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Research Article

RESEARCH ARTICLE

Targeted Multiplexed Selected Reaction Monitoring Analysis Evaluates Protein Expression Changes of Molecular Risk Factors for Major Psychiatric Disorders

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Abstract

Background: Extensive research efforts have generated genomic, transcriptomic, proteomic, and functional data hoping to elucidate psychiatric pathophysiology. Selected reaction monitoring, a recently developed targeted proteomic mass spectrometric approach, has made it possible to evaluate previous findings and hypotheses with high sensitivity, reproducibility, and quantitative accuracy.
Methods: Here, we have developed a labelled multiplexed selected reaction monitoring assay, comprising 56 proteins previously implicated in the aetiology of major psychiatric disorders, including cell type markers or targets and effectors of known psychopharmacological interventions. We analyzed postmortem anterior prefrontal cortex (Brodmann area 10) tissue of patients diagnosed with schizophrenia (n = 22), bipolar disorder (n = 23), and major depressive disorder with (n = 11) and without (n = 11) psychotic features compared with healthy controls (n = 22).
Results: Results agreed with several previous studies, with the finding of alterations of Wnt-signalling and glutamate receptor abundance predominately in bipolar disorder and abnormalities in energy metabolism across the neuropsychiatric disease spectrum. Calcium signalling was predominantly affected in schizophrenia and affective psychosis. Interestingly, we were able to show a decrease of all 4 tested oligodendrocyte specific proteins (MOG, MBP, MYPR, CNPase) in bipolar disorder and to a lesser extent in schizophrenia and affective psychosis. Finally, we provide new evidence linking ankyrin 3 specifically to affective psychosis and the 22q11.2 deletion syndrome-associated protein septin 5 to schizophrenia.
Conclusions: Our study highlights the potential of selected reaction monitoring to evaluate the protein abundance levels of candidate markers of neuropsychiatric spectrum disorders, providing a high throughput multiplex platform for validation of putative disease markers and drug targets.

Keywords: SRMstats, myelination, GSK3b, CamKII, microglia.

Introduction

Psychiatric disorders represent a considerable burden for healthcare providers around the world, affecting >20% of the global population. Despite extensive research efforts during the past decades, the aetiologies of the major psychiatric disorders

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schizophrenia (SZ), bipolar disorder (BD), and major depressive disorder (MDD) are largely unknown. These disorders are heterogeneous in nature, with genetic and environmental factors contributing to pathogenesis and aetiology (Levinson, 2006; van Os and Kapur, 2009; Kim et al., 2011; Craddock and Sklar, 2013; Klengel and Binder, 2013). Symptom profiles greatly overlap with regards to clinical psychopathology as well as putative pathophysiology (Smoller et al., 2013). So far, no single gene, mRNA transcript, or protein has been found that can account for the pathology of any of these disorders. However, the existence of a variety of risk genes and risk-associated protein alterations is now widely accepted. Genome-wide association studies, identifying a large number of single nucleotide polymorphisms in candidate genes and structural genetic differences such as copy number variations, as well as micro-array and proteomic analyses have greatly contributed to this knowledge (Pickard, 2011). However, at present, there is a lack of information regarding which of the genetic risk polymorphisms are associated with changes at the mRNA and protein levels.

Recent technological developments in proteomic methods have now made it possible to validate high-throughput findings with great accuracy and sensitivity (Wesseling et al., 2014). SRM is currently the most advanced targeted mass spectrometry-based technology. In contrast to shot-gun proteomics strategies, in which the goal is to simultaneously investigate abundance changes of hundreds to thousands of proteins at the expense of sensitivity, SRM mass spectrometry platforms allow monitoring of a predetermined selection of proteins/peptides with high sensitivity, reproducibility, and quantitative accuracy (Picotti et al., 2010; Picotti and Aebersold, 2012). In addition, the SRM approach has emerged as an alternative to affinity-based assays such as enzyme-linked immune-sorbent assays with the advantage of faster and more cost-effective assay development (Whiteaker et al., 2011). Protein quantification by SRM in complex samples using predefined assay coordinates is reproducible across different laboratories and instrument platforms, facilitating reproducibility of assays in follow-up studies. Developed assays are universally applicable to test hypotheses across a variety of samples of different origins (eg, brain tissue, blood) and easily adjustable to different matrices. Therefore, SRM is ideal to investigate whether previously reported candidate risk genes for psychiatric disorders translate to changes at the protein level, providing evidence of functional effects and clinical relevance.

We designed a targeted multiple isotope labelled SRM-assay, comprising 56 proteins that have been implicated in the major psychiatric disorders and screened a total of 89 postmortem brain tissue samples of 22 SZ patients, 23 BD patients, 11 MDD patients, 11 MDD patients with psychotic features (MDD-P), and 22 healthy controls (CTs). The proteins tested have previously been associated with psychiatric pathophysiology at the gene, mRNA transcript, or protein level. Our main objective was to investigate the potential of SRM to systematically elucidate the pathophysiology of neuropsychiatric disorders and to further evaluate putative risk markers as novel molecular targets for the next generation of drugs.

Materials and Methods

Clinical Samples

A total of 89 postmortem anterior prefrontal cortex (Broadmann Area 10) brain samples were provided by the Stanley Medical Research Institute (Bethesda, MD) (Torrey et al., 2000) and consisted of 22 SZ patients, 23 BD patients, 11 MDD patients without psychotic symptoms, 11 MDD-P patients with psychotic symptoms and 22 CTs. Tissue was collected postmortem from patients and controls with full informed consent obtained from a first-degree relative in compliance with the Declaration of Helsinki, and consent was obtained by questionnaires conducted over the phone and signed by 2 witnesses. The sample groups were matched for age of death, gender, and brain pH (t test). There were no significant differences in the brain side from which samples were obtained, secondary axis diagnosis of alcohol abuse/dependency, and drug abuse/dependency between patients and CTs (Fisher's exact test). Tissue was sectioned using a Leica Cryostat (Milton Keynes, UK) and stored at –80°C until use. All tissue samples used contained equal amounts of white and grey matter. A summary of the demographic details and statistical values is shown in supplementary Table S1. Additional information is provided in supplementary Table S2.

Sample Preparation

Approximately 50 mg of tissue per sample was used. Samples were added to fractionation buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ASB14, and 70 mM dithiothreitol at a 5:1 (vol/wt) ratio (Ernst et al., 2012). After sonication and vortexing for 30 minutes, protein concentrations of the lysates were determined using a Bradford assay (Bio-Rad, Hemel Hempstead, UK). Protein (approximately 100 µg) was precipitated using acetone. After dissolving the precipitate in 50 mM ammonium bicarbonate, protein concentrations were determined in quadruplets. Reduction of sulfhydryl groups on proteins was performed with 5 mM dithiothreitol at 60°C for 30 minutes and alkylation was carried out using 10 mM iodacetamide and incubating in the dark at 37°C for 30 minutes. Proteins were digested using trypsin at a 1:50 (wt/wt) ratio for 17 hours at 37°C, and reactions were stopped by the addition of 8.8 M HCl in a 1:60 (vol/vol) ratio. Sample aliquots were stored at –80°C until analysis.

Label-Based Selected Reaction Monitoring Mass Spectrometry

Abundance alterations of a panel of 56 candidate proteins implicated in the pathology of the major psychiatric disorders or associated with drug treatments were measured using targeted SRM mass spectrometry on a Xevo TQ-S mass spectrometer (Waters Corporation) coupled online through a New Objective nanoESI emitter (7 cm long, 10-mm tip; New Objective) to a nanoAcquity UPLC system (Waters Corporation). The system was comprised of a C18 trapping column (180 µm x 20 mm, 5-µm particle size) and a C18 BEH nano-column (75 µm x 200 mm, 1.7-mm particle size). The separation buffers were (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. For separation of peptides, the following 48-minute gradient was applied: 97/3% (A/B) to 60/40% in 30 minutes; 60/40% to 15/85% in 2 minutes; 5 minutes at 15/85%; and returning to the initial condition in 1 minute. The flow rate was 0.3 µL/min and the column temperature was 35°C.

SRM assays were developed following a high-throughput strategy (Picotti et al., 2010) (Figure 1a). We initially started with more than 200 selected proteins. Up to 12 unique peptides ranging from 6 to 20 amino acids in length containing tryptic ends and no missed cleavages were chosen for each of the selected proteins. All peptides containing amino acids prone to undergo modifications (eg, Met, Trp, Asn, and Gln), potential ragged ends, lysine/arginine followed by proline, or bearing NXT/NXS glycosylation motifs were avoided and selected only 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

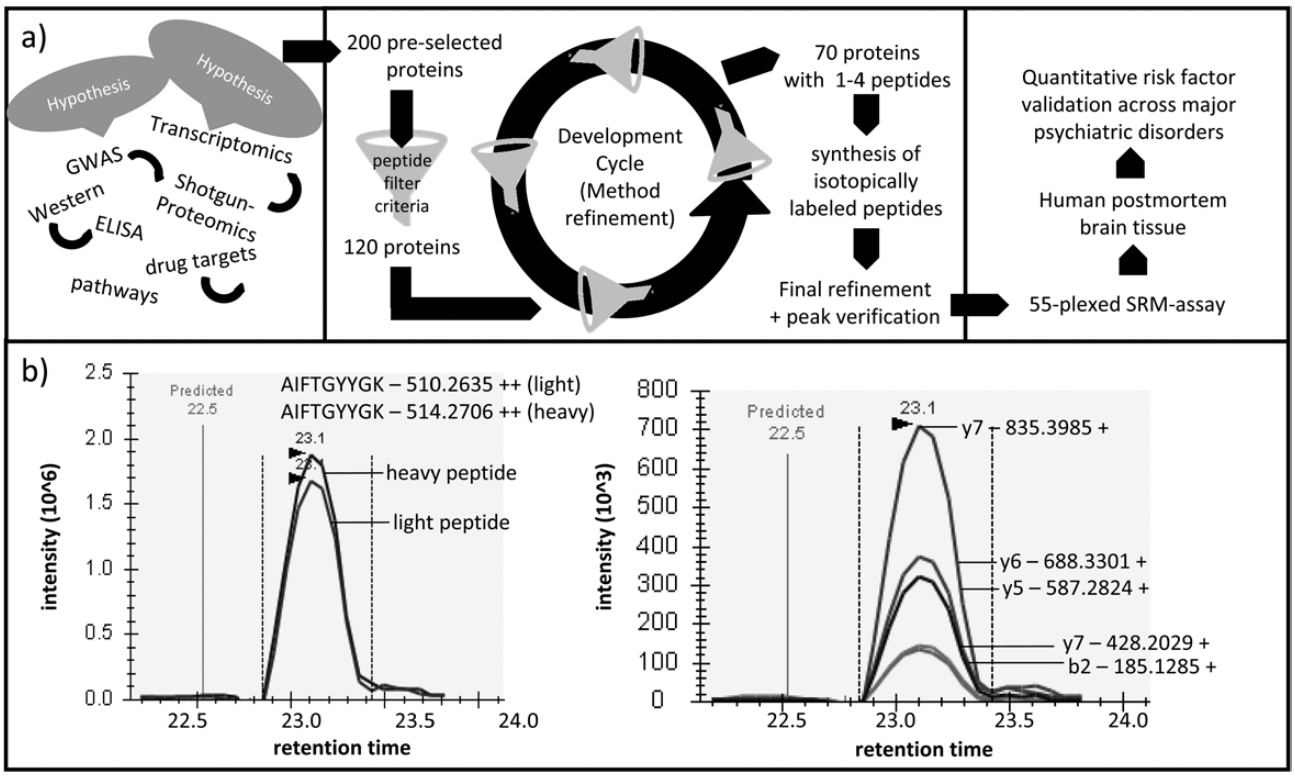

Figure 1. a, Schematic overview of the study design. b, left, Chromatographic SRM profile of the sample's endogenic tryptic peptide AIFTGYYGK and of the spiked heavy labelled reference peptide. Right, Chromatographic SRM profile of the transitions of the light peptide.

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 precursors in the range of 350 to 1250 Da. Transitions were selected based on software internal predictions, discovery proteomics data, and spectral data available through the Human NIST spectral libraries (Farrah et al., 2011). Method refinement was performed on quality control samples. For the final SRM assay, the 2 to 3 peptides with the maximal intensities and highest spectral library similarity (dotp) were selected. We also analyzed heavy-label spiked quality control samples (Figure 1b) in scheduled SRM mode to confirm identity via coelution, extracted the optimal fragment ions for SRM analysis, obtained accurate peptide retention times, and optimized collision energy and cone voltage for the quantification run applying skyline software (MacCoss Lab Software; Seattle, WA) (MacLean et al., 2010). Heavy labelled forms of the selected peptides (spiketides L) were chemically synthesized via SPOT synthesis (JPT Peptide Technologies GmBH, Berlin, Germany). The final transitions, collision energy, and retention time windows used for each peptide can be found in the supplementary information (supplementary Table S3).

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 labelled internal standard (JPT Peptide Technologies GmbH) was spiked in the peptide mixture for accurate quantification and identification. All SRM functions had a 2-minute window of the predicted retention time and scan times were 20 milliseconds. For each peptide, at least 3 transitions were monitored for the heavy and light version. Samples were run randomized and blocked (Oberg and Vitek, 2009) in triplicates, and blanks and quality control peptide injections (yeast alcohol dehydrogenase; supplementary Table S3) were performed alternating after every biological replicate. Resulting SRM data were analyzed using Skyline and explanatory data analysis (quality assessment), and model-based statistical analysis was conducted using SRMstats (Chang et al., 2011). The data preprocessing consisted of a $\log_2$ transformation of the data to stabilise the variance. A constant normalization was performed based on reference transitions for all proteins to equalize the median peak intensities of reference transitions from all proteins across all mass spectrometry 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 carried out using the linear mixed-effects model implemented in SRMstats. The linear mixed model function supported by SRMstats employs a "restricted" or "expanded" scope of conclusions (Chang et al., 2011; Surinova et al., 2013). In the restricted scope model, the individual samples being modelled are the population of interest, whereas in the expanded scope model, the samples being modelled are treated as a random sample from the population of interest. Consequently, the expanded scope model allowed us to draw conclusions about the population from which the samples were drawn, and the restricted scope allowed conclusions within the data itself. Initially, we assumed the restricted scope, taking into account the measurement error of transitions across runs (technical variation), to quantify protein abundance changes in our sample cohorts. Subsequently, we continued with the expanded scope, accounting for technical variation and considering the individual biological replicates (biological variation) of our sample cohorts as random selection of their respective populations of origin to test which protein changes could

be considered as representative of their underlying populations. The *P*-values were adjusted to control the false discovery rate at a cut-off of 0.05 according to Benjamini and Hochberg (Chang et al., 2011).

Results

Applying label-based SRM mass spectrometry, we measured differential protein abundance levels of 56 predefined proteins (Table 1) in postmortem brain samples from 22 SZ, 23 BD, 11 MDD, and 11 MDD-P patients compared with 22 CTs. Additionally, MDD-P was compared directly with MDD to identify proteins associated with affective psychosis in MDD. We detected common and unique alterations in individual protein levels across the disorders using a statistical modelling framework for protein significance analysis based on the SRM spectral data. The model required the scope of conclusion validity to be specified as either "restricted" or "expanded" corresponding to drawing conclusions about the data itself or about the population from which the individual samples were drawn.

Statistical analysis using the restricted model resulted in the identification of 12 differentially expressed proteins in the SZ/CT, 27 in BD/CT, 11 in MDD/CT, 6 in MDD-P/CT, and 15 in the MDD-P/MDD group comparisons (Table 2).

Most alterations in protein abundance levels were found in the BD/CT comparison in which the majority of proteins were increased, with the exception of oligodendrocyte-specific proteins (CN37, MBP, MOG, MYPR) and neurofilament light polypeptide, which were decreased. A less pronounced decrease in oligodendrocyte-specific proteins was found in the SZ/CT and MDD-P/CT comparisons. In MDD/CT, lower levels of the astrocyte marker glial fibrillary acidic protein and the microglia marker coronin 1a were identified. In the BD/CT comparison, the Wnt signalling pathway appeared to be upregulated. The MDD/CT comparison showed increases in calmodulin-dependent protein kinase 2 subunits β and γ as well as ERK signalling. All proteins found to be significantly different in MDD-P/MDD were decreased similar to the finding in the SZ/CT comparison, although this involved different proteins. In the MDD-P/MDD comparison, all 3 calmodulin-dependent protein kinase 2 subunits (CAMK2α, CAMK2β, CAMK2y) were decreased. To test for potential medication effects, we correlated the SRM intensity estimates and fluphenazine milligram equivalents and found no significant effect on any measured protein (Spearman correlation $P > .1$). However, effects of other psychotropic medication such as antidepressants and mood-stabilizing agents cannot be ruled out. To exclude further confounding effects, we correlated the SRM intensity estimates and additional demographic characteristics (brain pH, postmortem interval, age of death, age of disease onset, disease duration). We were unable to find any correlation (Spearman correlation $P > .1$). For further information, see supplementary Table S6.

Since these proteomic findings resulted from the analysis of our sample cohort, we subsequently applied an expanded model to determine the likelihood of finding similar results in a wider population. These results supported the decrease in oligodendrocytic proteins (MBP, MOG, MYP, CN37) in BD/CT and the decrease of Septin 5 (SEPT5) in SZ/CT and Ankyrin 3 (ANK3) in MDD-P/MDD (Figure 2).

Discussion

This study represents the first and largest label-based quantitative targeted proteomics investigation in human brain tissue to date, evaluating expression changes of high-risk genes and risk-associated proteins in 4 different psychiatric disorders.

Applying linear mixed effect models, we were able to detect a range of significantly altered proteins that have been implicated at the genetic, transcriptomic, and proteomic levels in psychiatric research. We provide evidence for microglial dysfunction in MDD (Frick et al., 2013; Kreisel et al., 2013) compared with controls as shown by a decrease in coronin 1A levels. The astrocyte marker glial fibrillary acidic protein was decreased in SZ and MDD, indicating a potential disturbance of neuronal maintenance and glutamate clearance, and all CamK2 isoforms were changed in the MDD-P/MDD comparison. In addition, we identified a decrease of proteins associated with energy metabolism in SZ and an increase in BD and MDD, in line with previous findings (Prabakaran et al., 2004; Marazziti et al., 2012). It is also of note that we were able to validate the widely reported expression changes of malate dehydrogenase in psychiatric disorders.

One of the most striking results in this study was the systematic decrease of all investigated oligodendrocyte-specific proteins in SZ, BD, and MDD-P. Myelin-related abnormalities have previously been observed in postmortem brain and imaging studies of SZ and BD (Davis et al., 2003; Dwork et al., 2007; Brambilla et al., 2009; Mcintosh et al., 2009; Andreasen et al., 2011; Bartzokis et al., 2011). In accordance with previous findings of mRNA changes (Tkachev et al., 2003), this is the first study to show a more prominent decrease in oligodendrocyte-specific proteins in BD compared with SZ. Myelination in the prefrontal cortex occurs predominantly in adolescence and early adulthood (Benes, 1989), consistent with the typical age of onset of SZ and BD. Interestingly, myelination deficits in SZ, BD, and MDD-P corresponded with changes in the expression of the GSK3β and Wnt signalling regulatory protein catenin β in SZ and BD. GSK3β is a negative regulator of oligodendrocyte differentiation and myelination and has been implicated in BD and SZ. GSK3β inhibitors such as lithium are widely prescribed as mood stabilizers in the treatment of BD and are known to increase oligodendrocyte differentiation and promote myelination (Azim and Butt, 2011). Furthermore, the NMDA receptor subunit NR1, which we found to be upregulated in BD, is a functional regulator of oligodendrocyte precursor cell differentiation and remyelination (Li et al., 2013). Consequently, the development and re-profiling of drugs that promote remyelination may represent novel pharmacological targets for psychiatric disorders, especially BD. The compound XAV939 has been shown to enhance oligodendrocyte differentiation and remyelination by stabilizing Axin2, an intracellular target of Wnt transcriptional activation (Fancy et al., 2011). In addition, synthetic and natural cannabinoids have been shown to protect oligodendrocytes and oligodendrocyte progenitor cells and to enhance myelination by promoting oligodendrocyte maturation in vivo and vitro (Molina-Holgado et al., 2002; Gomez et al., 2010; Solbrig et al., 2010; Mecha et al., 2012). They are currently being tested for efficacy in multiple sclerosis clinical trials (Zajicek and Apostu, 2011; Velayudhan et al., 2013) and could be evaluated for the treatment of SZ and BD (Deiana, 2012).

We also found that ANK3 protein levels were reduced in affective psychosis, supporting the finding on a wider population scale applying the expanded model. The ANK3 protein is involved in neuronal scaffolding and the formation and maintenance of the axon initial segment of neurons and nodes of Ranvier (Bennett and Lambert, 1999). Recently, a meta-analysis of the major psychiatric disorders suggested that the *ANK3* locus represents a shared risk gene for a number of psychiatric disorders (Smoller et al., 2013). ANK3 polymorphisms have been

Table 1. Overview of the Multiplex SRM Assay

Protein Name	UP-ID	Function Summary	CL	G	T	P	Link to SZ	BD	MDD
22q11.2 Deletion Syndrome									
Catechol O-methyl-transferase	COMT	Catalyzes inactivation, of catecholamine neurotransmitters and catechol hormones	22q11.21	√			[1–4]	[5, 6]	[7]
Proline dehydroge-nase 1, mito	PROD	Converts proline to delta-1-pyrroline-5-carboxylate	22q11.21	√			[1, 8, 9]		
Ran-binding protein 1	RANG	Inhibits GTP exchange on Ran. Increases GTP hydrolysis induced by RANGAP1	22q11.21	√			[1]		
Septin 5	SEPT5	Filament-forming cytoskeletal GTPase, may play a role in cytokinesis	22q11.21	√			[1]		
TCA transport protein, mito	TXTP	Involved in citrate-H^+/malate exchange	22q11.21	√			[1]		
Receptors									
Glutamate receptor 1	GRIA1	Ionotropic glutamate receptor (AMPA1)	5q31.1	√			[2, 10]		
Glutamate receptor 2	GRIA2	Ionotropic glutamate receptor (AMPA2)	4q32.1		√		[10–12]	[12]	[12]
Glutamate receptor 3	GRIA3	Ionotropic glutamate receptor (AMPA3)	7q21.1-q21.2		√		[10]		[12]
NMDA receptor 1	NMDZ1	NMDA receptor subtype, high calcium permeability, voltage-dependent sensitivity to Mg^{2+}	9q34.3	√	√	√	[10, 13, 14]	[15]	[16–18]
Scaffolding proteins									
Ankyrin 3	ANK3	Neuronal scaffolding protein in nodes of Ranvier and axon initial segments	10q21	√			[19, 20]	[19, 21, 22]	[19]
Disks large homolog 4 (PSD95)	DLG4	Interacts with the NMDA receptor subunits and shaker-type potassium channels	17p13.1	√	√	√	[13, 14, 23–31]	[13, 23]	
Shank3	SHAN3	Post-synaptic density protein, interconnects NMDA and metabotropic glutamate receptors	22q13.3	√			[32–34]	[35, 36]	
Downstream signalling									
Calcium signalling									
Calmodulin (CaM)	CaM	Calmodulin mediates the control of a large number of enzymes, ion channels by Ca^{2+}	14q32.11			√	[37, 38]		
CamK2α	KCC2A	Major protein kinase in the CNS, functions in LTP and neurotransmitter release	5q32		√	√		[39, 40]	
CamK2β	KCC2B	Function autonomously after Ca^{2+}/calmo-dulin-binding and autophosphorylation,	7p14.3-p14.1		√		[41, 42]		[41]
CamK2γ	KCC2G	involved in dendritic spine and synapse formation, neuronal plasticity	10q22						
Calcineurin subunit B type 1	CANB1	Regulatory subunit of calcineurin, Ca^{2+}/ calmodulin protein phosphatase	2p15			√	[43]		
IP3 receptor isoform 1	ITPR1	Intracellular channel, mediates calcium release from the endoplasmic reticulum	3p26.1					[44]	
Neurochondrin	NCDN	Involved in signal transduction, increases cell surface localization of GRM5	1p34.3				[45]		
Calmodulin-dep. calcineurin A subu-nit beta isoform	PP2BB	Ca^{2+}-dependent, calmodulin-stimulated protein phosphatase, may have a role in the calmodulin activation of calcineurin.	10q22	√		√	[46, 47]		
Protein Kinase									
Protein kinase C α type	KPCA	Ca^{2+}-act. Ser/Thr -protein kinase, involved in cell proliferation, apoptosis, differentiation, migration	17q22-q23.2	√		√		[48, 49]	[50]
Protein kinase C β type	KPCB	Ca^{2+}-act. Ser/Thr-protein kinase involved in oxidative stress-induced apoptosis & insulin signaling	16p11.2			√		[48, 49]	[50, 51]
Protein kinase C γ type	KPCG	Ca^{2+}-act. Ser/Thr-protein kinase, regulates neuronal receptor functions, mediates syn-aptic function	19q13.4			√		[48, 49]	
MARCKS	MARCS	MARCKS is the most prominent cellular substrate for protein kinase C	6q22.2			√		[52–54]	[55]

(*Continued*)

Table 1. Continued

Protein Name	UP-ID	Function Summary	CL	G	T	P	Link to SZ	Link to BD	Link to MDD
ERK-signalling									
ERK2	MK01	Serine/threonine kinase, essential component of the MAPK signal transduction pathway	22q11.21			√	[56–58]	[59]	
ERK1	MK03	Serine/threonine kinase, essential component of the MAPK signal transduction pathway	16p11.2			√	[56–58]		
PED	PEA15	Blocks Ras-mediated inhibition of integrin activation, modulates the ERK MAP kinase cascade	1q21.1	√			[60–62]		
mTOR									
mTOR	MTOR	Central regulator of cellular metabolism, growth and survival	1p36.2			√	[63]		
40S ribosomal protein RS3A	RS3A	Ribosomal subunit	8q24.3						
40S ribosomal protein S4, X iso.	RS4X	Ribosomal subunit	Xq13.1						
Wnt signalling									
Catenin β-1	CTNB1	Key downstream component of the canonical Wnt signaling pathway	3p21			√			[64]
Glycogen synthase kinase-3 β	GSK3B	Active protein kinase, neg. regulator in the hormonal control of glucose homeostasis,	3q13.3	√		√	[65–67]	[68–72]	[73, 74]
Phosphoprotein F1-20	AP180	Component of the adapter complexes which link clathrin to receptors in coated vesicles	6q14.2	√				[75]	
Cell-type specific proteins									
Oligodendrocytes									
CNPase	CN37	Involved in RNA metabolism in the myelinating cell, third most abundant protein in CNS myelin	17q21	√	√		[76–79]		
Myelin basic protein	MBP	Most abundant component of myelin membrane, plays role in myelin formation and stabilization	18q23	√	√		[2, 80–84]	[83]	[84]
Myelin proteolipid protein	MYPR	Major myelin protein from CNS, plays role in multi-laminar myelin formation or maintenance	Xq22		√		[85, 86]		[87]
Myelin-oligodendrocyte glycoprotein	MOG	Minor component of the myelin sheath, myelin sheath completion/maintenance, cell-cell communication	6p22.1		√		[88–90]	[88]	[87]
Astrocytes									
Glial fibrillary acidic protein	GFAP	GFAP, a class-III intermediate filament, is a cell-specific marker for astrocytes	17q21		√	√	[91–94]		[95, 96]
Microglia									
Coronin-1A	COR1A	Crucial component of the cytoskeleton of highly motile cells	16p11.2	√		√	[94, 97–100]		
Energy metabolism									
ATP synthase subunit b	ATP5F	Mitochondrial membrane ATP synthase	18q21	√	√	√	[101]	[102]	[103]
Citrate synthase, mito	CISY	Pace-making enzyme in the first step of the tricarboxylic acid cycle (TCA)	12q13.2			√	[104]		
Complex I-75 kDa	NDUS1	Core subunit of the mitochondrial membrane respiratory chain, required for catalysis	2q33-q34		√	√	[105–107]	[107, 108]	[107]
Malate dehydrogenase	MDHC	Catalyzes the oxidation of malate to oxalacetate, involved in TCA, gluconeogenesis	2p13.3	√	√	√	[11, 78, 105, 109–112]		
Phosphoglycerate kinase 1	PGK1	Major ATP-generating enzyme in glycolysis, reversed reaction in gluconeogenesis	Xq13.3			√	[113]		[114]
Triosephosphate isomerase	TPIS	Glycolytic enzyme, seems to act as a polymerase alpha cofactor protein	12p13			√	[94, 113]		

(*Continued*)

Table 1. Continued

Protein Name	UP-ID	Function Summary	CL	G	T	P	Link to SZ	Link to BD	Link to MDD
Oxidative Stress									
Catalase	CATA	Protects cells from the toxic effects of hydrogen peroxide	11p13	√		√	[115, 116]		[117–120]
Glutathione peroxidase 1	GPX1	Protects the hemoglobin in erythrocytes from oxidative breakdown	3p21.3	√		√	[121–123]		[124]
Protein DJ-1	PARK7	Protects cells against oxidative stress and cell death	1p36.23			√	[104, 125]		
Peroxiredoxin-3	PRDX3	Redox regulation of the cell. Protects radical-sensitive enzymes from oxidative damage	10q25-q26						
Neuronal structure/plasticity									
Amyloid beta A4 protein	A4	cell surface receptor, plays role in neurite growth, neuronal adhesion and axonogenesis	21q21	√			[126]	[127]	
BDNF/NT-3 growth factors receptor	NTRK2	Receptor tyrosine kinase, involved in the nervous system development via regulation of neuron survival, proliferation, migration, differentiation, synapse formation and plasticity	9q22.1	√	√	√	[128–130]	[128, 131–133]	[128, 134–138]
Neural cell adhesion molecule 1	NCAM1	Cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation & outgrowth	11q23.1	√		√	[2, 139]		[140]
Neuromodulin	NEUM	Major component of the motile “growth cones”, role in axonal and dendritic filopodia induction	3q13.31			√	[141, 142]	[142]	
Neurofilament light polypeptide	NFL	Maintenance of neuronal caliber	8p21		√	√	[28]	[143]	
Transcription Factors									
Nuclear factor NF-kappa-B p105	NFKB1	Pleiotropic transcription factor, endpoint of a series of signal transduction events	4q24		√		[144]	[145, 146]	
Transcriptional activator protein Purα	PURA	Transcription activator, plays a role in the initiation of DNA replication and in recombination	5q31		√			[147]	

Abbreviations: CL, chromosome location; G, implicated at genetic level; P, implicated at protein level; T, implicated at transcript level; UP-ID, UniProt identification code.

Assays were selected based on literature findings. For references, see supplementary Table S4.

associated with cognitive and behavioral dysfunction, including increased risk taking (Ruberto et al., 2011) and startle response (Roussos et al., 2011a), decreased sustained attention (Ruberto et al., 2011; Hatzimanolis et al., 2012), impaired set-shifting (Linke et al., 2012), and increased novelty seeking and anhedonia (Athanasiu et al., 2010; Roussos et al., 2011a). Interestingly, common ANK3 risk alleles in BD and SZ have been linked to both increased and decreased mRNA expression, dependent on the brain region under investigation (Roussos et al., 2011b; Rueckert et al., 2012). The identification of ANK3 in our study further supports the pathological relevance of this gene/protein in multiple psychiatric disorders and especially in affective psychosis. Neuroimaging studies have associated ANK3 risk alleles with decreased white matter integrity in line with our findings of decreased oligodendrocyte markers in affective psychosis. BD is clinically characterized by a higher frequency of psychotic symptoms compared with MDD (Mondimore, 2005), whereas other studies have suggested an affective-psychotic continuum between MDD, BD, and schizoaffective disorder (Gershon et al., 1982).

Another interesting finding of our study was a 40% decrease in the levels of SEPT5 in SZ patients only, which was supported by the population-based model. SEPT5 belongs to a family of evolutionarily well-conserved polymerizing GTP-binding proteins that contribute to the lateral compartmentalization of membranes, cortical rigidity, and regulation of membrane trafficking (Kinoshita and Noda, 2001). Notably, SEPT5 has been colocalized with presynaptic vesicles (Beites et al., 1999; Kinoshita et al., 2000), plays a role in membrane fusion during neuronal exocytosis (Amin et al., 2008), and is critical for dendritic spine morphology (Xie et al., 2007). Decreased levels of SEPT5 in SZ prefrontal cortex may be associated with abnormal neurotransmitter secretion, for example glutamatergic hypofunction (Paz et al., 2008) and abnormal dendritic spine morphology in the prefrontal cortex of SZ patients (Glantz and Lewis, 2000; Broadbelt et al., 2002). In terms of pathophysiology, *SEPT5* is a strong new candidate gene, as it is located on chromosome 22q11.2. In this chromosomal region, heterozygous deletions comprising either 3 or 1.5 Mbp (affecting 90% or 10% of patients, respectively) cause the 22q11.2 Deletion Syndrome. The phenotype of this syndrome is variable, but characteristic signs and symptoms include learning disabilities, dysfunctional social behavior and SZ, and autism spectrum disorders. Approximately one-third of adults with 22q11.2 Deletion Syndrome develop SZ-like symptoms (Karayiorgou et al., 1995; Ivanov et al., 2003; Horowitz et al., 2005). Interestingly, 3 major SZ susceptibility genes (catechol

Table 2. Significantly Changed Proteins Identified By Label-Based LC-SRM in the SZ/CT, BD/CT, MDD/CT, MDD-P/CT, and MDD-P/MDD Comparisons

	SZ/CT			BD/CT			MDD/CT			MDD-P/CT			MDD-P/MDD		
Protein	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**
22q11.2 Deletion Syndrome															
Catechol O-methyltransferase	not significant			1.25	0.0038	0.0102	not significant			not significant			not significant		
Ran-binding protein 1	not significant			not significant			1.16	0.009	0.048	not significant			not significant		
Septin 5	0.66	7.0E-06	7E-05	not significant			not significant			not significant			not significant		
not significant in any comparison:	TCA transport protein, mito; Proline dehydrogenase 1, mito														
Receptors															
Glutamate receptor 2	not significant			1.16	0.0039	0.0102	not significant			not significant			not significant		
NMDA receptor 1	not significant			1.27	0.019	0.0418	not significant			not significant			not significant		
not significant in any comparison:	Glutamate receptor 1, Glutamate receptor 3														
Scaffolding proteins															
Ankyrin-3	not significant			not significant			1.17	0.003	0.019	0.80	4.9E-05	0.0006	0.68	1.1E-09	6E-08
Disks large homolog 4 (PSD95)	0.88	1.0E-03	0.006	1.13	0.002	0.006	not significant			not significant			not significant		
not significant in any comparison:	Shank 3														
Downstream signalling															
Calcium signalling															
CamK2α	not significant			1.12	0.0002	0.0007	not significant			not significant			0.88	0.0035	0.026
CamK2β	not significant			not significant			1.15	8E-04	0.008	not significant			0.87	0.00515	0.031
CamK2γ	not significant			1.22	2E-07	1E-06	1.21	6E-05	0.001	not significant			0.87	0.01239	0.05
PP2BB	not significant			not significant			not significant			not significant			0.83	0.00447	0.03
Calcineurin subunit B type 1	not significant			1.19	0.0052	0.012	not significant			not significant			not significant		
Neurochondrin	not significant			1.19	0.0051	0.012	not significant			not significant			not significant		
not significant in any comparison:	Calmodulin, IP3 receptor isoform 1														
Protein Kinase															
Protein kinase C α type	not significant			1.17	0.0015	0.0048	not significant			not significant			not significant		
Protein kinase C γ type	not significant			not significant			not significant			not significant			0.85	0.01158	0.05
MARCKS	not significant			1.10	0.0086	0.02	not significant			not significant			not significant		
not significant in any comparison:	Protein kinase C, β type														
ERK-signalling															
ERK1	not significant			1.11	5E-05	0.0002	1.10	0.002	0.015	not significant			0.88	0.00046	0.005
not significant in any comparison:	ERK2, PED														
mTOR															
40S ribosomal protein S4, X isoform	not significant			1.16	0.0004	0.0014	not significant			not significant			0.85	0.00853	0.043
not significant in any comparison:	mTOR, 40S ribosomal protein RS3A														
Wnt signalling															
Catenin β-1	not significant			1.21	1E-06	7E-06	not significant			not significant			not significant		
Glycogen synthase kinase-3 β	0.90	7.5E-03	0.041	1.13	0.0037	0.0102	not significant			not significant			not significant		
'not significant in any comparison	Phosphoprotein F1-20														

(*Continued*)

Table 2. Continued

Protein	SZ/CT			BD/CT			MDD/CT			MDD-P/CT			MDD-P/MDD		
	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**	Ratio	*p*	*p**
Cell-type specific proteins															
Oligodendrocytes															
CNPase	0.79	1.1E-07	2.2E-06	0.53	<1E-16	<1E-16	not significant			0.79	1.8E-05	0.0003	not significant		
Myelin basic protein	0.79	2.3E-06	2.7E-05	0.64	<1E-16	<1E-16	not significant			0.79	1.4E-04	0.0014	not significant		
Myelin proteolipid protein	0.79	2.1E-11	6.2E-10	0.61	<1E-16	<1E-16	not significant			0.82	2.1E-06	4E-05	not significant		
Myelin-oligodendrocyte glycoprotein	0.79	2.4E-12	1.5E-10	0.56	<1E-16	<1E-16	not significant			0.82	1.5E-06	4E-05	0.86	0.00131	0.011
Astrocytes															
Glial fibrillary acidic protein	0.89	1.7E-04	0.001	not significant			0.83	5E-07	3E-05	0.79	8.4E-11	5E-09	not significant		
Microglia															
Coronin-1A	not significant			not significant			0.77	4E-04	0.005	not significant			not significant		
Energy metabolism															
Citrate synthase, mito	not significant			1.17	0.0001	0.0004	not significant			not significant			not significant		
Complex I-75 kDa	not significant			1.25	0.0002	0.0006	not significant			not significant			not significant		
Malate dehydrogenase, mito	0.93	8.5E-03	0.042	1.19	1E-10	1E-09	1.11	0.002	0.015	not significant			0.91	0.01183	0.05
not significant in any comparison	ATP synthase subunit b, Phosphoglycerate kinase 1, Triose phosphate isomerase														
Oxidative Stress															
Peroxiredoxin-3	not significant			1.19	1E-06	7E-06	not significant			not significant			not significant		
not significant in any comparison	Glutathione peroxidase 1, Protein DJ-1, Catalase														
Neuronal structure/plasticity															
Neuromodulin	0.89	3.6E-04	0.003	not significant			not significant			not significant			1.17	0.0007	0.007
Neurofilament light polypeptide	0.91	6.8E-04	0.005	0.84	5E-11	6E-10	1.10	0.002	0.015	not significant			0.86	6.3E-05	0.002
not significant in any comparison:	APP A4; Neural cell adhesion molecule 1; BDNF/NT-3 growth factors receptor														
Transcription factors															
Transcriptional activator protein Pur-α	not significant			1.13	2E-05	1E-04	1.18	1E-05	2E-04	not significant			0.85	0.00016	0.002
not significant in any comparison:	Nuclear factor NF-kappa-B p105														

Abbreviations: BD, bipolar disorder; CT, controls; MDD, major depressive disorder; SZ, schizophrenia.

P-values were determined using SRMstats (fixed-subject effects) and corrected to control for multiple hypothesis testing after Benjamini-Hochberg (Chang et al., 2011). Significant findings using the mixed subject effect model of the SRMstats framework are indicated by grey shading. For reasons of clarity, only ratios and significance levels of significantly changing proteins are shown. For full information, see supplementary Table S5.

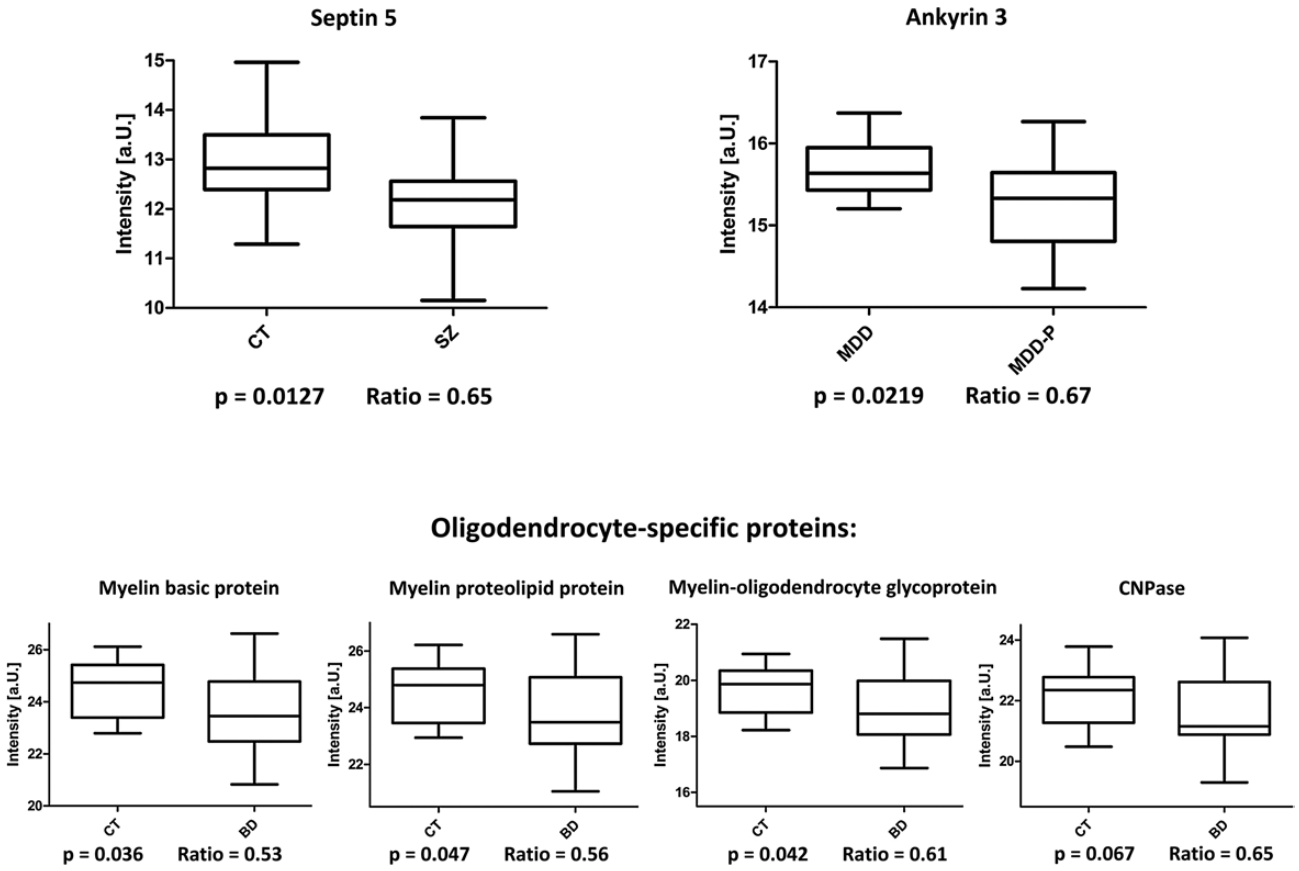

Figure 2. Box plots of the normalized SRM estimates illustrating the proteins detected as significantly changed in the expanded model. Ratios represent disease/CT or MDD-P/MDD, respectively.

O-methyltransferase, probable palmitoyltransferase, and mitochondrial proline dehydrogenase 1) are located in this chromosome region. However, it is still unclear which of the deleted genes or gene interactions contribute to the risk of developing the neuropsychiatric phenotype. The current study provides evidence that *SEPT5* may represent a novel risk gene for psychiatric disorders. As the SEPT5 protein is phosphorylated by cyclin-dependent kinase 5 to modulate exocytotic secretion (Amin et al., 2008), drugs targeting this kinase might represent a novel treatment approach for SZ. A *SEPT5* knockout mouse (Dent et al., 2002) and a patient case report of SEPT5 deficiency (Bartsch et al., 2011) demonstrated reduced dense granule secretion in platelets, suggesting that there may be a potential link of this protein to the periphery. Three independent studies showed reduced platelet-dense granule secretion in first-episode psychosis individuals and SZ patients (Yao et al., 1994, 1996; Reddy et al., 2007).

In summary, we have employed a hypothesis-driven, label-based SRM approach and generated the largest quantitative targeted proteomics data set to date in human postmortem brain tissue and specifically for neuropsychiatric disorders. We were able to confirm changes in protein levels of previously reported risk factors for psychiatric disorders and identified novel putative risk factors for SZ and affective psychosis (SEPT5 and ANK3). Testing a wide range of previously described molecular risk factors for neuropsychiatric disorders, this is the first study to follow up risk genes and validate findings at the protein and functional level. We were able to demonstrate that myelination abnormalities are prominent in SZ, BD, and affective psychosis, as implicated by overlapping changes in several oligodendrocyte protein markers. We have also shown that GSK3b and Wnt signalling is altered in BD and SZ, and CamK2 abnormalities are prominently changed in BD and affective psychosis postmortem brain, indicating potential novel drug targets.

Our study highlights the potential of SRM to analyze protein abundance levels of candidate markers of neuropsychiatric spectrum disorders in a highly quantitative manner, thus providing a high-throughput multiplex method to validate and quantify potential disease markers and drug targets. So far, targeted proteomics has been employed in only a small number of clinical studies ranging from oncology (Cerciello et al., 2013) to neurology (Jia et al., 2012) and covering a range of biofluids (Shi et al., 2013). Because of increased throughput and sensitivity compared with shotgun approaches, SRM will enable the evaluation and validation of protein biomarkers during the hypothesis-generating phase of drug discovery. Universally applicable SRM assays for disease-associated proteins will also accelerate preclinical biomarker discovery and validation studies, facilitating the transfer of pathophysiological hypotheses towards clinical application and translation.

Supplementary Material

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

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Conflicts of Interest

S.B. is a consultant for Myriad Genetics Inc and Psynova Neurotech Ltd. H.W. and M.G.G. declare no conflicts of interest.

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