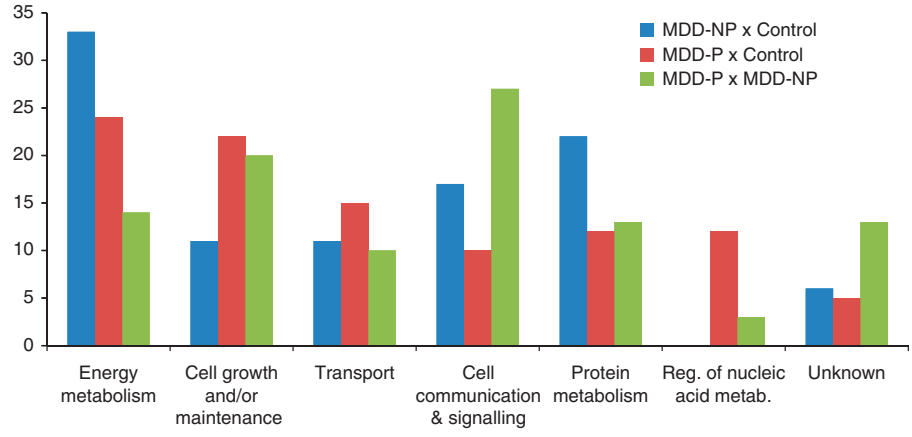

Figure 2 Distinct representation of biological processes in each of the comparisons including MDD-P, MDD-NP and controls. MDD, major depressive disorder.

diagnostic, prognostic and treatment-related biomarkers. GAP43 could not be validated most probably due to the different sensitivity of mass spectrometry and Western blot.[41]

One of the major pathways associated with the DLPFC proteomic differences in MDD patients was related to energy metabolism, consistent with previous imaging findings. Previous studies have shown a reduction in glucose metabolism in brains from MDD patients using a positron emission tomography approach.[42] This is interesting as an increase in glucose metabolism was found in this same brain region of MDD patients after administration of the anti-depressant paroxetine.[43] Such effects on energy are likely to be common in psychiatric disorders,[44] suggesting that these may be nonspecific features of these conditions. However, our data

Table 3 Differentially expressed proteins comparing healthy controls to MDD patients divided in psychotic or non-psychotic patients

Biological processes	*Gene name*	*Protein name*	*FC*	*IP*	P-*value*	q-*value*
(a) MDD-NP vs controls						
Cell growth/maintenance	TUBA4A	Tubulin alpha-4A chain	2.03	2	0.0321	0.3725
	TUBA4B	Putative tubulin-like protein alpha-4B	1.33	6	0.0196	0.3725
Cell communication/signalling	*HINT1*	Histidine triad nucleotide-binding protein 1	1.45	4	0.0063	0.3725
	RHOC	Rho-related GTP-binding protein RhoC	1.36	2	0.0396	0.3725
	SIRPA	Tyrosine protein phosphatase non receptor type substrate 1	1.19	12	0.0293	0.3154
Metabolism/energy	ATP5F1	ATP synthase subunit b mitochondrial	1.24	3	0.0245	0.3154
	ATP5I	ATP synthase subunit e mitochondrial	1.58	3	0.0237	0.3725
	COX7A2	Cytochrome c oxidase polypeptide 7A2 mitochondrial	1.55	2	0.0007	0.3725
	CYCS	Cytochrome c	1.59	13	0.0001	0.3725
	NDUFA2	NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit2	1.60	3	0.0401	0.3725
	NDUFS5	NADH dehydrogenase ubiquinone iron sulfur protein 5	1.67	2	0.0257	0.3725
Protein metabolism	*CST3*	Cystatin C	1.58	3	0.0333	0.3725
	HSP90AA1	Heat-shock protein HSP 90-alpha	1.22	5	0.0327	0.3725
	PPIA	Peptidyl-prolyl *cis-trans* isomerase A	1.37	3	0.0265	0.3725
	SNCG	Gamma synuclein	1.38	11	0.0215	0.3725
Transport	*FABP3*	Fatty acid-binding protein heart	1.45	6	0.0028	0.3725
	FXYD6	FXYD domain-containing ion transport regulator 6	1.77	4	0.0039	0.3725
Unknown	*CISD1*	CDGSH iron sulfur domain-containing protein 1	1.64	8	0.0134	0.3725
(b) MDD-P vs controls						
Metabolism/energy	TXN	Thioredoxin	−1.33	3	0.0005	0.2854
	MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	−1.45	2	0.0448	0.3654
	ACYP2	Acylphosphatase 2	−1.47	3	0.0344	0.2854
	COX5B	Cytochrome c oxidase subunit 5B mitochondrial	−1.56	7	0.0453	0.2854
	PRDX6	Peroxiredoxin-6, mitochondrial	−1.60	5	0.0169	0.2854
	NDUFA13	NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 13	−1.61	7	0.0477	0.2854
	NDUFA6	NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 6	−1.71	5	0.0022	0.2854
	ACOT13	Acyl coenzyme A thioesterase 13 Short Acyl CoA thioesterase 13	−1.88	2	0.0207	0.2854
	ATP6V1F	V type proton ATPase subunit F Short V ATPase subunit F	−2.26	2	0.0492	0.2854
Cell growth/maintenance	**TUBB2A**	Tubulin beta-2A chain	1.58	2	0.0428	0.2854
	TUBB6	Tubulin beta-6 chain	−1.24	5	0.0179	0.3654
	NEFM	Neurofilament medium polypeptide	−1.31	143	0.0079	0.1236
	PFN2	Profilin 2	−1.58	7	0.0209	0.2854
	SEPT2	Septin 2	1.25	20	0.0016	0.3298
	PFN1	Profilin-1	−1.59	6	0.0239	0.2655
Transport	ATP6V1H	V type proton ATPase subunit H Short V ATPase subunit H	−1.15	24	0.0052	0.3654
	SNAP25	Synaptosomal-associated protein 25	1.17	27	0.0048	0.2655
	FABP7	Fatty acid-binding protein brain	−1.47	4	0.0393	0.2854
	GABARAPL2	Gamma aminobutyric acid receptor-associated protein-like 2	−1.72	3	0.0275	0.2854
	FABP3	Fatty acid-binding protein heart	−1.85	6	0.0371	0.2854
Cell communication/signalling	*AMPH*	Amphiphysin	−1.16	40	0.0068	0.3024
	FKBP1A	Peptidyl prolyl *cis-trans* isomerise	−1.51	2	0.0230	0.4132
	RHOC	Rho-related GTP-binding protein RhoC	−1.63	2	0.0413	0.2854
	CALM1	Calmodulin	−1.66	2	0.0275	0.2854
Protein metabolism	**PPIA**	Peptidyl-prolyl *cis-trans* isomerase A	1.29	11	0.0123	0.3298
	SNCG	Gamma synuclein	−1.37	11	0.0107	0.2655
	SNCA	Alpha synuclein	−1.58	5	0.0235	0.2854
	CSTB	Cystatin-B	−1.70	2	0.0474	0.4132
	CST3	Cystatin C	−1.81	3	0.0357	0.2655
Regulation of nucleic acid metabolism	PBXIP1	Pre B-cell leukemia transcription factor-interacting protein 1	−1.31	2	0.0123	0.3298
	SATB2	DNA-binding protein SATB2	−1.64	3	0.0018	0.2854
	HIST1H2BL	Histone H2B type 1-L	−1.85	2	0.0412	0.4132

Table 3 *(Continued)*

Biological processes	*Gene name*	*Protein name*	*FC*	*IP*	P-*value*	q-*value*
	SSBP1	Single-stranded DNA-binding protein mitochondrial	−1.99	2	0.0269	0.4132
	HIST1H4A	Histone H4	−2.11	4	0.0314	0.2854
Unknown	FANCI	Fanconi anemia group I protein	−1.42	2	0.0314	0.3298
	SVIP	Small VCP p97-interacting protein	−1.43	2	0.0121	0.4132
(c) MDD-P vs MDD-NP						
Cell communication/signalling	ARF1	ADP-ribosylation factor 1	1.37	8	0.0119	0.3001
	ARF3	ADP-ribosylation factor 3	1.69	8	0.0163	0.3001
	EEF1AL3	Putative elongation factor 1-alpha-like 3	−1.16	11	0.0031	0.3612
	FKBP1A	Peptidyl prolyl *cis-trans* isomerase	−1.45	2	0.0414	0.4091
	PRKCA	Protein kinase C alpha type	1.32	2	0.0101	0.3579
	SIRPA	Tyrosine protein phosphatase non receptor type substrate 1	1.24	12	0.0135	0.3248
	STIP1	Stress-induced phosphoprotein 1	1.58	2	0.0252	0.3579
	SYT2	Synaptotagmin 2	2.10	4	0.0113	0.3579
Cell growth/maintenance	**SEPT2**	Septin 2	1.33	20	0.0417	0.2311
	TUBA1B	Tubulin alpha-1B chain	1.15	17	0.0314	0.3001
	TUBA3E	Tubulin alpha-3E chain	1.15	22	0.0026	0.2311
	TUBA8	Tubulin alpha-8 chain	2.57	3	0.0389	0.4091
	TUBB2A	Tubulin beta-2A chain	1.93	2	0.0366	0.3001
	TUBB8	Tubulin beta-8 chain B	1.30	2	0.0291	0.3579
Metabolism/energy	SDHA	Succinate dehydrogenase, mitochondrial	1.68	3	0.0095	0.3579
	DLAT	Dihydrolipoyllysine acetyltransferase	1.39	14	0.0057	0.3248
	TXN	Thioredoxin	−1.79	3	0.0416	0.4091
	ATP6V1A	V-type proton ATPase catalytic subunit A	1.24	17	0.0057	0.3248
Protein metabolism	**CALR**	Calreticulin	−1.26	2	0.0314	0.3612
	SERPINB1	Leukocyte elastase inhibitor	−1.42	3	0.0222	0.3579
	PPIA	Peptidyl-prolyl *cis-trans* isomerase A	1.36	11	0.0291	0.3001
	UBE2V2	Ubiquitin-conjugating enzyme E2 variant 2	−2.57	2	0.0155	0.4091
Transport	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	1.83	18	0.0086	0.3248
	GOSR1	Golgi SNAP receptor complex member 1	1.36	2	0.0321	0.3579
	STXBP1	Syntaxin-binding protein 1	1.44	112	0.0002	0.1214
Regulation of nucleic acid metabolism	PAPOLA	Poly A polymerase alpha	−1.32	2	0.0399	0.3579
Unknown	FMNL2	Formin-like protein 2	1.83	2	0.0185	0.3579
	NCDN	Neurochondrin	1.25	20	0.0072	0.3248
	SAMD9	Sterile alpha motif domain-containing protein 9	−1.42	4	0.0241	0.3579
	SASS6	Spindle assembly abnormal protein 6 homolog	1.60	2	0.0467	0.4091

Abbreviations: Acc No, SwissProt accession number; FC, fold change; IP, number of identified peptides; MDD, major depressive disorder.
In gene name, italic/underline means the proteins that were also found differentially expressed comparing all MDD patients with all controls of this study as shown in Table 1. In bold, are proteins previously found in proteome analyses of schizophrenia. *P*-values for Wilcoxon test.

suggest some specificity in energy metabolism dysfunction at the protein level. Although in schizophrenia the main affected metabolic pathway appears to be glycolysis,[38] the most affected pathway in MDD appeared to be oxidative phosphorylation. However, it should be noted that some components of the oxidative phosphorylation pathway are also affected in schizophrenia,[45,46] but not to the same extent as glycolysis. We found that 20 different subunits of the oxidative phosphorylation complexes showed increased levels in MDD brains (Supplementary Material 6). Interestingly, oxidative phosphorylation proteins are mostly decreased in schizophrenia.[37] Considering that the main product of oxidative phosphorylation is ATP, we measured ATP concentrations in MDD DLPFC samples and verified that there were significantly lower levels in comparison with those found in control subjects (Figure 4). One possible explanation for this is that ATP may be depleted in this brain area of MDD subjects due to the presence of oxidative stress[47] and the oxidative phosphorylation complexes may be over-expressed to compensate for this effect.

Our findings also support an impairment of synaptic function in MDD, especially for components of soluble N-ethylmaleimidesensitive (NSF) sensitive factor attachment protein receptor (SNARE) function. For example, we identified and validated changes in SNAP25, which is a pre-synaptic membrane protein involved in anchoring synaptic vesicles in the SNARE complex. We also found changes in the GABARAPL2, a cytosolic regulator of SNAREs involved in stimulation of the ATPase activity of NSF that signals disassembly and recycling of SNARE complexes.[48] Syntaxin 1B (STX1B), which was found significantly upregulated ($P=0.0291$, fold change 1.07), but discarded from our list

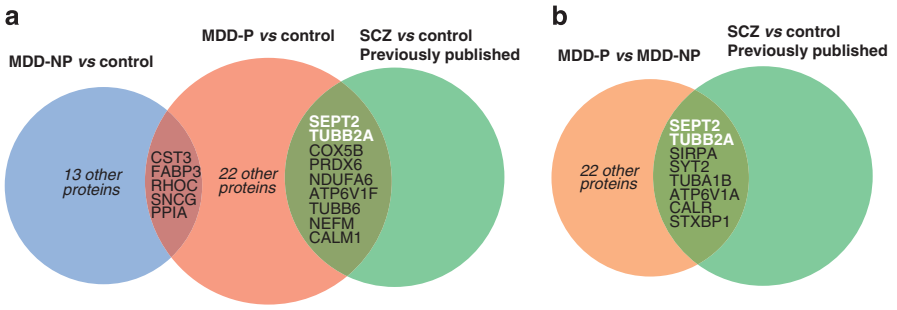

Figure 3 Venn diagrams representing (**a**) overlaps of differentially expressed proteins in MDD with and without psychosis versus controls; (**b**) overlaps of differentially expressed proteins between MDD with psychosis versus MDD without psychosis. The overlaps of differentially expressed proteins with previous analyses of schizophrenia DLPFC tissue are also indicated. DLPFC, dorsolateral prefrontal cortex; MDD, major depressive disorder.

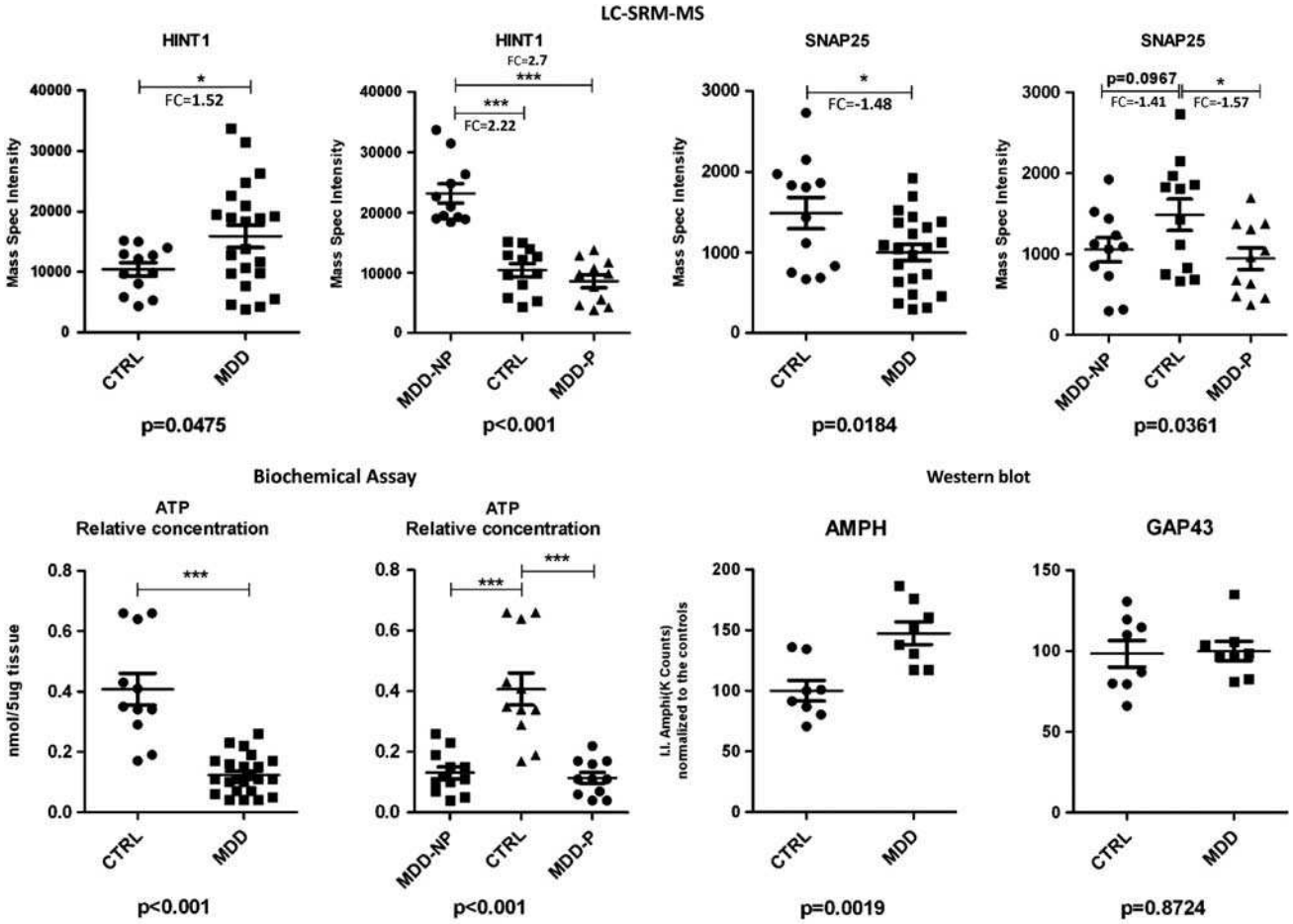

Figure 4 Validation of differentially expressed proteins and metabolites in the DLPFC from MDD patients and controls using different techniques as described. *P* values were obtained by Student's *t*-test statistical analysis. Differences with $P<0.05$ were considered significant. DLPFC, dorsolateral prefrontal cortex; FC, fold change; MDD, major depressive disorder.

according to our fold change cutoff, is a cellular receptor for transport vesicles (Figure 1), which inhibits SNARE function[49,50] and thereby controls neurotransmitter release.[51] STX1B has a role in the calcium-dependent synaptic transmission[52] and has also been found to be increased in schizophrenia.[53] Changes were also found and validated for AMPH, which is present on the cytoplasmic surface of synaptic vesicles.[54] In addition, we found alterations in alpha-synuclein (SNCA) and SNCG, which are involved in integration of presynaptic signaling and membrane trafficking.

Altered levels of these proteins has been associated previously with Parkinson's and Alzheimer's diseases.[55]

Dysregulation of phospholipids and fatty acids, which are critical components of synaptic vesicle membranes, has also been linked to depression.[56] Our proteomic findings identified changes in expression of arachidonic acid and phospholipase D2 (PLD2), which are components in membrane structure and function. Antidepressant treatment reduces arachidonic acid turnover,[57] and the release of this molecule is decreased when PLD2 is inactivated.[58] In this regard, we found that the levels of SNCA were increased, which inhibits PLD2. Moreover, PLD2 is inhibited by AMPH,[59] which was found to be increased. PLD2 interacts directly with protein kinase C (PKC). PKC is a component of intracellular signal transduction and its activity has been shown to be reduced in depression.[60] PKC is also activated in the cytoplasm by diacylglycerol, which is anchored in the membrane, facilitating PKC translocation from the cytosol to membranes. Diacylglycerol and inositol trisphosphate are hydrolytic products of membrane phospholipid phosphatidyl inositol-bisphosphate resulting from activity of the enzyme phospholipase C. In this study, we found changes in several proteins in MDD brains, which are involved in PKC regulation, including PFN1, PFN2 and actin-binding proteins[61] (Figure 1).

We found that the levels of HINT1 were increased in MDD patients, which was confirmed by SRM mass spectrometry analysis. Interestingly, the levels of this protein were found to be deceased in the DLPFC in previous studies of schizophrenia.[62] HINT1 was originally described as a PKC inhibitor, although its precise function has yet to be confirmed. It is widely expressed in a number of tissues including liver, kidney and brain, where it has been associated with control of transcriptional processes along with tumor suppression and susceptibility.[63] A potential role of HINT1 in MDD and anxiety disorders was described recently from studies of Hint1 knockout (KO) mice. The absence of HINT1 expression resulted in altered postsynaptic dopamine transmission in striatum and nucleus accumbens as well as an elevation of circulating corticosterone levels, suggesting effects on the hypothalamic-pituitary-adrenal axis. In addition, anxiety-like behavior has been observed in this model.[39,40,64] Our findings not only support those of previous studies, which suggested that HINT1 may be a novel biomarker for MDD, but suggest that this could be specific for subjects without associated psychosis. This was shown by the fact that this protein was only increased in those MDD subjects without psychosis.

It has been known for decades that most neuropsychiatric disorders have a significant overlap of symptoms. It is likely that this will be reflected by an overlap of molecules and pathways in affected tissues such as the brain and only subtle differences are likely to be responsible for differentiating one disorder from another. For example, psychotic symptoms, which are a hallmark for schizophrenia, may occur in the course of MDD, although in a slightly different manner. Even so, the current findings suggest that there may be common molecular changes in MDD-P and patients with schizophrenia. In all, 11 of the 36 differentially expressed proteins in DLPFC of MDD-P patients compared with controls were identified in previous studies of schizophrenia.[38,65,66] This included calmodulin (CALM1), which is involved in synaptic metabolism and calcium homeostasis and regulates the function of D1 and D2 dopamine receptors[67] and the neurofilament medium polypeptide (NEFM), which belongs to the dopamine receptor-interacting protein (DRIP) family of proteins.[67] Another protein that was altered in common between MDD-P and schizophrenia subjects was peroxiredoxin-6 (PRDX6).[66,68] This protein has been associated with schizophrenia susceptibility in Taiwanese families[69] and has a role in phospholipid metabolism with phospholipase A2 (PLA2) activity.

The identification of proteome fingerprints has the potential to lead to the development of more specific classification tests compared with those depending on single molecules. In this case, testing for correlative changes in these protein fingerprints in peripheral tissues such as CSF and even serum might lead to the development of multiplex biomarker assays for psychosis. Moreover, direct comparison of MDD-P and MDD-NP patients resulted in identification of 12 differentially expressed proteins, which have also been found previously in schizophrenia (Table 3c). It was also of interest that comparison of MDD-P patients to controls identified differentially expressed proteins that were associated with energy metabolism, whereas comparison of MDD-NP patients and controls resulted in identification of proteins, which were mostly involved in cell growth and maintenance. This suggested that different mechanisms may be involved in the development of these two forms of MDD. Further experiments in this area should be conducted, which may eventually help with patient stratification for generating more personalized treatments.

Interestingly, 53.7% of the altered proteins in MDD-P overlapped with those in total MDD patients. This suggests that there may be a proteomic signature in MDD that exists independent of the presence of psychosis. Of the proteins unique to the MDD-P patients, two could not be associated with a biological class, although there are some leads about their function. One of these proteins was fanconi anemia group I protein (FANCI) that participates in protein ubiquitination and its differential expression can lead to defective DNA repair.[70] The other was small VCP p97-interacting protein (SVIP), which is an 8.5 κDa membrane protein involved in ubiquitination of endoplasmic reticulum misfolded proteins.[71] Considering the novelty of these proteins in psychiatric studies and their role in ubiquitination, their function could be further investigated in MDD-P.

Curiously, none of the proteins that were found to be differentially expressed between MDD-P and MDD-NP patients were altered in total MDD patients. This suggested that the MDD-P vs MDD-NP signature was more strongly related to psychosis and also more similar to previous findings in schizophrenia brain proteomes. In the MDD-P and MDD-NP comparison, we also identified protein targets with unknown biological function such as formin-like protein 2 (FMNL2), sterile alpha motif domain-containing protein 9 (SAMD9), spindle assembly abnormal protein 6 homolog (SASS6) and neurochondrin (NCDN). NCDN seems to interact with phosphatidic acid, which is a metabolite related to phospholipid metabolism (Figure 1) and SASS6 is likely to be involved in ubiquitination.[72]

One protein changed in common in all comparisons shown in Table 3 was PPIA (also known as cyclophilin A). PPIA

accelerates the folding of proteins that are involved in cyclosporin A-mediated immunosuppression.[73] Interestingly, this protein has also been found to be increased in schizophrenia thalamus,[38] which suggests some relationship with the psychosis status. However, considering the fact that this protein is also differentially expressed in MDD-NP compared with controls, it does not seem to be a specific biomarker candidate to psychosis, but most likely associated to psychiatric conditions. On the other hand, the protein peptidylprolyl *cis-trans* isomerase (FKBP1A), which is from the same family of PPIA, was specifically altered in MDD-P samples. FKBP1A acts on immunoregulation and cellular processes involved in protein folding and trafficking and it also interacts with several intracellular signal transduction proteins.[74] Moreover, FKBP1A binds to the immunosuppressants FK506 and rapamycin. Interestingly, the mammalian target of rapamycin (mTOR) signaling pathway in the prefrontal cortex is compromised in MDD,[75] and mTOR interacts with FKBP1A bound to rapamycin.[76] FKBP1A also interacts with the transforming growth factor (TGF)-beta receptor, which is critical for modulation of GABA synaptic transmission and dendritic homeostasis.[77] Moreover, FMNL2, which we also found increased in MDD-P, is also part of TGF-beta pathway.[78] These findings warrant further studies on the involvement of these proteins in psychiatric disorders.

Figure 2 represents a broader perspective of the unique findings of each of the compared groups. Changes in energy metabolism are notable in both MDD-P and MDD-NP. Although protein metabolism processes are more related to MDD-NP, changes in cell growth/maintenance, transport and regulation of nucleic acids are more related to MDD-P. When the two groups of MDD patients are compared, defects in cell signaling are pronounced so as are proteins with unknown biological processes. This last group of proteins should be further studied for providing more leads about the stratification of different MDD subtypes.

It is widely known that factors such as age, gender, postmortem interval, drug treatment and others may have confounding effects on global proteomic studies involving *post-mortem* samples.[79] None of the factors considered here seem to have had a significant effect on the analyses. Compared groups are matched for demographic variables and these have not shown significant differences (Table 1). In addition, no unusual segregation of subjects using principle component analyses has been observed (Supplementary material 3). However, it should be noted that information was not available regarding the number of patients who were relapsed or who were on or off medication at the time of death. Although some effects on proteomic changes were observed when comparing MDD patients that committed suicide ($n=17$) with non-suicide MDD patients ($n=6$), we could not explore this any further due to the low numbers of subjects who did not die from suicide. Moreover, in the non-suicide group, some of these subjects actually attempted suicide although they were not successful.

The static nature of *post-mortem* brain tissue and limited sample sizes can be a drawback in studies such as the one presented here. Therefore, a replication of this study in an independent sample would be essential. However, we concur with a recent report that the analysis of *post-mortem* tissue from patients in brain disorders is indispensable, especially considering that it has generated important and unique insights for psychiatric studies.[80]

Our systems biology analyses of the MDD brain proteome showed that some of the differentially expressed proteins found in subjects with MDD have been associated previously with other diseases such as Huntington's and Alzheimer's disease and schizophrenia. This supports the notion that neurological and psychiatric disorders may share common pathways at the molecular level. Therefore, identification of multiple components of these conditions may be required in order to identify unique biomarker fingerprints. This may require identification of differences in the expression of several genes and proteins, together with consideration of potential environmental factors. Nevertheless, we and others have identified peculiarities for different diseases, as seen by differential expression of specific proteins. In fact, we showed that the changes in HINT1 expression were specific for MDD-NP in the DLPFC. Moreover, there appears to be distinct energy metabolism signatures for MDD and schizophrenia, with MDD affected more by changes in oxidative phosphorylation and schizophrenia through glycolysis-related pathways.

The current findings support the known impairment on synaptic transmission and especially on SNARE-related proteins in MDD. We also attempted to identify biomarker-specific signatures for subgroups of MDD through studies on depression associated with psychosis and even with suicide. Interestingly, we found a significant overlap of differentially expressed proteins in *post-mortem* brain tissue from MDD subjects with psychosis, with that of schizophrenia subjects.

These findings suggest that it may be possible to distinguish different subtypes of MDD patients based on differences in brain proteomic profiles. Translating such findings to the periphery might lead to novel personalized medicine strategies based on patient stratification according to molecular profiles. Also, the identification of new models for MDD based on brain and serum molecular profiles could lead to the development of such models for use in drug discovery. This could lead to more targeted treatment approaches and reduce the rate of drug attrition within the field of neuropsychiatric disorders.

Conflict of interest

PCG, HR and SB are consultants for Psynova Neurotech Ltd.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)