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Research Article

RESEARCH ARTICLE

Proteomic Enrichment Analysis of Psychotic and Affective Disorders Reveals Common Signatures in Presynaptic Glutamatergic Signaling and Energy Metabolism

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Abstract

Background: Although genetic studies suggest an overlap in risk alleles across the major psychiatric disorders, disease signatures reflecting overlapping symptoms have not been found. Profiling studies have identified candidate protein markers associated with specific disorders of the psychoaffective spectrum, but this has always been done in a selective fashion without accounting for the entire proteome composition of the system under investigation.
Methods: Employing an orthogonal system-based proteomic enrichment approach based on label-free liquid chromatography mass spectrometry, we analyzed anterior prefrontal human post-mortem brain tissue of patients affected by schizophrenia (n = 23), bipolar disorder (n = 23), major depressive disorder with (n = 12) and without psychotic features (n = 11), and healthy controls (n = 23). Labeled selected reaction monitoring (SRM) was used to validate these findings on a pathway level. Independent in silico analyses of biological annotations revealed common pathways across the diseases, associated with presynaptic glutamatergic neurotransmission and energy metabolism. We validated the proteomic findings using SRM and confirmed that there were no effects of post-mortem confounders.
Results: Schizophrenia and affective psychosis were linked to a hypoglutamatergic state and hypofunction of energy metabolism, while bipolar disorder and major depressive disorder were linked to a hyperglutamatergic state and hyperfunction of energy metabolism.
Conclusions: These findings support recent investigations, which have focused on the therapeutic potential of glutamatergic modulation in psychotic and affective disorders. We suggest a disease model in which disturbances of the glutamatergic system and ensuing adaptations of neuronal energy metabolism are linked to distinct psychiatric symptom dimensions, delivering novel evidence for targeted treatment approaches.

Keywords: glutamate, glycolysis, proteomics, psychosis, systems biology

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Introduction

The etiology of the major psychiatric disorders still remains elusive. At the forefront of psychiatric research have been extensive genetic studies (Levinson, 2006; Owen et al., 2010; Craddock and Sklar, 2013), which have often generated contradictory findings. Consequently, this has impeded the development of a coherent disease model. Currently, schizophrenia (SZ), bipolar disorder (BD), and major depressive disorder are defined by symptom patterns and, to a lesser degree, disease course. However, some core symptoms such as psychosis, affective disturbances, and cognitive dysfunctions overlap between disorder categories. The question arises whether there are proteomic features shared between these complex disorders and if they converge onto the same pathways.

The largest genome-wide association (GWA) study of the major psychiatric disorders to date has identified a number of shared risk loci, mainly located within genes associated with calcium channel signaling (Smoller et al., 2013). However, it remains to be determined how genetic changes affect the expression and function of the encoded proteins and the biological system in question (Smoller et al., 2013).

Genetic analyses of patients presenting with either psychotic or mood disorders have suggested a greater overlap of risk loci between SZ and BD compared to BD and depression (Purcell et al., 2009; Williams et al., 2011; Smoller et al., 2013). However, twin and GWA studies imply shared susceptibility factors for BD and depression (Green et al., 2010; McMahon et al., 2010). These observations have promoted a dimensional understanding of psychotic, manic, and depressive symptoms (van Os and Kapur, 2009). Familial co-segregation of schizoaffective disorder, SZ, BD, and major depressive disorder (Tsuang and Faraone, 1990; Craddock et al., 2009; Lichtenstein et al., 2009), and shared susceptibility markers across diagnostic hierarchy boundaries (Cardno et al., 2002) argue for a critical review of the current psychiatric nosology. Given the polygenic etiology of the major psychiatric disorders, we investigated whether there are common changes in biological pathways and whether diagnoses with overlapping symptom dimensions also share similar pathophysiologies, employing an orthogonal system-based proteomic approach.

As the proteome represents the transcribed and translated genetic information following modifications at the epigenetic and mRNA levels, it has been suggested that proteomics is closer to reflecting the pathophysiological disease end point than genomics and transcriptomics (Bayes and Grant, 2009). However, the focus of molecular psychiatry research has predominantly remained on single disorders and sets of individual proteins rather than multiple disorders and protein networks. Such an approach runs the risk of missing effects at the system level. Findings in psychiatric proteomics have mostly been analyzed on the basis of an individual protein being involved in a given pathway (English et al., 2011), rather than testing for significant overrepresentation of dysregulated proteins sharing common pathways within the detected proteome (Wesseling et al., 2014).

Thus, in order to investigate psychotic and affective dimensions, we conducted a non-hypothesis–driven proteomic screen in post-mortem brain tissue (Brodman Area 10 [BA10]) from a total of 23 SZ, 23 BD, and 23 major depressive disorder patients and 23 controls. Major depressive disorder patients were further divided into subgroups with and without psychosis. Previous work has implicated the anterior prefrontal cortex in the integration and coordination of information processing and transfer in multi-operational cognitive tasks (Ramnani and Owen, 2004). This mediator position made BA10 a primary target in affective and psychotic disorders characterized by impairments in executive functioning and socio-emotional cognition.

The only study investigating BA10 protein changes in neuropsychiatric disorders used a conventional 2D-gel based technique (Johnston-Wilson et al., 2000). However, due to some limitations (Lilley, 2003), this method was superseded by highly sensitive mass spectrometry methods (e.g. liquid chromatography coupled tandem mass spectrometry [LC-MS E]) allowing quantitative protein profiling (Bayes and Grant, 2009). To overcome the bottleneck of validation in such screening studies, recent advances in mass spectrometry have led to the development of selected reaction monitoring (SRM) as a targeted proteomic approach to quantify levels of putative biomarkers—without the need of antibody-based methods like ELISAs and immunoblotting—in a highly accurate and quantitative manner (Picotti and Aebersold, 2012). To our knowledge, this is the first proteomic investigation which has attempted to integrate the spectrum of major psychiatric disorders in one single study, comprising 92 well-matched patient and control samples analyzed by label-free LC-MS^E proteomic profiling, combined with protein-set enrichment analysis and SRM validation.

Methods

Clinical Samples

A total of 92 post-mortem anterior prefrontal cortex (BA10) brain samples were obtained from the Stanley Medical Research Institute (Torrey et al., 2000). Samples were derived from 23 SZ patients, 23 BD patients, 12 major depressive disorder patients without psychotic symptoms (MDD), 11 major depressive disorder patients with psychotic symptoms (MDD-P), and 23 control (CT) subjects. Tissue was collected post-mortem from patients and controls with full informed consent obtained from a first-degree relative in compliance with the Declaration of Helsinki; consent was obtained by questionnaires conducted over the phone and signed by two witnesses. The patient samples were matched with control samples based upon age at death, post-mortem interval, and brain pH (t-test). There were no significant differences in brain side, gender or secondary axis diagnosis of alcohol abuse/dependency and drug abuse/dependency between patients and controls (Fisher's exact test). Samples contained equal amounts of grey and white matter, as determined by neuroanatomical analyses. A summary of the demographic details and p values is shown in Supplementary Table S1. Additional information is provided in Supplementary Table S2. Tissues were sectioned (25 µm) using a Leica Cryostat (Milton Keynes) and stored at -80 C until use.

Sample Preparation

Approximately 50 mg of tissue slices per sample were used in this study. Total lysis sample preparation was performed as previously described (Ernst et al., 2012). Briefly, tissue samples were added to a fractionation buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ASB14, and 70 mM DTT at a 5:1 (v/w) ratio. After sonication and vortexing for 30 min, lysates were centrifuged for 1 min at 5000 rpm to remove residual cell debris and protein concentrations of the supernatant were determined using a Bradford assay (Bio-Rad). Based on the lysates, two randomized, blinded, independent sample preparations (for all 92 samples) were performed for LC-MS^E and SRM to avoid bias in sample preparations.

Protein (approx. 100 µg) was precipitated using acetone. For quality control purposes, a pool of all lysates was split into ten aliquots and prepared in parallel with the samples. After dissolving the precipitate in 50 mM ammonium bicarbonate, protein concentrations were determined in quadruplicate. Reduction of protein sulfhydryl groups was performed with 5 mM DTT at 60°C for 30 min and alkylation was carried out using 10 mM iodacetamide and incubating in the dark at 37°C for 30 min. Digestion of the proteins was conducted using trypsin at a 1:50 (w/w) ratio for 17 h at 37°C, and reactions were stopped by addition of 8.8 M HCl in a 1:60 (v/v) ratio. All sample aliquots were stored at -80°C until analysis.

Label-Free LC–MS^E Analysis of Brain Tissue

Brain tissue samples were analyzed individually in technical duplicates using splitless nano-ultra-performance liquid chromatography (10 kpsi nanoAcquity; Waters Corporation), coupled online through a New Objective nanoESI emitter (7 cm length, 10-mm tip; New Objective) to a Waters Q-TOF Premier mass spectrometer. Data were acquired in expression mode (MS^E) and the total continuous run time was 31 days. The procedure, quality assessment and data processing were performed as described previously (Ernst et al., 2012). Data were acquired in MS^E positive V mode. The cycle time per scan was 1.25 s. During low-energy scans, the collision energy was set to 5eV and during high-energy scans this was ramped from 17eV to 40eV. LC-MS^E data were processed using the ProteinLynx Global Server v.2.5. (Waters Corporation), and Rosetta Elucidator v.3.3 (Rosetta Biosoftware) was used for time and mass/charge alignment of mass spectrometry data. The Swiss-Prot human reference proteome (Uniprot release, March 2013; 20 252 entries) was used for protein identification searches. To control the false discovery rate (FDR), data were searched against a decoy database, which was the randomized version of the database mentioned above to conserve amino acid frequencies. The FDR was set at the default maximum rate of 4%, as applied before (Lu et al., 2007; Krishnamurthy et al., 2011; Ralhan et al., 2011; Yang et al., 2011). The search parameters were: (1) enzyme = trypsin, (2) fixed modification = carbamidomethylation of cysteines, (3) variable modifications = oxidation of methionine and phosphorylation at serine, threonine, or tyrosine residues, (4) initial mass accuracy tolerances = 10 ppm for precursor ions and 20 ppm for product ions, and (5) one missed cleavage allowed. In addition, the following criteria were used for protein identification: (1) ≥ 3 fragment ions per peptide, (2) ≥ 7 fragment ions per protein, and (3) ≥ 1 peptide per protein. Raw data and ProteinLynx Global Server search results were imported into the Rosetta Elucidator software (build 3.3.0.1.SP3.19, Rosetta Biosoftware). Elucidator performed the retention time alignment and feature identification and extraction for all samples using the Rosetta PeakTeller algorithm. Dynamic background subtraction, smoothing in retention time and m/z dimensions and isotopic regions creation for peak-matching across all runs were calculated using an retention time correction of 4 min at the maximum. A single data file was randomly chosen as the master, and all other sample files were aligned to the master in form of a dynamic retention time shift. This procedure allowed the improved identification of peptides and proteins in each sample by taking the available data of all samples into account. Features were normalized based on total ion current to account for potential differences in protein loading and peptide ionization. Only features detected in both replicates and in >80% of samples were included in further analyses. Feature intensities were summed to generate peptide intensities, which were used for statistical analysis. Protein abundance changes for the five comparisons (SZ/CT, BD/CT, MDD/CT, MDD-P/CT, MDD-P/MDD) were determined using the MSstats package (Clough et al., 2012) based on linear mixed-effects models under defaults settings, following $\log_2$ transformation, and exclusion of intensity values deviating more than three standard deviations from the mean of each group. The p values were adjusted to control the FDR at a cut-off of 0.05 following the Benjamini Hochberg procedure (Chang et al., 2012).

Protein Set Enrichment Analysis

Protein set enrichment analysis was performed as described previously (Pan et al., 2009; Zhang et al., 2011). Briefly, for each disorder comparison, significantly-changed proteins were partitioned into six bins, according to fold change (FC): FC < 1.0; FC < -1.1; 1.1 < FC <1; 1.0 < FC < 1.1; FC > 1.1; FC > 1.0. The R-package database org.Hs.eg.db version 2.8.0 (17 959 entries) was used for gene ontology (GO) term annotation based on Entrez gene identifiers. Significant overrepresentation of an annotated GO term per bin was determined by the GOstats package (Falcon and Gentleman, 2007). For each bin, p values for the GO categories (Ashburner et al., 2000) of biological pathway (BP) and cellular compartment (CC) were calculated by a conditional hypergeometric test using the entire detected proteome as a background. These tests accounted for the hierarchical structure of the GO terms by first testing the child terms of any given GO category and filtering significantly-enriched proteins prior to analysis of the parents terms, as described previously (Alexa et al., 2006). This prevented the identification of directly-related GO terms with a considerable overlap of assigned proteins. GO terms with no significant enrichment in any bin ($p > 0.05$) and GO terms with less than two annotated proteins were removed. The remaining p values greater than 0.05 were replaced by a conservative p value of 1. P values were replaced by their negative logarithm to the base of ten and then converted to z-scores within their proteomic comparison for every remaining GO term. Finally, all significantly-enriched GO term associations were aligned across all five proteomic comparisons and arranged by one-way hierarchical clustering using Euclidean distance as distance function and the Average Linkage Clustering method available in the software Genesis (Sturn et al., 2002). The same enrichment analysis was repeated using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation (5 871 entries) in order to provide an independent *in silico* validation of our findings. Only significantly-enriched terms appearing in at least 3 disorder comparisons were considered to represent overlapping features across the psychoaffective spectrum (further data available upon request).

SRM Mass Spectrometry

Level alterations of proteins belonging to candidate pathways identified by label-free LC-MS^E profiling were validated using targeted SRM mass spectrometry on a Xevo TQ-S mass spectrometer (Waters Corporation) coupled online through a New Objective nanoESI emitter (7 cm length, 10 mm tip; New Objective) to a nanoAcquity nano-ultra-performance liquid chromatography system (Waters Corporation). The system was comprised of a C18 trapping column (180 um x 20 mm, 5 µm particle size) and a C18 BEH nano-column (75 um x 200 mm, 1.7 mm particle size). The separation buffers were 0.1% formic acid and 0.1% formic acid in acetonitrile. For separation of peptides, the following 48 min gradient was applied: 97/3% (A/B) to 60/40% in 30 min; 60/40% to 15/85% in 2 min; 5 min at 15/85%; returning to

the initial condition in 1 min. The flow rate was 0.3 µL/min with column temperature at 35°C.

SRM assays were developed following a general high-throughput strategy. Initially, up to 12 unique peptides ranging from 6 to 20 amino acids in length, containing tryptic ends and no missed cleavages, were chosen for the selected proteins. All peptides containing amino acids prone to modifications (e.g., Met, Trp, Asn, and Gln), potential ragged ends, or lysine/arginine followed by proline or bearing the NXT/NXS glycosylation motif were generally avoided and only selected when no other options were available (Lange et al., 2008). Peptides were checked by Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches to ensure uniqueness. For method refinement, up to 12 transitions per peptide were tested in SRM mode. Transitions were calculated using Skyline version 1.2.0.3425 (MacLean et al., 2010) and corresponded to singly charged y-ions from doubly- or triply-charged precursor ions in the range of 350–1250 Da. Transitions were selected based on prediction, discovery proteomics data, and spectral data availability through the Human NIST spectral libraries (Farrah et al., 2011). Method refinement was performed on quality control samples. For the final SRM assays, the 2–3 peptides with the maximal intensities and highest spectral library similarity (dotp > 0.9) were selected. A further development step of analyzing heavy-label spiked QC samples in scheduled SRM mode was used to confirm identity via co-elution, extract the optimal fragment ions for SRM analysis, obtain accurate peptide retention times, and optimize collision energy and cone voltage for the quantification run applying Skyline software (MacCoss Lab Software; MacLean et al., 2010). Heavy-labeled forms of these selected peptides (spiketides L) were chemically synthesized via SPOT synthesis (JPT Peptide Technologies GmbH). The final transitions, collision energy, and retention time windows used for each peptide can be found in the Supplementary Materials (Supplementary Table S3A).

Quantitative SRM measurements comparing patients and controls were performed in scheduled SRM acquisition mode, using the optimized parameters defined during the assay refinement. For each target peptide, a heavy isotope-labeled internal standard (JPT Peptide Technologies GmbH) was spiked in the peptide mixture for accurate quantification and identification. All SRM functions had a 2 min window of the predicted retention time and scan times were 20 ms, which ensured a dwell time of over 5 ms per transition. For each peptide, at least three transitions were monitored for the heavy and light versions. Samples were randomized and run in blocked triplicates (Oberg and Vitek, 2009). Blanks and quality control peptide injections (alcohol dehydrogenase; Supplementary Table S3B) were run alternately after every biological replicate. Resulting SRM data were analyzed using Skyline and protein significance analysis was performed using SRMstats (Chang et al., 2012). For the first stage, data pre-processing was performed by transforming all transition intensities into $\log_2$-values. Then a constant normalization was conducted based on reference transitions for all proteins, which equalized the median peak intensities of reference transitions from all proteins across all runs and adjusted the bias to both reference and endogenous signals. Protein-level quantification and testing for differential abundance among patient and control groups were performed using the linear mixed-effects model implemented in SRMstats. Each protein was tested for abundance differences between patients and healthy controls. The p values were adjusted to control the FDR at a cut-off of 0.05 following the Benjamini Hochberg procedure (Chang et al., 2012).

Results

Label-Free LC-MS^E Proteomic Analysis of Post-Mortem Anterior Prefrontal Cortex (BA10)

Proteomic analysis identified a total of 1 310 unique quantifiable proteins, based on 22 629 unique peptides, across all brain samples. Based on linear modelling, we found that 241 proteins (54 proteins ≥ 2 unique peptides) were differentially expressed in SZ patients, 664 proteins (368 proteins ≥ 2 unique peptides) in BD, 524 proteins (274 proteins ≥ 2 unique peptides) in MDD, and 224 proteins (44 proteins ≥ 2 unique peptides) in MDD-P, in comparison to CT individuals. In order to identify characteristic changes associated with psychotic features in affective disorders, we compared MDD-P to MDD and found 437 proteins (193 proteins ≥ 2 unique peptides) significantly differentially expressed. Characteristic expression profiles for each comparison are shown in Figure 1A–E. Based on similarities in the fold change direction (e.g. BD and MDD showed a tendency for positive fold changes) and to identify shared significant protein alteration patterns across disease entities, we cross-compared the findings using linear correlation (Figure 1F; correlation plots can be found in Supplementary Figure S1A–J). This enabled us to detect disease-specific and overlapping disease signatures at the protein level (Supplementary Table S4; Supplementary Figure S2). As expected, protein changes reflecting affective psychosis (MDD-P/MDD) overlapped with markers of MDD with psychotic features (MDD-P/CT), although no significant correlation between MDD-P/CT and MDD/CT was found. Proteins significantly changed in the MDD-P/MDD comparison showed opposite directional change in BD/CT, MDD/CT and SZ/CT. BD/CT and SZ/CT did not show significantly overlapping protein changes, yet biomarker patterns for both disorders were positively correlated to changes identified in MDD/CT (Figure 1F).

Functional Annotation Enrichment Analysis and SRM Pathway Validation

In an attempt to investigate functional implications of the identified overlapping changes in expression patterns, we performed protein-set enrichment analysis based on GO terms for each disorder, using the total of significantly changed proteins regardless of the magnitude of change. We assumed that similar pathways can be affected by distinct underlying proteomic signatures and that slight variation in the expression of multiple proteins may result in significant pathway alterations. Based on the fold-change distribution of the significant protein-abundance changes, we analyzed six different bins for overrepresentation of GO terms reflecting BP and CC, as determined by hypergeometric testing (Figure 2). The highest annotation overlap of biological pathways was found between the comparisons of BD/CT, MDD/CT, and MDD-P/MDD. In the cellular compartment analysis the highest annotation overlap was found between SZ/CT, BD/CT, and MDD-P/MDD. In order to independently validate our *in silico* findings, we performed the same analysis with KEGG pathway annotation. Two major points of overlap were identified between both analyses. Firstly, annotations related to glutamate, represented in the GO BP analysis by glutamate catabolism and glutamate secretion, in the GO CC analysis by clathrin-sculpted glutamate transport vesicle membrane, and in the KEGG pathway analysis by alanine, aspartate, and glutamate metabolism. Secondly, annotations related to energy metabolism, represented in the GO BP analysis by respiratory electron transport chain, gluconeogenesis, carbohydrate

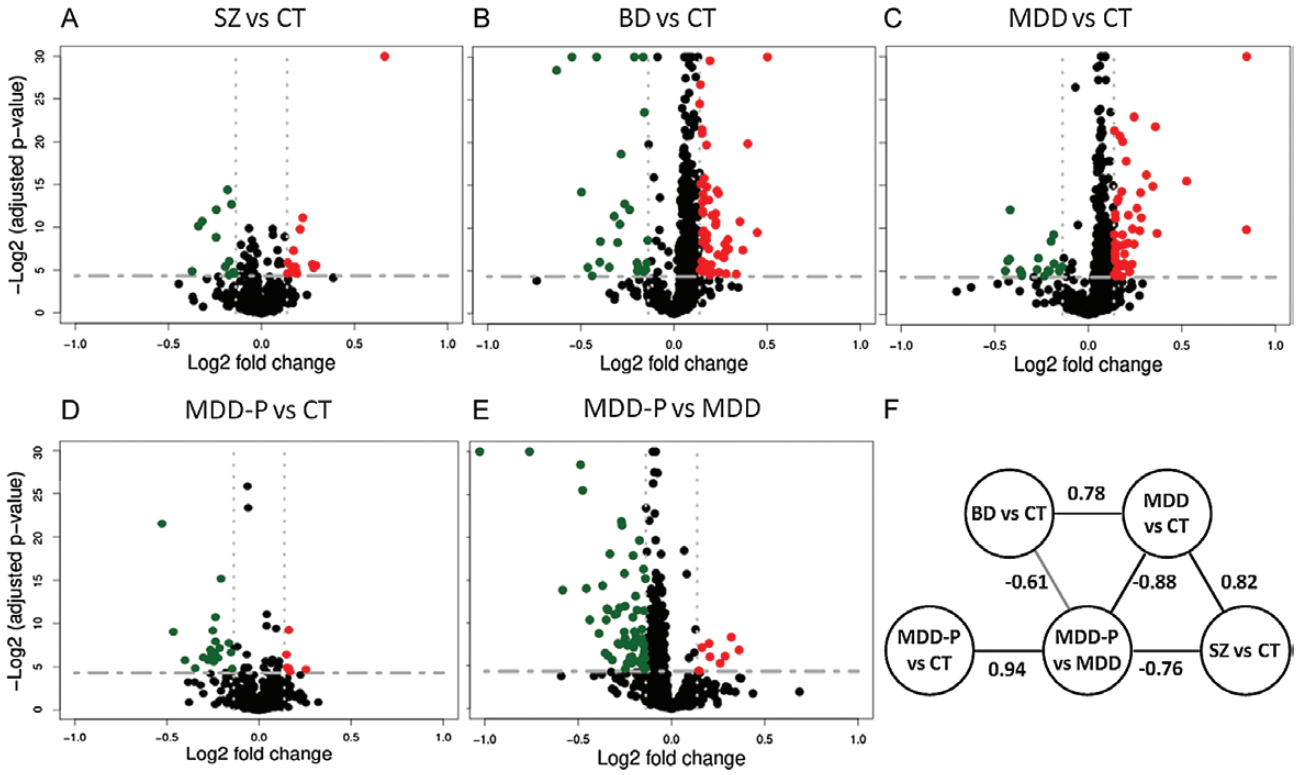

Figure 1. (A–E) Volcano plots of group comparisons showing the adjusted significance p value (log_2) versus fold change (log_2). The plots show an increased magnitude of differential protein abundances in BD/CT, MDD/CT, and MDD-P/MDD compared to SZ/CT and MDD-P/CT. BD/CT and MDD/CT revealed a prominent trend towards increases in protein levels and decreases in the MDD-P/MDD comparison. Horizontal grey lines indicate an adjusted p value threshold of 0.05; vertical grey dotted lines indicate a fold change threshold of 10%. Red dots indicate positive fold changes; green dots indicate negative fold changes. (F) Correlation map identifying overlapping protein abundance patterns between diseases. Spearman correlation coefficents ($p < 0.001$) were estimated for the fold changes of all significantly-changed proteins overlapping between any two comparisons (see Supplementary Figure S1A–J). Positive correlations indicate fold change patterns in the same direction.

catabolism, glycolysis, lipid catabolism, and ATP synthesis, and in the KEGG pathway analysis by oxidative phosphorylation and metabolic pathways.

Based upon the overlap between the enrichment analyses described above, we selected 10 proteins for the 5 biological pathways (2 proteins/pathway) for SRM validation, particularly focusing on presynaptic glutamatergic neurotransmission and energy metabolism (Figure 3). Additionally, we decided to evaluate whether we can confirm the significant over- or under-representation of certain pathways by including proteins not detected in the initial LC-MS^E analysis yet implicated in the same pathways. To avoid a technical bias resulting from the initial protein precipitation, this validation was performed on samples generated by an independent precipitation. Spearman correlation confirmed a positive relationship between predicted LC-MS^E and SRM estimates for all measured samples (see Supplementary Figure S3). To exclude the possibility of a medication effect in our findings we correlated the fluphenazine mg equivalent with the measured SRM intensity estimates for each protein, but were unable to find any significant correlation (Spearman correlation $p > 0.05$). The effects of other psychotropic medication (mood stabilizers and antidepressants) cannot be accounted for, as the respective dosages were not available and the samples and medication compliance at time of death does not allow extrapolation of the appropriate durations of treatment. SRM intensity estimates were also tested for correlation with further demographic characteristics (post-mortem interval, brain pH, age of death). However none of these variables showed significant correlations (all Spearman correlation $p > 0.05$; for r and p values see Supplementary Table S5). SRM validation of the GO BP term glutamate secretion and the CC term clathrin-coated vesicle (a parent term of the various neurotransmitter vesicle membrane terms identified), both indicating presynaptic disturbances, confirmed significantly elevated abundance levels in BD/CT (vesicular glutamate transporter 1 [VGLU1] FC 1.17, synaptotagmin [SYT] FC 1.18, synaptophysin [SYPH] FC 1.13, and synapsin 1 [SYN1] FC 1.19) and MDD/CT (VGLU1 FC 1.19, SYT FC 1.12, and SYN1 FC 1.14), while decreased levels were validated in SZ/CT (VGLU1 FC -1.12, SYT FC -1.13, SYPH FC -1.22, and SYN1 FC -1.15) and MDD-P/MDD (VGLU1 FC -1.08, SYT FC -1.16, and SYN1 FC -1.16; see Figure 3 for overview and p values). A similar pattern, as indicated by GO and KEGG enrichment analysis, was replicated for glutamate catabolism with increased protein abundance levels in BD/CT (glutamate decarboxylase 2 FC 1.28 and aspartate aminotransferase [AATM] FC 1.19) and MDD/CT (AATM FC 1.19) and decreased levels in SZ/CT (glutamate decarboxylase 2 FC -1.13 and AATM FC -1.07) and MDD-P/MDD (AATM FC -1.14). SRM validation of the GO BP terms glycolysis and respiratory electron transport chain and the KEGG term oxidative phosphorylation, all primary components of cellular energy metabolism, confirmed elevated abundance levels in BD/CT (mitochondrial pyruvate kinase FC 1.08, fructose-bisphosphate aldolase A FC 1.09, and cytochrome C FC 1.17, mitochondrial ATP synthase subunit beta FC 1.17]) and MDD/CT (mitochondrial pyruvate kinase FC 1.09, fructose-bisphosphate aldolase A FC 1.09, cytochrome C FC 1.09, and mitochondrial ATP synthase subunit beta FC 1.09), while decreased levels were validated in SZ/CT (cytochrome C FC -1.08 and mitochondrial ATP synthase

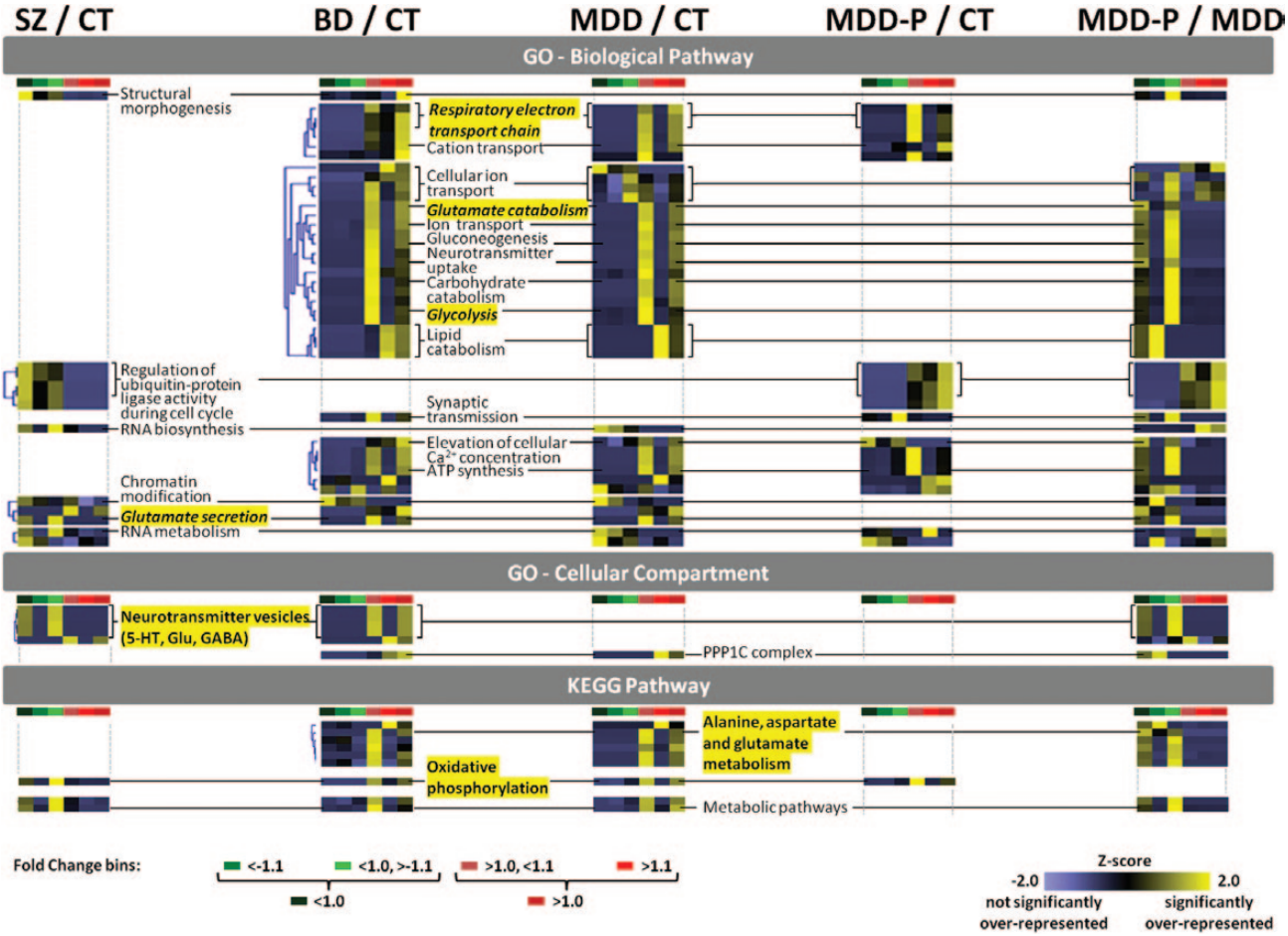

Figure 2. Functional enrichment analysis of GO biological pathway, GO cellular compartment,and KEGG pathway annotations based on conditional hypergeometric testing of significantly changed proteins in the anterior prefrontal cortex of four different major psychiatric disorders. Only comparisons with significant enrichment in at least three different comparisons are displayed. The fold change bins resulting from the quantitative proteome comparison were separately analyzed for enrichment and subsequently clustered and ordered to render terms comparable if they were shared across disorders. Downregulations are depicted in green and upregulations are shown in red. Color-coded z-score–transformed p values indicate the significance of enrichment for every bin. Representative enriched GO terms are annotated. Pathways highlighted in yellow were chosen for SRM validation. For visual purposes the z-score scale and GO terms were truncated.

subunit beta FC -1.10) and MDD-P/MDD (mitochondrial pyruvate kinase FC -1.10, fructose-bisphosphate aldolase A FC -1.10, and mitochondrial ATP synthase subunit beta FC -1.08).

Discussion

This comprehensive study is the first to combine non-hypothesis–driven label-free LC-MS^E with an enrichment analysis in post-mortem brains (anterior prefrontal cortex, BA10) from psychotic and affective disorder patients. We identified shared affected pathways across these major psychiatric disorders and validated them by targeted labeled SRM mass spectrometry analyses, a combination which has previously not been applied in neuropsychiatric research. This novel integrative approach delivers proof that systemic differences and commonalities exist within the psychoaffective disease spectrum, substantiating previous findings (for a review see van Os and Kapur, 2009).

Comparing the altered proteins and pathways and their inter-disease correlations, we identified molecular similarities across all major neuropsychiatric disorders analyzed. Our results imply that pathological hallmarks of these disorders may derive from similar effects at the protein level affecting overlapping biological pathways. This supports the view that the pathologies of major neuropsychiatric disorders represent a disease spectrum. Integration of the quantitative proteomic and molecular pathways suggests that BD and MDD are most closely related, whereas the comparison between MDD and MDD-P showed alterations in similar pathways with opposite directional change. SZ appeared to be more distinct when compared to affective disorders. Interestingly, pathways identified in affective psychosis (MDD-P/MDD) showed similar directional patterns as in the SZ/CT comparison, suggesting a common signature of psychotic features.

For all disease groups, overlapping GO biological pathway and cellular compartment annotations indicated alterations in presynaptic glutamatergic neurotransmission (glutamate secretion; clathrin-sculpted glutamate transport vesicle membrane; and glutamate catabolism) and energy metabolism (respiratory electron transport chain; gluconeogenesis; carbohydrate catabolism; glycolysis; lipid catabolism; and ATP synthesis) as primary comparison nodes. This was further supported by a second enrichment analysis based on KEGG pathway annotation (alanine, aspartate, and glutamate metabolism; oxidative phosphorylation; and metabolic pathways). Consequently, proteins for SRM validation were selected from these pathway categories to validate our findings. To expand the systemic scope of our

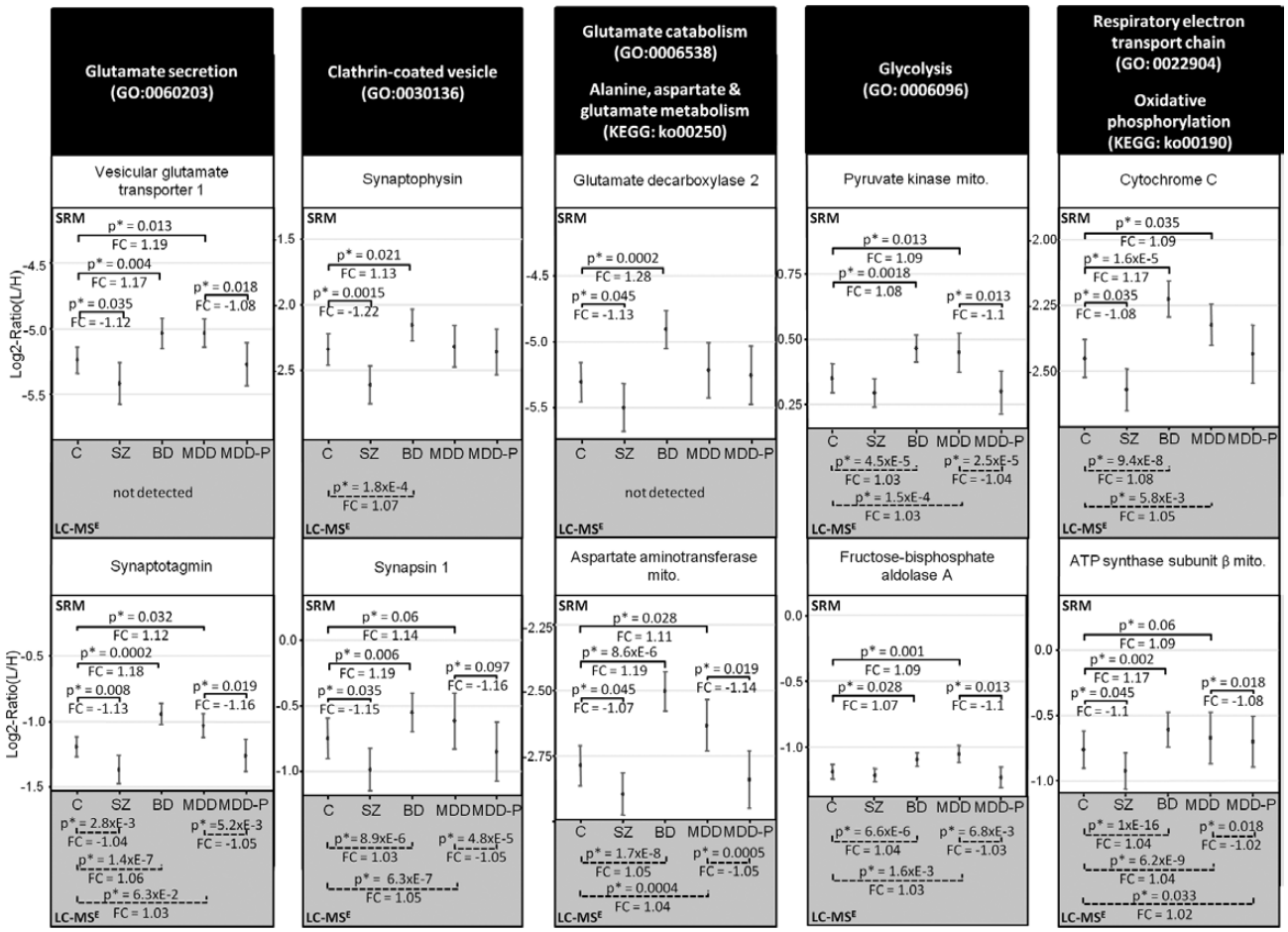

Figure 3. Multivariate analysis of SRM estimates as shown by condition plots illustrating the systemic difference between the disorders. X-axis is condition and y-axis is log ratio of endogenous (L = light) over reference (H = heavy). Dots represent the mean of $\log_2$ ratio for each condition and error bars indicate the confidence interval with 0.95 significance. The interval is not related with model-based analysis. Significant changes, as measured by LC-MS^E, are indicated below each protein. SRM was able to validate abundance changes in GO term associated proteins as suggested by LC-MS^E analysis. Corrected p values (p*) were determined by post hoc correction after Benjamini Hochberg.

functional network discoveries, this included the quantification of proteins implicated in the same biological pathways, which were not detected in the LC-MS^E profiling analysis.

Our integrated analysis suggests decreased glutamatergic neurotransmission in SZ and affective psychosis, while an increase in the same domain was found in BD and MDD. This is consistent with the postulated importance of glutamate in the pathophysiology of psychotic states and is supported by previous findings which reported reduced glutamate metabolism in SZ patients in the frontal cortex and other brain regions (Tsai et al., 1995) and findings of alterations in glutamate signaling in transcriptomic (Tkachev et al., 2007) and genomic studies (Miyatake et al., 2002; Timms et al., 2013). Also, a decrease in medio-fronto-cortical glutamate levels has been confirmed in a meta-analysis investigating 166 SZ patients and 171 CTs (Marsman et al., 2013). The importance of glutamatergic signaling has been demonstrated by pharmacological studies, which have shown that blocking glutamatergic signaling with the N-methyl-D-aspartate (NMDA)-receptor antagonists PCP (Luby et al., 1962) or ketamine (Adler et al., 1999) in healthy subjects induces a hypoglutamatergic psychosis-like state with cognitive disturbances and sensory dysfunctions indistinguishable from SZ (Umbricht et al., 2000). Additional support for the importance of glutamatergic neurotransmission in psychotic states comes from clinical studies evaluating NMDA-receptor agonists (e.g., glycine and D-serine) and glycine transporter inhibitors (e.g., sarcosine) in combination with typical and atypical antipsychotics in SZ, resulting in marked improvements in negative and cognitive symptoms (Javitt, 2004).

Conversely, in BD and MDD release inhibition and receptor blockade of the glutamatergic system has been shown to improve affective symptoms, providing clinical evidence for the glutamatergic hyperfunction of these disorders, as identified in our analysis. This dates back to the discovery of antidepressant effects of NMDA-receptor antagonism (Crane, 1961). Lamotrigine, a known inhibitor of glutamate signaling, ameliorates episodes of persistent or treatment-resistant depression in BD (Berk, 1999; Boylan et al., 2002), as does ketamine (Diazgranados et al., 2010). Furthermore, an inhibitor of glutamate release, riluzole (Lamanauskas and Nistri, 2008), which has been shown to be effective in treatment-resistant MDD (Zarate et al., 2004), has also demonstrated efficacy in BD (Zarate et al., 2005). In addition, single doses of ketamine also show a rapid antidepressant effect in treatment-resistant cases (Berman et al., 2000; Krystal et al., 2002; Zarate et al., 2006). Recently, these findings were followed up by the successful evaluation of lanicemine, a low-trapping NMDA-receptor antagonist suitable for long-term treatment without the dissociative and psychotomimetic side

effects of ketamine (Sanacora et al., 2013). Further evidence for increased levels of glutamate in the frontal cortex of mood disorder patients is provided by proton magnetic resonance spectroscopy studies (Gigante et al., 2012) in living patients, as well as in post-mortem brain tissue (Hashimoto et al., 2007) and in serum (Kim et al., 1982) and plasma (Altamura et al., 1993; Hoekstra et al., 2006).

Our results implicate a presynaptic dysfunction in the frontal cortex of all investigated disorders, as indicated by the cellular compartment annotation in the enrichment analysis. This was validated by SRM mass spectrometry analyses, which showed changes in a number of presynaptic vesicle proteins (synapsin 1 SYN1, synaptotagmin 1 SYT1, and synaptophysin SYP). Furthermore, we were able to show that altered glutamatergic signaling (glutamate secretion) across all comparisons might be due to glutamate release abnormalities by measuring the abundance of a bottleneck protein for this GO term, the vesicular glutamate transporter 1 (Daniels et al., 2006). This implies that our pathway analysis can be expanded beyond the pool of proteins identified by the LC-MS^E profiling experiments. Increased vesicular glutamate transporter 1 expression has been linked to mood disorders (Eastwood and Harrison, 2010), and novel compounds inhibiting the crucial step of vesicle loading are currently being evaluated for their potential efficacy for treatment of affective disorders (Thompson et al., 2005).

Glutamate is the most abundant excitatory neurotransmitter in the brain and a key component of intermediary energy metabolism (Erecinska and Silver, 1990). Approximately two thirds of the energy derived from brain glucose metabolism is invested into glutamatergic neurotransmission and related events (Shulman et al., 2002). This link between synaptic transmission and glutamate release with glycolysis and ATP biosynthesis is consistent with the second major outcome of our pathway enrichment analysis. Besides the presynaptic glutamatergic abnormalities, we detected a dysregulation of the glutamate catabolism, which is interconnected with the energy metabolism found to be dysregulated across all investigated disorders. This finding was validated by SRM (glycolysis; respiratory electron transport chain; and oxidative phosphorylation). The detected abnormalities follow a similar fold-change directional pattern to those observed for the presynaptic glutamatergic neurotransmission. The relevance of this finding has been demonstrated in a clinical study, in which rapid antidepressant response to ketamine was linked to increased pre-treatment fronto-cortical metabolic activity during exposure to negative emotional stimuli (Salvadore et al., 2009).

In vivo neuroimaging studies have already demonstrated reduced frontal ATP levels (Volz et al., 2000) and metabolic rates in SZ patients (Ingvar and Franzen, 1974; Buchsbaum et al., 1984). Additionally, MDD patients with psychotic features were found to display reduced prefrontal activation during cognitive encoding tasks compared to CTs (Kelley et al., 2013), while the ameliorating effects of electroconvulsive therapy in psychotic depression patients were correlated with an increase in fronto-cortical metabolism (McCormick et al., 2007). This supports our findings of a hypofunction of energy metabolism in SZ and affective psychosis. Previous targeted SZ post-mortem brain studies have identified reduced transcript and protein levels (Cavelier et al., 1995; Bubber et al., 2011), along with reduced activity (Maurer et al., 2001) of the mitochondrial electron transport chain, further supported by the results of several proteomic (Beasley et al., 2006; Clark et al., 2006) and transcriptomic (Prabakaran et al., 2004) studies.

Consistent with our proteomic findings of increased energy metabolism in BD, previous studies reported elevated fronto-cortical markers of metabolism in medication-free BD patients (Kato et al., 1998; Dager et al., 2004), increased frontal activity in patients undergoing a manic episode (Blumberg et al., 2000), and a raised metabolism of glucose in the frontal lobe of treatment-resistant mildly depressed and euthymic BD patients (Ketter et al., 2001). Similar signs of increased energy metabolism (Gruber et al., 2003) have also been reported for MDD, in line with our proteomic and bioinformatic findings. Higher prefrontal metabolism in MDD patients has been shown to be normalized by several antidepressant drug classes (Drevets et al., 1992) and psychotherapy (Brody et al., 2001; Kennedy et al., 2007), providing *in vivo* proof for a prefrontal hypermetabolic state.

In terms of a systems biological perspective, this study provides evidence that abnormalities in energy metabolism are secondary effects of presynaptic abnormalities in glutamatergic neurotransmission. This is the first global and systemic proteomic approach which has provided molecular evidence for a hyperglutamatergic state in affective disorders and a hypoglutamatergic state in psychosis, associated with increased or decreased brain energy metabolism, respectively. Although we aimed to exclude methodological biases by carrying out independent protein extraction for the SRM validation phase, further validation using extracts from independent sample sets is still required. Our results provide evidence for the potential of drug interventions specifically focusing on glutamatergic neurotransmission counteracting these dysregulations. These findings also encourage the re-conceptualization of the psychiatric classification system supported by characteristic disease-specific pathological hallmarks, if similar distinctive signatures for affective and psychotic traits can be validated in larger patient cohorts by *in vivo* measurements. The presented enrichment analysis offers a comparative insight in the spectrum of major neuropsychiatric disorders while suggesting candidate pathways for future investigations to evaluate their diagnostic and therapeutic potential.

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

Supplementary Figure S1 (*a-j*): Correlation of $\log_2$ protein fold changes between all possible disease comparisons across all proteins. Spearman correlation for all proteins significantly changed in both given comparisons. Coloured dots indicate fold change directions in both comparisons: green=both negative; red=both positive; grey=opposite directions. Vertically and horizontally dotted lines indicate a fold change threshold of 10%. Uniprot identifiers are given for selected protein fold changes of higher magnitude for a given comparison.

Supplementary Figure S2: Venn diagram displaying the overlap of significantly changed proteins across all comparisons.

Supplementary Figure S3: Spearman correlation of predicted, normalized SRM and LC-MS^E estimates.

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Statement of Interest

Prof. Bahn and Dr Guest are consultants for Myriad Genetics. M.G.G. and H.W. declare no conflict of interest.

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