Proteomics and Schizophrenia: The Evolution of a Great Partnership

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Abstract

The mass spectrometer is an instrument that observes particular masses of molecules of interest. Over the past century, it has grown to become a highly sensitive and robust tool in laboratorial and clinical research to identify and quantify thousands of proteins in a given sample in an unbiased manner leading to the quick rise in its use. This unbiased and high-throughput nature is extremely important in discovery-based studies, since no preset targets can be selected, as is the case with several other proteomic methods. In studying multifactorial diseases such as schizophrenia, mass-spectrometry-based proteomics has been frequently used and new improvements to the technique have been quickly taken advantage of. Over the past 15 years, mass spectrometry has evolved greatly, and with it, the proteomic analyses and data have evolved. In this chapter, a brief history of the evolution of mass spectrometry is covered along with how schizophrenia research has grown alongside this valuable methodology.

Keywords

Proteomics · Shotgun proteomics · Schizophrenia · Mass spectrometry

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1 Introduction

The continuous evolution of mass spectrometers in terms of their sensibility, velocity of ion acquisition, capacity for ion separation, and fragmentation methods has improved the quantity and reliability of the data generated by mass spectrometry (MS). One main consequence of these improvements is the spread of this methodology from laboratorial research to the biomedical field (Banerjee, 2020). Even with liquid chromatography separation, multiple peptides can coelute, and, in the case of shotgun proteomics, often all possible fragmentation transitions are measured. This causes extreme complexity of the MS spectra, demanding improvements not only in the capabilities of mass spectrometer analyzers, instrument settings, and software analysis tools but also in the methods by which samples are prepared (Wang et al., 2014). Many of these improvements include ways to minimize sample complexity and remove any possible confounding contaminants (such as salts, detergents, cell debris, etc.) from the peptide mixture; this results in less background noise during an MS analysis, a factor which suppresses the obtention of low abundant peptide ions.

Schizophrenia is a multifactorial disorder in which dysregulations in the expression of various proteins trigger the development of the disease. In this type of disorder, information is often lacking as to how the biochemical mechanisms and pathways interact to generate the pathological phenotype, as is the case with schizophrenia. Currently, no molecular target has been found to be definitive of the disorder, and its diagnosis is performed exclusively clinically. As such, finding biomarkers to assist in earlier and more confident diagnoses is a critical focus of study. Shotgun proteomics, a broad-reaching approach to proteomics, can identify qualitatively and quantitatively thousands of proteins in a single experiment, which can be a great advantage in studies without any predefined targets or with multiple targets to be investigated (Li et al., 2017). Due to this capability, shotgun proteomics is an important tool in the study of schizophrenia and other multifactorial diseases and has been proven to be much more suitable for discovery-based studies of molecular mechanisms than other proteomic methods, immunoprecipitation, antibody-dependent analyses, and multiplex techniques. There is no need for any previous knowledge about the sample or dysregulations of the biological system to be analyzed, therefore removing the need for preexisting molecular targets or protein panels to focus the investigation (Meyer & Schilling, 2017).

Several laboratories have applied shotgun proteomics to many facets of schizophrenia, discovering and investigating dysregulations in several metabolic pathways and molecular disturbances in schizophrenia. These investigations have been performed for well over a decade with *postmortem* brain tissue (Martins-De-Souza et al., 2010; Mei et al., 2006; Nascimento & Martins-de-Souza, 2015; Reis-de-Oliveira et al., 2020; Saia-Cereda et al., 2015, 2017; Velásquez et al., 2019), induced pluripotent stem cells (Brennand et al., 2011, 2015; Notaras et al., 2021; Pedrosa et al., 2011), and peripheral fluids such as blood, plasma, and cerebrospinal fluid (Campeau et al., 2021; Garcia et al., 2017b; Herberth et al., 2011; Huang et al., 2006; Jiang et al., 2003; Li et al., 2012; Liu et al., 2015; Sabherwal et al., 2016; Vasic et al., 2012; Yang et al., 2006). Information about the mechanisms and development of the disease has been mounting, implicating neurotransmission, oxidative stress, neurodevelopment, glycolytic pathways, and cell signaling, among other dysregulations. Although a considerable number of discoveries have already been made in this field, there is still much more to be done, taking advantage of new methods and technologies in mass spectrometry and combining them to obtain new insight into the bases of schizophrenia.

2 Evolution of Mass Spectrometers for Proteomics

Despite mass spectrometers having been used for over a century, J.J. Thomson's first parabola spectrograph (Thomson, 1912) and later modifi-

cations over the following decades (Kingdon, 1923; Smythe et al., 1934) came far from being able to work with macromolecules like polypeptides. This was only first achieved in 1988 by Tanaka and colleagues using matrix-assisted laser desorption ionization (MALDI) with a time-of-flight (TOF) instrument (Tanaka et al., 1988). Over the years, mass spectrometers have grown exponentially in their capabilities and applications with more complex samples. Multiple methods of ionization have also been developed, though not all are compatible with proteomics due to high energy levels that cause extensive fragmentation. Softer ionization methods such as ESI (electrospray ionization) enable clean polypeptide ionization without generating extraneous fragments, thereby preserving structural information; ESI has therefore become the most commonly used ionization method for proteomics.

Even with the capability of analyzing polypeptides, mass spectrometry was only applied to discovery-based, large-scale studies at the turn of the century with high-capacity and fast, full-scan data acquisition of the quadrupole time-of-flight (Q-TOF) instrument, which quickly gained popularity. The first of these studies that aimed to understand alterations in molecular pathways in schizophrenia were carried out by MS analysis after separation by two-dimensional electrophoresis (2-DE), whereupon the Q-TOF identified proteins of interest on the gels (Beasley et al., 2006; Behan et al., 2009; Clark et al., 2006; English et al., 2009; Johnston-Wilson et al., 2000; Martins-de-Souza et al., 2009a, b, c, d, e, 2010; Pennington et al., 2008; Prabakaran et al., 2004; Sivagnanasundaram et al., 2007). This two-dimensional separation was required due to the level of resolution available to TOF analyzers of the 2000s, restricting protein identification.

Increasingly higher resolutions were then achieved with the development of mass analyzers such as the Orbitrap – invented in 2005 – and the creation of hybrid instruments such as the QTOF (quadrupole time-of-flight) instrument, applied to proteomics in 2000, all of which led to increases in sensibility/resolution and also the velocity of the duty cycle. These improvements made it possible to isolate and subsequently fragment a larger number of ions, thereby conferring the ability to analyze more complex samples without losing information. Hybrid instruments also solved some of the shortcomings of prior instruments, such as the mechanical complexity and size of Fourier-transform ion cyclotron resonance (FT-ICR) analyzers; the low sensitivity, limited dynamic range, and (at that time) lower resolution of orthogonal time-of-flight (TOF) analyzers; and the limited mass accuracy of ion trap analyzers.

High-throughput proteomics quickly discovered the need for higher resolutions to detect parent ions with faster and more sensitive fragmentation (MS/MS) analyses, a need that was met by the linear ion trap mass analyzer. The architecture of Orbitrap hybrid instruments has filled this need and quickly becomes a crucial analytical tool in the development of the field, providing both the speed and sensitivity necessary for online liquid chromatography (LC) coupling and full MS/MS scans. With increasing accuracy and acquisition capacity, many of other mass spectrometry advances followed suit, resulting in the development of multiple new Orbitrap hybrid instruments (Eliuk & Makarov, 2015). In parallel, other methods for ion separation and isolation have emerged without using an Orbitrap analyzer, including ion mobility separation (IMS). Different from the Orbitrap analyzer, ion mobility technology does not use Fourier transformation to determinate the position of the injected ions; it takes physical properties like size and charge of each ion into account to determine how they drift through a gas under the influence of an electric field. Ion mobility technology is usually coupled with a TOF analyzer and used in data-independent acquisition (DIA) mode, providing bias-free and more reproducible data. Accompanying this evolution of mass spectrometers, proteomic studies of schizophrenia have also evolved, increasing not only the number of identified proteins but also unlocking high-throughput quantitative proteomics without the need for labels or tags (Distler et al., 2016).

3 Advantages and Disadvantages of Acquisition Modes for Proteomics

A great majority of studies using mass spectrometry-based proteomics employ shotgun approaches, as already mentioned above; digested peptides are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The first methods analyzed these molecules in a data-dependent acquisition (DDA) manner (Link et al., 1999). DDA works by selecting the most intense peptide ions from a full mass spectrum (MS) scan, also called precursor or parent ions; these peptides are then filtered for further fragmentation (MS/MS). Several MS and MS/MS parameters (e.g., mass resolution, monoisotopic precursor selection, preview mode for FT-MS master scans, and ion population) are defined by the user, allowing flexibility to decide which of these parameters will be used and which values will be selected. Despite being seemingly advantageous, at the same time, these various parameters and their broad ranges of possible values are compounding factors in the design of a DDA experiment (Chapman et al., 2014). The main advantage of this method is its high accuracy and sensibility for the selected targets; however, proteins not included in the list of the most intense peptide ions may not be acquired, resulting in a lack of data on proteins with very low abundance and short elution profiles.

DDA is juxtaposed with data-independent acquisition (DIA), in which all precursor ions within an LC-MS scan can be destined for fragmentation, independently of their intensity or charge state (Chapman et al., 2014). MSe (stylized as MS^E) is one such DIA method that was first commercialized in 2005 by Waters Corporation, in which two alternating MS scans are recorded at low and high collision energies along the full MS spectrum, generating precursor and product ion information, respectively (Bateman et al., 2002; Silva et al., 2005). The greatest advantage of DIA is the ability to have complete information about all the peaks detected in the full MS scans, increasing the chances to identify low-abundance proteins in complex samples. It is for this reason that DIA has been largely used for recent proteomics studies. Despite its benefits, the data generated by DIA in proteomics are extremely complex and difficult to deconvolute, making it challenging to correlate fragment ions with their precursor ions.

To overcome this problem, database-driven peptide and subsequent protein identification use the chromatographic elution profile to assign fragment ions to their respective precursor from the MS scan. Nevertheless, samples from biological tissues, cells, and fluids have a very complex protein profile, resulting in the coelution of peptides with similar chromatographic characteristics. In this way, fragments derived from coeluted peptides could be misassigned to multiple precursor ions present at that specific retention time, causing incorrect peptide and protein identification. This issue was solved through the application of ion mobility technology, which provides an additional dimension of separation that increases the overall system peak capacity in multiple LC-MS workflows, even allowing for the separation of isobaric precursor ions (Ogata & Ishihama, 2020; Shliaha et al., 2014; Sturm et al., 2014). The combination of DIA MSe with ion mobility was named HDMSe by Waters Corporation and has been a valuable tool in many proteomics studies (Baker et al., 2010; Fenn et al., 2009; Valentine et al., 2005).

With continuous modifications and improvements, other DIA methods have been developed to increase the reliability, sensibility, and accuracy of data acquisition. This includes SWATH-MS (Gillet et al., 2012); sequential windows are selected for fragmentation; however, this method is less sensitive than targeted methods (Gillet et al., 2012; Liu et al., 2013; Schmidlin et al., 2016). Complementary to MSe and using a windowed selection method similar to SWATH-MS is SONAR (Brannan et al., 2016). SONAR uses pre-selected windows at the quadrupole going from low to high m/z, generating a continuum scan of the precursor mass range of interest during each MS cycle. By alternating between low and high collision energies from one scan to the next, data can be collected from

both precursor and fragmented ions with lower levels of convolution, making precursor and fragment alignment easier and more reliable (Zhang et al., 2020). To date, no proteomic studies employing SWATH-MS have been performed to study the bases of schizophrenia; current applications have been limited to lipidomics (Yan et al., 2018) and proteomics (Heald et al., 2020) of antipsychotic response in schizophrenia. SONAR has also not been applied to any schizophrenia-related research at this time.

These DIA methods have allowed for a manifold increase in the use of proteomics in discovery studies, ranging from biochemical mechanisms and pathway dysregulation to biomarker discovery. Increases in other capacities have also enabled deeper and more delicate studies such as studying the immunoaffinity-depleted proteome (depletome) of serum from patients with depression and schizophrenia (Carlson et al., 2018; Costa et al., 2018; Garcia et al., 2017a), identifying 10,390 proteins in MO3.13 oligodendrocytic cells (Cassoli et al., 2017), and proteomic studies of post-translational modifications (PTMs). Due to the transient and partial modification of proteins, post-translationally modified proteins are difficult to study, requiring the sensitive equipment that years of advances have provided, along with carefully planned experimental designs.

4 Proteomics and Schizophrenia Research Evolving Together

The continued evolution of mass spectrometry has constantly allowed for better correlations and collaborative studies with other omic techniques. The main result is an increase in knowledge about the origin of schizophrenia and its various hypotheses, including but not limited to the dopaminergic, GABAergic, and neurodevelopmental theories (reviewed by Bansal & Chatterjee, 2021). Proteomics as a whole provides important information about the physiological state of a cell, tissue, or organism due to the transcriptional, epigenetic, translational, and degradative levels of regulation that control protein expression. For this reason, genetics nor transcriptomics can be considered great indicators of the current biological state. A PubMed search showed that since 2006 after the development of DIA, 546 articles have been published in the field of schizophrenia [search terms (schizophrenia) AND (proteomics)], with only 30 publications before this point, showing how proteomics has grown together with the methodology (Fig. 1).

One example of this growth in experimental complexity was as a screening of the proteomic profiles of subcellular compartments of *postmortem* tissue of the cerebellum, posterior cingulate cortex, and caudate nucleus (Reis-de-Oliveira et al., 2020). Significant alterations were found in energy metabolism, oxidative phosphorylation, neuron development, the myelin sheath, and the MAPK signaling pathway. Energy metabolism has been repeatedly implicated in studies of schizophrenia, bipolar disorder, and major depressive disorder (Zuccoli et al., 2017).

5 Induced Pluripotent Stem Cells (iPSCs) in Proteomics

One of the biggest challenges in neuropathies and psychiatric disorders is access to the tissue of interest; only *postmortem* tissue can be feasibly obtained and biofluids don't always represent the biological state of the disease (Pedrosa et al., 2011). iPSCs enable the study of mature brain cells and the neurodevelopmental processes that occur during their differentiation, which is a great boon to disorders with neurodevelopmental characteristics. In addition, iPSCs can be generated from patients with the disease or disorder in question and the influence of a patient's genetic background on the development of the disease (Marchetto et al., 2010). Over the past decade, iPSCs have proven themselves to be a highly useful model to study schizophrenia, resulting from improvements of the technique itself as well.

It is precisely modern proteomics and its discovery-based analyses and high sensitivity that have offered justifications for such complex and costly models. The high costs of time and

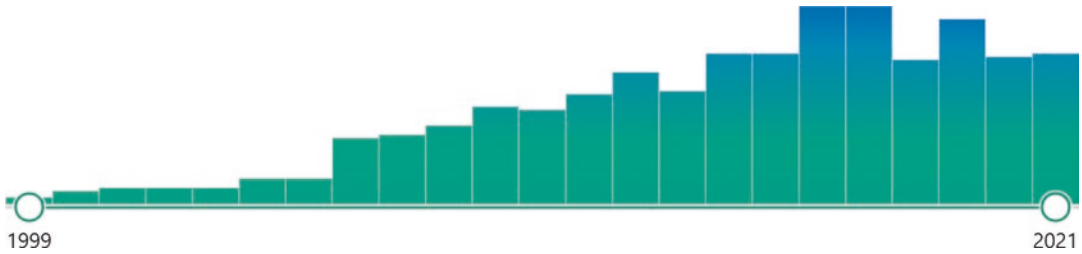

Fig. 1 PubMed search for publications involving schizophrenia and proteomics [keywords (schizophrenia) AND (proteomics)]

resources to generate iPSC models are now counterbalanced by the amount of information that can be extracted through unbiased, shotgun proteomics. Data can be analyzed and reanalyzed in multiple ways even after collection as databases and bioinformatic tools evolve. iPSC models hold great potential to continue to reveal information about the molecular mechanisms and signaling pathways that are behind schizophrenia and its symptoms and treatment. With recent discoveries involving post-translational modifications (PTMs), previously collected data can be looked at from a different point of view without the need to reacquire the data. Previous studies involving the phosphoproteome of plasma (Jaros et al., 2012), *postmortem* tissue (Saia-Cereda et al., 2016), HEK cells in vitro (Martín-Guerrero et al., 2021), and a murine model (Hwang et al., 2021), though they represent investigations into only a single of many interesting PTM targets of study for schizophrenia, have all highlighted their importance in schizophrenia research.

iPSCs also show potential in generating more personalized treatment options, unlocking the ability to test therapeutic options on the patient's own cells in a controlled environment before administration to the patient (Avior et al., 2017). As costs for generating and analyzing cells decrease over time, it becomes more feasible to apply LC-MS-based proteomics to clinical settings and applications (Smith & Martins-de-Souza, 2021). Other future applications are the use of iPSCs to create molecular signatures for a patient and creating molecular signatures for a patient for anticipating interventions and accompanying treatment (Nascimento & Martins-de-Souza, 2015).

The technique has been further extended to produce iPSC-derived brain organoids from patients with schizophrenia and 3D structures that more closely represent the in vivo state of the human body. These organoids were analyzed with isobaric labeling, whereupon 3705 proteins were identified and multiple dysregulated proteins were found in the organoids derived from patients with schizophrenia when compared to healthy controls (Notaras et al., 2021) and in iPSC-derived cerebral organoids to model the first trimester of in utero brain development (Stachowiak et al., 2017). These dysregulated proteins included neuron-related development factors GAP43, CRABP1, NCAM1, and MYEF2, as well as cell-specific factors MAP2, TUBB3, and SV2A. Such molecular findings can indicate a disruption in neurogenesis, resulting in a lower number of neurons in the affected cerebral organoids. One of the most important discoveries with iPSCs was that proteomic findings from this model are highly correlated with the dysregulated pathways that have been found in fetal tissue, reinforcing its strength as a model for molecular alterations in schizophrenia (Nascimento et al., 2019). Over the years the use of iPSCs as a model to study schizophrenia have increased, drastically expanding our knowledge about the development and molecular mechanisms of the disease (Fig. 2).

6 Conclusions

Evolutions in mass spectrometer setups have conferred higher resolution, faster acquisition, and a diversity in the methods available for a

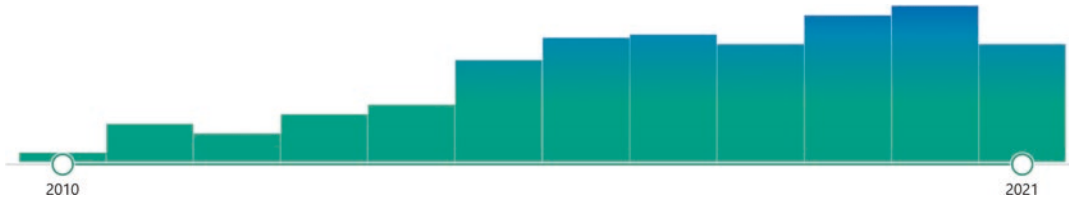

Fig. 2 PubMed search for publications involving iPSCs and schizophrenia [keywords ("induced pluripotent stem cells") AND (schizophrenia)]

given experiment. This has increased the capacity to perform high-throughput experiments with unbiased acquisition methods, resulting in an increase in robustness, reliability, and replicability of the acquired data. In turn, a broader panorama of the proteomic landscape in multifactorial diseases such as schizophrenia has been made possible, along with more complex experimental designs. This has all contributed to a better understanding of the molecular bases of the disease, its onset, its symptoms, and its treatment. Over the past decade, shotgun proteomics has grown to be extensively applied to discover new potential biomarkers and elucidate ever more subtle dysregulations in biomolecular pathways, providing insight into how to better diagnose, treat, and prevent schizophrenia, and holds great potential for even more discoveries in the coming years.

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