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REVIEW

Technological advances for deciphering the complexity of psychiatric disorders: merging proteomics with cell biology

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Abstract

Proteomic studies have increased our understanding of the molecular pathways affected in psychiatric disorders. Mass spectrometry and two-dimensional gel electrophoresis analyses of post-mortem brain samples from psychiatric patients have revealed effects on synaptic, cytoskeletal, antioxidant and mitochondrial protein networks. Multiplex immunoassay profiling studies have found alterations in hormones, growth factors, transport and inflammation-related proteins in serum and plasma from living first-onset patients. Despite these advances, there are still difficulties in translating these findings into platforms for improved treatment of patients and for discovery of new drugs with better efficacy and side effect profiles. This review describes how the next phase of proteomic investigations in psychiatry should include stringent replication studies for validation of biomarker candidates and functional follow-up studies which can be used to test the impact on physiological function. All biomarker candidates should now be tested in series with traditional and emerging cell biological approaches. This should include investigations of the effects of post-translational modifications, protein dynamics and network analyses using targeted proteomic approaches. Most importantly, there is still an urgent need for development of disease-relevant cellular models for improved translation of proteomic findings into a means of developing novel drug treatments for patients with these life-altering disorders.

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Introduction

Over the last decade, proteomics has gone through rapid developments in many different areas. These include improvements in mass spectrometry techniques, peptide identification algorithms, biostatistics and bioinformatics applications. There is now considerable scope in applying these methods to answer important medical issues. In doing so, the advantage of proteomic methods over traditional targeted approaches lies in the unbiased nature of looking globally at cellular system dynamics in disease and healthy states. This is of interest as most medical studies focus on single protein abnormalities rather than considering the high interconnectivity of the proteome, and that the whole network dynamics might be hampered in the disease state.

There have been a number of recent advances using novel proteomic profiling methods to uncover the pathways affected in psychiatric disorders such as schizophrenia. Until recently, studies of these conditions using the long standing targeted methods have been hampered due to the heterogeneous aetiology, presumed polygenetic architecture (Stefansson et al., 2009; Group, 2011; Kim et al., 2011) and the emerging concept that these are whole body diseases which can affect not just the brain but multiple organ systems as well (Harris et al., 2012). Current hypotheses now suggest that these diseases can result from a complex interaction of genetic predisposition and environmental factors, which ultimately lead to the observed molecular alterations in the brain and other parts of the body (Caspi et al., 2003; Karg et al., 2011).

These interactions are dynamic in nature and are likely to introduce numerous proteomic alterations that converge on similar pathways. Likewise, increased risk for a particular psychiatric disorder is more likely to be conferred by the emergent properties of the pathway itself rather than by a single gene product (Sullivan, 2012). Therefore, application of multiplex proteomic profiling methods seems especially suited to elucidating affected pathways, furthering our understanding of the disease mechanisms and facilitating drug discovery in psychiatric disorders (Barabasi et al., 2011). This is also of importance for the phenomenology of psychiatric disorders which are

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increasingly coming to be considered as a continuous spectrum. The overlap of shared symptoms is likely to be manifested at the level of protein networks compared to similar genetic vulnerabilities.

Taking these factors into consideration, numerous quantitative proteomic methods have already been applied for the study of different brain regions, peripheral body tissues and fluids which have been implicated in psychiatric disorders. These studies initially involved the use of two-dimensional polyacrylamide gel electrophoresis methods, and have been followed by a variety of more in-depth mass spectrometry-based approaches. In addition, multiplex immunoassay panels have been used to investigate changes in the concentration of low-abundance proteins such as cytokines, hormones and growth factors in bio-fluids, including serum and plasma. The approach of profiling bio-fluids is most likely to lead to a biomarker signature with prognostic, diagnostic and theranostic value, as these are readily accessible and amenable for study in the clinical environment. Ultimately, proteomic bio-fluid signatures might be used to enhance our knowledge of disease mechanisms and drug actions and to derive new biomarker tests for improved diagnosis, prediction of drug response and for monitoring drug efficiency and side effects.

The aim of this review is to discuss the recent progress on proteomic and cytomic methods and their application to studies of the brain and peripheral systems. As the brain presents unique challenges for proteomic analyses due to its regional and cellular heterogeneity, as well as its obvious inaccessibility in living patients, we will also describe new approaches based on the systemic nature of psychiatric disorders to circumvent these issues. Proteomic and cytomic approaches will be described using peripheral blood cells and reprogrammed skin cells, both of which share many properties of neuronal cells of the brain. We will also discuss the potential of using such cell-based systems in combination with proteomic biomarkers as novel pre-clinical models for use in drug discovery.

Quantitative proteomic methods in psychiatric research

Current quantitative proteomics methods in psychiatric research have mainly involved the measurement of relative protein abundances between different disease and health states or the effects of different drug treatments on proteomic profiles.

The first generation of proteomic approaches employed two-dimensional gel electrophoresis (2-DE) approaches such as difference gel electrophoresis (2D-DIGE) or direct mass spectrometry-based methods for simultaneous quantitation and identification of potential protein biomarkers. These methods have been and are still widely employed in psychiatric research. However, this approach is limited in terms of the types of proteins which can be identified and the capacity for comparing large numbers of samples. To overcome these limitations, direct mass spectrometry-based methods have been developed for simultaneous quantitation and identification of potential protein biomarkers. This has been facilitated by technological advancements in mass spectrometer design and by combining electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) ion sources with ion traps or quadrupole and time-of-flight (TOF) mass analysers.

In addition quantitative capacity has been enhanced using label-free platforms such as liquid chromatography mass spectrometry in expression mode (LC-MS^E) (Levin et al., 2011), the advances of travelling wave-based ion-mobility separation coupled with mass spectrometry (Bond et al., 2013), selected reaction monitoring (SRM) (Kuhn et al., 2004) and sequential window acquisition of all theoretical fragment-ion spectra (SWATH) (Hopfgartner et al., 2012), and labelling methods including isotope-coded affinity tags (ICAT) (Gygi et al., 1999), isotope tagging for relative and absolute quantitation (iTRAQ) (DeSouza et al., 2005), and stable isotope labelling by amino acids in cell culture (SILAC) (Ong et al., 2002). Multiplex immunoassay platforms have improved in accuracy and throughput via developments using dye-containing microspheres combined with flow cytometric analysis for simultaneous identification and quantitation of analytes (Liu et al., 2005) as well as in the development of aptamer-based detection systems (Kraemer et al., 2011; Yoshida et al., 2012). Nevertheless, the approaches have mainly led to the identification of changes in overall levels of proteins, without offering novel insights into changes in function at the systems level or taking post-translational modifications into account. This is of great importance as proteins are regulated in a systems biology manner through interactions with other proteins or molecules in complex networks (Silverman and Loscalzo, 2012). The following two sections summarize the major reproducible findings of these analyses in studies of psychiatric disorders.

Summary of proteomic alterations in psychiatric research

Brain tissue profiling

In psychiatric research, proteomic tissue profiling has been predominantly used for analysis of brain regions which have been implicated in disease (Table 1). Most of these studies have led to identification of proteomic abnormalities in energy metabolism (aldolase, fructose–bisphosphate, creatine kinase, enolase, lactate dehydrogenase), oxidative stress (heat shock proteins, peroxiredoxins, superoxide dismutase), synaptic transmission (protein kinase C, inositol monophosphatase) or cell maintenance and structure (alpha internexin, neurofilaments, dynamin, glial fibrillary acidic protein, actin and tubulin) (Johnston-Wilson et al., 2000;

Table 1. Summary of brain proteomic studies in neuropsychiatric research. Included studies had at least 10 *biological replicates in the disease group*

Ref	Brain region	Samples/Disease	Method	Altered proteins	CPA	VD	Linked pathways/biological functions
[1]	PFC, BA10, gray matter	24 SZ, 23 BD, 19 MDD, 23 HC	2DE	8	NO	NO	Cytosolic and metabolism
[2]	DLPFC, BA9, gray/white matter	10 SZ *vs.* 10 SZ	2D-DIGE	215	NO	NO	Metabolism and oxidative stress
[3]	ACC, BA24, gray matter	15 SZ *vs.* 15 BD *vs.* 15 MDD *vs.* 15 HC	2DE	19	NO	IB	Mitochondrion and cytoskeleton
[4]	ACC, BA24, gray matter	10 SZ *vs.* 10 SZ	2DE	39	NO	TR	Metabolism, oxidative stress, synaptic, signalling, glial proteins
[5]	DLPFC	17 SZ *vs.* 20 BD *vs.* 20 HC	MALDI-TOF	24 peaks (21 SZ, 7 BD)	NO	NO	Cell metabolism, signalling, chaperones
[6]	DLPFC, BA9, gray matter	34 SZ *vs.* 32 BD *vs.* 30 HC	2DE	15 (SZ) +51 (BD)	NO	IB	Septin family
[7]	ACC, BA24, white matter	10 SZ *vs.* 10 HC	2DE	32	NO	NO	Cytoskeleton, metabolism
[8]	Corpus callosum	11 SZ *vs.* 10 HC	2DE	64	NO	NO	Cytoskeleton, signal transduction, metabolism
[9]	DLPFC, BA9, gray matter	10 SZ *vs.* 10 BD *vs.* 10 HC	2D-DIGE GELC-MS/MS	96 56	NO	TR, IB	Synaptic, cytoskeletal proteins and metabolism
[10]	IC, gray matter, layer 2	12 SZ *vs.* 13 HC	2D-DIGE	57	NO	NO	Neuronal plasticity, neurite outgrowth, synaptic proteins
[11]	Hippocampus	20 SZ *vs.* 20 BD *vs.* 20 HC	2D-DIGE	108 SZ +165 BD	YES	NO	Cytoskeleton, metabolism
[12]	Thalamus	11 SZ *vs.* 8 HC	ITRAQ, 2DE	41, 10	NO	WB, FA	Energy metabolism, oligodendrocytes, cytoskeleton
[13]	DLPFC	11 MDD-NP, 12 MDD-P, 24 HC	LC-MS^E	28 (MDD-NP), 36 (MDD-P), 31 (MDD-NP *vs.* P)	NO	IB, SRM, FA	Energy metabolism, synaptic function
[14]	DLPFC, BA9	10 SZ *vs.* 10 HC	LC-MS^E	34	NO	IB	Synaptogenesis, vesicle dynamics, energy buffering systems
[15]	DLPFC, BA9	10 SZ *vs.* 10 HC	LC-MS^E	53	YES	FA	Long-term potentiation, cellular assembly organization, cytoskeleton

Ref=reference; PFC=prefrontal cortex; DLPFC=dorsolateral prefrontal cortex; BA9=Brodmann area 9; BA10=Brodmann area 10; BA24=Brodmann area 24; ACC=anterior cingulated cortex; IC=insular cortex; SZ=schizophrenia; BD=bipolar disorder; MDD=major depressive disorder; HC=healthy control; P=psychotic; NP=non-psychotic; 2DE=two dimensional electrophoresis; 2D-DIGE=two dimensional difference gel electrophoresis; MALDI-TOF=matrix assisted laser desorption/ionization-time of flight mass spectrometry; GELC-MS/MS=in gel digestion in 1 dimensional gels followed by tandem mass spectrometry; iTRAQ=isobaric tagging for relative and absolute quantification mass spectrometry; LC-MS^E=liquid chromatography mass spectrometry in expression mode. CPA=computational pathway analysis, VD=Validation, IB=immunoblot, TR=technical replication, FA=functional assay, Refs: [1] Johnston-Wilson et al. (2000), [2] Prabakaran et al. (2004), [3] Beasley et al. (2006), [4] Clark et al. (2006), [5] Novikova et al. (2006), [6] Pennington et al. (2008a), [7] Clark et al. (2007), [8] Sivagnanasundaram et al. (2007), [9] Behan et al. (2009), [10] Pennington et al. (2008b), [11] Focking et al. (2011), [12] Martins-de-Souza et al. (2010), [13] Martins-de-Souza et al. (2012), [14] Chan et al. (2011), [15] Wesseling et al. (2013).

Prabakaran et al., 2004; Beasley et al., 2006; Clark et al., 2006; Novikova et al., 2006; Sivagnanasundaram et al., 2007; Pennington et al., 2008a, b; Behan et al., 2009; Martins-de-Souza et al., 2010, 2012; Chan et al., 2011; Focking et al., 2011; Wesseling et al., 2013). These proteomic alterations are more and less robust across different studies and psychiatric disorders. However, it still remains to be determined whether the effects seen on these pathways represent true disease modifications, or if they are detected due to the higher relative concentrations of such proteins in neuronal cells, thus biasing the interpretation of the findings at the pathway level. In addition, the proposed pathways fall into broad categories. One way of overcoming this problem would be to carry out bioinformatic pathway analysis using geneset enrichment analyses applied on a protein level. This could also lead to identification of shared biological functions across different studies. In addition, the low sample numbers associated with most post-mortem brain studies have resulted in uncertainty in the statistical robustness of the findings. Thus, most of the functional and pathway data inferred from such studies still require validation. Nevertheless, most of the findings from these investigations show significant convergence with candidate genes, which have been implicated in genomic (English et al., 2011) or transcriptomic (Prabakaran et al., 2004) studies of the same or related post-mortem brain samples.

There are other limitations which make interpretation of these findings difficult. Firstly, with the use of post-mortem material comes the potential of confounding effects, including differences in post-mortem intervals, time and method of storage and other variables. Furthermore, virtually all psychiatric patients are likely to have received various medications and suffered co-morbidities prior to death. Also, few studies have carried out technical validation of the findings using orthogonal proteomic methods or functional follow up studies, due to the low availability of high quality post-mortem brain tissues. We suggest that the application of multiple platforms in combination will not only provide a deeper insight into the affected protein pathways, but this will also enable cross-validation of the findings and investigation of the abnormalities in a system-based way. Furthermore, we propose that future studies employ biomaterials which can act as surrogates of brain tissue and can be obtained easily from living patients (see below). This will help to overcome the numerous confounding factors associated with post-mortem materials and should also lead to increased statistical power of the studies.

Proteomic profiling of serum and plasma

Serum and plasma have been used increasingly in proteomic studies of psychiatric disorders. The rationale for this stems from the emerging fact that psychiatric disorders are whole body diseases. The fields of endocrinology, immunology and biochemistry have shown that the brain is integrated in fundamental bodily functions, which is also reflected in changes in the composition of blood proteins and other bioactive molecules. One of the best examples is the fight-or-flight reflex (Tsigos and Chrousos, 2002) which begins with perception of danger, followed by release of a cascade of hormones such as corticotrophin releasing factor, adrenocorticotrophic hormone and cortisol. These hormones circulate throughout the body to increase blood pressure and glucose levels in preparation for the muscular actions required in the response. Other well known processes mediated through the bloodstream which can affect both brain and peripheral function include the regulation of food intake (Mastorakos and Zapanti, 2004), immune system dysfunction (Spathschwalbe et al., 1994), metabolic disorders and insulin resistance (Pasquali et al., 2006; Reagan, 2007; Solas et al., 2010). Furthermore, the bloodstream comprises a large repository of proteins and metabolites which are secreted or leaked from surrounding tissues, blood cells and organs (Anderson and Anderson, 2002; Zhang et al., 2007).

One advantage of using bio-fluids is that the majority of the constituent proteins are soluble. Therefore, the usual solubilization steps prior to proteomic analyses of tissues are not needed. However, there are other major challenges associated with their use, such as the high dynamic range of protein and peptide abundances, which span more than 10 orders of magnitude (Anderson and Anderson, 2002). Accordingly, optimized methodologies have been established to address this technical limitation using extensive fractionation (Guerrier et al., 2005; Pan et al., 2007) and depletion of the 12–14 most abundant proteins. The latter removes approximately 98% of the total protein mass (Levin et al., 2010a) and allows increased identification and quantification of larger numbers of low-abundance proteins (Liu et al., 2006; Schutzer et al., 2010). However these approaches introduce technical variability and can also lead to unwanted depletion of some proteins through protein–protein interactions with depleted high abundance proteins, as described previously (Koutroukides et al., 2011).

One way of overcoming these problems is through the use of several assay systems which offer a means of targeted proteomic profiling of several hundred analytes in serum or plasma with high sensitivity, such as the multiplex immunoassay platforms. These methods have already been successfully applied in clinical studies of various diseases, including psychiatric disorders (Chandler, 2003; Domenici et al., 2010; Schwarz et al., 2012). In these methods, the samples are added to red/infrared dye-coded microspheres containing covalently bound antibodies that target specific proteins. After subsequent incubation with a secondary antibody with a covalently-bound fluorescent label, the mixtures are passed through a flow cytometry instrument for identification of the coded antibody-microspheres and quantitation of the bound analytes. This method has the

advantage of high sensitivity, high throughput capacity and ease of use in the clinic.

Most of the mass spectrometry-based analyses of serum and plasma from psychiatric patients have resulted in identification of high abundance proteins involved in molecular transport, including apolipoproteins, ferritin and transthyretin, and the clotting cascade, such as complement components (Fleming et al., 2009; Levin et al., 2010b; Jaros et al., 2012; Li et al., 2012). In a complementary manner, the use of multiplex immunoassay platforms allows analysis of molecules such as hormones, growth factors and cytokines, which are lower in abundance. Two separate groups have used this platform for profiling plasma (Yang et al., 2006; Levin et al., 2010b) and serum (Schwarz et al., 2010, 2012; Guest et al., 2011) from schizophrenia patients and controls, and found common changes, including effects on brain-derived neurotrophic factor (BDNF), acute phase response proteins, insulin, prolactin and growth hormone.

However, considerable further work is required to maximize the impact of these findings, as none of these potential serum biomarkers have been developed for routine use in the clinical or pharmaceutical company environments. The following sections indicate novel technological approaches which may help to achieve better translation of proteomic findings into clinical use, or for applications in drug discovery and development.

Innovative approaches

Phosphoproteomics

Post-translational modifications such as phosphorylation are critical for altering the activity, cellular localization, turnover and interaction of proteins. For example, changes in the phosphorylation levels of the cAMP response element-binding protein have been observed in patients who respond to psychiatric medications compared to non-responders (Koch et al., 2002). Improvements in mass spectrometry methods have now made it possible to identify thousands of phosphorylation sites on proteins with high precision (Huttlin et al., 2010), and recent data suggest that more than half of the proteome might be regulated by phophorylation and de-phosphorylation cycles (Lemeer and Heck, 2009).

Multidimensional liquid chromatography (MDLC) mass spectrometry methods have been employed in 'bottom-up' workflows (Fig. 1). These methods combine prior enrichment of phosphopeptides or phosphoproteins using strong cation exchange (SCX) (Ballif et al., 2004), hydrophilic interaction chromatography (HILIC) (Albuquerque et al., 2008; McNulty and Annan, 2008), electrostatic repulsion liquid chromatography (ERLIC) (Alpert, 2008) and strong anion exchange (SAX) (Nuhse et al., 2003). Common methods for phosphopeptide enrichment include chemical affinity tag derivatization, selective chromatographic enrichment of phosphopeptides or the application of linked-scan mass spectrometer acquisition methods relying on diagnostic ions specific to phosphopeptides. Chemical derivatization techniques exploit the reactivity of the phosphate functional group. For example, beta-elimination of phosphoserine and phosphothreonine, yielding dehydroalanine or beta-methyldehydroalanine, can be induced by high pH conditions. The products are then modified by chemical addition of affinity tags (Oda et al., 2001) or stable isotopes (Goshe et al., 2002) for quantitation purposes.

Chromatographic methods for phosphopeptide enrichment include immobilized metal ion (e.g. Fe^{3+} (Posewitz and Tempst, 1999; Villen and Gygi, 2008), Ga^{3+} (Posewitz and Tempst, 1999), Zr^{4+} (Zhou et al., 2006), Ti^{4+} (Zhou et al., 2008)) affinity chromatography (IMAC), TiO2-based phosphopeptide enrichment (Pinkse et al., 2004), phosphotyrosine immunoprecipitation (Rush et al., 2005) and soluble polymer-based phosphopeptide enrichment. More advanced technologies include the use of Ti^{4+}-based IMAC enrichment (Zhou et al., 2011) or immunoprecipitation of peptides containing the target sequences of specific kinases (Moritz et al., 2010). The Zr^{4+}/Ti^{4+} IMAC approaches use a phosphate group as the coordinating ligand, which confers higher specificity compared to traditional metal oxide and Fe^{3+} IMAC approaches (Zhou et al., 2006, 2008). Most remarkably, a recent direct comparison showed that a novel Ti^{4+}-based IMAC approach was superior to other methods by enabling identification of around 5000 unique phosphopeptides from 400 μg of HeLa cell lysate digest (Zhou et al., 2013). This approach holds great potential to provide greater insight into alterations affecting phosphorylation cascades in neuropsychiatric research (Martins-de-Souza et al., 2011).

Although there have been considerable advances in the development of phosphoproteomics techniques there are still limitations that need to be considered. Sample preparation is often complex and requires relatively large quantities, and the correct interpretation of phosphorylation dynamics always requires normalization by protein expression changes (Wu et al., 2011) In addition, the scientific community still have not reached a consensus regarding standardization of phosphoproteomic data and the approaches needed for data gathering, analysis, storage and sharing. Finally, appropriate follow-up experiments are required to ascertain the functional significance of identified phosphorylation sites.

Problems can be addressed by targeted phosphoproteomic analysis using MRM coupled with automated sample preparation methods. This has shown promise for improving sensitivity and throughout. The future development of such MS-based assays could enable this technique to become an alternative approach in clinical applications when antibody reagents are not easily generated.

Although mass spectrometry methods are useful for phosphoproteomic investigations, a recent study used a

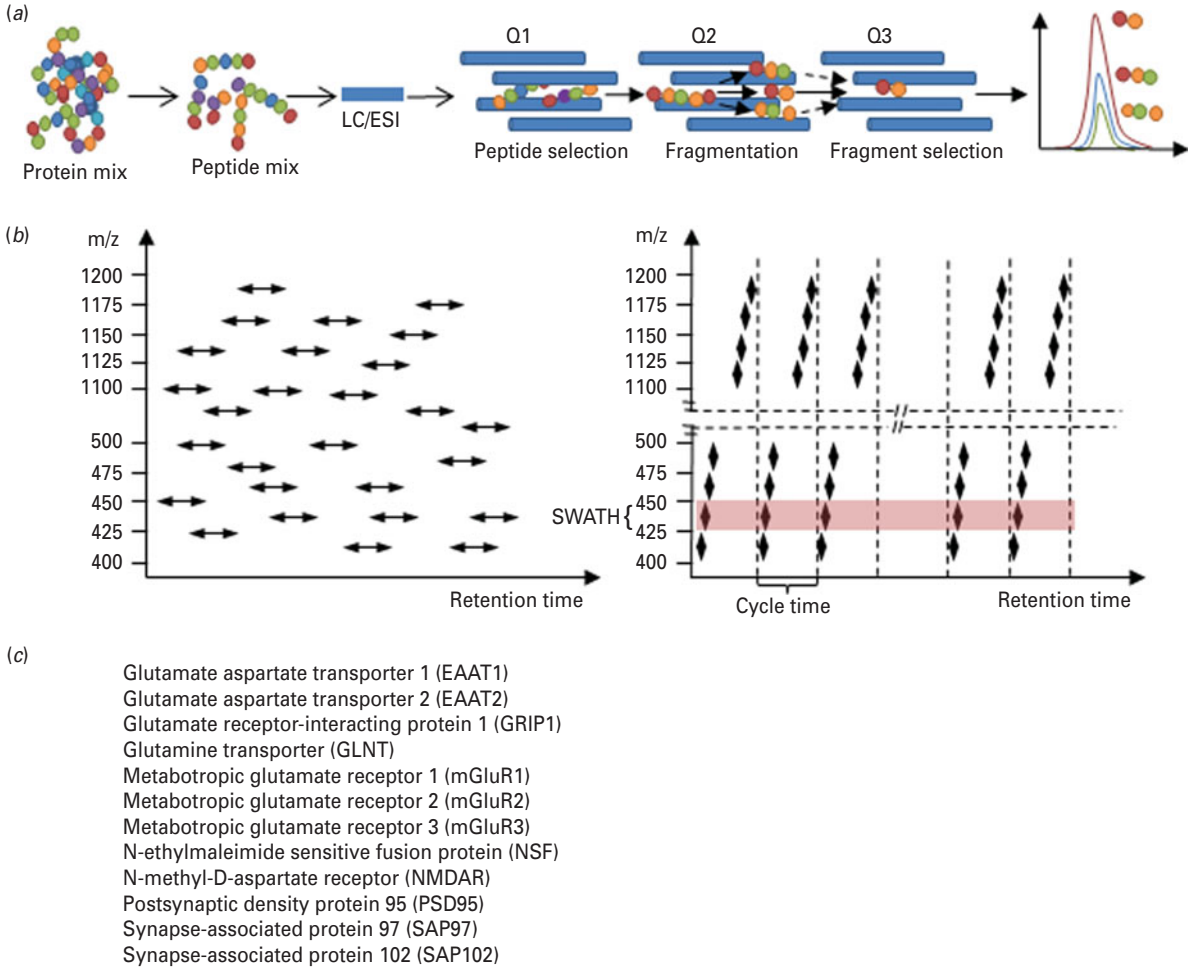

Fig. 1. (*a*) In SRM, a triple quadrupole MS filters selected predefined mass-to-charge (m/z) values corresponding to intact and fragment ions of the peptide. The second quadrupole serves as a collision cell. (*b*) Comparison of SRM and SWATH-MS data-independent acquisition. Left: SRM monitors unique combinations of multiple peptide and fragment ions in specific time windows (horizontal black arrows). Right: The SWATH-SRM method involves consecutive acquisition of high resolution, accurate mass fragment ion spectra during the entire chromatographic elution (retention time) range. It repeatedly steps through discrete precursor isolation windows of 25 Da width (black double arrows) across the 400–1200 m/z range. The series of isolation windows acquired for a given precursor mass range is referred to as a 'swath' (red shading). (*c*) SRM and SWATH-MS approaches can be used to simultaneously investigate multiple components of a single protein network. The example shows the targets in the glutamate receptor signalling pathway, which is known to be affected in schizophrenia.

phospho-specific flow cytometry technique as an alternative for identification of disease-associated signalling abnormalities (Krutzik and Nolan, 2003; Perez and Nolan, 2006). In this method, cells derived from patient and control samples were stimulated together to activate intracellular signalling cascades. Subsequently, the cells are fixed with paraformaldehyde to freeze the signalling events for analysis and then permeabilized for staining with fluorescently-labelled antibodies specific for cell surface markers or for the phosphorylated forms of specific signalling proteins before flow cytometry analysis.

Selected reaction monitoring

A current bottleneck in the discovery of protein biomarkers for disease is the development of suitable methods for validation. This is critical before too much time and money are invested in biomarker candidates which turn out to be non-reproducible. Thus far most studies have used antibody-based methods such as immunoassays and Western blot analyses for confirming the results of proteomic profiling studies. However, these methods require the availability of specific antibodies, which is not always a viable option.

Over recent years, a 'bottom-up' liquid chromatography SRM mass spectrometry approach has emerged which aims to overcome this bottleneck for targeted quantification of protein biomarker panels. This method is already in use for quantitation of low molecular weight analytes (<1000 Da) in pharmaceutical, clinical and environmental applications (Gergov et al., 2003). However, it has recently been optimized for peptides and is being

used increasingly as a targeted mass spectrometry method to determine relative and absolute protein levels in biological samples (Anderson and Hunter, 2006; Keshishian et al., 2007, 2009). SRM experiments are typically run on triple quadrupole mass spectrometers. The first quadrupole (Q1) is used to scan and filter ions, while the second quadrupole (Q2) is used as a collision cell to fragment the peptide and to transmit ions to the third quadrupole (Q3), where further scanning and filtering occurs. Transitions of the precursor and fragment ions in Q1 and Q3, respectively, can then be selected, which gives SRM a high dynamic range, accuracy and sensitivity for peptide detection in complex samples compared to traditional approaches (Addona et al., 2009). A number of targeted SRM assays have been developed which can analyse up to 100 proteins in a single experiment. For example, SRM was used for measurement of 67 putative cardiovascular disease biomarkers over the concentration range of 100 ng/ml to 41 mg/ml (Domanski et al., 2012). Absolute quantitation can be achieved by incorporation of synthetic stable isotope-labelled standard peptides spiked into the samples designated for analysis. The low development costs, multiplexing capability and high sample through-put of SRM should help in the verification and validation stages of the protein biomarker pipeline and provide a potential platform for clinical use. However, it should be noted that immunoassay approaches can still outperform SRM methods in terms of dynamic range and sensitivity of protein biomarkers in serum and plasma. Thus, a major step forward would be an increase in the sensitivity of SRM-based assays and a move towards more user friendly configurations to facilitate ease of use in the clinic.

In attempts to improve the lower limit of the detection range of SRM, techniques such as stable isotope standards, capture by anti-peptide antibodies (SISCAPA) and high-pressure high-resolution separations with intelligent selection and multiplexing (PRISM) (Whiteaker et al., 2010; Shi et al., 2012) have been developed. Both these methods use immune-affinity isolation of the targeted peptides to enhance sensitivity. In addition, a novel targeted data analysis strategy has emerged which allows consistent and accurate quantification of proteomic data produced in SRM experiments by mining the complete fragment ion records generated during data-independent acquisition (Gillet et al., 2012; Hopfgartner et al., 2012). This alternative method is called sequential window acquisition of all theoretical fragment-ion spectra (SWATH) mass spectrometry. In this technique, sequential precursor ion windows can be recorded over the entire chromatographic range to collect the same spectra of precursor and fragment ions over a defined collision energy range (Fig. 1). The resulting high-specificity fragment ion maps can be queried for the presence and quantity of protein targets using *a priori* information contained in spectral libraries containing fragment ion signals, their relative signal intensities and chromatographic concurrence. This offers the advantage of increasing the potential number of peptide targets 10-fold in a single mass spectrometry run compared to standard SRM approaches, and circumvents the tedious manual development of SRM assays.

Further development of these methods to investigate multiple components of protein networks should help to advance our knowledge in the systems biology nature of diseases such as schizophrenia. For example, a number of studies have indicated effects on myelin (Walterfang et al., 2011) and oligodendrocyte (Edgar and Sibille, 2012) function in schizophrenia. This could be investigated further in studies of post-mortem brain tissues from schizophrenia patients and as multiplex readout in studies of preclinical models by developing SRM panels targeting multiple components of these pathways. In this case, this could include assays for myelin proteolipid protein, myelin basic protein, myelin-associated glycoprotein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Fulton et al., 2010).

Cell based models

The development of novel drugs for psychiatric illnesses has come to a standstill due to difficulties of classifying symptoms and an inadequate understanding of the affected molecular pathways in patients. Moreover, a high drug attrition rate has resulted from a current focus on pathophysiologies identified in animal models, which are not readily translated to the human disease. Recent studies indicate that data with higher translational relevance can be obtained using biological samples such as blood serum and cells, which can be obtained directly from patients. Serum contains molecules such as hormones and cytokines, which can act as molecular readouts of brain function, and peripheral blood cells (PBMCs) express important targets which are found in the brain including neurotransmitter, hormonal and cytokine receptors, and the corresponding signalling pathways (Gladkevich et al., 2004). Previous studies have shown that PBMCs can be used for identification of biomarkers related to altered energy metabolism in first-onset antipsychotic–naïve schizophrenia patients and healthy controls (Herberth et al., 2011). The main objective is now to test such cells as potential novel screening platforms for drug profiling, using reporter systems for activation of receptor signalling cascades. The functional responses measured using this cell-based system include calcium flux, phosphorylation of signalling cascades, mitochondrial membrane potential, receptor and transporter expression/internalization, GPCR ligand binding, apoptosis, oxidative stress, proliferation and cell cycle properties (Valet, 2006). All of these processes are known to be affected in schizophrenia and bipolar disorder (BD). For example, disease signatures can be identified by comparison of specific protein kinase signalling

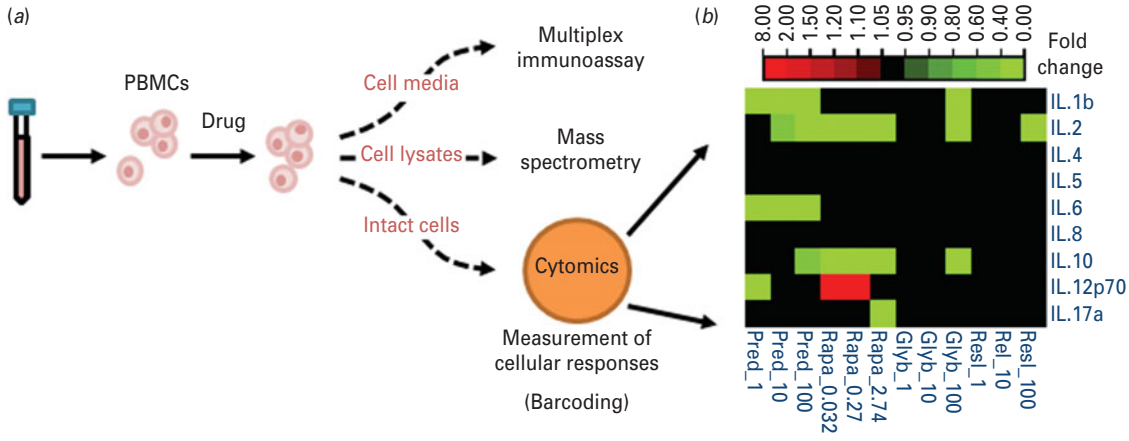

Fig. 2. (*a*) General scheme to explore functional aspects of drug effects on cells using a combination of proteomic (mass spectrometry and multiplex immunoassay) and cytomic methods. (*b*) Generation of cellular barcodes showing impact of different drugs and doses (*x*-axis) on cytokine response (*y*-axis) in PBMCs isolated from four control subjects (unpublished findings). Drugs were tested under stimulated conditions using SEB/anti-CD28/LPS. Only significant changes ($p<0.05$, Wilcoxon rank-sum test) of at least 5% are shown. The colours correspond to fold changes as shown in the legend. Black indicates that respective hit was not available (NA), not significant (NS) or FC was too low to have relevant biological effect (FC<5%). Comparison of barcodes from control subjects and psychiatric patients can give a new insight into the affect pathways and also identify potential drug targets for development of novel pharmaceutical treatments. Pred=prednisolone; Rapa=rapamycin; Glyb=glybenclamide; Res=resveratrol.

cascades using PBMCs from psychiatric patients and controls after addition of control drugs. Testing can also be carried out after addition of current psychiatric medications or potential novel therapeutic approaches including anti-inflammatory, anti-diabetic and anti-oxidant drugs (Fig. 2). The resulting activation patterns can then be considered as a functional barcode which can be used to stratify patients with respect to diagnosis, prognosis, treatment response and side effects. Likewise, screening for novel targets will be possible with this system. Taken together with traditional proteomic signatures obtained by LC-MS^E analysis of cell lysates, novel phosphoproteomic approaches and multiplex immunoassay profiling of cell supernatants, this could lead to a preclinical model with companion biomarker read-outs for use in studies of psychiatric disorders and in the discovery of new drug targets and medications.

Another potential model which can be obtained directly from living patients is functional neuron-like cells from reprogrammed fibroblasts. This is achieved by introduction of key transcription factors into fibroblasts to produce induced pluripotent stem cells (iPSCs) which can be differentiated into neuronal cells (Marchetto et al., 2010; Qiang et al., 2011; Israel et al., 2012). A proof-of-principle study generated iPSC-derived neurons from schizophrenia patients with a disrupted in schizophrenia 1 (*DISC1*) mutation, and found that these cells recapitulated features found in schizophrenia, such as reduced neuronal connectivity, reduced outgrowths from soma and reduced post-synaptic density 95 (PSD95) levels relative to controls (Brennand et al., 2011; Chiang et al., 2011). Interestingly, the gene expression data indicated effects on pathways which have not been described previously in schizophrenia, including notch signalling, cell adhesion and Slit-Robo-mediated axon guidance. Pedrosa and co-workers generated iPSCs from three schizophrenia patients and reported that the resulting neurons expressed a number of transcription factors, chromatin remodelling proteins and synaptic proteins relevant to schizophrenia (Pedrosa et al., 2011).

It is likely that iPSC-derived neuronal cells from psychiatric patients and controls can also be profiled using the combined proteomics and cytomics approach described above. However, in the case of the derived neuronal studies, other cellular processes such as differentiation can be investigated to potentially shed light on hypotheses regarding schizophrenia as a neurodevelopmental disorder (Piper et al., 2012).

Mass cytometry

There have been numerous studies using flow cytometry methods in the study of psychiatric diseases (Baier et al., 2009; Brito-Melo et al., 2012; Muller et al., 2012). Recently a rapid quantitative cell-counting method for frozen unfixed post-mortem brains using a flow cytometer was developed (Nihonmatsu-Kikuchi et al., 2011). Using this approach, the authors were able to count stained nuclei and measured their sizes in frontopolar and inferior temporal cortices from patients with schizophrenia and BD. Overall, this provided simple means of rapid cell-counting for quantifying the densities of neurons, oligodendrocytes, astrocytes, microglia and endothelial cell nuclei comprehensively. A newly-developed technology, called mass cytometry, combines fluorescence-based flow cytometry with inductively coupled plasma

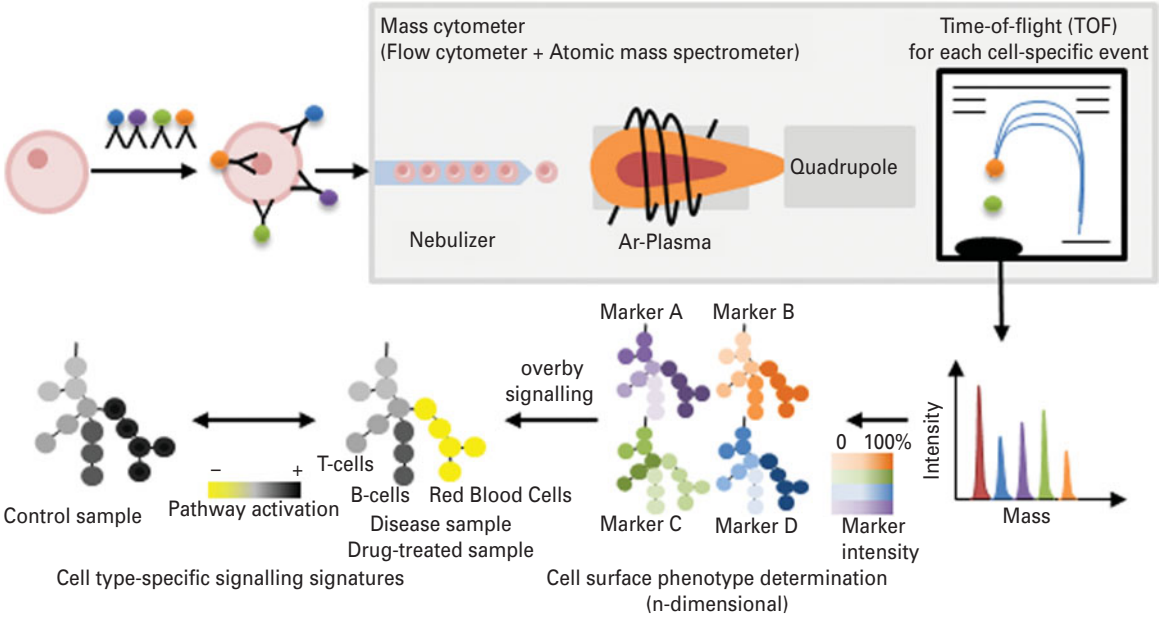

Fig. 3. Mass cytometry enables high-dimensional immuno-phenotyping of signalling behaviour in single cells. Antibodies coupled to distinct, stable transition element isotope conjugates are sprayed as single-cell droplets into inductively-coupled argon plasma at 5500 K to vaporize each cell and ionize the atoms. Resulting elemental ions are sampled by MS-TOF and quantified, enabling measurement ~1000 cells/s. The approach is able to discriminate between cell types and analyse intracellular signalling pathways in response to treatment. Data can be subjected to unsupervised cluster analysis (SPADE), which identifies distinct phenotype populations and determines the relationships based on nearest neighbour populations. Spade plots are shown representing the expression of specific markers across all clusters. The plots for associated markers can be overlaid to create plots to visualize pathway activation.

time-of-flight mass spectrometric analysis of single cells. This method makes use of transition element isotopes as chelated antibody tags for target epitopes on and within cells (Fig. 3). The method enables the simultaneous measurement of 34 cellular parameters instead of the 6–10 parameters normally obtained using standard flow cytometry platforms (Perfetto et al., 2004). This is due to the fact that mass cytometry is affected less by interference from spectral overlap compared to standard flow cytometry approaches (Lou et al., 2007; Bandura et al., 2009).

This method has been successfully employed for measurement of 34 parameters in cells derived from healthy human bone marrow, resulting in a system-wide view of normal human hematopoietic and immune signalling following *ex vivo* stimulation and inhibition using various compounds (Bendall et al., 2011). This facilitated identification of cell-specific signalling phenotypes of drug action which could be mapped to specific pathways. The resulting dataset of bone marrow cells captured snapshots of the cell types and corresponding regulatory signalling responses present throughout development from early progenitors to lineage-committed cells. Given that this technology allows determinations of up to 100 parameters per cell (Ornatsky et al., 2010), it should help to increase our understanding of cell type-specific signalling responses in complex networks such as the immune system (Bandura et al., 2009). The method also helps to overcome some of the existing challenges in flow cytometry with regards to spectral interference, fluorescent dye quenching and autofluorescence. Although there is still room for improvement due to low sensitivity and inadequate availability of antibodies, the advantages include high multiplicity of biomarker detection, absolute quantification, absence of detection channel overlap, no sample matrix effects, simplified measurement protocols and lower sample and reagent consumption. These factors should help to revolutionize the use of flow cytometry methods in psychiatric research by leading to the identification of system-wide views of abnormal signalling in humans suffering from these disorders. Furthermore, the methods could also be applied to studies of the disease and psychiatric drug mechanisms of action using PMBCs or iPSC-derived neurons, as described above.

Subcellular proteomics

Current proteomic techniques normally look at the proteome at specific endpoints as in post-mortem brain studies. Although such studies are valuable, psychiatric disorders are considered to be neurodevelopmental disorders and thus studies over distinct time frames could lead to novel insights into the aetiologies. The use of novel cellular models, such as patient-derived iPSC and

neuronal cells mentioned above, makes it possible to investigate differences in the levels of proteins and their subcellular location during the differentiation process. This is also of interest in other fields of medicine, since protein dynamics and localization can determine cellular function (Schurov et al., 2004; Dranovsky and Hen, 2007; Mackie et al., 2007).

Approaches studying the subcellular distribution of proteins include the purification of specific organelles and characterization of their protein compositions. A previous fractionation study investigated the protein composition of human nucleoli over a series of time points following various drug treatments (Andersen et al., 2002). Although organelle-based approaches provide valuable information about specific subcellular compartments in isolation, methods have now been developed to obtain a system-wide view of proteome dynamics. With this in mind, a stable isotope labelling of amino acids in cell culture (SILAC) -based approach has been developed for quantifying cellular subproteomes and for measuring the dynamics of proteome translocation in response to stimulation. The method involves subcellular proteomic comparison of parallel cell lines grown on different SILAC isotopic label-containing media. The ratio of SILAC labels for each peptide then reflects the relative levels of the corresponding protein in each compartment. In the case of the above study, the authors were able to identify proteins which were translocated in response to p53-dependent DNA damage (Boisvert et al., 2010). Another spatial proteomic method has combined subcellular fractionation with pulse-SILAC to measure the synthesis, degradation and turnover rates of proteins (Boisvert et al., 2012). Such methods could also be applied to obtain information about abnormalities in neuronal differentiation and synaptic dynamics in patient-derived differentiated neurons or in whole brains of animal models.

Another approach for investigating subcellular proteomic changes is matrix assisted laser desorption/ionization–time of flight (MALDI-TOF) MS imaging. This method allows investigation of the cellular distribution of proteins, peptides, lipids, drugs and metabolites in intact tissue sections. It provides important insights into biological processes since the native distribution of various proteins are minimally disturbed and histological features remain intact throughout the analysis (Seeley et al., 2011). Various forms of MALDI-TOF MS imaging have already been successfully applied to characterize the expression of proteins and other organic biological compounds in diseased and normal brain slices (Stoeckli et al., 2001; Todd et al., 2001; Coughenour et al., 2004). The approach can also be used for detection of pharmaceutical compounds in tissues, thereby providing target information (Reyzer et al., 2003; Khatib-Shahidi et al., 2006; Hsieh et al., 2007). This approach has been used to identify changes in protein expression in neurodegenerative disorders including Parkinson's disease (Pierson et al., 2004) and Alzheimer's disease (Stoeckli et al., 2002). The MALDI-TOF MS imaging approach also allows high-resolution single-cell analysis and the combined application of mass spectrometry scanning can be used in a discovery mode to identify new protein constituents of organelles and to determine how cells respond to external cues such as different drug treatments (Stoeckli et al., 2002). This could be particularly useful in studies of the targets of psychiatric medications, as the mechanisms of action of these drugs have not been fully elucidated.

Future perspectives

This review has described recent advances using proteomic biomarkers for increasing our understanding of the molecular nature of psychiatric disorders. The ultimate goal is to improve translation of preclinical findings to clinical studies to enable development of improved treatment strategies. One of the most important phases of this endeavour is the validation of proteomic findings using separate samples and through the use of orthogonal technologies such as SRM combined with SWATH mass spectrometry. Using such methods it will be possible to investigate effects on whole cellular pathways, such as glutamate, serotonin and dopamine signal transduction, which have been implicated in psychiatric disorders. With this in mind, this review has discussed how it is also important to associate biomarker changes with functional read-outs based on whole cell analyses as the most critical form of validation. Studies at the level of whole cell biology can help to provide insights into systems biology. This could lead to a more integrative view of the perturbed biological pathways, which are now thought to affect many organ systems throughout the body. In support of this, proteomic alterations have been identified in cerebrospinal fluid (Bartolomucci et al., 2010), serum (Guest et al., 2011; Schwarz et al., 2012), plasma (Domenici et al., 2010), fibroblasts (Wang et al., 2010) and peripheral blood cells (Freudenreich et al., 2010; Herberth et al., 2011) from living patients and from post-mortem pituitary tissues (Krishnamurthy et al., 2012). In addition, this review also described how the combined use of subcellular analyses with proteomic profiling can lead to insights into effects on translocation of proteins between different cellular compartments in disease. Most importantly, better translation of proteomic findings to the clinic may be achieved using cellular models such as PBMCs or iPSC-neurons, which can be obtained directly from living patients. Analysis of these cells using cytomic platforms can lead to functional barcodes depicting changes in the state of cells in disease or in response to drug treatment. In turn, this should lead to development of novel therapeutic targets for drug development and to the individualization of treatment approaches, thereby increasing the chances of positive therapeutic outcomes.

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Statement of Interests

PCG and SB are consultants for Myriad-RBM.

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