Advances in Experimental Medicine and Biology 1400
Proteomics, Metabolomics, Interactomics and Systems Biology

Daniel Martins-de-Souza *Editor*

Neuroproteomics as a Tool for Understanding Schizophrenia

Springer

Advances in Experimental Medicine and Biology

Volume 1400

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISSN 2730-6216 ISSN 2730-6224 (electronic)
Proteomics, Metabolomics, Interactomics and Systems Biology
ISBN 978-3-030-97181-6 ISBN 978-3-030-97182-3 (eBook)
https://doi.org/10.1007/978-3-030-97182-3

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Schizophrenia is one of the most disabling diseases of humankind, alongside with other psychiatric disorders. It affects about 20 million people worldwide, and in the last few years, more than 1 million new cases are reported every year. Schizophrenia is incurable, and most patients – generally young adults – are incapable of working, studying, and having proper relationships with their family and friends. Antipsychotic medication is the main way of managing the disease, but its efficacy is debatable: a considerable number of patients give up medication because of severe side effects in relation to poor efficacy; in general, only part of the symptoms are managed and not all patients considered good responders are able to live their lives as prior to the disease.

All these hurdles are because schizophrenia still needs to be better understood from a molecular perspective, despite all efforts in the past decades. In the post-genomic era, proteomics has emerged as a tool to depict molecular mechanisms through the large-scale analyses of expressed proteins and their post-translational modifications, enabling the quantification of differentially expressed proteins involved in a given disease and the connection of the dysfunctional biological processes and biochemical pathways. Generated data have the potential to enlighten the molecular features of schizophrenia, which could be helpful down the road for the development of innovative medication and more effective treatment.

Proteomics has been largely explored by scientists involved in schizophrenia research in the last 20 years. Several laboratories across the globe engaged on this regard, and most of these efforts are compiled here. In ten chapters, authors approached proteomic studies (and metabolomics) in postmortem brain tissue, animal, and cellular pre-clinical models and as well as in the context of treatment, discussing how classical antipsychotics and newly proposed treatments modulate protein expression in different brain cell types.

Knowledge contained in this book will help drawing new roads to be travelled from here on in the search of molecular comprehension of schizophrenia. While these are rather basic information, distant for a clinical application, they are indispensable to build strategies based in precision medicine concepts which will in turn have an impact in patients' lives.

Campinas, SP, Brazil — Daniel Martins-de-Souza

Contents

Postmortem Brains: What Can Proteomics Tell us About the Sources of Schizophrenia?

Guilherme Reis-de-Oliveira, Bradley J. Smith, and Daniel Martins-de-Souza

Contents

Abstract

Modeling schizophrenia is challenging due to the uniquely human component of psychiatric disorders. Despite several advances in cellular and animal modeling, *postmortem* brain tissue derived from patients is still one of the extremely few sources of information that comprises brain complexity, human genetics, and patient experiences. Additionally, postmortem tissue from patients with schizophrenia can be used to drive hypotheses that can then be validated in other models, involving either other animals or an in vitro approach. While evaluating high-throughput and sensitive techniques, shotgun proteomics allows for the identification and quantitation of thousands of proteins present in biological

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*, Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_1

systems. In the context of schizophrenia, proteomics can map differentially regulated proteins throughout brain regions of patients with schizophrenia, generating a large amount of information regarding the disorder's pathophysiology. In this chapter, our aim is to bring the literature up to date regarding proteomics tools applied to *postmortem* brains from patients with schizophrenia, additionally discussing new findings, roads, and perspectives for the comprehension of this severe disorder.

Keywords

Psychiatry · Schizophrenia · Postmortem · Proteomics · Systems biology · Database

1 Schizophrenia: Multifactorial Disorders Require Multifaceted Approaches

The spectrum of psychiatric disorders is composed of heterogeneous and multifactorial disorders that comprise schizophrenia, bipolar disorder, autism, and depression, among others (Adam, 2013). Within this spectrum of disorders, schizophrenia forms a pivotal category that presents psychotic and negative prodromal and residual-phase symptoms lasting for over 6 months (Lieberman & First, 2018), in addition to lifelong symptoms affecting the central nervous system, periphery, and cognition.

Studying the molecular mechanisms associated with – and potentially behind – the physiopathology of schizophrenia is particularly challenging since studying and otherwise accessing the brains of living patients are limited by ethical and practical reasons, even more so in controlled conditions. In response to this difficulty, several models have been developed to better understand the various molecular aspects of this disorder, varying from complex systems (e.g. in vivo models, using rats, mice, or other animals) to more biologically delimited systems (e.g. in vitro models, using immortalized cell lines, induced pluripotent stem cells, cultured organoids, and other similar models). Despite the advances that have been made in creating more robust models, the diagnosis of schizophrenia still relies on examining human-exclusive characteristics through interviews between psychiatrists and patients, in which the latter will describe their view of the world to reveal personal delusions, hallucinations, and other symptoms.

In addition to animal models, postmortem brains from patients with schizophrenia are complex systems that can improve our knowledge about this disorder since it allows us to obtain molecular information directly from the brains of patients that were diagnosed by specialized professionals. Postmortem brains also originated from a complete biological system and therefore present a higher degree of similarity to living patients than induced animal models or in vitro models. Overall, results from postmortem human tissues can be used to complement existing and support new hypotheses in other, more manageable, and environment-controlled models.

In order to get as much information as possible from the rare and precious samples, that is, postmortem brains from patients with schizophrenia, various "omic" tools have the potential to highlight new molecular features and pathways that can be used, respectively, in biomarker research and in drug discovery for novel treatments. Shotgun proteomics identifies and quantifies proteins in a given sample in an unbiased manner and is therefore, by definition, a powerful strategy to elucidate the molecular mechanisms of heterogeneous and multifactorial disorders. Quantitative proteomics is capable of measuring the abundance of thousands of proteins among conditions in a single experiment, enabling a deeper investigation of protein abundance, turnover, spatiotemporal distribution, post-translational modifications, and other characteristics (Bludau & Aebersold, 2020).

This approach has been extensively used by many research groups around the world that have published original articles using proteomics with postmortem brain samples from patients with schizophrenia. In 2017, our group reviewed the literature published until then and, in doing so, compiled proteomic results from studies using

postmortem brain tissues from patients with schizophrenia, bipolar disorder, and major depressive disorder (Saia-Cereda et al., 2017a). According to PubMed (accession date: 21/11/2021, searched term: "*(Proteomics) and (Schizophrenia) and (postmortem)*"), since that publication, 25 other publications have been deposited in the database. Of these, we manually filtered for original articles that carried out proteomic analyses of human postmortem brain tissues from patients with schizophrenia.

In this chapter, we updated the list of proteins differentially regulated in schizophrenia with more recently published articles. In the following sections, this newer list was used to discuss the pathophysiology of schizophrenia with a protein-centric focus, followed by a systems biology analysis of this new data. Bringing these approaches together, throughout this chapter, we aim to show the advances made in the field of schizophrenia that were brought about by postmortem brain tissues using proteomics. We also intend to show new perspectives and reveal potential paths to better understand psychiatric disorders, particularly schizophrenia, and, finally, highlight the role of and methods for sharing and integrating the knowledge that has been generated thus far.

2 Proteomics and Postmortem Tissue: News About This Ideal Union

Since our first review in 2017, seven new articles have been published using shotgun proteomics as the main tool to better understand schizophrenia in the context of postmortem brains from patients. These articles included the cerebellum (CER, (Vera-Montecinos et al., 2021; Reis-de-Oliveira et al., 2020), caudate nucleus (CN, (Reis-de-Oliveira et al., 2020)), posterior cingulate cortex (PCC, (Reis-de-Oliveira et al., 2020)), primary auditory cortex (PAC, (MacDonald et al., 2020)), corpus callosum (Cc, (Saia-Cereda et al., 2017b)), anterior temporal lobe (ATL, (Saia-Cereda et al., 2017b)), dorsolateral prefrontal cortex (DLPFC, (Martins-de-Souza et al., 2021; Zeppillo et al., 2020)), and hippocampus (HIP, (Zuccoli et al., 2021)) from patients with schizophrenia and the respective matched control groups. In addition, some of these studies carried out subcellular fractionation experiments, enriching certain organelles, such as myelin, nuclei, mitochondria, and synaptosomes. The human brain is a highly heterogeneous organ, both in function and in biological activity. For this reason, investigating variations in individual brain regions before comparing them can not only allow tracing back to upstream causes of such dysregulations but can also help visualize clusters of dysregulated proteins and pathways that might otherwise be overlooked in a whole-brain system.

In our previous report, we described 407 proteins differentially regulated in the brains of patients with schizophrenia. Joining the results from the recently published data with the original publication, we added 1159 new differentially regulated proteins in schizophrenia, now totaling 1566 proteins that have been found dysregulated in this disorder. In addition, we can see that, to date, 13 brain regions have been studied following a proteomic approach in the context of schizophrenia. In Fig. 1a, it is possible to see the distribution of regions analyzed throughout the brain, covering eight cortical regions (Fc, DLPFC, IC, PAC, WA, ATL, ACC, and PCC), MDT, CN, HIP, Cc, and CER. All these regions had been associated previously with schizophrenia, and in focused studies, it is possible to link psychotic and behavioral symptoms with protein abundance dysregulations in individual investigations.

Among all these regions, the PCC presented the highest number of differentially regulated proteins, presenting 728 proteins affected in schizophrenia (Fig. 1b). The posterior cingulate cortex has been widely recognized for its highly metabolic activity for the maintenance of default mode networks in the brain, playing a pivotal role in daydreaming and mind-wandering behaviors (Leech & Sharp, 2014). Dysfunctions in default mode networks have already been described to be related to schizophrenia, being associated with the severity of positive symptoms (extensively reviewed in (Hu et al., 2017)), as well as a reduc-

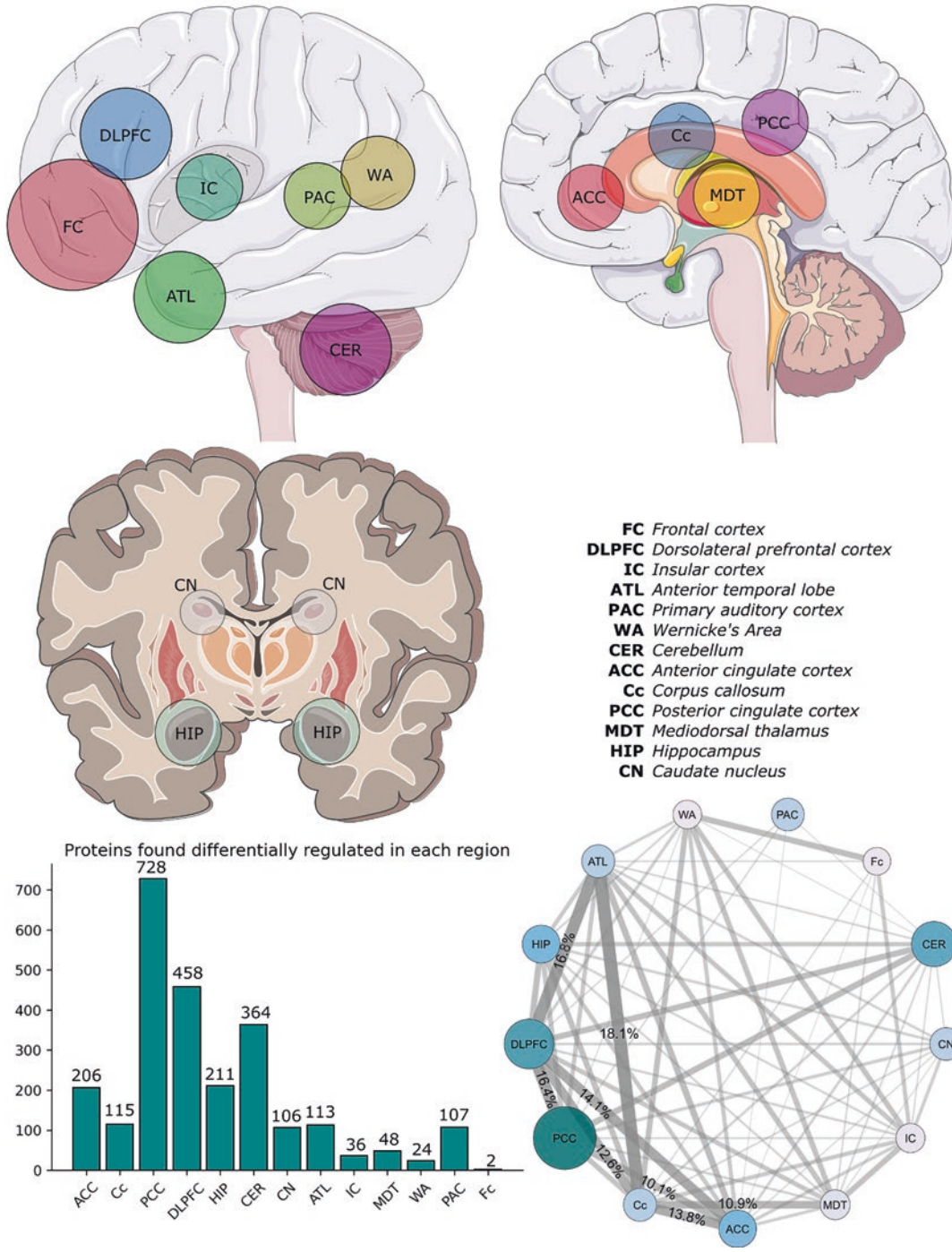

Fig. 1 *Postmortem brain regions with proteome dysfunctions in schizophrenia.* (**a**) Schematic representation of the brain showing the regions analyzed using proteomic approaches. Circles represent approximately the region reported by articles. (**b**) Number of differentially regulated proteins in each brain region according to our data. (**c**) Network of overlapping dysregulated proteins by region. Line thickness represents the percentage of proteins dysregulated between the respective regions

tion in PCC metabolism that has also been observed in these patients (Haznedar et al., 2004). In fact, the proteomic analysis of the mitochondrial fraction of PCC from patients with schizophrenia presented 47 proteins differentially regulated in the disorder, highlighting that disturbances in metabolism also can reach proteome-level dimensions.

According to our data, the second-highest dysregulated region was the DLPFC, which is one of the most studied cerebral regions in the context of psychotic disorders (Fig. 1b), presenting 458 proteins dysregulated in schizophrenia. Being part of the mesocortical pathway of dopamine, it is hypothesized that the DLPFC is related to negative and cognitive symptoms due to the reduced activity of D1 receptors in this region. In addition to this pharmacology-based theory, proteomic data reveals that proteins present in myelin and synaptosome fractions of the DLPFC are differentially abundant in patients with schizophrenia. Dysregulations in these subcellular compartments integrate another phenomenon described in schizophrenia: the disconnectivity among the brain regions.

When compared with mentally healthy control groups, patients with schizophrenia present different patterns of communication among brain regions, which is seen by magnetic resonance imaging while these patients are performing a broad range of tasks. To evaluate whether protein abundance has the potential to reflect connectivity between the analyzed regions, we crossed the differentially abundant proteins among annotated brain parts and constructed a network based on the percentage of genes shared between two regions (Fig. 1c). According to this analysis, eight interactions presented more than 10% overlap in proteins found differentially regulated among all regions, of which the Cc and DLPFC together total seven of these interactions.

The corpus callosum enables communication between the brain hemispheres, and dysfunction in this region may be one reason behind the disconnectivity reported in schizophrenia. Interestingly, the Cc shares several proteins differentially regulated with four other regions that are known to be anatomically linked to it: ATL (18.1%), ACC (13.8%), DLPFC (12.6%), and MDT (10.9%). These cortical regions and the thalamus are associated with the development of positive, negative, and cognitive symptoms in schizophrenia, potentially driven by disconnections either between these regions or in the same region, in an interhemispheric way.

Despite the differentially abundant proteins being an interesting starting point in formulating hypotheses to explain the pathophysiology of schizophrenia, the advent of the "omics" era also has enabled the consolidation of an entirely pivotal and independent area: bioinformatics. From classical statistical analyses to machine learning-based approaches, the big data generated by proteomics can be much more valuable than a simple list of differentially regulated proteins.

3 Bioinformatics: New Roads, New Perspectives

In this section, we used the proteins dysregulated in schizophrenia to perform an overrepresentation analysis (ORA). Also known as enrichment analysis, this computational experiment performs a Fisher's exact test that determines if genes/proteins from predefined sets (e.g., pathways from KEGG or Reactome) are more present than expected by pure chance in a subset of experimental data (such as proteins differentially regulated in a proteomic analysis) (Boyle et al., 2004). In the context of postmortem brains from patients with schizophrenia, this approach allows us to see how differentially regulated proteins would be affecting canonical pathways inside those living brains. Besides the unveiling of molecular pathways associated with schizophrenia, these analyses also can drive drug discovery based on these target pathways, which holds the potential to improve the efficacy and specificity of antipsychotic-based treatment.

First, we used as input the 1566 proteins of our dataset against the DisGeNET discovery platform (Piñero et al., 2017), an integrative database containing genes and variants involved in human diseases (Fig. 2a). Interestingly, the first term

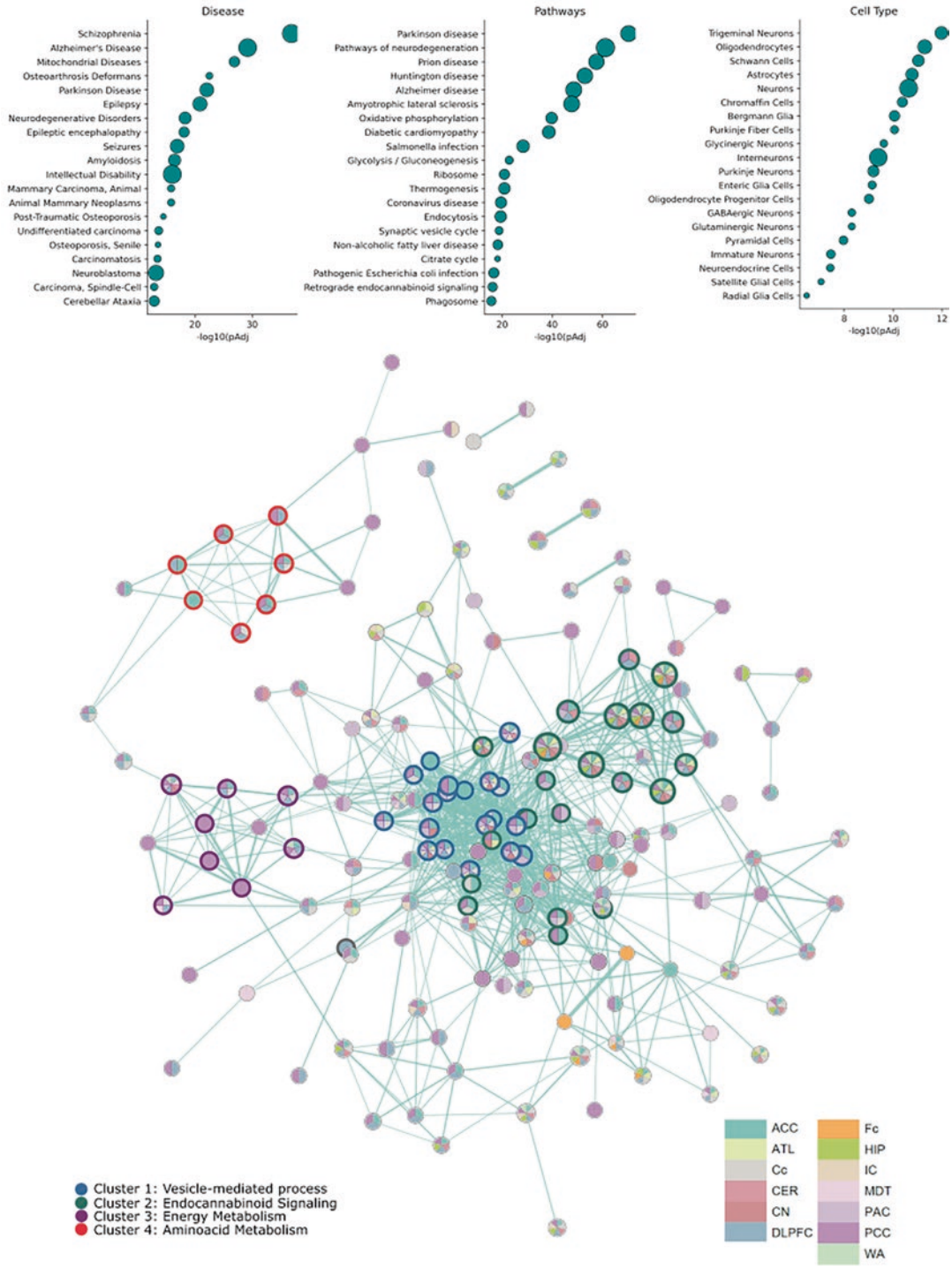

Fig. 2 *Enrichment analysis of differentially abundant proteins in schizophrenia.* (**a**) Top 20 diseases associated with our datasets, showing several neurological diseases, including schizophrenia. (**b**) Top 20 terms in KEGG that are related to our dataset, highlighting several classes potentially dysregulated in schizophrenia: neurodegenerative disorders, energy metabolism, synaptic vesicles, and others. (**c**) Top 20 terms according to ORA, carried out against Panglao DB, showing the cell types that are over-represented by the differentially regulated proteins in schizophrenia. (**d**) Enrichment map of pathways associated with our dataset, highlighting each brain region in the pie chart and respective clusters in donuts

enriched for this analysis was "Schizophrenia" (p-Adj = 1.4e-37), containing 319 genes already described for this disorder. Besides confirming the specificity of our dataset, the proteins that were not assigned for schizophrenia were instantly highlighted as potential targets to be further studied in the context of this disorder, using genetic and/or proteomic approaches. In addition, other neurological diseases were highlighted in this analysis, such as Alzheimer's and Parkinson's diseases, epilepsy, and seizures. Neurodegenerative disorders and epilepsy are known to be diseases that can lead to psychosis, and this phenotype is associated with a worsening of the patient's condition (Lieberman & First, 2018; Murray et al., 2014; Samudra et al., 2016; Clancy et al., 2014). Nonetheless, the association between schizophrenia and neurodegeneration is still under debate. In fact, a study with 329 patients with schizophrenia showed that this subgroup presented a loss of gray and white matter in an age-dependent manner (Cropley et al., 2017). These findings can be crossed with other results that showed microglia-mediated neuroinflammation in patients with schizophrenia (Bloomfield et al., 2016; Howes & McCutcheon, 2017). Microglial activation has been linked with the pathophysiology of Alzheimer's disease, being one of the key players in the induction of neurodegeneration (Hansen et al., 2018).

We also performed an ORA against the Kyoto Encyclopedia of Genes and Genomes (KEGG, (Kanehisa et al., 2021)), which is a database of canonical and manually curated pathways (Fig. 2b). When considering all the proteins from our dataset, several terms were linked with pathways described for neurodegenerative diseases, reinforcing the importance of further studies to better understand neurodegeneration and schizophrenia. Another class of pathways is related to energy metabolism, mainly the glucose catabolism processes, including glycolysis, citric cycle, and oxidative phosphorylation. Dysfunctions in energy metabolism have been linked with schizophrenia in several studies using postmortem brain tissues, animal models, and an in vitro approach (Martins-de-Souza et al., 2011a; Saia-Cereda et al., 2017a). The brain is the organ that, proportionally, has the largest consumption of glucose in the human body (Hyder et al., 2013), and changes in glucose and oxygen uptake can lead to cognitive deficits and psychosis (Hirai et al., 2021). In addition, the role of energy metabolism is not exclusive for neuronal cells. Some studies have revealed that schizophrenia and antipsychotics are able to affect energy metabolism in oligodendrocytes (Seabra et al., 2020) and astrocytes (Martins-de-Souza et al., 2011b).

While performing ORA against PanglaoDB (Franzén et al., 2019), proteins found differentially regulated in schizophrenia were enriched for glial cells, such as oligodendrocytes and astrocytes (Fig. 2c). Oligodendrocytes are the cells that make up the myelin sheath of neurons and therefore assist in the propagation of nerve impulses, as well as provide axonal energy support (reviewed in (Duncan et al., 2021). In the context of schizophrenia, oligodendrocytes were first brought onto the scene in the mid- to late 2000s with several studies reporting reduced oligodendrocyte density in various brain regions of patients (Uranova et al., 2004; Uranova et al., 2010; Vostrikov et al., 2007). During the same period and with independent cohorts, proteomics was also being applied to *postmortem* brain tissue; in these studies, links were formed between schizophrenia and dysfunctions in oligodendrocytes (Martins-de-Souza, 2010; Martins-de-Souza et al., 2009). Since then, more discoveries have been made providing evidence that both genes and proteins related to oligodendrocytes ontogeny (e.g., MBP, CNP, and MOG) have associations with schizophrenia, often being differentially expressed. Additionally, reductions in white matter and in oligodendrocytic proteins were recently reported with data from a proteomic assay using organoids derived from induced pluripotent stem cells from patients with schizophrenia (Notaras et al., 2021). Together, these data suggest that the maturation of these cells may be compromised in schizophrenia, leading to subsequent neuronal malfunctioning from myelin damage and associated axon energy deficits. It also highlights the importance of looking at schizophrenia as a multifaceted disorder that not only presents dysfunctions in classically

accepted neurons and neuronal transmission but also includes dysfunctional activity in glial cells and in energy metabolism.

To evaluate how molecular pathways can be dysregulated throughout the brain regions of patients with schizophrenia, we performed an ORA against the KEGG database with the differential proteome of each region. The results from this analysis were used as the input to construct a network, in which each enriched term is a node and the line thickness is a coefficient of similarity between the terms. Due to the complexity of this enrichment map, we also applied the MCODE algorithm to find highly interconnected regions (Bader & Hogue, 2003) in order to find clusters that help us to better explain the data for each region. In Fig. 2d, cluster 1 is composed of processes that are mediated by vesicles, such as dopaminergic synapses and long-term potentiation; and in all cases, the enriched terms were associated with the ACC, an important component of limbic systems and cognitive functions. In the context of schizophrenia, the ACC has been associated with a reduction of gray matter (Fornito et al., 2009) and with treatment-resistant psychosis (Mouchlianitis et al., 2016). Since the ACC is related to the negative and cognitive symptoms in schizophrenia, understanding the molecular mechanisms that are affected in this brain area would enable the manipulation of molecular cascades as well as the treatment of prodromal symptoms.

The second cluster identified was associated with endocannabinoid signaling, which is emerging in the field of psychiatric disorders. Cannabis abuse is one of the environmental risks associated with the onset of schizophrenia in late adolescence and the beginning of adult life (Desfossés et al., 2010). According to the Human Protein Atlas, cannabinoid receptor type 1 (CNR1) is expressed throughout the brain in humans and mice ("Brain Tissue Expression of CNR1 – Summary – The Human Protein Atlas", n.d.). Although we did not identify CNR1 as differentially regulated in this dataset, proteins enriched for endocannabinoid signaling were dysregulated in the ACC, DLPFC, PCC, PAC, and PCC. Recently, Rodríguez-Muñoz and colleagues have demonstrated that CNR1 influences the activity of NMDA receptor (NMDAr) subunits and its downstream signaling pathway (Rodríguez-Muñoz et al., 2017). NMDAr is pivotal in the theory of the psychopathology of schizophrenia, since its downregulation in parvalbumin-positive GABAergic neurons can lead to the hyperactivation of dopaminergic neurons of mesolimbic pathways (Balu, 2016). Linking the endocannabinoid system with the current theories of schizophrenia represents an interesting target for future research, mainly due to the increasing development of drugs based on cannabis compounds.

In the enrichment map, cluster 3 is mainly associated with the PCC and energy metabolism. As mentioned above, the PCC is associated with the default mode network and is highly metabolically active in daydreaming and mind-wandering behaviors (Leech & Sharp, 2014). Since this region is highly connected with other brain areas and is also associated with learning, memory, reward, and task engagement (Pearson et al., 2011), issues in metabolism within the PCC can lead to several brain dysfunctions and, consequently, behavioral changes. Proteomic data indeed reinforces that patients with schizophrenia present a disturbance in PCC energy metabolism, supporting findings aforementioned in molecular terms. Future studies with more complex in vitro models (e.g., human brain organoids) will be able to see how energy metabolism in cortical areas could affect the neurodevelopment and psychopathology of schizophrenia.

The last cluster emphasized here is cluster 4, which mainly includes pathways associated with amino acid metabolism. For each theory of schizophrenia, there is one neurotransmitter (or more) that plays a pivotal role, and at their core, all these molecules have amino acids as precursors or are amino acids in their own right. For example, while serotonin and dopamine are generated from tryptophan and tyrosine, respectively, for proper NMDAr function, glutamate (neurotransmitter), glycine, and D-serine (ligands) are all crucial (Yu & Lau, 2018). Since proteomic

data shows alterations in amino acid metabolism in some brain regions (ACC, PCC, DLPFC, and Cc), the generation and release of neurotransmitters also might be affected in these areas, which are highly connected throughout the brain. This finding is reinforced in Fig. 2b, in which "synaptic vesicle cycle" is enriched for all proteins dysregulated in the schizophrenia dataset.

Despite these aforementioned topics, regarding postmortem brains from patients with schizophrenia, this compiled information still has much information to be extracted. To achieve this goal, it is essential to create a virtual environment where this knowledge can be shared and used by other researchers worldwide. Therefore, in the next section, we intend to talk about the role of publicly available databases in the research community and how they are fundamental to any knowledge field, from proteomics to neuropsychiatric research.

4 Knowledge: Sharing and Multiplying

When shotgun proteomic samples are collected, a large amount of raw data is generated, consisting of the thousands of peptide ions and fragments that reach the detector of the mass spectrometer along the course of a sample run. Processing these data into points that are usable for downstream analyses relies on multiple parameters that have not been standardized, nor can they easily be, due to variations in the capabilities of mass spectrometers and chromatographic systems. Nonetheless, some recommendations have been made in broad sweeps by international organizations, such as the guidelines described by the Human Proteome Organization (HUPO) (Omenn, 2021). As part of these guidelines, sharing proteomic data is not just a good research practice, but it has been required during the submission process of proteomic-based studies in scientific journals.

Publicly available proteomic data are stored in repositories, such as PRIDE (the PRoteomics IDEntification database (Perez-Riverol et al., 2018)), part of the ProteomeXchange Consortium (Deutsch et al., 2017), with the intent of transparency and easy availability for future studies. Additionally, other members of the ProteomeXchange Consortium have specific purposes, such as jPOSTdb (Moriya et al., 2018), specifically dedicated to reanalyzing previously published data and iProX (Ma et al., 2019), a repository dedicated to the Chinese human proteome. Multiple challenges exist when compiling multiple datasets, however, such as variations in instruments, sample processing workflows, and chromatographic separation steps. Nonetheless, proteomic datasets can be invaluable tools for understanding dysregulations in biological pathways, especially when looking at a system from various viewpoints.

In addition to these proteomic repositories, databases have been constantly improved and updated in the most diverse areas of biochemistry and genetics, such as human protein sequences (e.g., UniProt (UniProt Consortium,, 2018)), protein-protein interactions (e.g., IntAct (Hermjakob et al., 2004)), and ontological information (e.g., Gene Ontology (the Gene Ontology Consortium, 2019)), which highlights the potential in reusing proteomic data for future analyses. Also, with advances in bioinformatic capabilities, customized databases for protein identification can now be generated using genetic information from subpopulations (Cao & Xing, 2021), such as alleles common in patients with schizophrenia.

Regarding schizophrenia in particular, a new database called SZDB (Wu et al., 2017) was published using data from genomic, transcriptomic, and methylomic data from patients with schizophrenia. In this database data from genetic, gene expression, network-based, brain expression quantitative trait loci (eQTL), and single nucleotide polymorphism (SNP) sources can be found, manually curated from schizophrenia-related research. Frequently updated, this is one of the most comprehensive databases dedicated to understanding schizophrenia. Despite this level of comprehensiveness, proteomic data from postmortem brains of patients with schizophrenia were not included, which could be a future implementation or even an incipient idea for schizophrenia databases.

5 Far Away from Conclusions

Since the first description of schizophrenia in the 1910s by Paul Bleuler (Ashok et al., 2012), we have never accumulated as much data as we have now. The increase in the amount of information regarding this disorder has arisen mainly due to the "omics" era, in which overwhelming amounts of big data have been produced and need to be interpreted to their fullest. In this context, proteomics has been used as an important tool to discover new molecular aspects of diseases and to unravel the puzzle of complex disorders such as schizophrenia. When combined with complex models such as *postmortem* brains from patients, proteomics indeed allows us to better characterize the disorder and formulate new hypotheses that can be tested in several other models or be confirmed by clinical data.

In this chapter, we compiled and updated the literature that applied proteomic approaches to study postmortem brains from patients with schizophrenia. This information was used to visualize similarities among the brain regions studied thus far, highlighting potential connections or differences among them. The associations between brain regions and potential validation of these data could be performed in the future by, for instance, functional magnetic resonance imaging (fMRI) experiments in patients with schizophrenia. These studies will be crucial to know if the findings from postmortem brains are true depictions of the disorder or are rather artifacts resulting from a limitation of postmortem brains as a model, such as the fact that patients are frequently under the effect of antipsychotics and have greatly varying demographics.

In addition, the data presented here also allow us to investigate whether the differentially regulated proteins in a patient's brain could affect non-neuronal brain cells. According to these analyses, we reinforced the role of studying glial cells in the context of schizophrenia, since these cells seem to present proteins dysregulated in these datasets. Despite many challenges, determining the role of astrocytes and oligodendrocytes in schizophrenia is essential to search for new explanations of the pathophysiology of schizophrenia and for new drugs that can target these cells during antipsychotic treatment. For this purpose, the utilization of complex in vitro models – such as induced pluripotent stem cell (iPSC)-derived astrocytes and oligodendrocytes, as well as cerebral organoids, all from patients with schizophrenia – could be interesting models for understanding the role of these players in this disorder.

Our data also presented some pathways dysregulated in the context of schizophrenia. Among these pathways, we highlighted the endocannabinoid system, energy and amino acid metabolism, and vesicle-mediated signaling. We also briefly discussed some features of schizophrenia that overlap with neurodegenerative disorders, providing further evidence of the level of complexity of schizophrenia. For all of the points raised in these discussions, animal models or cellular cultures should be employed for subsequent validations, the elucidation of molecular mechanisms, and drug discovery for the treatment of psychosis.

In conclusion, studying schizophrenia and other psychiatric disorders is a challenge that has been investigated by scientists for a long time. Despite the large theoretical framework regarding the psychopathology of schizophrenia, we still need to understand how this disorder develops throughout the patient's life and, finally, how to better treat these patients to improve their quality of life and life expectancy.

Acknowledgments GRO, BJS, and DMS would like to thank FAPESP (São Paulo Research Foundation) for funding (grants 2016/07948-8, 2017/25588-1, 2018/01410-1, 2019/25957-2, 2019/05747-3), and BJS would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES, grant number 88887.495565/2020-00).

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What Can We Learn from Animal Models to Study Schizophrenia?

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Abstract

Schizophrenia is a complex and heterogeneous neurodevelopmental psychiatric disorder characterized by a variety of symptoms classically grouped into three main domains: positive (hallucinations, delusions, and thought disorder) and negative symptoms (social withdrawal, lack of affect) and cognitive dysfunction (attention, working and episodic memory functions, and processing speed). This disorder places an immense emotional and economic pressure on the individual and society-at-large. Although the etiology of schizophrenia is not completely known, it is proposed to involve abnormalities in neurodevelopmental processes and dysregulation in the signaling mediated by several neurotransmitters, such as dopamine, glutamate, and GABA. Preclinical research using animal models are essential in our understanding of disease development and pathology as well as the discovery and advance of novel treatment choices. Here we describe rodent models for studying schizophrenia, including those based on the effects of drugs (pharmacological models), neurodevelopmental disruption, demyelination, and genetic alterations. The advantages and limitations of such models are highlighted. We also discussed the great potential of proteomic technologies in unraveling the molecular mechanism of schizophrenia through animal models.

Keywords

Schizophrenia · Animal models · Psychosis · Glutamate · Dopamine · Proteomic

1 Introduction

Schizophrenia is a chronic and debilitating mental disorder that affects around 1% of the population worldwide. Patients with schizophrenia may present various symptoms that are classically divided into three main groups: positive, negative, and cognitive symptoms. Positive symptoms include hallucinations, delusions, and thought disorders. Negative symptoms include blunted affect, alogia, anhedonia, social withdrawal, and avolition (Marder & Galderisi, 2017). Cognitive dysfunction involves deficits in working memory, attention, and processing speed and trouble focusing (Bora et al., 2010). The main strategy for the management of the symptoms relies on the use of antipsychotics. However, these drugs are not effective in treating all symptom domains (Woodward et al., 2005) and one-third of the patients do not respond to antipsychotics (Ackenheil & Weber, 2004).

Although the etiology of schizophrenia is still poorly understood, it is proposed that it arises from the interaction between genetic predisposition and socio-environmental risk factors, such as exposure to stressors and adversities, as well as cannabis abuse (Forti et al., 2019; Gomes et al., 2019). Dysregulation in the signaling mediated by several neurotransmitters, including dopamine, glutamate, and gamma-aminobutyric acid (GABA), and abnormalities in neurodevelopmental processes have been implicated in the pathophysiology of schizophrenia (Yang & Tsai, 2017).

Animal models of psychiatric disorders are valuable tools to investigate pathophysiological alterations observed in patients and new drugs with therapeutic potential (Nestler & Hyman, 2010; Winship et al., 2019), providing a translational framework for clinical research. Indeed, part of our current knowledge on the pathophysiology of schizophrenia and the effects of drugs to treat this complex disorder is derived from studies using animal models. However, one of the major criticisms for studying schizophrenia using animal models is that several of the symptoms, such as hallucinations, delusions, blunted affect, and language disturbances, are considered uniquely human. Thus, only through observing distinct behavioral phenotypes that changes in animals' behavior may be somewhat correlated with disease symptoms. However, due to the limited understanding of the etiology of schizophrenia, there is a continued need for improved animal models recapitulating a schizophrenia-like phenotype.

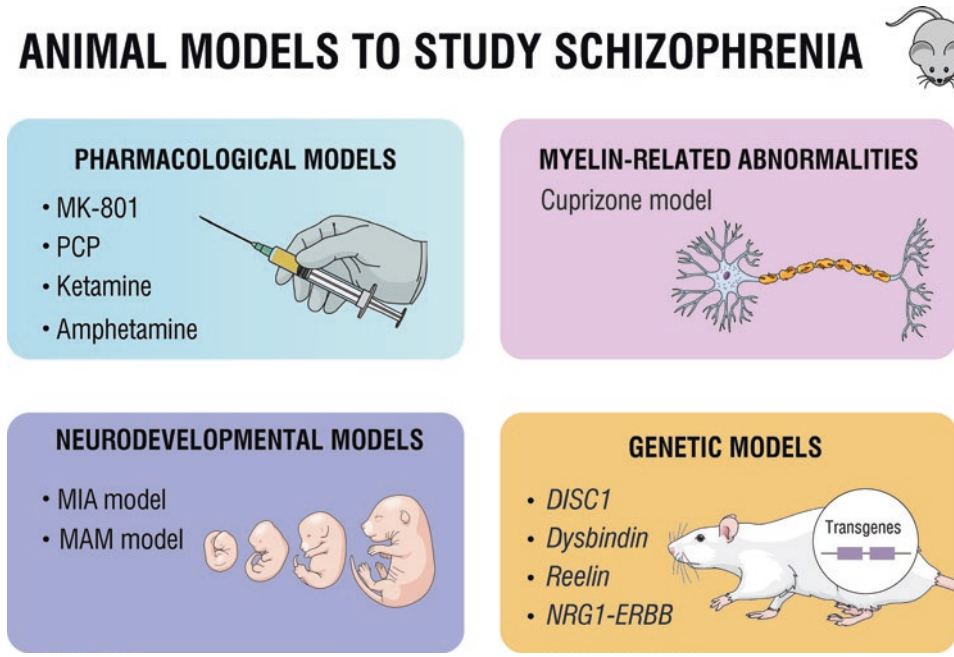

Fig. 1 Animal models to study schizophrenia. The animal models described were divided into the following categories: drug-based (pharmacological), neurodevelopmental disruptions, demyelination, and genetic alterations

Another critical issue in schizophrenia is its pharmacological treatment. First-generation (also called typical) antipsychotics, such as chlorpromazine and haloperidol, produce its effects by blocking the dopamine D2 receptor. These drugs effectively treat only the positive symptoms, an effect that is often accompanied by extrapyramidal side effects (Leucht et al., 2009; Meyer & Simpson, 1997). On the other hand, second-generation (or atypical) antipsychotics, such as quetiapine and clozapine, can also interact with serotonin 5-HT2A receptors. Still, the core mechanism by which these drugs produce antipsychotic effects also relies on the antagonism of D2 receptors. Second-generation antipsychotics effectively attenuate positive symptoms and cause a slight improvement in negative symptoms and cognitive dysfunction, the latter being a matter of debate in the field (Hill et al., 2010; Kaneko, 2018). Furthermore, they also induce limiting side effects such as metabolic syndrome, sedation, and the risk of agranulocytosis for clozapine (Stępnicki et al., 2018)

This chapter aims to provide an updated overview of the most common animal models used to study etiopathological mechanisms related to schizophrenia and new drugs to treat this complex disease. We focused on comparing proteomic profiles of animals and schizophrenia patients. The animal models described below were divided into the following categories: based on the effects of drugs (pharmacological models), neurodevelopmental disruption, demyelination, and genetic-environmental interaction (Fig. 1). The advantages and limitations of these models were discussed.

2 Pharmacological Models

The first animal models to study schizophrenia were based on the effects of drugs in humans. During the 1950s–1970s, it was observed that the administration of drugs such as amphetamine, phencyclidine (PCP), and ketamine to healthy subjects produced behavioral changes resembling schizophrenia (Domino & Luby, 2012;

Sargant, 1958). While amphetamine produces only changes related to the positive symptoms, PCP and ketamine cause changes related to positive and negative symptoms as well as cognitive impairments. In rodents, these drugs produce behavioral changes that are proposed to reflect some aspects of schizophrenia (Krystal et al., 1994, 2005; Javitt, 2007).

The psychotic-like effects induced by drugs that potentiate dopamine neurotransmission, such as amphetamine, along with the fact that antipsychotics act through the antagonism of D2 dopamine receptors are the basis of the "dopaminergic hypothesis of schizophrenia". Also, the discovery that PCP and ketamine act as antagonists of glutamate N-methyl-D-aspartate (NMDA) receptors led to the "glutamatergic hypothesis of schizophrenia" (Steinpreis, 1996; Thaker & Carpenter Jr., 2001). In the light of these hypotheses, animal models based on the pharmacological manipulation of dopamine and glutamate neurotransmitter systems were proposed as useful tools for exploring the pathophysiology of schizophrenia and drug discovery. The stimulation of the dopaminergic system with drugs such as amphetamine or methamphetamine, and the administration of NMDA receptor antagonists, such as PCP, ketamine, and dizocilpine maleate (MK-801), have been extensively used as an animal model to study schizophrenia.

Animal models based on the administration of amphetamine are thought to mimic the hyperdopaminergic state found in schizophrenia (Jones et al., 2011). In rodents, acute amphetamine increases locomotor activity and impairs sensorimotor gating, both reversed by antipsychotics (review by Heal et al. 2013; Moszczynska & Callan 2017). These amphetamine-induced changes have been associated with the psychomotor agitation and deficits in sensorimotor gating found in some schizophrenia patients (Elst Van Der et al., 2007; Mansbach & Geyer, 1989). Methamphetamine and cocaine have also been used to study schizophrenia-related changes in rodents (Wearne & Cornish, 2018).

Drugs such as PCP, ketamine, and MK-801 produce schizophrenia-related behaviors in healthy subjects and exacerbate symptoms in schizophrenia patients (Bird et al., 2001; Lipska & Weinberger, 2000; Weickert et al., 2013). In rodents, both the acute and repeated administration of these drugs produce behavioral changes that recapitulate several schizophrenia symptoms, such as increases in locomotor activity, decreases in social interaction, deficits in sensorimotor gating, and impairments in cognitive function (Gomes et al., 2014; Long et al., 2006; Pedrazzi et al., 2021; Van Der Staay et al., 2008, 2011). However, the effects induced by repeated administration of NMDA receptor antagonists, unlike those observed after acute administration, can be long-lasting (Rodrigues da Silva et al., 2020) and are proposed to more closely resemble the behavioral, neurochemical, and neuroanatomical changes found in schizophrenia (Jentsch & Roth, 1999). Among these changes, a decreased number of GABAergic interneurons containing the calcium-binding protein parvalbumin in the prefrontal cortex and hippocampus has been observed in rodents after repeated treatment with NMDA receptor antagonists (Gomes et al., 2014; Jenkins et al., 2010). This is consistent with findings from the *postmortem* brains of schizophrenia patients (Beasley & Reynolds, 1997; Zhang & Reynolds, 2002) and has been proposed as a consequence of a hypofunction of NMDA receptors located in these interneurons (Gonzalez-Burgos & Lewis, 2012). However, the exact mechanisms underlying this hypofunction remains unknown.

In the scope of MK-801, a proteomic study demonstrated alterations in the expression of several proteins (HSP60, HSP72, albumin, DRP-2, aldolase, and malate dehydrogenase) in the thalamus of rats submitted to repeated MK-801 (Paulson et al., 2004). Furthermore, under similar conditions, treatment with the second-generation antipsychotic clozapine reversed MK-801-induced proteomic changes, both in the thalamus and prefrontal cortex. The first-generation antipsychotic haloperidol also reversed MK-801-induced proteomic changes, but to a lesser extent than clozapine (Paulson et al., 2004, 2007). Furthermore, several similarities were found when comparing the proteomic profile of *postmortem* prefrontal cortex of schizophrenia

patients with different schizophrenia models based on the NMDA receptor hypofunction, including acute and chronic PCP, acute ketamine, and NMDA receptor knockdown by liquid chromatography-coupled tandem mass spectrometry with the data-independent acquisition ($LC\text{-}MS^E$). Functional pathways identified in schizophrenia were presented in all animal models studied. Among the altered pathways were those related to intracellular signaling and regulation, development and differentiation, intracellular transport and localization, biosynthetic processes, energy metabolism, nucleic acid metabolism, and ATP/GTPase activity. However, the chronic PCP treatment caused the highest similarity across pathological features of schizophrenia (Cox et al., 2016). Taken together, studies involving brain proteomics profiles induced by MK-801, PCP, and ketamine in rodents have provided insights on cellular responses triggered by these drugs, and, due to similarities with data from schizophrenia patients, they have improved the construct validity of models based on the NMDA receptor antagonism. However, it should be taken into account that despite being useful for studying the consequences of a manipulation that may be of relevance to schizophrenia, pharmacological models provide limited value in understating the etiology of the disease.

3 Neurodevelopmental Models

To better understand the neurodevelopmental disturbances in schizophrenia, experimental models are helping to understand how the exposure to risk factors in distinct developmental periods can cause schizophrenia-like alterations (Jones et al., 2011). Most of the animal models for schizophrenia based on neurodevelopmental disruption originated from clinical evidence indicating the relevance of early exposure, mainly during gestation and the perinatal period, to adverse environmental factors, such as viral infections, maternal immune activation (MIA), maternal stress, maternal malnutrition, obstetric complications, and childbirth injuries, that may lead to schizophrenia development later in life (Fatemi & Folsom, 2009; Lewis & Levitt, 2002).

3.1 The Maternal Immune Activation (MIA) Model

The MIA model is proposed to mimic a state where environmental insults, infectious or not, promote a maternal systemic immune response typically of innate nature, disrupting the placental-blood barrier and reaching the fetus (Smith et al., 2007). From the clinical perspective, some historical evidence has highlighted the impact of MIA on schizophrenia incidence. Indeed, an increased incidence of schizophrenia was observed in the offspring of mothers exposed to great famines, such as the Dutch Hunger Winter (1944–1945) at the end of the Second World War (Hoek et al., 1998). Similarly, increased cases of schizophrenia were associated with gestational exposure to several virus epidemics, such as the influenza virus (H1N1) in the Spanish Flu Pandemic in 1918–1919 (Kępińska et al., 2020), H2N2 (1889–1892) in the Russian influenza pandemic (Honigsbaum, 2013); H2N2 (1957–1958) in the Asian influenza pandemic, H5N1 in the "bird flu" epidemic in 2003; and H1N1 (2009–2010) in the "swine flu" pandemic (Selten et al., 2010). Not only this but other pathogen gestational infections, such as *Toxoplasma gondii*, *Herpes simplex virus*, rubella, mumps, and other seasonal respiratory viruses, have also been associated with higher risk for schizophrenia (Krause et al., 2010; Pedersen et al., 2011). Thus, infections or "sterile" immune events can activate a systemic maternal inflammatory response and alter the fetal environment, with consequential effects on brain function and behavior in the offspring (Macêdo et al., 2012; Meyer, 2013).

In rodent studies, the MIA model has typically been induced by the inoculation of pathogen antigens, namely, pathogen-associated molecular patterns (PAMPs), which act on pattern recognition receptors, such as toll-like receptors (TLRs), mediating a strong systemic inflammatory response (Woods et al., 2021). The most

commonly used PAMP to induce MIA is the polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA that acts as an agonist of toll-like receptor 3 (TLR3) and is used experimentally to model viral infections, and the lipopolysaccharide (LPS), an endotoxin from gram-negative bacteria with TLR4 agonist properties (Aguilar-Valles et al., 2020).

Mice exposed in utero to poly I:C have shown several changes resembling schizophrenia (Meyer, 2014). However, these changes seem to be strongly modulated by the exact period of the immune stimulus (Chow et al., 2016). The early challenge with poly I:C in the gestational day (GD) 9, corresponding to the first gestational trimester in humans, results in sensorimotor filter deficits and psychotic-like abnormalities, such as prepulse inhibition (PPI) dysfunction, amphetamine-hypersensitivity, and hyperlocomotion (Arsenault et al., 2014). On the other hand, exposure to poly I:C later in the GD 17, corresponding to the third gestational trimester, results predominantly in cognitive deficits and changes related to the negative symptoms (Purves-Tyson et al., 2019). Other studies have used different developmental periods, such as GD 12.5, referring to the beginning of the human second trimester of pregnancy (Chow et al., 2016), or even during the first postnatal week (neonatal exposure), and identified the emergence of behavioral changes compatible with all spectra of schizophrenia symptoms (Monte et al., 2017; Ribeiro et al., 2013).

Neurotransmitter abnormalities during decades have been the core theory for schizophrenia neurobiology. In the poly I:C model, several abnormalities in dopaminergic signaling have been described in limbic and frontocortical circuits, for instance, behavioral hypersensitization to dopamine agonists (Borçoi et al., 2015), increased striatal dopamine release (Winter et al., 2009; Zuckerman et al., 2003), increased dopamine turnover rate, and reduced binding to striatal D2 receptors, indicating increased synaptic dopamine bioavailability (Ozawa et al., 2006). Changes in other neurotransmission systems have also been reported in poly I:C MIA models, such as reduced hippocampal GABA content (Bitanihirwe et al., 2010), decreased expression of the enzyme glutamic acid decarboxylase (GAD) and $GABA_A$ receptor subunits (Richetto et al., 2017), and impairment of long-term potentiation (LTP) and excitatory transmission in the hippocampus (Bagot et al., 2012; Khan et al., 2014).

Robust evidence has indicated a similar ability of other agents, such as LPS, to activate maternal immune systems and promote behavioral changes related to schizophrenia and other developmental disorders in the fetus (Aguilar-Valles et al., 2020). LPS injection on GD 15 induced impairments in cognitive function and PPI in adulthood. The NMDA receptor antagonist ketamine exacerbated these deficits in a dose-dependent manner (Simões et al., 2018). Additionally, gestational LPS administration on GD 15 elevated pro-inflammatory cytokine gene expression in the maternal serum, amniotic fluid, and fetal brain that remained at least for 24 hours. Microarray transcriptomics of fetal brain 4 hours after LPS exposure showed increased mRNA expression of cellular stress and cell death genes and reduced expression of developmentally regulated and brain-specific genes, mainly those regulating the migration of GABAergic interneurons (Oskvig et al., 2012). These findings may be associated with the decreased number of parvalbumin-positive GABAergic interneurons found in animals exposed to LPS in utero (Boksa, 2010; Wischhof et al., 2015).

Proteomics is a powerful tool for investigating a broad picture of biological and cellular alterations involving schizophrenia and MIA models (Rodrigues-Amorim et al., 2019). Despite this, few studies have applied the proteomics approach in MIA models. Kitagawa et al. (2019) reported that mice exposed to poly I:C in utero showed altered levels of aldehyde dehydrogenase family 1 member L1 (ALDH1L1) and collapsin response mediator protein 5 (CRMP5) in the hippocampus (Kitagawa et al., 2019). In another proteomics study, abnormalities in over 1000 protein pathways were found in the prefrontal cortex of poly I:C prenatally exposed rats. Particular reductions in the expression of myelin-related proteins, such as myelin basic protein isoform 3 (MBP1) and

rhombex 29, were observed. These changes were reversed by the treatment during adolescence with the second-genereation antipsychotic risperidone (Farrelly et al., 2015). Moreover, a recent study using mass spectrometry-based proteomics reported increased activation of mTORC1 and its upstream regulator, AMPK signaling, in the placenta 6 hours after the poly I:C challenge. Interestingly, these changes were sex-specific and only occurred in male offspring placenta (McColl & Piquette-Miller, 2021). Therefore, despite promising as a biological and powerful tool to understand the complex pathophysiology of schizophrenia, proteomics on the molecular mechanisms of the MIA model need to be better clarified. For instance, brain proteome profiles obtained from this model at different ages could provide important insights based on the changes observed during neurodevelopment in this model. Finally, it is worth mentioning that, in addition to schizophrenia, MIA models have also been used to study autism spectrum disorders (review by Zawadzka, Cieślik, and Adamczyk (2021)).

3.2 The Methylazoxymethanol Acetate (MAM) Model

Another rodent model based on neurodevelopmental disruption revelant for schizophrenia is the methylazoxymethanol acetate (MAM) model. The administration of the short-acting DNA-alkylating agent MAM on GD 17 causes selective disturbance of proliferation and migration of neuronal precursor cells interfering with the development of specific brain regions in the offspring that displayed many of the characteristics associated with schizophrenia in late adolescence/adulthood (Hradetzky et al., 2012; Modinos et al., 2015). This included thinning of cortical regions homologous to those showing thinning in the *postmortem* brain of schizophrenia patients (Moore et al., 2006). As in schizophrenia, a selective loss of parvalbumin-positive GABAergic interneurons is observed in the hippocampus and prefrontal cortex in the MAM model (Lodge et al., 2009). MAM-exposed animals also show deficits in sensorimotor gating, sociability, and cognitive function and enhanced locomotor response to amphetamine and NMDA receptor antagonists (Gomes et al., 2016; Takahashi et al., 2019). The increased response to psychostimulants is consistent with a dysfunction in the dopamine system in schizophrenia (Grace & Gomes, 2019). Indeed, a hyperdopaminergic state is one of the main changes observed in the MAM model. MAM rats have shown ventral hippocampal hyperactivity that is proposed to drive an increase in the number of active dopaminergic neurons in the ventral tegmental area, which is consistent with the overactivity of striatal presynaptic dopamine terminals indicated by neuroimaging studies with schizophrenia patients (Laruelle & Abi-Dargham, 1999; Lodge & Grace, 2011). These findings suggest that the MAM model presents considerable face and construct validities. In addition, this model has also been applied in studies aiming at understanding the mechanisms of current antipsychotics and new treatments (Grace & Gomes, 2019).

Consistent with the onset of psychosis in schizophrenia patients, most of the changes related to the positive symptoms in the MAM model become evident only in late adolescence/early adulthood, while social withdrawal and cognitive deficits are present both before and after these periods (Gomes et al., 2016). Thus, the MAM model offers the possibility to study the link between the disruption in neurodevelopment and the transition into a schizophrenia-like state in the adult. Recently, it was found that an enhanced stress responsivity during adolescence in MAM rats may contribute to the emergence of the schizophrenia-like phenotype in adulthood, since it was prevented by interventions applied during adolescence that relieve stress, such as anxiolytics and environmental enrichment (Du & Grace, 2013; Zhu & Grace, 2021; Zimmerman et al., 2013). Supporting the role of stress, normal animals exposed to stress during adolescence present changes similar to those found in the MAM model (Gomes et al., 2020; Gomes & Grace, 2017).

Proteomic approaches have also been used to study biological and cellular alterations involving

schizophrenia in the MAM model. Hradetzky and colleagues, using mass spectrometry proteomics combined with magnetic resonance metabolomics, found major alterations in AMPA receptor subunit expression and phosphorylation, clathrin-mediated receptor internalization, and calcium signaling in the hippocampus of MAM rats. In addition, MAM rats presented marked changes in the glutamine/glutamate cycle since reduced *scyllo*-inositol, phosphocholine, glutamate, *N*-acetyl aspartyl glutamate (NAAG), and glutamine (Hradetzky et al., 2012). Such studies have the potential to indicate molecular mechanisms potentially implicated in schizophrenia and novel targets for alleviating schizophrenia symptoms.

4 Models Based on Myelin-Related Abnormalities: The Cuprizone Model

Mounting evidence suggests a connection between oligodendrocyte metabolism, the impaired white matter integrity, and brain disconnectivity seen in schizophrenia (Clark et al., 2007; Gouvêa-Junqueira et al., 2020; Saia-Cereda et al., 2017). *Postmortem* and brain imaging studies showed alterations in the myelination of white matter tracts and cortical areas, as well as a reduced number of oligodendrocytes in schizophrenia patients (Hof et al., 2003; Jørgensen et al., 2016). Furthermore, oligodendrocyte integrity (Hof et al., 2003; Uranova et al., 2001) and the expression of myelination-related proteins and genes are also altered in schizophrenia (Hakak et al., 2001; Martins-de-souza et al., 2009; Tkachev et al., 2003). Hence, animal models can be a useful tool to investigate myelination dysfunctions in schizophrenia.

The main model to investigate (de)myelination processes in schizophrenia is based on the oral administration of the copper-chelating agent cuprizone (bis-cyclo-hexanone oxaldihydrazone) (Vega-Riquer et al., 2019). The standard protocol consists of feeding adult rodents with 0.2% cuprizone for 4–6 weeks, which results in marked demyelination. However, following cuprizone withdrawal, spontaneous remyelination can be observed (Praet et al., 2014). While demyelination over 4–6 weeks is usually reversible, exposure to cuprizone for more than 12 weeks leads to chronic demyelination, resulting in oligodendrocytic impairment and incomplete remyelination after cuprizone withdrawal (Torkildsen et al., 2008).

Cuprizone-induced toxicity has been postulated to originate from copper deficiency (Benetti et al., 2010). Copper is a critical transition metal that acts as an essential enzymatic cofactor, and, on average, 0.3% of the eukaryotic proteome has been estimated to consist of copper-binding proteins, which are mainly related to copper homeostasis and the catalytic process of redox reactions or electron transfer (Andreini et al., 2008). Moreover, copper participates in a wide array of biochemical reactions involved in several brain functions, such as neurotransmitter metabolism, neuron depolarization, and maintenance of redox balance (Ackerman & Chang, 2018; Rihel, 2018).

The hallmark of the cuprizone model is oligodendrocyte damage that leads to extensive apoptosis of these cells, causing brain demyelination (Praet et al., 2014). Demyelination due to cuprizone exposure has been shown in the corpus callosum and other brain areas, including the hippocampus, cortex, and cerebellum (Liu et al., 2020; Skripuletz et al., 2008; Groebe et al., 2009; Steelman et al., 2012). Reductions in the synthesis of myelin proteins and the levels of classical myelin-related genes have also been reported (Han et al., 2020; Zhan et al., 2020), along with increases in the number of Iba1-positive microglia and GFAP-positive astrocytes after 4 weeks and 6 weeks of feeding cuprizone, respectively (Han et al., 2020).

There is accumulating evidence that the immune system is involved in the cuprizone-induced demyelination model, indicated by increased activation of microglia and astrocytes that hampers oligodendrocyte apoptosis. Severe microglia reactivity was observed as early as during the third week of cuprizone feeding (Zhan et al., 2020). In addition, several inflammatory mediators have also been implicated in the cuprizone-induced demyelination model, pro-

duced both by microglia and astrocytes, including nitric oxide, interleukin-6, interleukin-17, TNF-α, and IFN-γ (Vega-Riquer et al., 2019).

The altered behavioral responses of cuprizone-treated animals have been associated with the cognitive impairments observed in schizophrenia patients. Animals treated with cuprizone show cognitive disturbances, including alterations in spatial memory, accompanied by PPI deficits and reduced social interaction (Makinodan et al., 2009; Xu et al., 2010). Concerning the role of white matter in cognition, multiple image studies confirm the presence of white matter abnormalities in the pathophysiology of schizophrenia (Flynn et al., 2003; Hof et al., 2003; Jiang et al., 2019; Schultz et al., 2017). Proteomic approaches have also provided an overview of individual proteins and constituent pathways associated with these alterations, highlighting the role of oligodendrocytes in this disorder (Clark et al., 2007; Saia-Cereda et al., 2017). Therefore, the cuprizone-induced demyelination model has been used to elucidate the mechanisms underlying white matter impairments in schizophrenia. For instance, the expression of the oligodendrocyte transcription factor *OLIG2*, usually expressed in progenitor cells, is increased by cuprizone administration (Liu et al., 2017). This transcription factor regulates neuronal and glial differentiation from progenitor cells (Gaber & Novitch, 2011). In addition, *OLIG2* silencing attenuates the behavioral alterations in cuprizone mice, suggesting that this gene may be an important candidate for understanding schizophrenia-like symptoms (Liu et al., 2017). Multiple reaction monitoring (MRM) analyses using a triple quadrupole mass spectrometer were performed using the hippocampi of mice exposed to cuprizone; a prominent reduction of serine signal levels was observed in these animals, while a 300% increase of GABA was observed (Hayakawa et al., 2019). A mass spectrometry-based lipidomic approach found alterations in lipid composition following cuprizone administration in several brain areas. The results revealed that cuprizone caused changes mainly in glycerophospholipids and sphingolipids, with the most distinct alterations occurring in the prefrontal cortex (Zhou et al., 2020). Quetiapine, a second-generation antipsychotic, ameliorated the cuprizone-induced cognitive impairments and, in part, normalized lipidomic alterations. These findings highlight the potential for the cuprizone model to unravel mechanisms related to antipsychotics. Nonetheless, further translational studies are needed to better understand mechanisms underlying schizophrenia. In addition to cuprizone, other methods to study myelin-related conditions using chemical induction models, such as lysolecithin and ethidium bromide, and transgenic models that mimic genetic alterations are available. These models are also valuable tools when studying other myelin related-disorders, including multiple sclerosis (Torre-Fuentes et al., 2020).

5 Genetic Models

Schizophrenia is a polygenic disorder with more than 250 risk genes implicated in the etiology of schizophrenia but with all conferring a small effect on the phenotype, even though advanced genetic models offer the possibility to study candidate genes implicated in disease susceptibility. Among these genes, we will highlight the disrupted-in-schizophrenia 1 *(DISC1)* and genes related to the neuregulin 1 (*NRG1*)-*ERBB* signaling. Insights derived from animal studies into the mechanisms on how these candidate genes increase the risk of schizophrenia will be provided.

The *DISC1* gene encodes for an intracellular scaffolding protein that interacts with several other proteins, forming complexes (Camargo et al., 2007; Morris et al., 2003) that play a key role in neurodevelopment and synaptic regulation (Tomoda et al., 2016). Changes in the *DISC1* gene were proposed as a risk factor for schizophrenia after the discovery of a translocation between chromosomes 1 and 11 in a Scottish pedigree (St Clair et al., 1990). Further evidence has indicated that changes in biological pathways associated with *DISC1* may be relevant to the pathophysiology of severe mental disorders, such as schizophrenia, autism, and mood disorders

(Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013; Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Given DISC1 functions and its potential role in mental disorders, mouse models based on *DISC1* dysfunction have been created through genetic manipulation. There are three variants of the haploinsufficiency model based on the disruption of the *DISC1* gene, leading to a functional loss due to nonsense-mediated mRNA decay. The first variant, named Δ25bp, causes impairments in cognitive function, particularly in working memory (Koike et al., 2006). The second variant, named Δex2/3, is characterized by a higher impulsivity phenotype, threshold shift in long-term potentiation (Kuroda et al., 2011), and altered endoplasmic reticulum calcium response (Park et al., 2015). The third variant is the Disc1-LI and is associated with aberrant trafficking and processing of amyloid precursor protein (Shahani et al., 2015). It must still be determined if haploinsufficiency models can help unravel the molecular mechanisms underlying schizophrenia (Koszła et al., 2020).

Two missense models – Q31L and L100P – were developed from point mutations in the *DISC1* gene (Clapcote et al., 2007). While the Q31L is characterized by a depressive-like phenotype, the L100P is characterized by a schizophrenia-like phenotype indicated by impairments in PPI and decreased working memory. Further studies using the L100P model revealed enhanced dopamine function, altered neurexin function, deregulated glycogen synthase kinase-3α (GSK3A) activity in synapses, and deficits in interneuron development (Brown et al., 2011; Lee et al., 2011, 2013; Lipina et al., 2010; Su et al., 2014; Tomoda et al., 2016). The phenotypic alterations observed in this model were reversed by the inactivation of GSK3 (Clapcote et al., 2007).

Another model focused on *DISC1* mutation is based on the expression of putative dominant-negative (DN) isoforms of *DISC1* in the mouse brain (Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008; Shen et al., 2008). In this model, mice, similar to schizophrenia patients, present asymmetrical brain anatomy with enlarged lateral ventricles, interneuron impairments, deficits in PPI, and social withdrawal (Brandon & Sawa, 2011; Leung & Jia, 2016). One study using a transgenic model overexpressing *DISC1* analyzed synaptosomal preparations of the dorsal striatum with mass spectrometry-based proteomics. Functional enrichment analysis revealed that an increased *DISC1* expression modifies proteins and pathways related to membrane trafficking, ion transport, synaptic organization, and neurodevelopment. Thus, proteomic tools applied to study DISC1-related alterations in animal models have led to discovering protein networks relevant to schizophrenia (Sialana et al., 2018).

Neuregulin 1 (NRG1) is the best characterized in the family of neuregulins, which are epidermal growth factors that activate ErbB receptor tyrosine kinases and are involved in plasticity development and oncogenesis (Leung & Jia, 2016). *NRG1* was identified as a candidate gene for schizophrenia (Karl et al., 2007; Stefansson et al., 2002) that modulates glial development, neuronal migration, synaptic transmission, and plasticity (Harrison & Law, 2006). As a result, several transgenic animal models have been generated targeting *NRG1* and its receptor ErbB to investigate the molecular and behavioral changes to study abnormalities related to the neuregulin 1 (*NRG1*)–*ERBB* signaling. The most commonly studied mouse model of NRG1 dysfunction is the *NRG1* heterozygous mouse, which shows increased locomotor activity and PPI deficits (Stefansson et al., 2002; Karl et al., 2007; C. M. Spencer et al., 2006). One study showed that clozapine administration and environmental enrichment reversed the increased locomotor activity in this model (Duffy et al., 2008; Karl et al., 2007). Besides targeting a deletion of *NRG1*, other studies have investigated the effects of *NRG1* overexpression in transgenic animals. These animals presented behavioral alterations, such as increased locomotor activity and impaired social interaction, and molecular alterations, such as increased expression of parvalbumin in the frontal cortex (one of the phenotypic markers for cortical GABAergic neurons), as well as increased

expression of myelination markers in the frontal cortex (Kato et al., 2010).

Another transgenic model was developed in which the human isoform *NRG1-IV* is overexpressed in neuronal cells. Behavioral alterations relevant to schizophrenia, such as alterations in sensorimotor gating, discrimination memory, and social behaviors, were observed in these animals. In addition to these behavioral changes, abnormal dendritic development, a synaptic pathology, and a prefrontal cortical excitatory-inhibitory imbalance were also observed (Francesco Papaleo et al., 2016). Overexpression of the *NRG1-III* isoform also led to behavioral alterations related to impaired sensorimotor gating and social and cognitive deficits (Olaya et al., 2018). One study used the transgenic *NRG-1* heterozygous mouse model to examine the hippocampal proteomic differences compared to wild-type animals. They used a 2D gel-based approach and discovered alterations in the levels of schizophrenia-associated proteins associated with serotonergic neurotransmission, growth factor pathways, and vesicular release of neurotransmitters, exemplified by the SNARE proteins (Spencer et al., 2013). More proteomic studies are needed to establish a connection between proteins related to the molecular changes in schizophrenia and NRG1. Besides *DISC1* and *NRG1*, several other transgenic animal models relevant to schizophrenia have been developed, including models based on dysfunction in dopamine and glutamate systems (Duncan et al., 2002; Kellendonk et al., 2006; Mohn et al., 1999), dysbindin (Papaleo et al., 2012; Karlsgodt et al., 2011) and reelin (Krueger et al., 2006; Tueting et al., 2006).

In addition to genetic involvement in the etiology of schizophrenia, the interactions between genetic and adverse socio-environmental factors have mounting evidence for their roles in the development of schizophrenia (Kahn et al., 2015). Urban environment, migration status, prenatal and perinatal events, as well as drug abuse have been reported as risk factors of the disorder (Kahn et al., 2015). Stress exposure during early adolescence in rodents recapitulates behavioral, physiological, and histological disturbances observed in schizophrenia (Gomes et al., 2020). Moreover, cannabis use has been associated with increased vulnerability to the development of psychiatric illnesses, including schizophrenia (Forti et al., 2019; Lowe et al., 2019).

6 Concluding Remarks

Animal models are valuable preclinical tools to investigate the neurobiological bases of complex psychiatric disorders, such as schizophrenia (Winship et al., 2019). These models allow the study of how genetic and molecular changes and environmental factors can act alone or through their interaction to produce abnormalities resembling schizophrenia. In addition, the use of laboratory animals offers the opportunity to perform ethically invasive monitoring of structural and molecular changes to investigate the development and progression of a disease-like state (Powell & Miyakawa, 2006). In addition, despite the advances in stem cell technology, animal models represent the complexity of behavioral and physiology that cannot be simulated in in vitro models (Prytkova & Brennand 2017). The advantages of using animal models for studying schizophrenia are due to anatomical and physiological similarities between humans and animals, particularly mammals, which have prompted researchers to investigate a range of mechanisms potentially related to schizophrenia and new drugs to treat this pathology before applying their discoveries to humans (Barré-Sinoussi & Montagutelli, 2015).

It is challenging to define which animal model would be the most effective to study schizophrenia as each has its advantages and limitations. As such, we propose that combining phenotypic and proteomic data, thereby comparing the proteomes of animal models with data from schizophrenia patients, could be a powerful tool for increasing the validity of these models and providing insights into the biological and cellular aspects underlying schizophrenia. This has the potential to contribute to a better understanding of the pathophysiology of this complex disorder and indicate novel therapeutic targets.

Funding FC, CBT, GSZ, GMV, JAC, EDB, and FGV are supported by the São Paulo Research Foundation (FAPESP, grant numbers 2019/22398–2; 2018/14666–4, 2017/25055–3, 2017/25588–1, 2018/03673–0, 2018/17597–3). JFCP is supported by the Coordination for the Improvement of Higher Level Personnel (CAPES/BRAZIL, grant number 2020–969).

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Modeling Schizophrenia In Vitro: Challenges and Insights on Studying Brain Cells

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Contents

Abstract

One of the challenges in studying neuropsychiatric disorders is the difficulty in accessing brain tissue from living patients. Schizophrenia is a chronic mental illness that affects 1% of the population worldwide, and its development stems from genetic and environmental factors. In order to better understand the pathophysiology underlying schizophrenia, the development of efficient in vitro methods to model this disorder has been required. In addition to several in vitro models, induced pluripotent stem cells (iPSCs) arose as a powerful tool, enabling access to the genetic background of the donor. Moreover, genetic modification of these cells can improve studies of specific dysfunctions observed in the pathophysiology of several neuropsychiatric disorders, not only schizophrenia. Here, we

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*, Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_3

summarize which in vitro models are currently available and their applications in schizophrenia research, describing their advantages and limitations. These technologies in the cell culture field hold great potential to contribute to a better understanding of the pathophysiology of schizophrenia in an integrated manner, in addition to testing potential therapeutic interventions based on the genetic background of the patient.

Keywords

Schizophrenia · In vitro cell model · iPSC · 3D brain models

1 Introduction

Schizophrenia is a severe psychiatric disorder with a complex pathophysiology. The first report of the disorder was postulated to be dementia praecox by Emil Kraepelin in the nineteenth century, who pioneered a combinatorial way of looking at symptoms to define mental illnesses (Berrios & Hauser, 1988). Recent definitions of the symptomatology of schizophrenia includes positive symptoms (hallucinations and delusions), negative symptoms (impaired motivation and social withdrawal), and cognitive deficits. Though several hypotheses for the etiology of schizophrenia exist, the dopaminergic hypothesis enhanced by the glutamatergic hypothesis is adept at explaining, in part, the psychopathology of schizophrenia. The dopamine hypothesis postulates that an overactivity of dopaminergic neurotransmission contributes to the etiology of the positive symptoms (Lau et al., 2013; Uno & Coyle, 2019). Glutamatergic projections are thought to participate in the regulation of dopaminergic neurons, making them sensitive to changes in glutamate release (Howes et al., 2015). Several studies have provided evidence for disruptions of the glutamatergic system, mainly associated with the hypofunction of NMDA receptors. One widely accepted hypothesis that more comprehensively explains the overall neurotransmitter alterations in this disorder is the neurodevelopmental hypothesis (Murray et al., 1991; Weinberger, 1987), in which a combination of both genetic and environmental risk factors of schizophrenia promotes early insults to the developing brain, leading to prodromal symptoms during childhood/early adolescence and to the establishment of the disorder in late adolescence/early adulthood (Insel, 2010; Owen & O'Donovan, 2017).

Most of the molecular studies conducted so far on schizophrenia come from *postmortem* brain samples from patients (Williams et al., 2020), and those studies were fundamental to bring insights into the cellular processes altered in this disorder. However, *postmortem* tissue reflects only the final stage of schizophrenia, including any chronic medication, psychosocial factors, and environmental variables, providing limited and potentially confounded data regarding the pathophysiological mechanisms of psychiatric disorders. In addition, this model doesn't allow genetic or pharmacological modulations nor the study of the developmental course of the illness in living cells. In this way, developing appropriate in vivo and in vitro models for schizophrenia is key to enable a deeper understanding of the pathophysiology and mechanisms of the disease. Different animal models for the study of schizophrenia have been established, based on pharmacological or genetic approaches, but there are challenges in working with in vivo models to study neuropsychiatric disorders, given the difficulty of interpreting the complex symptoms manifested in schizophrenia, such as delusions and paranoia (St Clair & Johnstone, 2018).

In this way, in vitro approaches emerge as an important tool to study disease mechanisms, neurodevelopment, and possible pharmacological interventions. A series of recent advances in cell culture techniques hold promise to accelerate the understanding of neuropsychiatric diseases. Arising from genomics, genome-wide association studies (GWAS) have identified a large number of schizophrenia risk loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Though animal models have been valuable to study phenotypes induced by genetic mutations, human induced pluripotent stem cells (hiPSCs) have arisen as a tool for elu-

cidating regulatory processes during neurodevelopment in a human genetic context (De Vrij et al., 2019). Culturing in suspension, hiPSCs can self-organize and give rise to three-dimensional (3D) cell cultures, including subsequent differentiation toward a neural lineage, producing reliable, brain-like structures (Dosso et al., 2020). Moreover, techniques that allow the modulation of specific genes may help to better understand the pathomechanisms of psychiatric disorders. Considering the growing interest in studying psychiatric disorders such as schizophrenia, in this book chapter, we overview the main in vitro models for studying schizophrenia and the current advances in this area (Fig. 1).

2 2D and 3D Cell Models of Schizophrenia

2.1 Neuronal Cell Lines

Numerous cell lines have been established from human malignant tumors and are widely applied as in vitro models. The most commonly used to study schizophrenia are neuronal cell lines, for example, SH-SY5Y. This cellular model has the advantage of being reproducible, inexpensive, and procedurally easy, as well as having proven biochemical and functional similarities with human neurons (Koszła et al., 2020). The SH-SY5Y neuroblastoma cell line is a sublineage of SK-N-SH cells derived from a metastatic bone tumor. Undifferentiated SH-SY5Y cells resemble immature catecholaminergic neurons, expressing immature neuronal markers such as nestin (Lopes et al., 2010). Several mature neuron markers are expressed by fully differentiated SH-SY5Y cells, including MAP2, βIII-tubulin, synaptophysin, and NeuN, providing a valuable model of mature human neurons (Shipley et al., 2016). SH-SY5Y cells treated with MK-801, a glutamatergic N-methyl-D-aspartate (NMDA) receptor antagonist, has been used as a neuronal impairment model to study schizophrenia (Unal et al., 2019; Zhu et al., 2016). Moreover, as SH-SY5Y cells represent catecholaminergic neurons, it has been accepted as a valuable model to study the dysregulation of dopamine (Brenner-Lavie et al., 2008).

The ease of manipulating cell lines such as SH-SY5Y makes genetic manipulation a convenient tool for a deeper understanding of the biological roles of genes at schizophrenia-associated risk loci. For example, this model was used to investi-

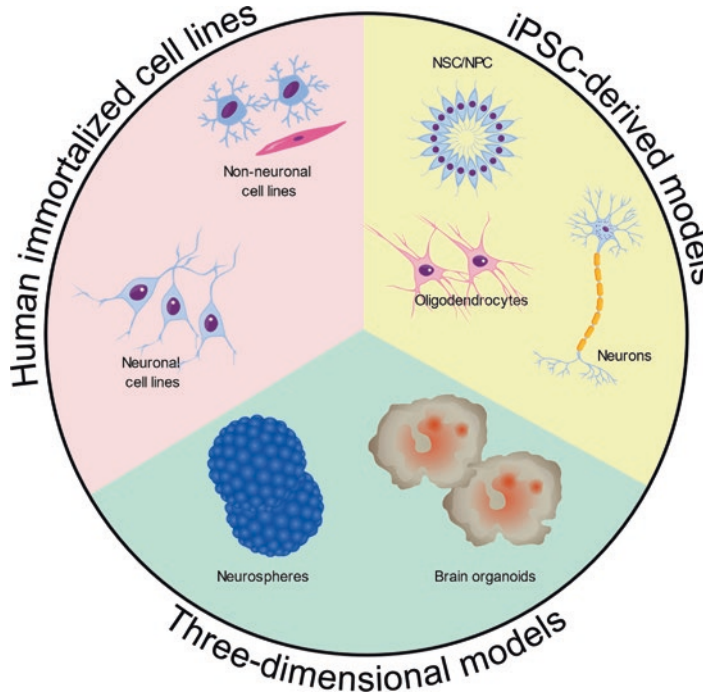

Fig. 1 Schematic of cell culture models to study schizophrenia in vitro

gate the disruptions regarding disrupted-in-schizophrenia 1 *(DISC1)*, a well-known susceptibility gene for schizophrenia (Ogawa et al., 2005). Furthermore, across multiple studies, the SH-SY5Y cell line has been used to elucidate the mechanisms underlying antipsychotics in neuronal cells (Arun et al., 2008; Deslauriers et al., 2011; Hu et al., 2021; Sánchez-Wandelmer et al., 2009). Despite the many advantages of the SH-SY5Y cell line, however, there are considerable disadvantages that should be mentioned. Because this cell line was established from tumor cells, unlimited growth time is a hallmark of this lineage. As a result, this generates genetic aberrations that affect their biological functions across passages (Koszła et al., 2020).

Another important immortalized neural cell line to study the mechanisms behind psychiatric disorders is HT22. This cell line is a sublineage cloned from HT4 cells, which were immortalized from primary mouse hippocampal neurons (Liu et al., 2009). Differentiated HT22 cells have cholinergic neuronal properties and are able to release acetylcholine (ACh) in response to neuronal depolarization (Liu et al., 2009). Multiple new pieces of evidence have emphasized hippocampal alterations in the pathophysiology of schizophrenia, including alterations in glucose metabolism and glutamatergic and dopaminergic dysfunctions (Collado-Torres et al., 2019; Grace & Gomes, 2019; Schubert et al., 2015; Zuccoli et al., 2021). Alterations in the levels of glycolytic enzymes were reported in HT22 neuronal cells after MK-801 treatment (Guest et al., 2015). HT22 cells have additionally been used to modulate gene expression regarding the pathophysiological processes of schizophrenia (Plach et al., 2019; Zhang et al., 2015).

2.2 Non-Neuronal Cell Lines

Application of the right in vitro cell model is essential to create a better environment for studying specific cellular processes, such as myelination. There is a great deal of evidence in the fields of proteomics (Saia-Cereda et al., 2017), transcriptomics (Gandal et al., 2018), and genomics via GWAS (Schork et al., 2019) that supports the involvement of glial cells in the pathophysiology of schizophrenia. Thus, the use of non-neuronal lineages has significantly increased. MO3.13 is a cell line of human progenitor oligodendrocytes and is widely used in schizophrenia studies. It has been applied to study schizophrenia-associated stimuli such as glutamatergic dysfunction (Brandão-Teles et al., 2017; Cassoli et al., 2016), to test therapeutic interventions (Brandão-Teles et al., 2019), as well as to study the specific mechanisms underlying oligodendrocyte metabolism (Fioramonte et al., 2021).

In addition to oligodendrocytic cells, there are some human immortalized microglia cell lines available, for example, HMC3, HMO6, and HM1900, all of which express microglial markers, such as IBA1 and MHC class II proteins (reviewed by Zhang & Cui, 2021). However, the number of human microglial cell lines is limited by the restricted availability of resources to make human primary microglia. Thus, primary mouse microglia have been widely used to study microglial functions (Timmerman et al., 2018). As with any non-human model for psychiatric disorders, the results obtained through rodent microglial cell lines, while invaluable, should always be explored with caution.

2.3 Human Induced Pluripotent Stem Cells (hiPSCs)

In addition to studies that have used different in vitro models to better understand the pathomechanisms of schizophrenia, induced pluripotent stem cells (hiPSCs) have risen as an important tool in the field of in vitro studies. hiPSCs can be generated from adult human somatic cells through the expression of four transcription factors (*Sox2*, *Oct3/4*, *Klf4*, and c-Myc) (Takahashi et al., 2007). Advanced methods in stem cell research have allowed the generation of hiPSCs from patient cord blood cells and peripheral mononuclear cells, as well as epithelial cells from urine (Okita et al., 2013; Zhou et al., 2012). As a result, hiPSCs derived from schizophrenia patients have the genetic background of their

donors, which constitutes one of the main advantages of using these cells. Moreover, the ability for genetic modification expands the utility of hiPSCs in studying gene function during normal human neurodevelopment and disease. The CRISPR-Cas9 system is a highly specific technique for gene editing, which is for this reason a powerful tool for disease modeling (Tian et al., 2019; Wang et al., 2014). Studies integrating the CRISPR technique and hiPSC technology can provide high-quality data about the molecular mechanisms associated with psychiatric disorders (Tian et al., 2019).

The first reports that studied hiPSCs derived from a schizophrenia patient with the *DISC1* mutation represent pioneer work with hiPSC technology. These studies paved the way for studying specific and individual dysfunctions that are observed in the pathophysiology of psychiatric disorders (Brennand et al., 2011; Chiang et al., 2011). Schizophrenia patient-derived iPSCs can be differentiated into several brain cell types, therefore representing a powerful tool to study the pathophysiology of schizophrenia. Once differentiated, these cells provide a reliable model for generating disease-relevant cell types, carrying out drug discovery research, and performing clinical studies (Nakazawa et al., 2019).

2.3.1 Neural Stem Cells (NSCs) and Neural Progenitor Cells (NPCs)

During neural induction protocols, hiPSCs can undergo highly controlled differentiation toward neural lineages. After embryoid body (EB) formation, under adherent conditions, neural stem cells (NSCs) and later neural progenitor cells (NPCs) migrate from the EB (Casas et al., 2018; Karus et al., 2014). As in all stem cell techniques, both the resulting NSCs and NPCs preserve the genetic material from their hiPSC donors, opening possibilities to recapitulate neurogenesis in vitro (Brennand et al., 2015; Casas et al., 2018; Marchetto et al., 2011). In response to this potential, NSCs and NPCs have been successfully used as a model to study schizophrenia-related biological mechanisms. For example, increased oxygen consumption and higher levels of reactive oxygen species (ROS) were reported in NPCs from schizophrenic patients (Paulsen et al., 2012). In another study, through miRNA expression analysis, the levels of miR-9 were significantly decreased in a subset of schizophrenia NPCs, and alterations in the expression of this microRNA may constitute a risk for developing schizophrenia (Topol et al., 2016). Furthermore, schizophrenia patient-derived NSCs revealed reduced migration and impairments in the secretion and expression of neuro-angiogenic factors (Casas et al., 2018).

2.3.2 hiPSC-Derived Neurons

Differentiation of hiPSCs into neuronal cells has been carried out to unravel the molecular disruptions regarding neurotransmission pathways, as observed in schizophrenia. Several protocols have therefore emerged to create specific hiPSC-derived neuronal types, such as glutamatergic and dopaminergic neurons (Lin et al., 2016). Based on gastrulation processes, these advanced protocols use small molecules to drive the differentiation into the neural path. Bone morphogenetic protein (BMP) signaling inhibition and the activation of the fibroblast growth factor (FGF) pathway contributed to the efficiency of neuroepithelial inductions; as a result, NPCs can differentiate into various types of neurons (Wang et al., 2020).

The dopamine hypothesis of schizophrenia has become one of the most strongly supported ideas for the pathophysiology of the disorder (McCutcheon et al., 2019). Excessive dopamine release in the mesolimbic pathway adds to the knowledge that antipsychotic medications mainly act by blocking dopamine D2 receptors and, in part, can thereby explain the positive symptoms of schizophrenia (Stahl, 2018). Strategies to develop hiPSC-derived dopaminergic neurons are hampered by the lack of protocols to generate a homogeneous dopaminergic neuronal culture. Besides developing efficient protocols, the CRISPR/Cas9-based editing strategy could also overcome this limitation (Calatayud et al., 2019).

hiPSC-derived dopaminergic neurons from schizophrenic patients showed morphological alterations, mitochondrial dysfunction, and

impairments during differentiation processes at the neural precursor stage; for example, none of the schizophrenia-derived dopaminergic neurons expressed dopamine transporter (DAT), a marker expressed by mature dopaminergic neuronal cells (Robicsek et al., 2013). Furthermore, dopaminergic neurons derived from hiPSCs with 16p11.2 deletion showed an overexpression of synaptic markers and an increase in the soma size, whereas dopaminergic neurons derived from hiPSCs with 16p11.2 duplication showed decreased levels of synaptic markers and impairments during neuronal differentiation. The variation of 16p11.2 has been strongly associated with several neuropsychiatric disorders, including schizophrenia (Sundberg et al., 2021).

As schizophrenia is not explained by just one hypothesis, other subtypes of induced neurons remain to be considered to more wholly investigate the molecular mechanisms underlying the disorder. Synaptic dysfunction observed in schizophrenia patients has been proposed to be a result of dysfunctional synaptic transmission across multiple neurotransmitter systems, including dopamine, glutamate, and gamma-aminobutyric acid (GABA) (Frankle et al., 2003). Several hiPSC-derived neurons have been used for disease modeling, such as glutamatergic neurons, GABAergic neurons, and cortical interneurons.

One such additional hypothesis is the glutamate hypothesis, in which the glutamatergic dysfunction observed in patients is proposed to be mainly due to the hypofunction of NMDA receptors (Egerton et al., 2020). Standardized protocols available to differentiate glutamatergic neurons result in a mainly heterogeneous culture, with a majority of glutamatergic cells and fewer GABAergic neurons (Abashkin et al., 2021). Abnormalities in GABA neurotransmission have been widely reported in schizophrenia (Kumar et al., 2021; Schmidt & Mirnics, 2015). As GABA is the most prevalent inhibitory neurotransmitter, these alterations may underpin the imbalance of excitatory/inhibitory networks observed in schizophrenia. Schizophrenia patient hiPSC-derived neurons composed of a mixture of GABAergic inhibitory neurons and glutamatergic excitatory neurons showed impairments in excitatory synaptic transmission and alterations in the expression of potassium-chloride cotransporter KCC2. This transporter contributes to excitatory-to-inhibitory GABA polarity during neuronal maturation (Toritsuka et al., 2021). Moreover, differentiation of hiPSC into cortical interneurons demonstrated that schizophrenia-derived interneurons have reduced expression levels of GAD67 and reduced synaptic puncta density in co-culture with excitatory neurons (Kathuria et al., 2019).

2.3.3 Glial Cells

In addition to the many studies that have reported alterations in gray matter, a great deal of evidence has shown that white matter deficits are also implicated in the pathophysiology of schizophrenia (Fornito et al., 2009; Kanaan et al., 2017; Menon et al., 1995). Thus, glial involvement has since been widely considered in the mechanisms of schizophrenia (reviewed by Dietz et al., 2020).

Astrocytes are the most numerous cell type in the human brain and have high morphological diversity. Beyond the metabolic support given to neurons and oligodendrocytes, astrocytes also participate in a range of processes including neurogenesis, synaptic plasticity, molecular homeostasis, and the operation of the lymphatic system (reviewed by Verkhratsky & Nedergaard, 2018). Evidence from astrocytic impairments in schizophrenia has been supported by *postmortem* studies from multiple brain regions, such as the prefrontal cortex, frontal cortex, and cingulate cortex (Tarasov et al., 2019; Trépanier et al., 2016; Zhang et al., 2020). Regarding astrocyte participation in the pathophysiology of schizophrenia, several studies have used hiPSC-derived astrocytes to model neurodevelopmental abnormalities underlying these cells.

Szabo et al. (2021) used an RNA-seq approach combined with hiPSC-derived astrocytes from schizophrenia, revealing several alterations in temporal patterns associated with astrocyte differentiation. They showed dysregulations in the expression of genes related to functional characteristics of astrocytes, such as axogenesis, signal release from synapses, regulation of metalloexo-

peptidase, and ion channel activity. These divergences became more salient in the more advanced stages of in vitro differentiation (Szabo et al., 2021). hiPSC-derived astrocytes have also been used as a model to study the effects of inflammatory modulation on astroglia (Akkouh et al., 2020; Akkouh, Hughes, et al., 2021). Using IL-1β as an inflammatory stimulus, the authors showed that schizophrenia hiPSC-derived astrocytes exhibit significant differences in the expression of several genes, including important genes for regulatory T cell migration (Akkouh et al., 2020). Using the same stimulus, they performed a miRNome, comparing hiPSC-derived astrocytes from schizophrenia and healthy controls; lower levels of four miRNAs in schizophrenia astroglia were identified, mainly related to the regulation of inflammation (Akkouh, Hughes, et al., 2021). Moreover, using hiPSC technology, Akkouh, Hughes, et al. (2021) investigated the astrocyte diversity between healthy individuals and schizophrenia, including responders and nonresponders to the antipsychotic clozapine. Sifting through the many alterations, the authors found a significant reduction in the levels of intracellular glutamate in schizophrenia astrocytes and decreased levels of D-serine (Akkouh, Hribkova, et al., 2021).

Impairments in white matter integrity have been considered a hallmark pathology in schizophrenia. A prominent reduction in oligodendrocyte density occurs in the prefrontal cortex of schizophrenia patients (Hof et al., 2003; Uranova et al., 2004). Furthermore, brain imaging and *postmortem* brain studies have also shown white matter dysfunctions in schizophrenia at a molecular level (Bora et al., 2011; Saia-Cereda et al., 2017; Uranova et al., 2020). Decreases in oligodendrocyte-related proteins (e.g., MOG, MAG, and CNP) and alterations in myelin-associated genes have been proposed to be promising candidates for targeting oligodendrocyte dysfunction in this disorder (Martins-de-Souza et al., 2009; Tkachev et al., 2003). Although there are several protocols to generate mature oligodendrocytes from hiPSCs, most require a long period in culture or the ectopic expression of key oligodendroglial transcription factors (Douvaras et al., 2014; García-León et al., 2018; Piao et al., 2015; Wang et al., 2013). Nonetheless, the generation of hiPSC-derived mature oligodendrocytes has been shown to be possible, and there are a few studies involving these cells and schizophrenia. For example, differentiation of hiPSC-derived oligodendrocytes from schizophrenia patients showed decreased production of OPCs and oligodendrocytes compared to healthy controls (McPhie et al., 2018). Moreover, using neonatal transplantation of glial progenitors from an hiPSC-derived schizophrenia patient to investigate glial dysfunction, alterations were seen in migration leading to hypomyelination when compared to controls (McPhie et al., 2018; Windrem et al., 2017).

The extent of the involvement of microglia in the etiology of schizophrenia remains uncertain. Nonetheless, one of the most accepted hypotheses postulates that the disruption of microglia may affect brain development and maturation, resulting in impairments in synaptic pruning. Moreover, aberrant microglia activation in schizophrenia is hypothesized to be an exacerbating factor for symptoms in patients (Laskaris et al., 2016). There are several methods to differentiate microglia from hiPSCs, with the majority of protocols inducing hiPSCs to a mesodermal fate, guiding them to hematopoietic progenitors, before leading them to maturation along the myeloid lineage (reviewed by Hanger et al., 2020). Combining hiPSC-derived neurons and monocyte-derived microglia-like cells to create a model of synaptic pruning, schizophrenia-derived microglia showed exacerbation of synaptic pruning as a result of impairments in both microglia and synaptic structure (Sellgren et al., 2019). However, to date, to the best of our knowledge, no study has been reported analyzing hiPSC-derived microglia from individuals with schizophrenia.

2.4 Three-Dimensional Models

Neurospheres and organoids are 3D aggregates of neural cells obtained from human stem cells (NSCs or hiPSCs) that have the potential to dif-

ferentiate into distinct neural cell types, reproducing brain development. These cells are maintained under optimal culture conditions with specific growth factors to stimulate their growth, maturation, aggregation, and specificity (National Academies of Sciences, Engineering, and Medicine, 2021).

The symptoms of schizophrenia frequently begin in adolescence and early adulthood, and there is an association between alterations during neurodevelopment in utero and the etiology of schizophrenia. Neurospheres and organoids enable the study of neurodevelopment and the establishment of schizophrenia models with interventions in the early phases of brain cell development (Brown, 2012). Both organoids and neurospheres generated from schizophrenia patient-derived stem cells allow the study of pathogenic mechanisms and disease development, in addition to testing potential therapeutic interventions based on the genetic background of the patient.

2.4.1 Neurospheres

Reynolds and Weiss (1992) were the first authors that described the generation of neurospheres from isolated NSCs (Reynolds & Weiss, 1992). Nearly two decades later, Matigian et al. (2010) strongly highlighted the advantages of neurospheres over 2D stem cells as models for brain disorders (Matigian et al., 2010). The authors generated neurospheres from olfactory mucosa biopsies of schizophrenia patients, controls, and Parkinson's disease patients and found dysregulated neurodevelopmental pathways in schizophrenia patient-derived neurospheres. Since then, patient-derived olfactory neurospheres (ONSs) have been widely used in the field of 3D cellular systems for studying schizophrenia. Among the pathways found dysregulated in schizophrenia patient-derived ONSs cited above were genes involved in the cell cycle control pathway, such as the G1/S phase transition gene (Matigian et al., 2010). These findings were also reported in schizophrenia patient-derived and healthy control-derived ONSs, showing increased proliferation associated with a shorter cell cycle period and higher expression of the G1/S phase cyclins (D1, E, and A2) (Fan et al., 2012). Patient-derived ONSs were also used to evaluate focal adhesion dynamics, cell motility, and cell adhesion in schizophrenia patients. Schizophrenia patient-derived ONSs were less adhesive and more motile when compared with the healthy control-derived ONSs, mainly due to a dysregulation of focal adhesion kinase (FAK) signaling (Fan et al., 2013).

In the field of proteomics, a study integrated discovery-based proteomic, transcriptomic, and functional assays in schizophrenia patients and healthy control-derived ONSs to elucidate the causes of dysregulated protein synthesis in schizophrenia. Seventeen ribosomal proteins were reported to be expressed at lower levels, with an 18% reduction in the total ribosomal signal intensity in schizophrenia patient-derived ONSs compared to healthy controls. Transcriptomic and genomic data confirmed these findings and highlighted the dysregulation of the upstream translational control pathways eIF2/4 and mTOR (English et al., 2015).

Epigenetic alterations have been explored in schizophrenia patient-derived ONSs to evaluate the DNA methylation profile in distinct cell types from schizophrenia patients and have investigated its association with genetic risk factors very early in development (Vitale et al., 2017). Comparing neurosphere, fibroblast, and hiPSC-derived patient profiles, schizophrenia risk genes may be acting during the early stages of development, and each cell type is affected by the disorder differently.

Mice-derived neurospheres have also been useful in investigating cellular processes related to schizophrenia. Neurospheres from mice ventral rostral hindbrain at embryonic day 12 were established to study the molecular mechanisms that direct the induction and specification of serotonergic (5-HT) neurons (Osterberg & Roussa, 2009). Recently, the role of the Kv4.2 voltage-gated potassium channels (KCND2) and its accessory subunit dipeptidyl peptidase-like protein 6 (DPP6) was reported in the deficit of neurotransmission in schizophrenia patient-derived neurospheres (Naujock et al., 2020).

2.4.2 Organoids

Organoids are more complex structures than neurospheres because of their cell types and structure that more closely recapitulates a corresponding organ's characteristics, organogenesis, and architecture (Abashkin et al., 2021; National Academies of Sciences, Engineering, and Medicine, 2021). Neural organoids are derived from human iPSCs and can be classified as "self-patterning" or "whole-brain" organoids, in which the cells will have features of multiple brain regions, or as "pre-patterned" organoids, in which cells will characterize a specific brain region (National Academies of Sciences, Engineering, and Medicine, 2021). As the first "whole-brain" organoid was generated from hiPSCs, several protocols have arisen describing modifications that improve characteristics of brain development, such as migrations of specific cell types, neurogenesis from progenitor zones, implementation of endothelial cells, and generation of electrical activity (Abashkin et al., 2021; Bail et al., 2021; Lancaster et al., 2013; Pașca, 2018; Quadrato et al., 2017). "Pre-patterned" organoids, however, are exemplified by several organoids that recapitulate characteristics of multiple brain regions such as the forebrain, midbrain, hypothalamus, striatum, retina, thalamus, cerebellum, and spinal cord (Andersen et al., 2020; Ballabio et al., 2020; Cowan et al., 2020; Kadoshima et al., 2013; Miura et al., 2020; Ueno et al., 2006; Velasco et al., 2019). Another method to use 3D cultures with different neural cells is to culture aggregates from distinct brain regions in close proximity, deriving what are considered "assembloids" (National Academies of Sciences, Engineering, and Medicine, 2021).

A study using 20 individual organoids demonstrated that these produced a large variety of cell types with high organoid-to-organoid reproducibility and a variability comparable to that of individual endogenous brains (Velasco et al., 2019). Therefore, cerebral organoids can be used as an experimental platform to access early human cortical development and study disease mechanisms such as schizophrenia. During one study, organoids derived from three schizophrenia patients and four controls were used as a model to study molecular alterations related to the disease in the first trimester of in utero brain development (Stachowiak et al., 2017). Alterations in fibroblast growth factor receptor 1 (*FGFR1*) signaling were hypothesized to have a role in schizophrenia, based on the impaired cortical development of the schizophrenia organoids (Stachowiak et al., 2017). Cerebral organoids also provided insights about the etiology of schizophrenia through the exposure of organoids to the cytokine TNF-α, which disrupts brain organoid development, reproducing phenotypes such as the dysregulation of nFGFR1 signaling that occurs by genetic changes in schizophrenia (Benson et al., 2020).

Using forebrain organoids derived from schizophrenia patients with a mutation in the *DISC1* gene, this mutation has been shown to lead to cell cycle alterations during mitosis of radial glia caused by disruption of the DISC/Ndel1 complex (Ye et al., 2017). Moreover, patient-derived organoids from monozygotic twin pairs discordant for schizophrenia were developed as a tool to access a well-controlled genetic background of the disease. It was proposed that during early cortical development, there is an unbalanced specification of excitatory and inhibitory neurons, which is one of the endophenotypes of psychoses such as schizophrenia (Sawada et al., 2020).

The transcriptome landscape of schizophrenia patient-derived organoids has also been revealed through RNA sequencing (RNA-seq) of schizophrenia patient-derived organoids. The RNA-seq analysis showed differentially expressed genes related to neurodevelopment, synaptic biology, immune response, mitochondrial function, and modulation of excitation/inhibition balance compared to healthy control organoids. Some of these dysregulation pathways were validated with functional assays in which oxygen consumption and the response to electrical stimulation were measured. The results confirmed the disruption of mitochondrial function, which showed a lower basal consumption rate, reduced adenosine triphosphate production, proton leak, and nonmitochondrial oxygen consumption, in addition to a decreased response to electrical stimulation and

potassium chloride depolarization in schizophrenia organoids (Kathuria et al., 2020).

High-throughput quantitative proteomics was also applied to cerebral organoids derived from schizophrenia patients and healthy controls. The cutting-edge isobaric barcoding chemistry was used and adapted to condense the samples and analytically deconstruct them simultaneously via liquid chromatography/mass spectrometry (LC/MS). As a result, 43 proteins were found upregulated, and 54 were downregulated in schizophrenia patient-derived organoids, highlighting the depleted neuronal factors MAP2, TUBB3, SV2A, GAP43, CRABP1, and NCAM1, along with an alteration in pleiotrophin (PTN) and podocalyxin (PODXL), two novel GWAS factors. Among the altered pathways in the enrichment analysis, several are related to nervous system development, such as morphogenesis pathways regulating neuronal differentiation, axon guidance and development, axonogenesis, and substantia nigra development (Notaras, Lodhi, Fang, et al., 2021). Cerebral organoids derived from schizophrenia patients also revealed ventricular neuropathology with disrupted neuronal differentiation and altered progenitor survival. Through an adapted, unbiased mass-spectrometry protocol, they reported the modified expression of developmental factors belonging to POU-domain transcription factors, such as POU3F2/BRN2, and four GWAS factors (PTN, COMT, PLCL1, and PODXL). The molecular diversity of schizophrenia and control organoids was nonetheless similar. Furthermore, single-cell RNA-seq confirmed the depletion of *BRN2* and *PTN* in progenitors and neurons. These findings lead to speculations that schizophrenia is defined by cell-specific neuropathology and multiple neurodevelopmental mechanisms that occur during early corticogenesis (Notaras, Lodhi, Dündar, et al., 2021).

One of the limitations of using organoids is the lack of blood vessels as a nutritional and oxygen supply, leading to hypoxic areas inside the organoid, along with the fact that organoids cannot include the characteristics of all brain regions, therefore lacking the complex networks of connections that occur in the brain (National Academies of Sciences, Engineering, and Medicine, 2021). However, vascularized organoids have been proposed by embedding endothelial cells and brain organoids together in the Matrigel matrix and transplanting the organoids into immunodeficient mice brains to induce the growth of blood vessels (Mansour et al., 2018; Matsui et al., 2021; Pham et al., 2018; Shi et al., 2020). Furthermore, the development of cortical organoids using a slicing method has been proposed, which would prevent interior cell death caused by hypoxia, sustain organoid growth over long-term culture, and lead to the formation of distinct upper and deep cortical layers for neurons and astrocytes, recapitulating late-stage human cortical developmental features (Qian et al., 2020).

3 Advantages and Limitations of in Vitro Models to Study Schizophrenia

The complex etiology of schizophrenia creates several limitations in current research models, which makes this disorder challenging to study experimentally. Advances in the development of viable models for studying complex psychiatric disorders, especially in the field of stem cells, have made in vitro research crucial for understanding central nervous system diseases. The use of a range of experimental models such as hiPSCs, neural stem cells, 3D brain organoids, and embryonic cells has significantly advanced in neuropsychiatric research (Review in Koszła et al., 2020; Marchetto et al., 2011).

Patient-derived cell lines offer the advantage of capturing each individual's whole genetic background and environmental factors. The hiPSC-derived neuronal subtypes have already proven to be useful for high-throughput screening for genetic variables, pharmacological targets, and biochemical aspects of the disease (reviewed by Sloan et al., 2018; Koszła et al., 2020). The comparison of several cell types from schizophrenia patients and control individuals, for example, may indicate cell populations more frequently or strongly affected in a given condition. For instance, human iPSCs offer the advan-

tage of working with different patient cohorts: within families or individuals who are treatment-resistant or treatment-responsive (reviewed in Vadodaria et al., 2020). Furthermore, human iPSCs can be differentiated into 2D or 3D in vitro model systems with the additional benefit of a retained epigenetic memory. 3D brain organoids are a promising tool to study the early steps of human brain development, as well as neuron–astrocyte communication, and neuronal activity patterns (Unterholzner et al., 2021), all of which are key targets of study in schizophrenia.

Recently, the progress in the generation of 3D brain organoids derived from human pluripotent stem cells has complemented 2D cell culture models and animal studies to investigate aspects of human brain development and neuropathology. In this context, 3D brain organoids offer new cellular platforms for investigating neuropsychiatric disease etiology and understanding the impact of genetic variants associated with mental diseases, their development, and overall functions of the human brain (Koszła et al., 2020). These systems permit the reproduction of cellular microenvironment contexts as well as interactions between glia and neurons. Moreover, these models can serve as platforms for drug screenings for potential schizophrenia treatments.

Despite the many advantages of stem cell in vitro models, there are several limitations that should be mentioned. The most glaring is that protocols for the induction of stem cells and 3D brain organoids are still in the development phase. They have not been fully characterized, and protocols come at considerable expense, both in time and resources (Bray et al., 2012). Furthermore, while human iPCS limit environmental variables that can confound investigation of pathogenic mechanisms, it also loses the effects of environmental factors that contribute to neuropsychiatric disease (reviewed by Koszła et al., 2020; Abashkin et al., 2021).

Naturally, all cell models have their shortcomings. 3D brain organoids may represent a simplification of in vivo neural tissue, being relatively immature and more heterogeneous than developed human brains (Paşca et al., 2015; Quadrato et al., 2016). This creates limitations regarding neuronal connections and the proportions of cells. For example, astrocytes in 3D brain organoids are fewer than in primary tissues. Another drawback is the lack of certain cells that are normally present in brain tissues, such as endothelial cells, meninges, white matter regions, and blood vessels (Buchsbaum & Cappello, 2019).

Despite these limitations, 3D brain organoids derived from human pluripotent stem cells have successfully been used to elucidate mechanisms underlying neuropsychiatric disease – especially when combined with in vivo models. Broadly speaking, on account of the high complexity of the interactions between genetic and environmental factors linked to the development of schizophrenia and its symptoms, cell models can help elucidate the molecular and cellular bases of this disorder, though they should be recognized as a single level of inquiry.

4 Concluding Remarks

Several studies have used in vitro approaches to unravel the pathomechanisms behind schizophrenia. The complexity of schizophrenia imparted by the heterogeneity of the disorder makes studying its molecular bases, both in vitro and in vivo, increasingly more challenging. Moreover, since neuropathological events that begin during pregnancy and continue to adulthood can contribute to the onset of schizophrenia, studying these processes can be difficult given the infeasibility of directly accessing neuronal tissues. New technologies in the cell culture field, such as hiPSCs and 3D brain organoids, have provided the opportunities to create brain cells from patients, enabling an in vitro model for the pathogenesis of schizophrenia, taking that patient's genetic background into account. Despite the many challenges in establishing hiPSCs derived from patients, hiPSC-based research holds great potential to contribute to different aspects of the disease, enabling a better understanding of the pathophysiology of schizophrenia in an integrated manner.

Acknowledgments CBT, GSZ, GMV, and FC are supported by the São Paulo Research Foundation (FAPESP) grants 2017/25055-3, 2018/14666-4, 2017/25588-1, 2018/03673-0, 2019/22398-2, and 2021/06699-2. BJS is supported by CAPES grant 88887.495565/2020-00.

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Schizophrenia Outside the Brain

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Contents

Abstract

Schizophrenia is a multifactorial mental disorder, characterized by positive symptoms (delusions, hallucinations), negative symptoms (anhedonia, social withdraw), and cognitive symptoms (impairment of memory, learning, and executive functions). Despite the classic symptoms being related to the central nervous system, schizophrenia has been described by recent studies as a systemic disease, affecting other organs, tissues, and systems out of the brain. In this chapter, we summarize the main tissues and systems found affected in schizophrenic patients, both before and after antipsychotic administration. We offer an overview of the recent findings in the field about musculoskeletal system, metabolism, and immune system dysfunctions found in patients as well in models in vitro. We also discuss some of the side effects of certain antipsychotics often related to increased risk of comorbidities in patients with schizophrenia during the treatment.

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*,
Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_4

Keywords

Schizophrenia · Adipose tissue · Metabolic syndrome · Immune system · and Psychiatry

1 Introduction

Schizophrenia is a multifactorial disorder that primarily affects the brain, causing the classically recognized symptoms in the central nervous system (positive, negative, and cognitive symptoms) (Andreasen et al., 1995). In the past few decades, however, studies have indicated that concomitantly with neurological symptoms, other symptoms manifest in several other biological systems of the body (Malhotra et al., 2013; Nielsen et al., 2021; Bahorik et al., 2017), conferring an even greater degree of complexity to the etiology of an already complex disorder. These peripheral symptoms contribute to even further decreased quality of life of patients with schizophrenia (Karow et al., 2014; Padmavati, 2016) though the exact degree of the contributions has not been studied in comprehensive depth.

In addition to these peripheral effects that stem from the disorder itself, the principal line of treatment for several of the symptoms of schizophrenia is the use of antipsychotics, which also induce side effects that further impair the functioning of many of the same systems (Muench & Hamer, 2010; Freudenreich, 2020; Miller et al., 2008). Therefore, treatment of the symptoms of schizophrenia must also consider the side effects that develop from each medication, some of which may not be known until weeks after treatment begins; robust biomarkers for treatment response are still in their infancy (Martins-de-Souza et al., 2020; Blessing et al., 2020; Steiner & Guest, 2017).

In this chapter, we will address the physiological context of patients with schizophrenia in three different aspects: impairments of the musculoskeletal system, inflammation and immune system modulations, and metabolic syndrome. We will then discuss the most commonly observed side effects of the main medications used for the treatment of schizophrenia within these three physiological categories.

2 Schizophrenia as a Systemic Disorder

The physiological effects of schizophrenia are not restricted to the brain, and patients with this disorder present molecular alterations in various other systems, even before beginning any pharmacological treatment. However, due to the heterogeneity of such symptoms, the prodromal phase of schizophrenia is clinically recognized mostly by assessment of psychosocial behavior, though more complex models involving patient demographics and risk factors are under development (Conroy et al., 2018). Nonetheless, patients even before treatment still present alterations in the musculoskeletal system, categorized by problems with locomotion and motor coordination (Haralanov et al., 2018; Walther & Strik, 2012).

In addition to neurological symptoms, neuroinflammation also occurs in schizophrenia (Buckley, 2019), and that inflammation has been found to extend to the immune system as a whole (Meyer, 2013; Kirkpatrick & Miller, 2013; Müller et al., 2015; Mongan et al., 2020). This has led to the hypothesis that the overall inflammatory landscape may not only be a factor associated with schizophrenia but perhaps a factor responsible for the disorder.

Lastly, in addition to the musculoskeletal and immune systems, patients with schizophrenia also exhibit a number of significant metabolic changes. These include insulin resistance (Guest, 2019), impaired glucose tolerance (Spelman et al., 2007), adipose tissue dysfunctions (Ruppert et al., 2018; Smith et al., 2021), and diabetes (Suvisaari et al., 2016). Moreover, one promising new adjunct therapy for treating schizophrenia is aerobic exercise, which has been shown to improve both psychological and physiological characteristics of the disorder (Maurus et al., 2019; Girdler et al., 2019).

Due to the multifactorial nature of schizophrenia, such generalized dysregulations in these biological systems could potentially be only consequences of genetic alterations that occur in patients with schizophrenia, occurring only concomitantly with no causative nature. However, more recent studies have hypothesized that dysfunctions in the immune system and in adipose tissue may not only be parallel factors but instead potentially causative of the disorder (van Mierlo et al., 2020; Benros & Mortensen, 2020), highlighting the need to study schizophrenia in the human body as a whole and within individual biological systems, instead of solely within the central nervous system.

3 The Musculoskeletal System

Dysregulations in the musculoskeletal system have been found to be copresent with mental disorders, including schizophrenia (Tyrovolas et al., 2020). Specifically, several studies over the past decades have found connectivity problems with the motor system in patients with schizophrenia, even when antipsychotic-naïve (Walther et al., 2017; Danckert et al., 2004; Wolff & O'Driscoll, 1999; Walther et al., 2020).

This has been linked with multiple causes, such as a dysregulation in the parietal cortex (Danckert et al., 2004) and thalamocortical hyperconnectivity, connectivity between the rostral anterior cingulate and caudate, and connectivity between the motor cortex and cerebellum (Walther et al., 2017). In one comprehensive review, sensorimotor and other movement dysfunctions were proposed to be responses to broad cerebello-thalamo-cortico-cerebellar network dysfunction (Hirjak et al., 2021). Yet other studies have suggested correlations with motor neurons (Crayton & Meltzer, 1979) as well as premotor and motor cortices, basal ganglia, thalamus, and the connecting white matter tracts (Walther & Strik, 2012).

In an investigation of electronic health data, no correlation was found between patients with schizophrenia and musculoskeletal diseases (Bahorik et al., 2017), signifying that the changes that are seen in these patients are in majority due to schizophrenia itself and not any comorbidities. Antipsychotic medication also can induce several sensorimotor and musculoskeletal side effects, supporting a possible overlap between these dysregulated systems and the pathophysiology of schizophrenia; these side effects will be discussed later in this chapter.

4 Immune System and Inflammation

The first evidence that the immune system could be involved in the pathophysiology of schizophrenia dates back to 1918–1919, during the influenza epidemic. In his study, Menninger describes the association between schizophrenia and other mental illnesses and points out a surge of psychosis simultaneously with the influenza epidemic (Menninger, 1994).

Since then, many studies investigating the link between schizophrenia and the immune system have raised the "immune hypothesis," in an attempt to understand if infections during pregnancy, as well as childhood or adulthood, could contribute to neurodevelopmental impairments, subsequently leading to the symptomatology of schizophrenia (Arias et al., 2012; Khandaker et al., 2013).

The "immune hypothesis" to explain the schizophrenia pathophysiology has gained many defensors, and much insight has been generated that inflammation and immune system dysregulations could be responsible for a significant part of the symptoms of schizophrenia, such as impaired memory and learning (Kipnis et al., 2004; Ziv et al., 2006), reduced stress resilience (Vidal & Pacheco, 2020), and social behavior impairment (Filiano et al., 2016; Torres et al., 2016; Kopec et al., 2018; Lehmann et al., 2019).

The strongest evidence so far is the relation between the risk for developing schizophrenia and mutations in the MHC (major histocompatibility complex) locus, in which structurally distinct alleles of the complement component 4 genes affect the expression of *C4A* and *C4B* in the brain (Sekar et al., 2016).

C4 is a secreted protein expressed by neurons and microglia; in mice, C4 promotes

synapse elimination during the maturation of neuronal circuits (Yilmaz et al., 2021). C4 has been hypothesized to play a role in excessive synaptic pruning by microglia during adolescence and early adulthood, raising the possibility that the complement cascade could be crucial in neuron-microglia interactions, therefore offering a potential mechanism for the onset of schizophrenia, similar to other genetic evidence involving genes encoding synaptic proteins (Sekar et al., 2016; Kirov et al., 2012; Lee et al., 2014).

Besides these hypotheses, there is evidence showing a relationship between dopaminergic dysregulation and immune system regulation. The dopaminergic dysregulation found in the schizophrenia brain has been argued to be intimately related to inflammation, since dopamine has been demonstrated to be a major regulator of inflammation (Tracey, 2009). In this way, dopamine could play a crucial role in immune cell proliferation, migration, activation, and differentiation and whose dysregulation could contribute to the impairment of cognitive functions (Ilani et al., 2004; Watanabe et al., 2006; Nasi et al., 2019). Also, it is important to note that many antipsychotic drugs induce an anti-inflammatory profile, raising the possibility that these drugs could be acting not only in neurons but also in the dopaminergic system in immune cells (Park et al., 2019; MacDowell et al., 2015).

However, despite much evidence supporting the immune hypothesis, sample sizes, the difficulty to match pairs properly (van Mierlo et al., 2020), and confounding factors sometimes lead to ambiguous outcomes, and so far there is no confirmed and unbiased evidence showing a direct or an indirect role of the immune system impairments in the schizophrenia pathophysiology (Birnbaum & Weinberger, 2020).

As it is crucial to understand the pathophysiology of schizophrenia, more studies in the field are necessary in order to explore the role of the immune system and generate new insights into clinical treatment, since immune modulation could be a powerful ally to manage symptoms and slow the development and progression of the disorder. Nonetheless, there is a need to understand the extent and the benefits of this intervention, since long-term use of anti-inflammatory drugs could increase the risk of infection and cancer (Vidal & Pacheco, 2020).

5 Metabolic Syndrome in First-Episode Drug-Naïve Patients

Metabolic syndrome is a set of metabolic disorders characterized by hypertension, glucose metabolism impairment, obesity, and lipid metabolism disorders (Stubbs et al., 2015; Vancampfort et al., 2015; Sullivan et al., 2009).

One of the greatest consensuses in the literature about the physiological scenario of patients with schizophrenia is the occurrence of metabolic disturbances, even in patients with first-episode drug-naïve schizophrenia (Pillinger et al., 2017; Shah et al., 2019), suggesting that the metabolic disturbances observed in patients are not drug-induced.

Recent studies with first-episode drug-naïve patients have shown that sex subjects (Zhou et al., 2021) and schizophrenia onset (Lang et al., 2021) are related to different patterns of metabolic abnormalities.

Zhou et al. describe that female patients have a lower prevalence of high body mass index and hypertriglyceridemia but have a higher prevalence of high density lipoprotein cholesterol, compared with male patients. Besides, female patients have the strongest relation between metabolic parameters and psychopathological dimensions, showing that body mass index, waist circumference, and glycosylated hemoglobin (HAb1c) were associated with positive symptoms, while insulin and insulin resistance were associated with positive symptoms. For the male subjects, only waist circumference is associated with positive symptoms and negative symptoms (Zhou et al., 2021).

There is also an interesting difference in schizophrenia onset, described by Lang et al. in a Chinese

population. This study observed that early-onset schizophrenia patients have lower rates of metabolic abnormalities and metabolic syndrome than adult-onset and that early- and adult-onset patients have different patterns of contributing components and risk patterns for metabolic syndrome, including body mass index, triglycerides, glucose, total cholesterol, high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC), hemoglobin A1c (HbA1c), insulin, and insulin resistance (Lang et al., 2021).

Another recent study accessed 67 first-episode drug-naïve schizophrenia patients and concluded that 10.4% were identified with hyperhomocysteinemia, 7.5% with hypertriglyceridemia, and 11.9% with hyperprolactinemia, indicating that even before any antipsychotic medication, schizophrenic patients already have metabolic abnormalities (Zhang et al., 2021).

This profile prone to metabolic syndrome identified in drug-naïve patients appears to have a genetic core. Darcin et al. (2015) studied a group of drug-naïve schizophrenic patients along with their siblings and found out that both patients and siblings had metabolic syndrome risk (Darcin et al., 2015). Similarly, glucose abnormalities were seen in the siblings of patients diagnosed with schizophrenia (Fernandez-Egea et al., 2008).

6 Drugs and Side Effects Outside the Brain

The symptoms of schizophrenia are controlled to varying degrees with the use of antipsychotics, which are able to alleviate neurological symptoms and improve a patient's quality of life. Despite the benefits of their use, antipsychotics can also induce several side effects that can often aggravate the preexisting dysfunctions (Tandon et al., 2020; MacKenzie et al., 2018; Correll et al., 2018).

The most classical cases of side effects reported in the clinic are extrapyramidal effects in response to the use of first-generation (also referred to as typical) antipsychotics, such as haloperidol (Ali et al., 2021), and metabolic syndrome in response to second-generation (also referred to as atypical) antipsychotics, such as clozapine (Ijaz et al., 2018; Jeon & Kim, 2017). Some second-generation antipsychotics also confer the risk of developing agranulocytosis, a deficiency of granulocytes in the immune system, leading to serious consequences, even death (Yoshida & Takeuchi, 2021).

These side effects, by reducing the quality of life of the patient, can lead to treatment inconsistencies or even treatment dropout (Ascher-Svanum et al., 2010), further decreasing quality of life and increasing the disability-adjusted life year (DALY), a measurement of the overall burden of a disease or disorder. As each antipsychotic targets neurotransmitter receptors with differing K_D (dissociation constant) values (Saklad, 2017), with particularly large differences between generations of antipsychotics, we have separated this discussion into two parts.

6.1 First-Generation Antipsychotics

Haloperidol was one of the first medications used to control positive symptoms in patients with schizophrenia (Ramachandraiah et al., 2009) and is still a commonly used choice in the clinic as a first line of treatment for psychotic episodes due to its sedative profile. Due to its low cost (Rosenheck et al., 2003), it is an attractive option for treatment of low-income patients and still presents a considerable rate of symptomatic remission (Kane et al., 2007).

However, despite this treatment efficacy, haloperidol and other first-generation antipsychotics are strongly associated with movement and motor coordination problems due to its propensity for extrapyramidal side effects, caused by the strong inhibition of the dopaminergic system (Sykes et al., 2017). As such, the benefits resulting from the use of this medication must be carefully weighed against these side effects, which must also be monitored to not worsen over time. Follow-up studies are important since some extrapyramidal side effects can last for years, if not permanently, when not recognized (Caroff et al., 2011).

6.2 Second-Generation Antipsychotics

Atypical antipsychotics are considered the most modern medications for the treatment of schizophrenia, usually causing less extrapyramidal effects than typical antipsychotics, due to their weaker binding to dopaminergic receptors (Lieberman, 2004).

Despite their efficacy in treating both positive and negative symptoms (Lieberman, 2004), atypical antipsychotics also are highly related to metabolic disturbances and impairment of glucose and lipid metabolism (Ventriglio et al., 2015), varying between 11 and 69% of the medicated patients (Malhotra et al., 2013).

There are many factors contributing to the risk of metabolic syndrome in patients treated with atypical antipsychotics, including sex, age, lifestyle, and physical inactivity (Spertus et al., 2018). However, there is strong evidence that these drugs increase appetite and food intake (von Wilmsdorff et al., 2010) and interact directly with the adipose tissue (Rojo et al., 2015), altering the lipid accumulation and the secretion of adipokines, which are adipose-derived proteins related to appetite, energy balance, and insulin resistance, such as adiponectin and leptin (Chen et al., 2021; Dias et al., 2021).

The atypical antipsychotics most usually associated with metabolic syndrome development are risperidone, olanzapine, and clozapine (Chen et al., 2021).

There is no consensus about the mechanisms underlying the risks of metabolic syndrome provoked by atypical antipsychotics, although most of them are related to increased serum levels of triglycerides, blood, glucose, glucose intolerance, and insulin resistance (Wirshing et al., 2002; Löffler et al., 2016), despite some studies have found no correlation between risperidone and metabolic syndrome risk (Wirshing et al., 2002; De Hert et al., 2011).

Clozapine and olanzapine appear to have the biggest impact on cell metabolism, even provoking glucose intolerance and insulin resistance (Vestri et al., 2007). They also have a larger effect upon the adipokine secretion from the adipose tissue, suggesting that metabolic disturbances could occur through adipokine signaling (Chen et al., 2021; Dias et al., 2021).

Besides their effectiveness in increasing the food intake (Chen et al., 2021), recent studies in vitro demonstrated that atypical antipsychotics can act directly on adipocytes, increasing lipid accumulation (Chen et al., 2017; Löffler et al., 2016), even in standard culture conditions, indicating that there is a direct role of these drugs on adipocyte metabolism, which offer a direct clue about why the patients have so much rapid weight gain.

One of the most used drugs to manage the metabolic effects of atypical antipsychotics is metformin (de Silva et al., 2016). However, given the direct role of these drugs on adipocytes, the most effective approaches to managing the side effects are regular physical exercise and a balanced diet (Schmitt et al., 2018; Gurusamy et al., 2018), once lifestyle interventions are safe, which have no side effects and efficiently improve the life quality of these patients.

7 Conclusion and Final Remarks

Schizophrenia is a complex mental and neurodevelopmental disorder that manifests mainly by neurological and psychotic symptoms. However, several other organs and biological systems present dysfunctions and disturbances in patients, even those who have never taken any antipsychotic medication.

Some hypotheses explain these dysregulations as the product of mutations and genetic variations that also lead to the classically recognized symptoms of schizophrenia, while others suggest that the central nervous system is more susceptible to the disorder precisely due to these dysregulations. Such is the case with the storm of cytokines observed in schizophrenia, which could be tolerated well by some organs and systems but is highly detrimental to the functioning of the human brain.

Some of these dysfunctions and dysregulations are exacerbated by the use of the main line

of treatment for the symptoms of schizophrenia: antipsychotics. As such, the side effects of this line of treatment must be carefully monitored and managed to ensure that there is no harm to the patient nor their quality of life. It is not yet known what causes some patients to respond negatively to antipsychotics and develop potentially life-threatening side effects, though one possibility is that these poor responders already had a genetic foundation for these dysregulations that were intensified into a critical state by drug administration.

Several ongoing studies are aiming to understand and elucidate the mechanisms by which a certain population is susceptible to certain treatment options so that, in the future, those treatment options can be better tailored to a given patient or improved upon for general use. Investigating the interplay between genetic and environmental factors is crucial in this understanding, as well as to determine if other, currently unknown factors also play into this disposition.

Therefore, studying and understanding how schizophrenia affects individuals beyond the nervous system, both before and during treatment, is important to understand why some people are more likely to exhibit some peripheral symptoms over others and how to use that information to better treat and rehabilitate patients with schizophrenia.

Acknowledgments The authors would like to thank the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP; grant number 2018/03450-0) and the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES; grant number 88887.495565/2020-00) for financial support.

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Molecular Features Triggered by Antipsychotic Medication in Brain Cells

Lívia Ramos-da-Silva and André S. L. M. Antunes

Contents

Abstract

Treating schizophrenia is a challenge currently handled with the use of antipsychotic drugs. Despite being the most applied treatment strategy, current antipsychotics present severe limitations and side effects which impact patients' health and quality of life. For instance, although these drugs target mainly the dopamine system, they present target promiscuity and work by distinct mechanisms of action. As a consequence, complete comprehension of their pharmacological properties remains elusive. This chapter highlights research from the past 5 years that contributed to our current understanding of the mechanism of action and molecular features triggered by antipsychotic drugs in brain cells. In addition, we briefly discuss potential new therapeutic targets and strategies to treat schizophrenia.

Keywords

Schizophrenia · Antipsychotic drugs · Molecular targets · Cellular targets

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1 Introduction

Schizophrenia (SCZ) is a severe neuropsychiatric illness that continues to grow and impact the quality of life and productivity of over 20 million people worldwide (Charlson et al., 2018). Medication with antipsychotic drugs (APDs) is currently the standard pharmacological treatment to relieve symptoms of patients, and despite being widely prescribed, comprehension of their pharmacological properties remains elusive.

In general, APDs are divided into first-generation, called typical antipsychotics (i.e., haloperidol), and second-generation antipsychotics (i.e., clozapine), called atypical (Andrade, 2016). The major difference between their mechanisms of action is that typical antipsychotics act by blocking mainly dopamine D2 receptors (D2R) and regulate symptoms like hallucinations and delusion. On the other hand, atypical APDs act mainly as antagonists of D2 and serotonin (5HT) receptors, being most effective against the lack of motivation, social withdrawal, and cognitive impairments of patients (Aringhieri et al., 2018).

Despite notable clinical effectiveness, medication with APDs has limitations such as poor patient long-term adherence to treatment and development of severe side effects (Ascher-Svanum et al., 2006; Stępnicki et al., 2018). The therapeutic mechanism of action underlying most APDs remains poorly understood, largely due to their extreme molecular promiscuity regarding targets in the central nervous system (CNS). This is possibly a reason for the marked disparity among patients with respect to symptoms, clinical responses, and side effects (Meltzer, 2010; Fusar-Poli et al., 2015).

In this chapter, we focus on studies from the last 5 years that contributed to our current understanding of the mechanism of action and molecular features triggered by antipsychotic medication in brain cells. We then highlight ligands, pathways, and mechanisms that still remain underexplored in the field and briefly discuss potential new therapeutic targets and strategies to treat schizophrenia.

2 Pathways and Regulatory Mechanisms Affected by APDs

Two of the broad post-receptor intracellular mechanisms triggered by APDs, and still need to be deepened, are ERK1/2 and AKT pathways. Both pathways are relevant to many neural processes that might be implicated in schizophrenia and in other psychotic disorders (Kyosseva, 2004; Zheng et al., 2012). Comparing typical and atypical APDs on cell cultures, clozapine was the most efficacious antipsychotic in activating ERK1/2 and AKT pathways. This effect was mediated by the activation of the 5-HT_{2A} receptor in a G protein-independent manner and involved effectors such as β-arrestin, through a mechanism called "biased agonism" (Aringhieri et al., 2017). Since clozapine is the most effective APD in the management of treatment-resistant SCZ (Nucifora Jr et al., 2019), this evidence might be relevant to explain the superiority of clozapine among the APDs.

The cAMP/PKA and MEK/ERK1/2 signaling pathways are also involved in the mechanism of action of atypical APDs. Through the activity of D1-class receptors, these pathways were responsible to phosphorylate CREB protein and trigger the transcription of *ADAMTS2*. In this regard, clozapine had a fast and sustained inhibition of its expression, which in turn could be prevented by D2 and 5-HT2A receptor antagonists (Ruso-Julve et al., 2019). This study confirmed the involvement of cAMP/PKA and MEK/ERK1/2 signaling as a molecular mechanism exerted by APDs in the control of *ADAMTS2* expression and their potential role in the clinical efficacy of these drugs. Moreover, these findings underscore the "biased agonism" of clozapine on $5HT_{2A}$ receptors.

Haloperidol and clozapine were also shown to induce a significant decrease in the active form of phospho-Akt(Ser473) protein in the brain cortex of rats, while no changes were found in total Akt. Moreover, haloperidol induced a significant increase in GSK3b total levels, while clozapine did not induce any significant changes (Ibarra-

Lecue et al., 2020). Increased phosphorylation was also seen for S6 ribosomal protein (S6RP) in the frontal cortex of rats treated with haloperidol (Chadha et al., 2021). This protein participates in intracellular processes that are particularly important for neuronal signaling and synaptic plasticity and play a key role in neuropsychiatric disorders (Costa-Mattioli & Monteggia, 2013). Altogether, these results suggest a potential novel mechanism of action for antipsychotics and future new targets in the treatment of SCZ.

Environmental factors play a key role in the onset of SCZ, and epigenetics has been recognized as a strong component in the manifestation of SCZ, bipolar disorder, and major depressive disorder (Network and Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015). MicroRNAs (miRNAs) target epigenetic proteins and have also been involved with the etiology and treatment of SCZ. Overexpression of miR-132 specifically decreased genome-wide levels of H3K27me3 and its histone methyltransferase enzyme EZH1, and long-term treatment with clozapine and haloperidol was shown to downregulate EZH1 in mouse mPFC (Johnstone et al., 2018). In another study, miR-223, a miRNA known to be secreted via exosomes, was shown to be dysregulated in subjects with SCZ and differentially regulated in mouse cortical neuronal and astrocytic cultures upon treatment with haloperidol and olanzapine (Amoah et al., 2020). Further studies will be necessary to determine how miR-132/EZH1 and miR-223 signaling contribute to SCZ treatment and pathology.

Although there is general agreement that altered epigenetic mechanisms play an important role in the etiopathogenesis of SCZ, little is known regarding the effects of APDs on epigenetic modifications in neurons of patients with SCZ (Grayson & Guidotti, 2013). Through the analysis of a neurodevelopmental epigenetic mouse model (PRS), which exhibits a similar epigenetic profile to those of *postmortem* brains from patients with SCZ, results showed that haloperidol and risperidone fail to normalize the altered behavioral endophenotypes or to reduce DNMT1, a major DNA methylation protein, promoter hypermethylation in the mice model. On the other hand, clozapine normalizes the altered endophenotypes and aberrant chromatin remodeling (Dong et al., 2019). Together, the data suggest that drugs that reduce DNA methylation by reducing DNMT levels, or by blocking DNMT binding, may be desirable to provide beneficial effects to patients with SCZ who have an underlying epigenetic etiopathogenesis.

Still, DNA methylation analysis from human neuroblastoma cell lines treated with perospirone, an atypical APDs, led to changes in epigenetic profiles of neural genes. Many of the hypomethylated results were related to neurogenesis, which suggests that perospirone may enhance transcription of these genes, and the majority of hypermethylation results were related to genes for brain development (Murata et al., 2019). Studies in vivo are necessary to confirm these effects on the epigenetic status.

3 Cellular Receptors Targeted by APDs

Although not completely understood, the participation of DA and 5HT receptors and G protein-coupled receptors (GPCR) on APDs' mechanism of action is well known. However, beyond DA and 5HT receptors, there are emerging studies showing the importance of the pharmacological action of these drugs in other binding partners and how it might explain more deeply its mechanism of action and clinical effects. The α2A- and α2C-adrenoceptors (ARs) are the primary α2-AR subtypes involved in CNS function. In addition, they are intimately involved in the function of the brain noradrenergic system and have been shown to be dysregulated in SCZ as well as in major depressive disorder (Uys et al., 2017).

In cell culture models, the APDs chlorpromazine, fluphenazine, and clozapine were able to bind α2A-AR with similar affinities, and none of the agents induced classical heterotrimeric G protein-mediated α2A-AR signaling. Instead, significant differences were observed with respect to arrestin-3 recruitment and receptor endocytosis. These APDs drive significant

α2A-AR endocytosis, but via differing clathrin-dependent and lipid raft-dependent pathways (Cottingham et al., 2017). Conversely, samples from prefrontal cortex postmortem tissue of antipsychotic-treated patients with SCZ showed increased α2A-AR expression in synaptosomal plasma membrane and postsynaptic density fractions with no significant changes in α2C-AR. Significant lower stimulation of Gαi2 and Gαi3 proteins in these subjects was also found, whereas Gαo protein stimulation was significantly decreased in both antipsychotic-free and antipsychotic-treated patients with SCZ (Brocos-Mosquera et al., 2021). Collectively, these studies reveal that APD treatment is able to modify both the protein expression and the functionality of α2A-AR in opposite directions in the cortex of patients with SCZ. Further investigations are required to understand if APDs can be considered as novel arrestin-biased ligands at the α2A-AR.

With regard to side effects, atypical APDs have the propensity of inducing severe metabolic alterations (Doménech-Matamoros, 2020). Transient receptor potential vanilloid type 1 (TRPV1) channels are an emerging therapeutic target for APD-induced metabolic disorders. In fact, mice treated with olanzapine display metabolic alterations with concomitant increased expression of TRPV1/TRPV3 in the hypothalamus. Interestingly, treatment with berberine, an isoquinoline alkaloidal phyto-constituent from plants of the *Berberis* genus, significantly decreases TRPV1/TRPV3 expression and attenuates olanzapine-induced metabolic alterations observed in mice, suggesting that a potential supplementation for patients could be a preventive approach to reduce the metabolic adverse effects of APDs (Singh et al., 2020). Serotonin syndrome is yet another side effect associated with atypical APDs. Through mechanistic analysis using computational methods, 5-HT_{2A} antagonism and 5-HT_{1A} agonism have been punctuated as common mechanisms that may explain the association between atypical APDs and serotonin syndrome (Racz et al., 2018). Confirmation of these mechanisms may contribute to a more careful screening of APD combinations and increased patient monitoring to reduce the risk of serotonin syndrome.

Still regarding 5HT receptors, pimavanserin is the first antipsychotic drug that shows selectivity for serotonin 5-HT_2 receptors without binding dopamine D2 receptors and has been studied as adjunctive therapy for SCZ (Meltzer et al., 2012). Native human and mouse *postmortem* brain tissues revealed that the atypical APD pimavanserin is highly selective for the 5-HT_{2A}R coupling to Gαi1-proteins, whereas the drug operates as neutral antagonist on the signaling pathway mediated by $G_{\alpha q/11}$ proteins (Muneta-Arrate et al., 2020). In the brain, the 5-HT_{2A}R is able to discriminate activation of $G_{\alpha q/11}$ and $G_{\alpha i/o}$ proteins and other signaling pathways in response to diverse agonists, and a recent study had pointed out that, perhaps, anti-hallucinogenic properties of 5-HT_{2A}R drugs depend on modulation of $G_{\alpha i/o}$ proteins and their downstream signaling pathways (González-Maeso et al., 2007; López-Giménez & González-Maeso, 2017). Additionally, the supersensitivity of the 5-HT_{2A}R coupling to $G_{\alpha i1}$ proteins but not to $G_{\alpha q/11}$-proteins in *postmortem* brains of subjects with SCZ has been described (García-Bea et al., 2019). Taken together, these results provide further support for the suggestion that selective 5-HT_{2A}R antagonists/inverse agonists with functional selectivity on $G_{\alpha i1}$ proteins might be useful tools as APDs.

Regarding DA receptors, clozapine is more efficacious than typical APDs for improving both positive (psychotic) and negative (emotional) symptoms of SCZ, and it also does not lead to extrapyramidal and dystonia side effects (Horacek et al., 2006). Previous studies have suggested that the antipsychotic effect of clozapine is mainly mediated through dual dopamine D2R and serotonin 5-HT2 antagonism, while D4R blockade may participate to a lesser extent (Ptacek et al., 2011). In fact, one study suggests that despite clozapine suppression of both the initiation and expression of methamphetamine behavioral sensitization, as well as hyperactivity in dopamine transporter knockdown mice, the blockade of D4R signaling cascades on the mouse mesolimbic region appears to be dispensable for clozapine's therapeutic effects (Liao &

Chen, 2021). These results may show that molecular targets other than D4R should be prioritized in the development of future therapeutics for treatment of hyperdopaminergia-dependent neuropsychiatric disorders.

4 APDs and Brain Cells

Myelination disturbances and oligodendrocytes have been put forward as potential new targets to treat negative symptoms in patients (Bartzokis, 2012). When it comes to APD treatment, both typical and atypical APDs are associated with an initial intracortical myelination increase (Bartzokis et al., 2009). However, *Tishler* et al. showed through in vivo neuroimaging methods that intracortical myelination of subjects with SCZ was higher during the first year of atypical APDs' treatment but declined thereafter (Tishler et al., 2018). Additional studies will be required to establish if there is a causal relationship between APDs and intracortical myelination alterations in patients with SCZ. Nevertheless, it can be an indication that the correction in these deficits may be an important mechanism of action for antipsychotics.

Oligodendrocytes are the myelinating cells in the CNS and therefore essential for adequate neuronal function and survival. Disruptions in oligodendrocytes have been associated with dysfunctions of perception, behavior, and cognition seen in patients with SCZ (Takahashi et al., 2011; Saia-Cereda et al., 2015). In fact, these cells are now seen as potential targets for the treatment of SCZ (Gouvêa-Junqueira et al., 2020; Seabra et al., 2020). Analyzing the effects of typical and atypical APDs on oligodendrocytes cell cultures, *Brandão-Teles* et al. showed that the APDs could alter proteins involved on spliceosome machinery, protein synthesis on ribosomes, mTOR signaling pathway, ubiquitination pathway, energy metabolism, and 14–3-3 family. In addition, exclusive alterations could also be seen in each specific treatment (Brandão-Teles et al., 2019). In line with these findings, cannabidiol, clozapine, and haloperidol were shown to modulate proteins related to metabolism, antioxidant system, and RNA splicing on oligodendrocytic cultures (Falvella et al., 2021). Moreover, in a cuprizone-mediated toxicity model, still in oligodendrocyte cell cultures, these drugs had bidirectional modulations in apoptosis and cell proliferation protein co-treatments, which were mostly downregulated in response to cuprizone insult, suggesting a potential ability from cannabidiol and APDs to prevent demyelination events (Falvella et al., 2021).

Several studies have pointed out the participation of neuroinflammation in the pathophysiology of SCZ (Köhler-Forsberg et al., 2020). Astrocytes are glial cells that are involved in a range of CNS functions, including inflammatory response, and their dysfunction has attracted attention in neuropsychiatric disorders (Verkhratsky & Parpura, 2016). In one study, risperidone showed an anti-inflammatory activity in astrocyte-like cell line, decreasing the release of tumor necrosis factor-α (TNF-α) and interleukins 1β (IL-1 β) and 6 (IL-6), and increasing interleukin 10 (IL-10) (Bobermin et al., 2018). It was also able to decrease the transcriptional activity of nuclear factor-κB (NFκB) and improve glutathione content, an important antioxidant defense mechanism in the CNS. On the other hand, haloperidol induced a pro-inflammatory response, increasing p65 NFκB nuclear levels and the activation of p38 MAPK, a molecule that signals upstream from NFκB (Bobermin et al., 2018). Additionally, it increased extracellular levels of TNF-α and IL-1β and decreased IL-10. It is reasonable to consider that haloperidol induces an inflammatory response by activating p38 mitogen-activated protein kinase (p38 MAPK)/NFκB pathways.

Regarding neurogenesis, neurogenic regions in the adult brain are located in the subventricular zone of the lateral ventricles and subgranular zone of the hippocampal dentate gyrus (Yuan et al., 2014). Such a process is modulated by many factors, including APDs. In fact, chronic administration of olanzapine had proneurogenic effects on the adult rat (Lasut et al., 2018). It suggests that long-term treatment with olanzapine may stimulate neurogenesis in the subventricular zone and perhaps could represent an improve-

ment in negative symptoms of patients with SCZ. Neurodegeneration and impaired neural development are also observed in patients with SCZ. It is known that atypical APDs display neuroprotective effects; however, the mechanistic aspects are not yet fully understood. To understand whether selective 5-HT_{2A} inverse agonists have neuroprotective properties, pimavanserin was applied to primary cultures of dopaminergic neurons which received an insult that induces cell death. It was seen that pimavanserin induces the release of neurotrophic factors and protects dopaminergic neurons against the insult in a glial-derived neurotrophic factor (GDNF)-dependent manner (Lavigne et al., 2021).

5 Novel Potential Antipsychotics

Although APDs are able to control the intensity of the symptoms experienced by patients with SCZ, diminish exacerbations of the illness, and decrease the risk of relapse, current APD therapies are associated with significant side effects and medical morbidity burdens that hinder progress toward treatment goals (Kane, 2007; Fusar-Poli et al., 2015). Furthermore, despite the complete pathomechanism of SCZ remaining unclear, it is widely accepted that it involves many neurotransmitter systems. The central hypothesis of SCZ is still the dopaminergic hypothesis, which is complemented by the glutamatergic hypothesis, and the involvement of other neurotransmitter systems, in particular serotonin and γ-aminobutyric acid (GABA), as well as cholinergic and endocannabinoid system (Stahl, 2018; Ranganathan & D'Souza, 2020). Taking it all into account, in this section we consider it is relevant to mention some molecules that have been evaluated as novel potential antipsychotics.

Briefly, there is the compound D2AAK3, which through structure-based virtual screening was identified to have high affinity to D2R on the nanomolar range and additional affinity to D1, D3, 5-HT1A, 5-HT2A and 5-HT7 receptors on the nanomolar or low micromolar ranges. Regarding behavioral studies, D2AAK3 decreased amphetamine-induced hyperactivity and improved memory consolidation in mice. Furthemore, it induced anxiogenic activity in mice 30 min after acute treatment, which in turn disappeared after one hour (Kaczor et al., 2021). D2AAK4 is yet another compound, which has an atypical APD profile and low affinity to off-targets. It interacts with aminergic GPCRs and is shown to decrease amphetamine-induced hyperactivity at the dose of 100 mg/kg, and improve memory consolidation. However, it also had anxiogenic properties in mice (Kaczor et al., 2020).

SYA16263 is a compound that was reported as having a moderate radioligand binding affinity to D2 and D3 receptors and high binding affinities to D4 and 5-HT_{1A} receptors. It also produced antipsychotic-like behavior without inducing catalepsy in mice. Additionally, a structurally modified analog of SYA16263 was able to demonstrate antipsychotic-like activity in mice, with no significant binding affinity to D2R and moderate binding affinity to D3 and D4 receptors (Onyameh et al., 2021). Finally, a series of fluorinated 3-aminomethyl derivatives of 2-phenylimidazo[1,2-a]-pyridine that display high affinity for GABAA receptor benzodiazepine site and positive allosteric modulator activity were indicated as potential antipsychotic agents. The most promising of them showed antipsychotic-like activity, reversing hyperlocomotion induced by amphetamine without inducing significant sedation (Marcinkowska et al., 2017).

6 Concluding Remarks

Molecular mechanisms triggered by APDs are not yet fully understood. So far, studies that aimed to address this question revealed the promiscuity of APD targets and broad cellular responses in the brain. Despite investigations having focused on neuronal cells, some studies have been shifting their focus toward the interaction between APDs and glial cells, and continued research in the field could provide further knowledge regarding the effects of these medications

on the brain as a whole. Furthermore, the effects of atypical APDs on ERK1/2 and AKT and cAMP/PKA and MEK/ERK1/2 signaling may be a key point to better comprehend the distinction between the treatments with typical and atypical APDs. Moreover, the ability of APD treatment to modify both the protein expression and the functionality of α2A-AR and TRPV1/TRPV3 receptors, beyond "biased agonism" of clozapine on $5HT_{2A}$ receptors, warrants further investigation. Finally, better understanding of the molecular features triggered by APDs is crucial for the development of more specific and efficacious medications and hence achieves improvements in the clinical realm. Despite some potential new molecules having been presented here, more studies are required to validate them as new APDs.

Acknowledgments LR is supported by the National Council for Scientific and Technological Development (CNPq, grant number 160231/2021-3). LR and AA are funded by FAPESP (São Paulo Research Foundation grants 2017/25588-1, 2020/10282-7 and 2021/03598-0).

Author Contributions LR conceived and wrote the manuscript. AA designed, supervised the process, and finalized the manuscript. All authors approved the final version of the manuscript.

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Known and Unexplored Post-Translational Modification Pathways in Schizophrenia

Bradley J. Smith and Victor C. Carregari

Contents

Abstract

Post-translational modifications (PTMs) of proteins occur in all domains of life, affecting various structural and functional properties. Multiple methods can be used to study PTMs depending on the biological question, which can vary widely. Schizophrenia is a widespread brain disorder that possesses many known contributing environmental factors and hundreds of genetic risk factors; however, a full picture of the mechanisms behind how and why this disorder occurs and how it can be treated remains unknown. Various PTMs have been found to be differentially expressed in several pathways that are dysregulated in schizophrenia, as seen in cell line and animal models, *postmortem* brain tissue from people with schizophrenia, and biological fluids like blood, plasma, and cerebrospinal fluid. Despite recent advances, several pathways have been completely left undisturbed by

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*, Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_6

PTMomics and show great promise for better understanding of protein dynamics in schizophrenia, how the disease state occurs, and how it may be better treated in future therapies.

Keywords

Post-translational modifications · Schizophrenia · Proteomics · Mass spectrometry

1 Protein Modifications in Humans

One of the many regulatory mechanisms that control the pathway between DNA and enzymatic activity is the post-translational modification of proteins. In addition to the hundreds of known functional groups that can be added to many amino acid residues, proteins can also be cleaved, degraded, and structurally modified such as in the formation of disulfide bonds, and the amino acid residues themselves can also be chemically modified (Ramazi & Zahiri, 2021). Important to the study of many diseases and disorders, protein modification very closely represents a cellular phenotype, since many PTMs can be added and often removed quickly in response to stimuli and affect multiple properties of a protein.

Different mechanisms exist to perform these modifications, which can be reversible or irreversible. Many modifications occur via enzymatic activity, wherein a regulatory protein affixes a chemical group to a site in a directed, site-specific manner; common examples of this type of modification are acetylation via acetyltransferases and phosphorylation via kinases. Within this class are other modifications that affect the structure of a protein, such as protein cleavage and disulfide bridge formation, and protein or peptide addition, as is the case with ubiquitination. Other cases of modifications can be spontaneous, occurring when a chemical group comes into contact with a suitable site of modification, such as carboxylation, succinylation, and malonylation when carbon dioxide, succinyl-CoA, or malonyl-CoA interacts with a lysine residue (Park & Hausinger, 1995; Peng et al., 2011; Weinert et al., 2013; Colak et al., 2015).

A single protein can have multiple sites of modification, each being potentially targeted by multiple PTMs. Site specificity for modifications and even sub-specificity for regulatory proteins allow different proteins to add and/or remove PTMs, each with their own regulatory stimuli, further adding complexity to this regulatory system. In addition to the physiochemical changes that occur when a modification is made to a protein, several other effects may occur, depending on the PTM, its target protein, and the site of modification. Some classic examples are an increase in activity that results from certain proteins being phosphorylated (Ardito et al., 2017) and how histone acetylation can increase gene transcription of nearby DNA (Gräff & Tsai, 2013).

When focusing on the most relevant effects of PTMs in the scope of human disease, PTMs can affect protein stability, intracellular localization and membrane insertion, extracellular export, enzymatic and receptor activity, degradation and turnover, and protein-protein interactions (Ramazi & Zahiri, 2021). Dysregulations in any of these properties of a protein can lead to widespread and even compounding effects on multiple biological pathways and systems, even though it may not be visible via other omic techniques such as genomics, transcriptomics, and even standard proteomics.

2 Investigating Post-Translational Modifications

Upon studying PTM profiles, PTMomes, multiple layers of information can be extracted from a sample, based on how those modifications affect certain proteins. Unfortunately, this area is still in its infancy, and it is not known how many – if not most – modifications affect their target proteins. Nonetheless, after determining the effect of a particular PTM at a given site of modification, it is possible to extract further information about the state of that cell, potentially even linking changes to pathological phenotypes. As previ-

ously mentioned, PTMs can affect protein expression, enzymatic activity, intracellular localization or exportation, interactions with other proteins, and degradation; and all of these characteristics can be interesting targets of study in certain diseases and disorders.

3 2-Dimensional Gel Electrophoresis

Multiple methods can be used to study post-translational modifications, each with certain limitations and strengths. One early method for identifying certain protein states employs two-dimensional gel electrophoresis. Many PTMs, such as phosphorylation, modify the charge state of a protein, thereby changing its isoelectric point (pI), generating two neighboring spots on a 2D gel (Mayer et al., 2015). This methodology gives an overall picture of a known protein's modification state; however, it does not possess any site specificity and cannot usually distinguish between modifications with similar mass and properties, nor can it easily and confidently be applied to modifications with large mass and pI differences like ubiquitination and SUMOylation. Nonetheless, 2D gel electrophoresis can be a powerful tool for isolating a protein and its modified state(s) for further analyses (Zong et al., 2008; Magdeldin et al., 2014; Jastorff & Turck, 2019), with the caveat that some modifications may not be stable enough to undergo certain in-gel digestion steps or staining, or can bind to amide groups in polyacrylamide gels (Bonaventura et al., 1994; Feng & Lu, 2011), potentially affecting yield in an unpredictable manner.

4 Ion- and Antibody-Based Enrichment

Another useful tool for studying PTMs is columns with metal ions (Fíla & Honys, 2012) or antibodies (Yao & Seger, 2001) to enrich a sample before further analyses. This step reduces unmodified proteins, making the samples less complex and more easily studied by other methods. Unfortunately, ionic enrichment is not applicable for all PTMs, and in these cases antibody-based enrichment can be employed, relying on pan-PTM antibodies, which are specific to a certain modified residue, regardless of the flanking sequences. Zhao and Jensen wrote a more detailed review of PTM enrichment methods that can provide further information about this step (Zhao & Jensen, 2009).

5 Liquid Chromatography and Mass Spectrometry

One of the most important methods for studying PTMs both for their identification and quantitation is the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This method can be applied to a wide array of experiments, depending on the parameters and instrument used. For example, shotgun proteomics, optionally coupled with an enrichment method above, can be used to visualize a wide array of post-translationally modified proteins at the peptide level. As shotgun proteomics is performed in a non-hypothesis-driven fashion, hundreds to thousands of PTMs can be identified in this manner. However, sites of modification can be revealed to a close, but not always exact, degree, though this depends on the resolution of the instrument. When two or more potential sites of modification are found in close proximity, the site that is truly modified may not be resolved if peptide fragmentation efficiency and fragment ion identification were not sufficiently high. Lastly, depending on the software that is used to analyze the raw data, a great deal of extra work may be necessary to identify more than a few modifications at a time. Each variable modification that is added to the search parameters increases the potential error, causing the FDR threshold to be reached more quickly and thereby decreasing the number of identified proteins. Other software algorithms, such as PeaksPTM (Han et al., 2011), use the unmodified peptide and known biological frequency of a given PTM to quickly and easily identify modified peptides without first specifying the modification or needing to increase the FDR threshold.

Another LC-MS/MS method for PTM studies is through targeted proteomics, a highly sensitive but more restrictive method choice. In targeted proteomics, a predetermined set of peptides is selected, and those peptides can be quantified with a high degree of sensitivity and specificity, even in more complex samples. Since the mass of a peptide and its fragments are being tested in such a specific manner, there is no identification step; as such, the trustworthiness of targeted data is also high. The drawback for this method, however, lies precisely in its specificity. Targeted proteomics is only capable of validating hypotheses and cannot be used on a global scale or to identify new sites or modifications.

Lastly, mass spectrometry can also be used to carry out what is called top-down proteomics, in which intact proteins are injected into a mass spectrometer instead of smaller, digested peptides. Though top-down proteomics is capable of identifying fewer proteins overall in a given sample than bottom-up methods when used globally (Fornelli et al., 2018), it is able to provide an important layer of data for PTMomics: PTM co-localization, proteoform modification, and protein stability and cleavage (Siuti & Kelleher, 2007). In bottom-up proteomics, since all proteins are digested together, it is impossible to know if two modifications are present on the same protein or if they are exclusive to certain isoforms, for example. Top-down proteomics allows for this type of analysis and, like other LC-MS/MS methods, can be used in conjunction with other enrichment or separation methods.

6 PTMs in Schizophrenia

6.1 The Hypotheses of Schizophrenia

Several hypotheses have been developed to explain the pathophysiology of schizophrenia, which include the dysregulation of several neurotransmitter systems and neurodevelopmental pathways. In each of these hypotheses, some of the symptoms of schizophrenia can be explained by changes to certain biological systems. For example, in the dopaminergic hypothesis, levels of dopamine and its receptors are dysregulated, leading to various symptoms, particularly psychosis (Davis et al., 1991; Howes & Kapur, 2009). In the glutamatergic hypothesis, glutamate pathways are dysregulated, as well as the NMDA receptor (Coyle, 1996). In the GABAergic (interneuron) hypothesis, it is a hypofunction of GABAergic receptors that is presumed to cause symptoms of schizophrenia, particularly in interneurons (Nakazawa et al., 2012). All of these hypotheses, however, are focused mainly on the symptoms of schizophrenia instead of the mechanisms behind the dysregulations in these neurotransmitter pathways. In contrast, the neurodevelopmental hypothesis of schizophrenia has aimed to understand why and how schizophrenia occurs by tying in genetic, developmental, and environmental risk factors and their interplay (Fatemi & Folsom, 2009; Owen et al., 2011; Rund, 2018).

6.2 Differentially Expressed PTMs in Schizophrenia

Multiple methods have been developed to study human diseases and disorders, ranging from cell and animal models to cultures and organoids developed from induced pluripotent stem cells, from postmortem brain tissue to biological fluids like blood, plasma, and cerebrospinal fluid. Each model presents its benefits and drawbacks, as in any research system. When studying PTMs, certain drawbacks and benefits appear that are not necessarily present in other lines of research. For example, in vitro cell models and drug-induced animal models may present similarities to a disease state, but these are based on exogenous factors, and no genetic factors are contributing to the model. Patient-derived cultures and organoids show great promise in this line of reasoning, as a patient's genetic factors also become under study. Nonetheless, these systems still do not take into consideration a full biological system, with interplay between tissue types and different organs. Moreover, as symptoms of schizophrenia only present themselves during adolescence, any cul-

tured system cannot ethically or experimentally be kept long enough to reach that stage of development. *Postmortem* brain tissue gives an image of a matured, fully interacting system, but the interval before sample collection can vary, and in that time, PTM profiles can change due to their rapid response to stimuli, losing valuable information. Biological fluids such as blood and plasma can be processed immediately under careful laboratory conditions and can be more homogenous in comparison to organoids and brain tissue, but the blood-brain barrier prevents protein level information from leaving the central nervous system, making it only possible to visualize systemic changes. Sample collections from brain surgery of living patients and cerebrospinal fluid are important sources of information but are too infrequent or invasive to use with high sample numbers or with frequency. Data regarding PTM differences and their potential biological impact and association with schizophrenia are described below in three overall model types: cell and animal models, postmortem brain tissue, and biological fluids.

6.3 Cell and Animal Models

Taking into consideration the various hypotheses of schizophrenia mentioned above, differentially modified proteins in related pathways have been seen in several models of schizophrenia as well as in *postmortem* brains and biological fluids of patients with schizophrenia. When schizophrenia-associated mutations of the polysialyltransferase ST8SIA2 were expressed in a mouse and human cell lines, this resulted in reduced polysialylation (Hane et al., 2016). This was also seen in a mouse model with ST8SIA2-knockout mice that are now used as a model for schizophrenia due to several similar phenotypes (Kröcher et al., 2015). This phenotype is hypothesized to be due to a resulting dysregulation in neural cell adhesion molecule (NCAM) polysialylation, leading to neurodevelopmental defects (Mueller & Meador-Woodruff, 2020).

Core fucose is another glycan structure implicated in schizophrenia. Levels of core fucose and its regulatory enzyme, FUT8, are both reduced in schizophrenia (Mueller et al., 2017). The core fucosylation of *N*-glycans seems to affect PSA-NCAM binding (Kojima et al., 1996) and impaired expression of FUT8 has led to a model system for schizophrenia (Fukuda et al., 2011, p. 6; Gu et al., 2015). A dysregulation of core fucosylation has been hypothesized to contribute to schizophrenia via AMPA receptor subunit modification (Mueller & Meador-Woodruff, 2020). A better understanding of why these dysregulations occur and how to reverse them may be able to provide therapeutic options to ensure proper cell-cell interactions and neurotransmission during neurodevelopment.

6.4 Postmortem Brain Tissue

In *postmortem* brain tissue, several other proteins and pathways have been found to be differentially modified. When studying the phosphoproteome of patients with schizophrenia, multiple GABA receptor subunits ($GABA_AR$) were discovered to have decreased *N*-glycosylation levels, despite the concentrations of the receptors themselves remaining unchanged (Mueller et al., 2014). Modifying the glycosylation profile of $GABA_AR$ subunits has been found to alter various subunit properties like stability and expression, as well as the function of the receptor overall (Buller et al., 1994; Tanaka et al., 2008; Lo et al., 2010; Gurba et al., 2012, p. 3).

Multiple studies involving *postmortem* brain tissue have found changes in histone modifications near multiple genes of interest. Hypoacetylation was found at glutamate decarboxylase 67 (*GAD67*), serotonin receptor 2C (*HTR2C*), mitochondrial import receptor subunit TOM70 (*TOMM70A*), and protein phosphatase 1E (*PPM1E*), leading to reduced expression of the proteins these genes encode in patients with schizophrenia (Tang et al., 2011). In another study, having a glutamate decarboxylase 1 risk haplotype changes the epigenetic landscape of the chromatin surrounding *GAD67* (Huang et al., 2007). Furthermore, upon investigating three-

dimensional chromatin interactions, dopamine receptor D2 (*DRD2*) and several acetylcholine receptors were implicated (Ripke et al., 2014).

Prenylation is a class of protein lipidation that is regulated by multiple enzymes, three of which (FNTA, PGGT1B, and RABGGTB) were found downregulated in a postmortem study of schizophrenia brains (Pinner et al., 2020). This modification causes protein localization to membranes, specifically endomembranes, and proteins in the Ras, Rac, Rho, and Rheb families are the most common substrates of prenylation (Leung et al., 2006; Resh, 2006, 2013; Jiang et al., 2018). A dysregulation of Ras (Stornetta & Zhu, 2011), Rac (Bai et al., 2015), Rho (Sekiguchi et al., 2020, p. 10; Mizuki et al., 2021), and Rheb (Potheraveedu et al., 2017) family proteins has all been implicated in schizophrenia and neurodegenerative processes with varying degrees of certainty. As such, prenylation may be a common mechanism behind these important proteins in neurodevelopment and neuronal health.

Another lipid modification is palmitoylation, which is reversible and occurs on cysteine residues; in conjunction with other hydrophobic post-translational modifications, palmitoylation can help to stabilize plasma membrane interactions (van't Hof & Resh, 2000; Resh, 2006, 2016). *Postmortem* tissue from patients with schizophrenia was found to express reduced levels of S-palmitoylated proteins (Pinner et al., 2016) despite there are no changes in the levels of enzymes that add and remove palmitoylation groups (Davis et al., 2003). As reviewed by Mueller and Meador-Woodruff, protein S-palmitoylation in the brain affects neurotransmitter receptor stability and clustering, affecting action potentials, and mutations in three different palmitoyltransferase genes represent genetic risk loci for developing schizophrenia (Mueller & Meador-Woodruff, 2020). The activity of the palmitoyl group-removing enzyme PPT1 was found to be correlated with Positive and Negative Syndrome Scale (PANSS) scores (Wu et al., 2019).

Returning healthy levels of PTMs on neurotransmitter receptors would, at least partially, return stability and function to the various dysregulated neurotransmitter symptoms that occur in schizophrenia, including the GABAergic, serotoninergic, and dopaminergic systems already implicated. Similarly, understanding the mechanisms behind the epigenetic modifications surrounding various genes of interest may allow deductions of upstream causes of the changes in gene expression. The various types of protein lipidation can also be better investigated to understand what neurodevelopmental defects are occurring in schizophrenia and potentially dampen or otherwise suppress the detrimental effects. That said, the arguments regarding the ethicality of treating for a disease that has yet to present itself based only on risk factors are valid but will not be discussed in this chapter.

6.5 Biological Fluids

Lastly, in biological fluids of patients with schizophrenia, other differentially modified proteins were found. In 1 study of the plasma of patients with schizophrenia, 72 proteins were differentially phosphorylated, 59 of which showed no change in overall protein expression (Jaros et al., 2012). Using the Reactome Pathway Database (Jassal et al., 2020), these proteins were highly concentrated in the complement cascade (p-value = 1.11E-16) and platelet degranulation (2.22E-16) and activation, signaling, and aggregation (8.5E-13). Overall, the complement pathway is elevated in schizophrenia, and C4 has been specifically implicated (Woo et al., 2020), and the complement cascade can affect neuronal proliferation and migration and synaptic pruning (Druart & Le Magueresse, 2019). Together, these data suggest that differentially phosphorylated proteins during the period of neurodevelopment may affect the complement system and healthy brain development.

As for platelet aggregation, it is much lower in patients with schizophrenia when induced by collagen, though much higher when induced by ADP, and antipsychotics reduce platelet aggregation (Dietrich-Muszalska & Olas, 2009).

Moreover, illness duration in patients with schizophrenia is inversely correlated with platelet serotonin concentration (Peitl et al., 2020), strengthening the link between the periphery and neurotransmitter dynamics. It is therefore possible that dysregulations in neurotransmitter systems can affect the periphery and lead to changes in blood and plasma dynamics, leading to the increase in cardiovascular disease, elevated risk of thrombotic complications, and overall blood clotting and aggregation dysregulations that are observed in schizophrenia (Asor & Ben-Shachar, 2012). Finally, the platelet microenvironment can cause changes in complement pathway protein phosphorylation, not only linking the two pathways but also potentially implicating both in response to neurotransmitter dysregulations in the context of schizophrenia (Eriksson et al., 2019).

Mutations of collapsing response mediator protein DPYSL2 (CRMP2) have been implicated in schizophrenia (Tabarés-Seisdedos & Rubenstein, 2009; Liu et al., 2015; Pham et al., 2016). This protein plays a role in regulating the microtubule cytoskeletal network and is also a mechanistic target of lithium chloride treatment in bipolar disorder by leading to decreased phosphorylation via GSK3β inhibition (Moutal et al., 2019, p. 2). Ankyrin 3 (*ANK3*) disruption in schizophrenia also leads to increased phosphorylation of DPYSL2, altering the microtubule cytoskeleton (Garza et al., 2018, p. 3).

PTM changes in biological fluids present the most promising targets for biomarkers due to their availability compared to brain tissue. Dysregulations in peripheral PTMs can therefore not only make strong targets for tracing back to upstream causes and understanding how to ameliorate various comorbidities, but they also hold more potential as diagnostic and therapeutic biomarkers. Visualizing the changes in the cytoskeleton and neurotransmitter differences between patients can also give insight into the type of treatment that may be necessary with the rise of personalized medicine, as is being done with proteomics (Martins-de-Souza et al., 2019; Garcia-Rosa et al., 2020) and lipidomics (de Almeida et al., 2020).

7 Uninvestigated Modified Proteins and Pathways

Several post-translational modifications and modification sites have been identified that have yet to be tested in relation to brain disorders and schizophrenia. In a protein model generated in silico from patient and control genomes, computational predictions suggested that a common mutation in the gene for dopamine receptor D2 (DRD2) affects DRD2 phosphorylation (Wasti et al., 2017). In silico analyses show great promise to link SNPs and genetic risk variants with known and hypothetical sites of protein modification to narrow the search window and help direct proteomic studies.

In the brains of patients with schizophrenia, actin protein expression has been found to be unaffected compared to controls (Bauer et al., 2009; Fatemi et al., 2011); however, the stability of the actin cytoskeleton is reduced, which has been hypothesized to lead to physiological changes in dendritic spine density and spine morphology (Bhambhvani et al., 2017). Since such changes have been hypothesized to lead to a hyperdopaminergic state (Lewis & Gonzalez-Burgos, 2006), a hypothesis that has been confirmed in a murine model (Kim et al., 2015), it is possible that actin polymerization defects can be directly behind some of the symptoms of schizophrenia. Multiple PTMs of actin are known to affect its interactions with actin-binding proteins, including acetylation, arginylation, and oxidation, causing actin cytoskeleton disassembly or aggregation in extreme cases (Varland et al., 2019). Targeted studies regarding actin modification states in schizophrenia have the potential to reveal the mechanisms behind actin cytoskeleton dysregulations and uncover potential therapeutic targets and genetic risk factors. Supporting the possibility as a therapeutic target, clozapine and haloperidol treatment in in vitro cell models has been shown to affect actin signaling and structure (Martins-de-Souza et al. 2011b; Kedracka-Krok et al., 2015; Seabra et al., 2020; Jankowska et al., 2021, p. 12) and has been hypothesized to be a mechanism behind restored synaptic plasticity in schizophrenia (Jankowska et al., 2021).

Several metabolic disorders occur alongside schizophrenia, including lipid abnormalities, weight gain, insulin resistance, glucose intolerance, and hypertension (Henderson, 2005; Thakore, 2005), and levels of various lipids are dysregulated in first episode patients of schizophrenia, with corrections occurring after antipsychotic treatment, though dysregulations do not continue throughout the course of chronic schizophrenia (McEvoy et al., 2013). Moreover, multiple studies using various models and methods have found that energy metabolism is also dysregulated, visible through proteomics, metabolomics, and magnetic resonance spectroscopy (Martins-de-Souza et al., 2009; Martins-de-Souza et al., 2011a; Konradi & Öngür, 2017; Duarte & Xin, 2019; Pruett & Meador-Woodruff, 2020). One potential and unexplored link between these processes includes several PTMs that are known to regulate metabolic proteins: succinylation in the TCA cycle (Yang & Gibson, 2019) and malonylation in fatty acid biosynthesis and energy metabolism (Qian et al., 2016). Perturbations in succinyl-CoA ligase (SCL), the main known producer of the precursor to protein succinylation, have been found to cause mitochondrial encephalomyopathy (Gut et al., 2020), while little is still known about the effects of protein malonylation on the brain.

The mitochondrially bound enzyme monoamine oxidase (MAO) exhibits differing levels of activity in multiple diseases and disorders, including schizophrenia, and has been studied as a biomarker in platelets (Asor & Ben-Shachar, 2012). This protein has long been known to break down serotonin (Sjoerdsma et al., 1955) though the mechanism of this process was only recently fully understood (Prah et al., 2020). Very few studies have investigated post-translational modification of MAO, though acetylation and phosphorylation are known PTMs (Edmondson et al., 2004; Mousseau & Baker, 2012). As the activity of MAO is known to be affected in schizophrenia, and its modification is known, it makes a plausible target for future investigations and can be studied in neurotransmitter dysregulation and as a diagnostic biomarker.

Dozens of genetic risk factors for schizophrenia have been associated with its development, progression, and even protection, each one hypothesized to contribute to risk to a small degree (Birnbaum & Weinberger, 2017). Certain single nucleotide polymorphisms (SNPs) and variants (SNVs) can tilt the scales in favor of or against developing schizophrenia; however, the reasons behind this are not always discovered, due to complex interplays between proteins and biological pathways. Computational power has been constantly increasing, making it possible to predict sites of modification and find overlaps between these sites and genetic risk variants (Yang et al., 2019). Investigating these affected PTM sites has the potential to reveal previously unseen pathway dysregulations and therapeutic targets, additionally highlighting specific targets for localization, activity, stability, and protein-protein interaction assays, among others.

8 Future Directions and Conclusion

As techniques for studying post-translational modifications advance, data can be gathered more quickly, more robustly, and from more targets. It is becoming more and more important to integrate data from other fields such as genomics, transcriptomics, and proteomics in "PTMomics" studies to direct such studies and also make the most of the data obtained. Understanding the complex interactions between a protein and its activation, regulation, localization, activity, binding, stability, and other factors holds great potential to understand the otherwise invisible differences that occur in multifactorial diseases. In addition, overall investigations of PTM sites, levels, and ratios can become important targets for biomarkers and biomarker panels. As discovery techniques have improved, it has become more and more possible to look not only directly at a single site, but at global profiles of the proteome, discovering changes that occur in response to genetics and environmental, internal, and therapeutic stimuli. Lastly, understanding how PTM

profiles change in disease states can lead to both PTMs and their regulatory proteins becoming targets for therapeutic strategies, potentially even as adjunctive targets to current or future treatments. With schizophrenia being an extremely complex disorder with hundreds if not thousands of contributing factors, PTMs have the potential to help understand facets of the disease that are not visible from other standpoints and are showing increasing promise as investigative techniques advance.

Acknowledgments The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES; grant number 88887.495565/2020-00) and the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP; grant numbers 2014/10068-4, 2017/25588-1, 2019/00098-7, and 2019/05155-9) for financial support.

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Molecular Findings Guiding the Modulation of the Endocannabinoid System as a Potential Target to Treat Schizophrenia

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Contents

Abstract

Schizophrenia is a psychiatric disorder of neurodevelopmental origin that is thought to result from the combination of genetic and socioenvironmental factors. Several studies have linked the endocannabinoid system with the pathophysiology of schizophrenia. Here, we provide a brief overview of the role of the endocannabinoid system (ECS) in the context of biological processes relevant to schizophrenia, such as neurodevelopment, synaptic plasticity, and brain energy metabolism. We also discuss alterations related to the ECS in schizophrenia and current efforts in both in vivo and in vitro studies that have provided a better understanding of the functioning of this system in the context of the disorder.

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Finally, we highlighted the modulation of the ECS as a potential for discovering novel therapeutic targets, suggesting new avenues for future research in the field.

Keywords

Cannabinoids · Schizophrenia · Animal models · Cell culture · Cannabidiol

1 Introduction

The endocannabinoid system (ECS) has been implicated in several biological processes, and it likely participates in the manifestation of neuropathological conditions, including neurodegenerative and psychiatric diseases (Cristino et al., 2020; Lu & Mackie, 2016). The ECS is a retrograde and neuromodulator system composed of cannabinoid receptors, endogenous lipid mediators (endocannabinoids), and the enzymes responsible for the synthesis and degradation of endocannabinoids (Kitchigina, 2021) (Fig. 1a).

The endogenous ligands of the cannabinoid receptors as anandamide (N-arachidonoyl ethanolamine) and 2-AG (2-arachidonoylglycerol) are produced on demand (Maccarrone, 2009; Puighermanal et al., 2012). Heretofore, there are two well-characterized cannabinoid receptors, type 1 (CB1) and type 2 (CB2), which are two major G protein-coupled receptors found in the human central and peripheral nervous systems located mainly in presynaptic terminals (Howlett et al., 2002; Matsuda et al., 1990; Munro et al., 1993). In addition, other receptors such as transient receptor potential (TRP) channels and peroxisome proliferator-activated receptors (PPARs) are also activated by endocannabinoids (Howlett et al., 2002; Lu & Mackie, 2016).

The activation of cannabinoid receptors causes inhibition of excitatory and inhibitory transmission in the brain by triggering intracellular signaling pathways that lead to the inhibition of calcium channels, the activation of potassium channels, and the inhibition of the adenylyl cyclase resulting in decreased levels of AMP cyclic and protein kinase A (PKA) and activation of mitogen-activated protein kinase (MAPK), such as ERK, JNK, and p38 (Howlett et al., 2002; Jean-Gilles et al., 2010; Lu & Mackie, 2016). To end their effects, after being internalized, endocannabinoids undergo enzymatic hydrolysis, which can regulate their synthesis and degradation. While anandamide is primarily degraded by fatty acid amino hydrolase (FAAH), 2-AG appears to be mainly metabolized by monoacylglycerol lipase (MAGL). FAAH is the primary catabolic enzyme for the family of N-acylethanolamines and tends to be postsynaptically localized, whereas MAGL is mostly found presynaptically, implying distinct sites of degradation for these different families of endocannabinoids (Herkenham et al., 1991; Katona et al., 1999; Mackie, 2007).

The ECS has been widely studied in psychiatric disorders due to its capacity to act as a central nervous system (CNS) modulator in different neurophysiological processes (Review by Di Marzo et al., 2014). Several studies have investigated the role of the ECS in schizophrenia, suggesting that modulation of the ECS could be a novel potential therapeutic target for its treatment. At the same time, the frequent use of cannabis with high levels of Δ9-tetrahydrocannabinol (THC) during adolescence has been associated with an increased risk for the development of psychotic disorders, such as schizophrenia (Di Forti et al., 2019; Lowe et al., 2019; Stark et al., 2021).

Schizophrenia is a multifactorial psychiatric disorder that involves environmental, genetic, and neurodevelopmental dysfunctions. Patients with schizophrenia can present a variety of symptoms commonly classified as negative (impaired motivation and social withdrawal), positive (hallucinations and delusions), and cognitive symptoms (Abel & Nickl-Jockschat, 2016; Kahn et al., 2015). The pathophysiology of schizophrenia has been associated with abnormalities in neurodevelopmental processes that lead to imbalances in the dopamine and glutamate neurotransmission (Murray et al., 2008; Nuechterlein et al., 2004; Uno & Coyle, 2019).

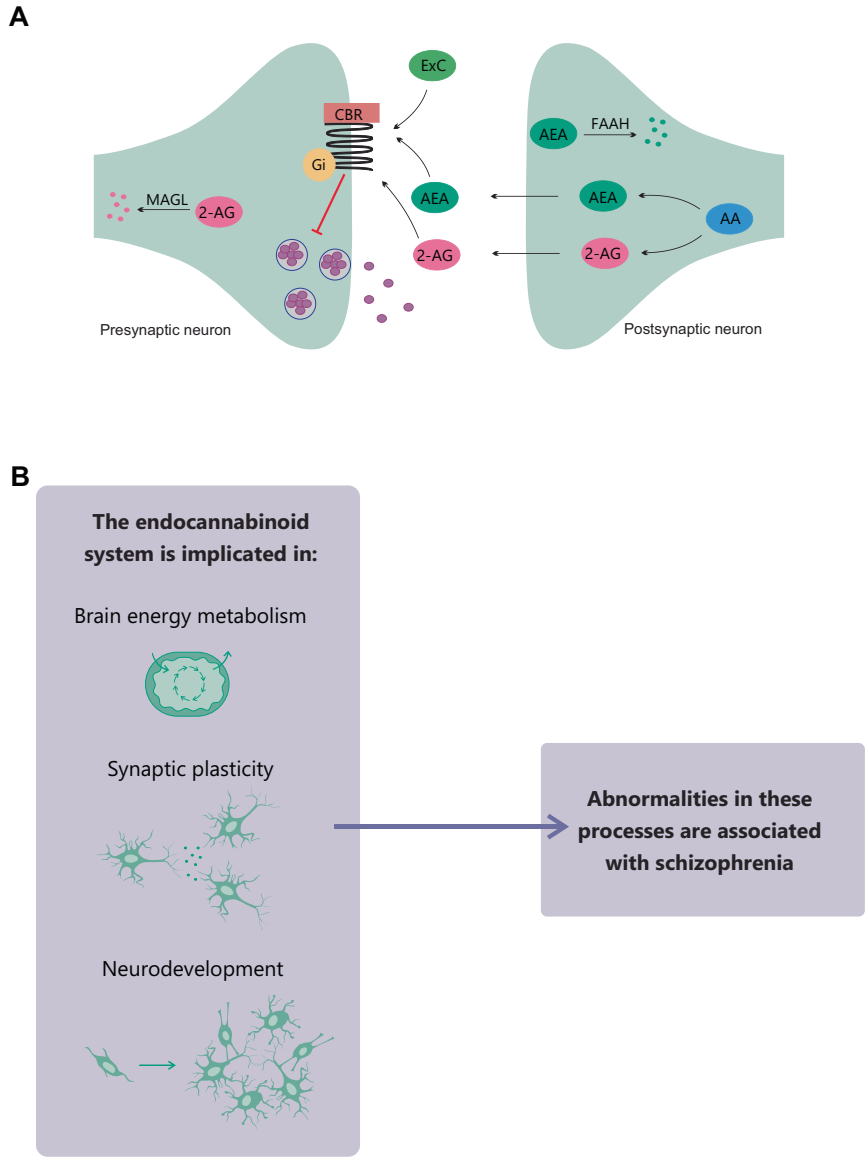

Fig. 1 (**a**) Schematic representation of the endocannabinoid signaling. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized from arachidonic acid (AA) on postsynaptic neurons. The endocannabinoids act in retrograde signaling, activating cannabinoid receptors (CBR) and inhibiting neurotransmitter release by the presynaptic neuron. Then, endocannabinoids undergo enzymatic hydrolysis, with AEA being primarily degraded by fatty acid amino hydrolase (FAAH) and 2-AG being mainly metabolized by monoacylglycerol lipase (MAGL). Exocannabinoids – synthetic cannabinoids and phytocannabinoids – (ExC) can also activate cannabinoid receptors. (**b**) Overview of the involvement of the endocannabinoid system in processes of brain energy metabolism, synaptic plasticity, and neurodevelopment and the implication that abnormalities in these processes are associated with the pathophysiology of schizophrenia

Currently, the major therapeutic strategy relies on the use of typical and atypical antipsychotic drugs that produce their effects mainly through the antagonism of D2 dopamine receptors. However, these drugs show limited efficacy against the negative and cognitive symptoms, and their benefits are sometimes obscured by their adverse effects, causing a lack of therapeutic options (Deng, 2013;

Stroup & Gray, 2018). Recently, several studies have pointed to the modulation of the ECS as a novel potential therapeutic target for schizophrenia treatment (Cortez et al., 2020; Leweke et al., 2012; McGuire et al., 2018; Volk & Lewis, 2019). Although the pathophysiology of schizophrenia is not completely known, it involves abnormalities in brain plasticity, synaptic transmission, and brain energy metabolism (Frankle et al., 2003; Prabakaran et al., 2004; Stephan et al., 2006). All these processes are affected through the modulation of the ECS (Maroon & Bost, 2018; Seabra et al., 2018), and several studies have supported the idea of an important role of the ECS in neuropsychiatric processes, especially in schizophrenia, and have suggested that the manipulation of this signaling system could represent a potential therapeutic benefit.

Here, we first discuss the role of the ECS in neurodevelopmental, synaptic plasticity, and brain energy metabolism processes and its involvement in abnormalities in them that are potentially associated with the pathophysiology of schizophrenia (Fig. 1b). Second, we discuss cannabis use and the ECS modulation in schizophrenia, describing evidence from *postmortem*, animal, and cell culture studies. Finally, we provide an overview of molecular findings of the ECS as a potential for the discovery of novel therapeutic targets, suggesting new pathways for future research.

2 Endocannabinoid System in Neurodevelopment, Synaptic Plasticity, and Brain Energy Metabolism

2.1 Neurodevelopment

During brain development, the generation of neural cells is achieved by the appropriate proliferation of neural progenitors and programmed cell death. The ECS and its lipid mediators regulate the commitment of neural progenitors and synaptic connectivity of the developing brain (Aguado et al., 2006; Guzmán et al., 2002), and the modulation of endocannabinoid signaling is an important aspect of neurodevelopment, evidenced by elevated levels of CB1 expression in the adolescent brain and fluctuation of 2-AG, AEA, and FAAH levels during this period (Meyer et al., 2018). Neural progenitor cells were shown to synthesize endocannabinoids, express the enzyme FAAH, and express CB1 receptors (Aguado et al., 2005). Studies revealed that the activation of CB1 receptors leads to neural progenitor cell proliferation, differentiation, and self-renewal (Harkany et al., 2007). The activation of CB2 receptors has also been linked to the proliferation of neural progenitor cells (Palazuelos et al., 2012). The participation of the ECS early in neurodevelopment is further supported by the observation that pharmacological activation of cannabinoid receptors leads to the modulation of other processes related to brain maturation, such as energy metabolism, arachidonic acid processing, and expression of neurotransmission-related proteins (Gomes et al., 2020; Fernández-Ruiz et al., 2004; Romero et al., 1997). The ECS also plays a key role in cell-fate decision-making processes in the context of neurodevelopment, with reported cases of its participation in astroglial differentiation (Aguado et al., 2006), neuronal differentiation, and maturation of neural stem cells (Maccarrone et al., 2014). Conversely, endocannabinoids, particularly anandamide, were shown to act on neuronal development by inhibiting neuronal differentiation through mechanisms related to the modulation of the ERK signaling pathway (Aguado et al., 2006; Rueda et al., 2002).

Neuronal migration is another important process during neurodevelopment that involves the ECS. A signaling interaction between CB1 receptors and the neurotrophin tyrosine receptor kinase B (TrkB) was observed, and TrkB signals are known to regulate neural development and maintenance of neuronal networks (Nakagawara, 2001). The activation of cannabinoid receptors by endocannabinoids activates the TrkB signaling pathway, promoting neuronal migration (Berghuis et al., 2005). Besides TrkB, the ECS interacts with growth factors, cytokines, and neurotrophins to induce cell migration, suggesting the key role of this system in the morphological,

physiological, and molecular aspects developed during neuronal differentiation (Gomes et al., 2020; Harkany et al., 2007).

Besides the role of the ECS in neurons, CB1 receptors are also located in glial cells, including astrocytes, microglia, and oligodendrocytes (de Almeida & Martins-de-Souza, 2018; Lisboa et al., 2016), where they also regulate neurodevelopmental processes, such as neurotransmission development, synaptic pruning, synaptogenesis, synaptic plasticity, and myelination. Due to the critical role of the ECS in neurodevelopment, it is proposed that exposure to cannabinoids during critical periods of neurodevelopment, such as the perinatal period through adolescence, may result in disruption of these processes, which in turn may lead to the development of psychiatric disorders, including schizophrenia (Fernández-Ruiz et al., 2000).

2.2 Synaptic Plasticity

The capacity of the mammalian brain to modify neural circuit function is based on activity-dependent changes in efficacy, number, and strength of synaptic transmission at the synaptic sites. This mechanism is defined as synaptic plasticity and is important for a wide range of brain functions, including memory, emotional responses, and behavior. Also, given the role of synaptic plasticity in modulating neural circuitry during development, impairments in this process could be associated with psychiatric disorders (Citri & Malenka, 2008).

The ECS is also critically involved in synaptic plasticity since, upon postsynaptic activation, the postsynaptic neuron initiates the production on demand of endocannabinoids that, as a retrograde messenger, act at the presynaptic terminal. Those messengers activate presynaptic cannabinoid receptors and interfere with neurotransmitter release in a transient or prolonged manner, mediating depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (Basavarajappa, 2007; Chevaleyre et al., 2006), which are important for short-term plasticity. It was found that while CB1 receptor antagonists inhibit both DSI and DSE, CB1 receptor agonists and drugs that increase the levels of endocannabinoids promote these processes (Kreitzer & Regehr, 2001; Maejima et al., 2001; Wilson & Nicoll, 2001).

The other two major synaptic plasticity processes regulated by the ECS are long-term potentiation (LTP) and long-term depression (LTD). Unlike DSI and DSE, LTP and LTD can persist for weeks (Gerdeman & Lovinger, 2003). Endocannabinoid-mediated LTD begins with the release of endocannabinoids from the postsynaptic cell mediated by increasing intracellular calcium levels and/or activation of group I mGluRs (Chevaleyre et al., 2006). Then, endocannabinoids activate CB1 receptors located in presynaptic terminals of original afferents (to produce homosynaptic LTD) or afferents at proximity (to produce heterosynaptic LTD), which in turn lead to a long-lasting reduction of neurotransmitter release (Heifets & Castillo, 2009). LTD is proposed to be a key process in establishing long-term changes in neural circuits, with reports of endocannabinoid-mediated LTD in the dorsal striatum, amygdala, nucleus accumbens, hippocampus, neocortex, cerebellum, prefrontal cortex, and ventral tegmental area (Heifets & Castillo, 2009; Sidhpura & Parsons, 2011).

2.3 Brain Energy Metabolism

Glucose is the obligatory energy substrate in the brain, and neuronal activity accounts for approximately 80% of the total brain energy requirements (Bélanger et al., 2011). Interestingly, neurons rely mostly on oxidative phosphorylation to perform metabolic activities, whereas glial cells – astrocytes and oligodendrocytes – are predominantly glycolytic (Hyder et al., 2006; Zhang et al., 2014). In this way, glial cells are responsible for providing lactate to the neighboring neurons, which can be further metabolized by the neuronal mitochondria to generate ATP (Magistretti & Allaman, 2015). Glucose metabolism is tightly coupled to brain activity. It has been shown that there is a modulation of brain glucose uptake upon administration of THC and

synthetic cannabinoids in both animal and human studies. This modulation was shown to vary based on the dose used and the brain region analyzed (Brett et al., 2001; Freedland et al., 2002; Margulies & Hammer, 1991; Miederer et al., 2017; Nguyen et al., 2012; Pontieri et al., 1999; Volkow et al., 1991; Whitlow et al., 2002).

In addition to the presynaptic terminal, CB1 receptors are also expressed at brain neuronal mitochondrial membranes (Bénard et al., 2012; Hebert-Chatelain et al., 2014, 2016; Koch et al., 2015), indicating a role of the ECS in regulating bioenergetic processes. Mitochondrial CB1 receptors directly regulate mitochondria energy metabolism by inhibiting electron transport and mitochondrial respiration, which leads to reduced ATP production (Bénard et al., 2012; Hebert-Chatelain et al., 2016). This was linked to changes in brain metabolism and deficits in memory formation induced by CB1 receptor agonists such as THC (Bénard et al., 2012; Hebert-Chatelain et al., 2016). Mitochondrial CB1 receptors were also recently found in astrocytes (Jimenez-Blasco et al., 2020), which are important players in brain energy metabolism by regulating energy supply to neurons. The activation of these receptors by THC altered cellular metabolism and animal behaviors by causing changes in glucose metabolism and a decrease in lactate production via a mechanism regulated by the decrease in the formation of reactive oxygen species by the complex I of the electron transport chain. This reduction in lactate production affected neuronal function and led to behavioral changes, particularly those related to social interaction (Jimenez-Blasco et al., 2020).

3 Cannabis Use, the Endocannabinoid System, and Schizophrenia

Adolescence represents a critical period of brain development characterized by synaptic reorganization, synaptic pruning, myelination, and other developmental processes (Stark et al., 2021). Evidence indicates that frequent cannabis use during adolescence is a major risk factor for psychotic disorders (Di Forti et al., 2015; Lowe et al., 2019; Stark et al., 2021). This has been linked to THC, since the higher the THC levels in cannabis, the higher the risk for psychoses (Di Forti et al., 2019). Also, laboratory studies with healthy individuals showed that intravenous administration of THC produces changes resembling schizophrenia, including positive, negative, and cognitive symptoms (D'Souza et al., 2005). Though the development of schizophrenia has been strongly associated with cannabis use, the effects of continued cannabis use on the symptoms and disease course are less clear. A long-term study showed patients with schizophrenia with a history of cannabis use had longer hospitalization periods and worse prognosis with a more severe disease course than patients who were non-cannabis users (Manrique-Garcia et al., 2014). Moreover, cannabis use has also been linked to treatment resistance to antipsychotics (Patel et al., 2015).

In addition to the relationship between cannabis use and schizophrenia, there is evidence of changes in the ECS in schizophrenia. Polymorphisms in *CNR1* and *CNR2*, genes that encode CB1 receptor and CB2 receptor, respectively, have been associated with schizophrenia susceptibility, progression, and treatment outcomes (Chavarría-Siles et al., 2008; Ferretjans et al., 2021; Ishiguro et al., 2010; Martínez-Gras et al., 2006; Suárez-Pinilla et al., 2015; Tsai et al., 2000). Furthermore, increased density of CB1 receptors in the dorsolateral prefrontal cortex, anterior cingulate cortex, and posterior-anterior cortex was found in the in the *postmortem* brain of patients with schizophrenia (Dalton et al., 2011; Newell et al., 2006; Wong et al., 2010; Zavitsanou et al., 2004). Regarding CB2 receptors, there is evidence for a decrease in their expression in the brain of patients with schizophrenia (Bioque et al., 2013; Ishiguro et al., 2010). Moreover, elevated levels of anandamide were reported in cerebrospinal fluid and plasma of patients with schizophrenia (De Marchi et al., 2003; Giuffrida et al., 2004; Leweke et al., 1999; Potvin et al., 2008; Reuter et al., 2017). However, negative and controversial findings have also been found, suggesting that the involvement of the ECS in schizophrenia is complex and far from being entirely understood. One study evaluated the proteomic

profile of olfactory neuroepithelium (ON) cells from patients with schizophrenia with and without a history of cannabis use. ON cells can help elucidate features related to neural systems and provide insights into potential biomarkers. The results of the proteomic study of patients with schizophrenia with a history of cannabis use revealed alterations in proteins related to RNA metabolism and protein metabolism. Moreover, the expression levels of two proteins involved in RNA metabolism were correlated with cognitive performance and clinical signs (Barrera-Conde et al., 2021). In this way, proteomic studies can bring insights into the pathways and proteins altered in schizophrenia in the context of cannabis use and upon modulation of the ECS.

4 Endocannabinoid System Modulation and Schizophrenia

4.1 Evidence from In Vitro Studies

In vitro models have provided critical insights on the role of the ECS in brain cells as well as the effects of cannabinoids in these cells. For instance, to mimic prenatal exposure to cannabinoids, one study evaluated the effects of CBD and THC on developing human neurons derived from induced pluripotent stem cells (iPSC). Disruption in neurodevelopmental processes, such as precocious neuronal and glial differentiation, was found after the treatment of iPSC for more than 30 days with THC (Miranda et al., 2020). In addition, the exposure of human iPSC-derived neurons to THC also leads to alterations in synaptic, mitochondrial, and glutamate signaling similar to those found in schizophrenia patient-derived hiPSC neurons (Guennewig et al., 2018). This is consistent with evidence suggesting cannabis with high THC levels as a major risk factor for schizophrenia (Di Forti et al., 2019).

Besides neurons, glial cells, including astrocytes, microglia, and oligodendrocytes, also express cannabinoid receptors and synthesize endocannabinoids (de Almeida & Martins-de-Souza, 2018; Lisboa et al., 2016). Despite the lack of studies directly investigating the ECS in glial cells in the context of schizophrenia, there are also some reports about the effects of cannabinoids on glial cells that may be related to schizophrenia pathophysiology. The treatment of astrocytes with WIN 55,212-2 leads to the activation of CB1 receptors, increasing glutamate levels (Navarrete & Araque, 2008). In addition, with the discovery of functional CB1 receptors in mitochondria and their role in controlling neuron-astrocyte metabolism and synaptic function, as previously described, endocannabinoid modulation of those receptors could be a promising therapeutic strategy in schizophrenia (Seillier, 2021), especially given that mitochondrial dysfunction is an important aspect of the disorder's pathophysiology (Ni & Chung, 2020). In microglia, CB1 and CB2 receptor agonists were reported to decrease their activation (Walter et al., 2003). Cannabinoids and agonists were reported to protect oligodendrocyte progenitor cells (OPC) from oxidative stress and apoptosis induced by inflammation (Mecha et al., 2012) and trophic deprivation (Molina-Holgado et al., 2002). In addition, endocannabinoids appear to have a role in oligodendrocyte differentiation by stimulating the ERK/MAPK pathway (Gomez et al., 2010). In vivo, cuprizone is known to induce oligodendrocytes apoptosis and demyelination, and CBD was shown to affect biochemical pathways similar to antipsychotics in an in vitro cuprizone model using the proteomics approach and the MO3.13 cell line, thus suggesting a potential protective role of CBD in oligodendrocytic cells after insults caused by cuprizone (Falvella et al., 2021).

Since ECS modulation affects different brain cells, 3D system cultures are a great alternative to explore the ECS mechanisms in the context of more than one cell type in schizophrenia studies. Human brain spheroids and organoids are 3D system cell cultures derived from hiPSC and neural stem cells and can develop different brain cell types, such as neurons, astrocytes, and oligodendrocytes. Furthermore, brain organoids recapitulate the characteristics, organogenesis, and architecture of the brain, providing powerful tools for modeling neurodevelopment and related diseases such as schizophrenia (National Academies of Sciences, Engineering, and Medicine; Policy and Global Affairs; Committee

on Science, Technology, and Law; Committee on Ethical, Legal, and Regulatory Issues Associated with Neural Chimeras and Organoids, 2021).

Recently, it was established that cortical spheroids express ECS components (Papariello et al., 2021), indicating that it may be an appropriate model for exploring ECS alterations in neurodevelopmental disorders, such as schizophrenia. In addition, rimonabant (SR141716A), a CB1 receptor partial agonist, selective antagonist of CB1 receptors, disrupted the excitatory and inhibitory synaptogenesis in cortical spheroids (Papariello et al., 2021), providing further evidence for the role of the ECS in synaptic function. One study using mouse embryonic stem cell-derived neurons and human iPSC-derived cerebral organoids investigated the role of the ECS in neurodevelopment and evaluated neuronal differentiation under CB1 receptor modulation (Paraíso-Luna et al., 2020). In the mouse-derived stem cells, CB1 receptor knockdown promoted the generation of upper-layer neurons and inhibited the differentiation of deep-layer neurons. This phenotype was rescued by CB1 receptor re-expression or with the CB1 receptor agonists THC and HU-210 and the MAGL inhibitor JZL-184. Also, THC and HU-210 applied during human iPSC-derived cerebral organoid development promoted the expansion of $BCL11B^{+}$ neurons (a deep-layer neuronal marker). These findings indicate that activation of CB1 receptors promotes mouse and human deep-layer cortical neuron development, contributing to the knowledge around the neurodevelopmental impact of cannabinoid exposure and the potential connection with psychiatric disorders, such as schizophrenia, since alterations in cortical-layer neuronal development are associated with the disorder (Paraíso-Luna et al., 2020; Whitton et al., 2018).

4.2 Evidence from Animal Models

Studies using animal models relevant for schizophrenia have pointed to alterations in the ECS as possible contributors to the disease's pathophysiology and target for new interventions. The involvement of changes in the ECS in schizophrenia has been evaluated in different animal models (Kucerova et al., 2014). For instance, reduced levels of CB1 receptors in the cerebellum, substantia nigra, hippocampus, and prefrontal cortex were found in a rodent model based on NMDA receptor hypofunction in which rats received repeated administration of the NMDA receptor antagonist phencyclidine (PCP) (Vigano et al., 2009). Changes in the levels of endocannabinoids are also altered in PCP-treated rats, such as elevated levels of 2-AG in the prefrontal cortex (Vigano et al., 2009) and decreased levels of anandamide in the amygdala and prefrontal cortex (Seillier et al., 2013). In addition, evidence indicates that some of the behavioral changes resembling schizophrenia in PCP-treated rats may result from deficient endocannabinoid neurotransmission. The decreased social interaction found in PCP-treated rats was reversed by the treatment with a drug that enhances anandamide levels through FAAH inhibition, URB597 (Seillier et al., 2013).

Changes in the ECS have also been observed in animal models for schizophrenia based on genetic changes and neurodevelopmental disruption. Mice with heterozygous deletion of neuregulin 1 (Nrg1 HET mice), a neurotrophic factor implicated in schizophrenia (Mei & Nave, 2014), show increased anandamide levels in the amygdala and 2-AG in the hippocampus. These changes were associated with cognitive impairments (Clarke et al., 2017). In addition, mice expressing a dominant-negative disrupted in schizophrenia 1 (DISC1) mutant (DN-DISC1) show decreased expression of hippocampal CB1 receptors. DISC 1 is thought to be one of the main candidate genes for schizophrenia (Kaminitz et al., 2014). Regarding rodent models based on neurodevelopmental disruption, when applying the maternal immune activation (MIA) model, impairments in endocannabinoid-related hippocampal plasticity that could underlie behavioral deficits were found (Guo et al., 2018). In the methylazoxymethanol acetate (MAM) rat model, which involves the prenatal exposure to the antimitotic agent MAM at gestational day 17, lower levels of CB1 receptor mRNA in the medial pre-

frontal cortex and higher levels in the dorsolateral striatum relative to controls were found (Gomes et al., 2018).

Besides CB1 receptors, animal models for schizophrenia based on NMDA receptor hypofunction have implicated CB2 receptors in schizophrenia. It was observed that the administration of CB2 receptor antagonists, such as AM630, worsened deficits in the prepulse inhibition (PPI) test and hyperlocomotion induced by the NMDA receptor antagonist MK-801 (Ishiguro et al., 2010; Kruk-Slomka et al., 2017). On the other hand, MK-801-induced PPI disruption was reversed by the CB2 receptor agonist JWH015 (Khella et al., 2014). Further evidence supports the involvement of CB2 receptors in schizophrenia. Mice lacking CB2 receptors present several schizophrenia-related behavioral changes, including PPI impairment, cognitive dysfunction, and increased locomotor response to psychostimulants (Ortega-Alvaro et al., 2011; Ishiguro et al., 2010; Kruk-Slomka et al., 2017). Given the role of CB2 receptors in modulating glutamatergic, dopaminergic, and immune systems, which are altered in schizophrenia, they have emerged as potential targets for intervention in schizophrenia (Cortez et al., 2020; Ortega-Alvaro et al., 2011).

Animal studies have also indicated that drugs targeting the ECS reverse schizophrenia-related changes (Mielnik et al., 2021; Seillier et al., 2013). Among these drugs, cannabidiol (CBD), a major non-psychotomimetic compound found in the *Cannabis sativa* plant, has received greater attention. CBD has a very low affinity for CB1 and CB2 receptors (Pertwee, 1997), and there is evidence for allosteric modulation of these receptors (Laprairie et al., 2015). CBD can also produce effects mediated by the activation of cannabinoid receptors due to increased anandamide levels through the inhibition of FAAH (Bisogno et al., 2001). In addition, a variety of other targets has been implicated in the CBD effects, such as TRPV1, PPARγ, and serotonin 1A (5-HT1A) receptors (Campos et al., 2012).

The antipsychotic properties of CBD have been indicated by preclinical and clinical studies (Leweke et al., 2012; McGuire et al., 2018; Volk & Lewis, 2019; Zuardi et al., 2012). In animal models, CBD was shown to reverse the increased locomotor activity and PPI deficits induced by amphetamine and MK-801 (Pedrazzi et al., 2015; Gomes et al., 2014; Moreira & Guimarães, 2005). CBD was also able to attenuate deficits in social interaction and cognitive function in animal models for schizophrenia (Osborne et al., 2017; Gomes et al., 2015; Rodrigues da Silva et al., 2020). The mechanisms in which CBD produces antipsychotic-like effects are currently unknown. Clinical studies indicated that the antipsychotic effects produced by CBD were associated with increased levels of anandamide (Leweke et al., 2012), which could increase the activation of cannabinoid receptors. On the contrary, CBD effects in attenuating impairments in social interaction and cognitive function in a mice model based on NMDA receptor hypofunction were mediated by the activation of 5-HT1A, but not CB1 and CB2 receptors (Rodrigues da Silva et al., 2020; Davies & Bhattacharyya, 2019).

5 Concluding Remarks

The ECS is involved with various key processes that occur while the brain is developing, synaptic transmission, and brain energy metabolism. Abnormalities in these processes are closely involved in the pathophysiology of schizophrenia. Several studies have tried to determine which cannabinoid receptor and endocannabinoid mediators are mostly involved in the disorder. Preclinical studies have provided insights into cellular mechanisms relevant to schizophrenia that are affected by cannabinoids, such as synaptic plasticity, energy metabolism, inflammation, and neuroprotection. They have also helped to elucidate the effects of the modulation of the ECS. However, a better understanding of the ECS dysregulation in schizophrenia is still needed. It has the potential to provide new perspectives into the pathophysiology of schizophrenia and the modulation of the ECS as a therapeutic approach to treat this complex disorder.

Acknowledgments GSZ, CBT, GMV, FC, and FGV are supported by the São Paulo Research Foundation (FAPESP) grants 2017/25055-3, 2017/25588-1, 2018/14666-4, 2018/03673-0, 2018/17597-3, 2019/22398-2, and 2021/06699-2.

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Metabolomics: A Powerful Tool to Understand the Schizophrenia Biology

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Contents

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Schizophrenia, as any other psychiatric disorder, is a multifactorial and complex illness whose etiology is not completely established. Therefore, studies involving strategies that are able to describe the molecular alterations caused by the disease and, consequently, indicate the altered metabolic pathways are of increasing interest. Metabolomics is a very suitable approach that can be applied for this task, since it consists of the evaluation of the set of metabolites contained in a biological system undergoing a biological process, such as a disease or treatment. In metabolomics, state-of-the-art analytical techniques (mass spectrometry and nuclear magnetic resonance) are employed to identify and quantify the metabolites present in the studied biological samples, and chemometric and bioinformatic tools are applied to determine the specific metabolites and metabolic pathways that are relevant to the biological process under investigation. The aim of this

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*,
Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_8

chapter is to describe the basic principles of metabolomics, how this strategy can improve the understanding of the schizophrenia biology, and the findings obtained so far.

Keywords

Schizophrenia · Metabolomics · Mass spectrometry · Nuclear magnetic resonance · Chemometrics · Bioinformatics

Abbreviations

AA	Amino acids
AAP	Atypical antipsychotics
ANOVA	Analysis of variance
BD	Bipolar disorder
BD1	Bipolar disorder type 1
CCU	Crack/cocaine user
CE	Cholesteryl ester
CE-MS	Capillary electrophoresis coupled to mass spectrometry
CNS	Central nervous system
CSF	Cerebrospinal fluid
DESI	Desorption electrospray ionization
ESI	Electrospray ionization
FEP	First-episode psychosis
FIA	Flow injection analysis
FT-IR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography coupled to mass spectrometry
HC	Healthy control
HMDB	Human Metabolome Database
HPLC	High-performance liquid chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCECA	Electrochemical coulometric array detection
LC-MS	Liquid chromatography coupled to mass spectrometry
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LLE	Liquid-liquid extraction
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSI	Mass spectrometry imaging
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PI	Phosphatidylinositol
PLS-DA	Partial least squares discriminant analysis
Q	Quadrupole
QC	Quality control
QqQ	Triple quadrupole
QTOF	Quadrupole time of flight
RDoC	Research Domain Criteria
SCZ	Schizophrenia
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
SV	Synaptic vesicle
TCA	Tricarboxylic acid
TG	Triglyceride
TOF	Time of flight
UHPLC	Ultra high-performance liquid chromatography
VLDL	Very low-density lipoprotein

1 Introduction

"Omics" sciences refer to a set of specific approaches that provide comprehensive information on biological systems. The term comes from the latin word "*ome,*" which means mass or many, designating the measurement of a large number of parameters per end point rather than one or a few, as in the case of classical molecular biology (dos Santos et al., 2020). Genomics, proteomics, transcriptomics, and, most recently, metabolomics have been developed in order to identify, characterize, and quantify the entire set of biological molecules on different biological levels (Vailati-Riboni et al., 2017), opening paths to understand how the biological dynamics operate. Significant progress of these strategies, over the last decades, has been achieved in the research of highly complex disease phenotype, especially across multi-

ple omic layers. Metabolomics represents the "ome layer" closely related to the phenotype (Humer et al., 2020), providing direct information on the progression and clinical symptoms of diseases, such as schizophrenia (SCZ).

Metabolomics comprises techniques that are able to assess the metabolome in biological matrices in order to evaluate the set of compounds with molecular masses lower than 1500 Da (metabolites) within a biological sample (cell, organelle, organ, tissue) (Drexler et al., 2011; Rampler et al., 2020). These molecules are responsible for building blocks of the genome, proteome, and cell membranes, for playing key roles in signaling processes and energy sources, and for being intermediates or final products of the cellular metabolism (Dunn et al., 2005; Kim et al., 2016; Villas-Boas et al., 2007). Metabolites are endogenous and/or exogenous molecules such as peptides, lipids, amino acids (AA), carbohydrates, vitamins, minerals, organic acids, and hormones, among others (Jacob et al., 2019). Together, these chemical keys provide integrative information on biological function and networks, enabling the assessment of biological system responses to different conditions such as diet, lifestyle, genetic, and environmental factors.

The knowledge of the gene sequence of an organism alone does not bring enough biological information for tracking biological system alterations. Besides, the transcriptome encoded by the genome is not always correlated to the proteome (e.g., not all mRNA synthesized in DNA transcription process leads to protein translation) and the translated proteome might not be functionally active (e.g., not all proteins that are translated have metabolic functions) (Graves & Haystead, 2002). Hence, the advantage brought by metabolomics to assess alterations in the metabolome composition caused by gene-environment interactions is relevant for adding biological information on phenotype and homeostasis and, thus, contributing for a holistic view of biological systems, which is particularly interesting for biomarker discovery and for the development of precision medicine (Shih, 2019).

In this chapter, the terms SCZ and metabolomics were combined for a systematic search in curated scientific databases, such as Web of Science and Scopus. In this search, 441 results were obtained, from which the titles and abstracts were analyzed considering only original studies based on metabolomics to be selected for further evaluation. Exclusion criteria consisted of reviews and clinical articles, as well as those from social sciences. This led to the selection of 134 articles published between 2004 and 2021.

From the 134 pre-selected articles, 45 covering a range of different issues about SCZ (e.g., first episode, schizoaffective disorder patients, nonviolent and violent SCZ patients, SCZ and major depression subjects) were arbitrarily assigned as study examples to be thoroughly evaluated in order to gather information about novel differential metabolites for SCZ. After a process of excluding reviews and papers regarding brain imaging and behavioral science of SCZ patients, 36 articles containing pertinent information for this chapter were employed in further analysis of the most cited metabolites and their pathways. Initially, aspects considering metabolomics will be described, and, subsequently, applications of this strategy to the study of SCZ will be discussed.

2 Metabolomics: Definitions and Related Analytical Techniques

The first description of the term metabolomics was introduced in 2001 by Oliver Fiehn, referring to the comprehensive and quantitative analysis of the metabolome in a biological system context. Before that, in 1999, the term "metabonomics" was defined by Nicholson et al. as the quantitative measurement of a biological system response following pathophysiological stimulus or under genetic modifications. Although these definitions are slightly different, both terms are commonly found interchangeably in the scientific literature (Klassen et al., 2017).

Besides, denominations such as "metabolic profiling" (Guo et al., 2020), analysis of previously selected metabolites and biochemistry pathways; "metabolic fingerprinting" (Cao et al.,

2020), sample classification according to source and biological relevance; and "footprinting" (Kell et al., 2005), analysis of compounds secreted by a cell in controlled conditions, can also be found.

In general, all of these definitions can be summarized into two main approaches: targeted and untargeted metabolomics (Kioroglou et al., 2020). Targeted metabolomics is characterized by the quantitative analysis of one (or more) previously selected metabolite(s) pertaining to a determined chemical class or which might be associated to specific biological pathways. It requires prior chemical knowledge of the analyzed metabolites and normally makes use of internal standards and employs selected reaction monitoring (SRM) mode for quantification by mass spectrometry (MS) techniques (Gonzalez-Dominguez et al., 2020). Untargeted metabolomics is commonly applied without an a priori study and relies on qualitatively identifying the greatest number of molecules as possible (Doğan et al., 2021). This is not an easy task, since "metabolites" englobe molecules with highly different chemical and physicochemical properties. For handling that, metabolomic approaches make use of different analytical techniques to identify and quantify metabolites. Mainly, nuclear magnetic resonance (NMR) and MS are used for their detection.

NMR is an excellent tool for metabolite analysis, since it is a nondestructive technique, except for the possible contamination with solvent (Giraudeau, 2020), since it requires the dilution of the samples with a solvent isotopically labeled with deuterium, which depending on its nature can be evaporated after NMR analysis (for hydrophobic metabolites, for example, deuterated chloroform – $CDCl_3$ – can be employed and easily removed). The platform needs few or no sample handling and enables the analysis of even solid or semi-solid samples. On the other hand, it presents low sensitivity and selectivity and spectral regions where different signals are superposed and cannot be resolved (Letertre et al., 2021).

MS, on the other hand, presents high sensitivity and selectivity, being the most applied analytical technique in metabolomic studies. It requires a very small amount of samples, providing opportunity for analyzing molecules in low concentrations in rare samples (Boutet-Mercey et al., 2018).

Due to matrix effect and ion suppression, phenomena that can hinder sensitivity and selectivity in MS, the use of a separation technique before detection is normally employed for improving experimental data quality. For volatile, less polar, and smaller molecules, gas chromatography coupled to mass spectrometry (GC-MS) is a very suitable strategy (Stettin et al., 2020). It normally requires a sample derivatization step in order to improve (or provide) volatility to metabolites. However, its robustness, reproducibility, and the application of electron ionization for MS analysis, though, give the technique the advantage of creating libraries containing data for retention time and for compound fragment ion spectra that are combined and can be used for an accurate identification of compounds in the samples.

Liquid chromatography coupled to mass spectrometry (LC-MS) is also widely employed for metabolomics (Depke et al., 2020). It presents high robustness, sensibility, and sensitivity and is a comprehensive technique regarding the compound classes that can be analyzed. The large variability on mobile and stationary phases permits the analysis of molecules with diverse physicochemical properties (Roca et al., 2021). Additionally, the chromatographic peaks from separation modules provide a means for metabolite quantification, which cannot be accurately measured when MS is used alone.

Another separation technique that has been applied for metabolomics is capillary electrophoresis coupled to mass spectrometry (CE-MS). It permits the separation in short time of ionic compounds with high resolution and needs injection volumes in order of nanoliters, being well suited for working with small amounts of biological samples.

Less used, but still a technique with high detection power, Raman and Fourier transform infrared spectroscopy (FT-IR) are useful tools when it concerns "fingerprinting" and "sample profiling" (Lelli et al., 2021).

Besides the techniques employed for the qualitative and/or quantitative analysis of metabolites approached in this section, other important analytical and computational steps are necessary for a complete metabolomic study. These stages will be described in the following section.

3 Metabolomic Workflow, from Sample Preparation to Bioinformatics

Several options concerning separation and detection techniques have been used in metabolomics, depending on laboratory suitability, the chemical nature of the detectable metabolites, and the biological question to be assessed (Zhang et al., 2012). Nevertheless, the metabolomic workflow starts with the biological or clinical question. This leads to the development of the experimental design (targeted or untargeted approach, number of samples and groups, etc.) and the choice of sample to be analyzed (fluids, tissues, cells). Metabolic quenching, i.e., the interruption of the enzymatic activity, is a crucial step and should be performed immediately after sample collection in order to interrupt the metabolism and preserve the samples (Guo et al., 2021). It is made by the addition of blockers and conservatives and/or refrigeration with dry ice or liquid nitrogen. For avoiding enzymatic activity and sample degradation, the samples should be stored at −80 °C to guarantee sample stability until proper analysis (Smith et al., 2020).

Depending on the biological sample (urine, blood serum, saliva, etc.) and on the class of metabolites of interest, different extraction protocols must be used (liquid-liquid extraction (LLE), solid-liquid extraction (SLE), and solid-phase extraction (SPE), among others) (Vuckovic, 2020). For liquid samples, the solid phase (proteins and salts commonly precipitated by the addition of organic solvents) must be removed, and there are cases in which the sample must be separated in its organic and aqueous phase, the first containing the hydrophobic metabolites (important for lipid analysis) (Züllig & Köfeler, 2021) and the latter the hydrophilic ones.

After sample preparation, the next step on the metabolomic workflow consists of acquiring data employing one of the analytical techniques described in the previous section. When MS is the technique of choice, in order to assess the equipment stability and performance, and proving its suitability for analysis (thus warranting data quality), a pool containing an equal small amount of all analyzed samples is prepared (Godzien et al., 2015). This consists of the quality control (QC) sample, which should be injected in predefined intervals throughout batch analysis to evaluate possible equipment fluctuations and tendencies on signal response. In the case of NMR, there is no need of using QC samples, since its robustness and high reproducibility allow comparison of spectra even acquired in different instruments (Letertre et al., 2021).

Metabolomic data are abundant and complex and require suitable tools for their treatment. For untargeted approaches, the data must undergo steps of spectral deconvolution, alignment, grouping, retention time and baseline correction (in the case of LC-MS or GC-MS analysis), and normalization (Domingo-Almenara & Siuzdak, 2020). For targeted metabolomics, internal and isotopically labeled standards are commonly used for quantification of preselected metabolites.

After data pretreatment, the statistical analysis is performed by univariate (Vinaixa et al., 2012) (ANOVA, Student's t-test, Mann-Whitney U) and multivariate (Liland, 2011) (principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA)) methods. These tools create statistical models from where the most important compounds that may discriminate two or more biological conditions (differential features) are ranked in a list and may be used to classify samples between the groups analyzed in the experiment (e.g., control vs. pathology, treatment 1 vs. treatment 2, etc.).

The putative identification of the differential features into metabolites is made by the comparison of data to libraries such as Metlin, MassBank, National Institute of Standards and Technology

(NIST), and Human Metabolome Database (HMDB) (Go, 2010). Identity confirmation is then performed by applying targeted approaches, 2D-NMR, or tandem mass spectrometry (MS/MS) analysis. For biological interpretation, correlation of metabolites to biochemical pathways can be made with the help of libraries such as Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and MetaboLights, among others. For confirmation of observed biochemical effects, a biological validation is necessary, in which new samples are submitted to metabolomic analysis.

The biological interpretation of highly complex disorders has as a starting point the investigation of different relationships at the innumerable molecular levels underlying dysfunctional pathways. The application of the aforementioned libraries on discovering and characterizing these dysfunctional molecular keys could improve the advances in diagnosis and treatment for many psychiatric disorders, such as SCZ, as proposed by the National Institute of Mental Health Research Domain Criteria (RDoC) project (Cuthbert & Insel, 2013).

SCZ is a devastating psychiatric disease characterized by symptoms of delusions, hallucinations, cognitive deficits, and thought disorders. The prevalence is about 0.8% around the world and the heritability is 80% (Gareeva & Khusnutdinova, 2018). Even being mostly hereditary, it is influenced by other factors, such as economic, gender, and psychosocial ones. Although a vast literature can be found about the disease, it remains poorly understood and points to compass a spectrum of diverse cases.

Due to the great complexity of this pathology, metabolomics presents itself as a proper tool for SCZ investigation, since it can provide information on SCZ phenotypes, on response to treatments, as well as on disease development and progression. In the following section, metabolomic studies regarding SCZ are summarized, and their most relevant results are presented, followed by a discussion about its current panorama, the scientific biological information acquired, and their gaps.

4 Metabolomics Applied to Schizophrenia Studies

One of the greatest challenges to metabolomics, and also to the omic sciences in general, is the access to samples used for molecular evaluation and validation considering clinical management of complex disorders (Escobar et al., 2021), such as SCZ. Many steps in the metabolomic workflow are considerably challenging. Problems associated with the low metabolite abundance, especially in complex matrices in which these molecules are encountered, make the analysis very laborious, despite the advances in analytical platforms for molecular analysis. Notwithstanding, metabolomics has a major role in clinical practice as it represents >95% of the workload in clinical laboratories worldwide (Jacob et al., 2019). Particularly in academic-scientific research, where the main analytical instruments are available, most of the analytical steps required to cover the high metabolite complexity are approached using different analytical platforms. In Table 1, articles from 2016 to 2021 were selected in order to extract information concerning the analytical strategies and platforms used for SCZ metabolomic research. Based on the results of these studies, together with other relevant ones, a discussion about the SCZ biology unraveled by metabolomics will be subsequently presented.

4.1 The Biology Behind Schizophrenia Metabolomics

Several approaches are able to design strategies for SCZ investigation, but it is a hard work process given the analytical tools limitations, as well as the biological variety and the disease dynamics complexity, as previously described. Then, it is important to carefully simplify the components, focusing on the knowledge regarding the central nervous system (CNS). Moreover, the interaction dynamics established at the neurotransmission process and drug treatments in psychiatric disorders, such as depression, SCZ, and

Table 1 Representative pool of selected research articles in SCZ metabolomics from 2016 to 2021: analytical platform(s) and metabolomic strategy employed

Reference	Title	Analytical platform(s)	Metabolomic strategy
S. Chen et al. (2020); X. Chen et al. (2020)	Dysregulation of amino acids and lipids metabolism in schizophrenia with violence	GC-TOF-MS	Untargeted
Dickens et al. (2020)	Links between central CB1-receptor availability and peripheral endocannabinoids in patients with first episode psychosis	LC-QqQ-MS	Targeted
Du et al. (2021)	Metabolomic identification of exosome-derived biomarkers for schizophrenia: a large multicenter study	UHPLC-MS/MS	Untargeted
J. H. Liu et al. (2021); Y. Liu et al. (2021)	Alteration of lipids and amino acids in plasma distinguish schizophrenia patients from controls: a targeted metabolomics study	LC-MS/MS	Targeted
L. Liu et al. (2020)	Metabolomics strategy assisted by transcriptomics analysis to identify biomarkers associated with schizophrenia	UHPLC-QTOF-MS/MS; ^{1}H-NMR	Untargeted
Parksepp et al. (2020)	Metabolomics approach revealed robust changes in amino acid and biogenic amine signatures in patients with schizophrenia in the early course of the disease	FIA-MS/MS; LC-MS	Untargeted
Tasic et al. (2019)	Peripheral biomarkers allow differential diagnosis between schizophrenia and bipolar disorder	^{1}H-NMR	Untargeted
Vendramini et al. (2016)	Pioneering ambient mass spectrometry imaging in psychiatry: potential for new insights into schizophrenia	DESI-MSI	Untargeted
Ward et al. (2018)	Atypical antipsychotic exposure may not differentiate metabolic phenotypes of patients with schizophrenia	^{1}H-NMR; GC-MS	Untargeted
Yang et al. (2020)	Assessing the causal effects of human serum metabolites on 5 major psychiatric disorders	UHPLC-MS; GC-MS	Untargeted
Yoshikawa et al. (2018)	Mutations of the glycine cleavage system genes possibly affect the negative symptoms of schizophrenia through metabolomic profile changes accepted	CE-MS	Untargeted

bipolar disorder (BD), among others, need to be considered in order to control the exceeding reductionist protocols and determine relevant gaps for the biological interpretation of these illnesses.

Neurons connect to glia cells, and these cells are the major cell types in the brain (Hellier, 2014). These connections established by the glial elements represent the main cellular dynamics that is able to recognize neuronal metabolic changes and trigger metabolic regulations by transferring metabolites from glia to neurons (Jha & Morrison, 2018). In other words, the glial cells act to modulate cross talking within the optimal for normal neuronal function, although they do not participate directly in neurotransmission (He & Wu, 2020).

The entire response of neuronal signal imbalances, as well as the metabolic regulations established by glial elements, is induced by the activity of chemical messengers secreted by canonical (i.e., membrane transporters) or non-canonical (i.e., activity-dependent release of small-molecule neurotransmitters packaged into synaptic vesicles, SVs) pathways to the composition related to neurotransmission. Different factors regulate the axon terminal chemical crossing to neurons; neurotransmitter synthesis (anabolism) and reuptake deactivation (catabolism) are the main processes that pinpoint the limits within the pathophysiological process, which is very relevant as a metabolomic workflow factor to be considered, mainly in the metabolic and the dynamic diversity context.

Besides the classical fields, as genetics, biochemistry, cell biology, neurology, neuropsychology, and even imaging, different assays have been proposed for the investigation of target molecules (Chantranupong et al., 2020). Although relevant, these methods have several caveats and low applicability for molecular detection concerning biological processes, i.e., low expression or intense turnover of mRNA to proteins related to the molecular machinery involved in synthesizing, packaging, or binding to neurotransmitters (Du et al., 2021; Hnasko & Edwards, 2012).

Advances in high-throughput techniques and sample preparation aiming at minimizing the biofluid complexity, e.g., exosome isolation (Dean et al., 2021; Du et al., 2021), allowed the metabolomic approaches to associate the use of multiple separation techniques as powerful strategies to deepen the knowledge of SCZ at a molecular dynamics level (Mussap et al., 2018).

In this chapter, we selected 134 articles on SCZ metabolomics, published in the period of 2006 to 2021. All research articles were selected and compiled in an .XML archive for information extraction about title, authors, year, and source of publication. Individually, these materials were analyzed to collect the following information: research objective, employed analytical platform(s), sample type, study subjects, and observed differential metabolite(s). The differential metabolites were organized by citation frequency, in each paper, and by their comparative trend among the subjects of the study. From the selection of 36 most relevant articles, it was possible to generate the graphic presented in Fig. 1, where we observe which differential metabolites display a greater frequency among the evaluated studies.

In summary, the list of metabolites related to SCZ, described in the literature until the present moment, was graphically represented within the frequency that these metabolites were reported in Fig. 1. Additionally, it is important to emphasize that these metabolites were determined in different sample sources and models and evaluated by metabolomics using different experimental approaches and analytical platforms, as can be observed in Table 2.

4.2 Hypotheses Generated from SCZ Metabolomic Studies

Metabolomics not only has the ability of screening the metabolite composition in biological matrices but also has the capability to provide clues on metabolic dynamics (Myint, 2012) and

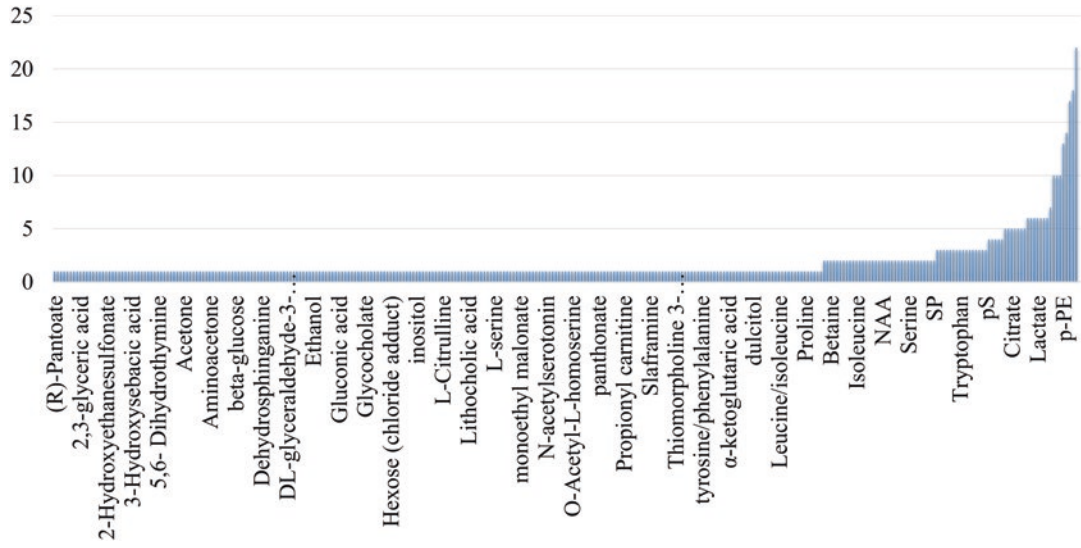

Fig. 1 Identified metabolites for SCZ. The metabolite list and their reported frequencies were used to construct the distribution of the metabolites already identified in SCZ metabolomic studies

Table 2 Examples of the variety of experimental approaches to search for differential SCZ metabolites

Reference	Sample source(s)	Model	Subject studies/analytical platform	Differential metabolite(s)
S. Chen et al. (2020); X. Chen et al. (2020)	Plasma	Human	Nonviolent and violent SCZ patients/GC-TOF-MS	Glycerol (increased)
Du et al. (2021)	Blood serum exosome	Human	SCZ patients/UHPLC-MS/MS	9,10-Dihydroxy-12Z-octadecanoic acid
Lamichhane et al. (2021)	Plasma	Human	First-episode psychosis (FEP) patients and individuals clinically high risk for psychosis/ UHPLC-QTOF-MS	TG (48:0)
M-L. Liu et al. (2015)	Peripheral blood mononuclear cells	Human	SCZ and major depression subjects/GC-MS	Glucose (increased)
J. H. Liu et al. (2021); Y. Liu et al. (2021)	Plasma	Human	First-episode drug-naive patients/LC-MS/MS	LDL-C (increased – SCZ4w[a])
O'Tuathaigh et al. (2017)	Post-mortem brain	Animal	Mice with heterozygous transmembrane-domain deletion of Nrg1/high-resolution MS	PI 38:4 (decreased)
Sha et al. (2012)	Post-mortem brain	Animal	Knockout mouse model/LC-MS	Glycerone phosphate
Tasic et al. (2017)	Blood serum	Human	Crack/cocaine users (CCU) and SCZ patients/^{1}H-NMR	Selenomethionine (HC+, CCU+, SCZ+)
Wood (2019)	Post-mortem brain	Human	SCZ patients/high-resolution MS	Ceramide (decreased)
Yang et al. (2013)	Blood serum; urine	Human	SCZ patients/GC-TOF-MS; ^{1}H-NMR	Glycerate (increased)
Yao et al. (2010)	Blood	Human	First-episode neuroleptic-naive SCZ schizophreniform or schizoaffective disorder patients/LCECA	Melatonin

Observations: [a] SCZ4w: the letter w represents week abbreviation (i.e., SCZ/SCZ4w is the comparison of the SCZ patient group without treatment related to SCZ patients groups after 4 weeks of treatment); + symbol indicates if the metabolite level comparison was present between the three groups

important directions to ultimate genome product comprehension about imbalanced mechanisms associated with highly complex diseases. Because of this knowledge, for decades omic studies have been conducted for the search of biomarkers, and psychiatric disorder studies have been driven into these demands. In the literature, the use of human serum and urine was the main sample inquiry. In the meantime, few studies have investigated this differentiation in brain samples or cerebrospinal fluid (CSF) in psychiatric disorders (Humer et al., 2020; Lozupone et al., 2017; Wesseling et al., 2013).

Tissue sample sources, such as brain, frequently come from the necropsy process, postmortem-derived samples, or SCZ animal models. The application of SCZ animal models is commonly directed to determine the progression of the disease and the effects of metabolic changes (Sobczuk et al., 2020) with regard to pharmacological and pharmacodynamics tests (Rezai et al., 2020). Despite some discussion, the combination of representative animal models and human studies using CSF (Humer et al., 2020) and the metabolomic approach correlations still make important contributions on the mental disorder understanding and guiding advances in new treatment strategies.

Besides the sample collection and the huge amount of sample preparation protocols, bioinformatics and public databases provide a very important contribution for the large amount of data to be interpreted according to biological contexts. Nowadays, studies in SCZ metabolomics, with characterized molecules or clear methodologies, may be revisited and tested for data mining revision using metabolomic public databases (e.g., Metabolomics Workbench and MetaboLights) (Haug et al., 2020; Kale et al., 2016; Powell & Moseley, 2021) or other omics database.

As an example, the metabolites already detected and identified in published studies, reported in this chapter, were evaluated in the web-based tool MetaboAnalyst (Pang et al., 2021; Xia et al., 2009; Xia & Wishart, 2011) to provide some clues in order to understand the biology of the listed SCZ-related metabolites. This web tool provide modules that help the analyst not only to carry out groupings related to the chemical nature of the metabolites, but also to enrich these molecules in altered biochemical pathways in the different cases of SCZ to be investigated. From the list of metabolites evaluated in this chapter (Fig. 1), the metabolites that were reported with a frequency of three times in the analyzed articles had their names converted into KEGG ID codes and grouped into the main chemical classes. Among these classes, AA and peptides, monosaccharides, and TCA acids are the most reported in the literature and involved in the CNS metabolism (Fig. 2).

AA and peptides are important metabolites for the synthesis of neurotransmitters, many of which are involved in events associated with the progression of SCZ episodes, e.g., basic AAs (arginine, lysine, histidine, and ornithine) and large neutral AAs (branched-chain AAs, citrulline, tryptophan, tyrosine, phenylalanine, and methionine) from blood to the brain. Among these AAs, some pathways are important keys to other classes of molecules (He & Wu, 2020). More specifically, the cross talk and downregulation in glutamate synthesis and unbalances in TCA cycle were previously reported (S. Chen et al. 2020; X. Chen et al. 2020; Marsman et al., 2013; Mei et al., 2018). These biochemical connections, among the proposed disturbance theories on the glutamate neurotransmitter system and energy metabolism, act negatively in neurotransmission, affecting mood (e.g., anxiety and depression), behavior, and food intake, thus triggering SCZ and other mood disorders (He & Wu, 2020).

5 Conclusion and Outlook

Metabolomics, among other omics, is a very promising field, especially for the study of highly complex diseases, considering that metabolites are connected to the phenotype level. However, when evaluating the protocols in this field, any condition of the biological system is very challenging and requires proper strategies to seek coverage and wide reproducibility on the greatest

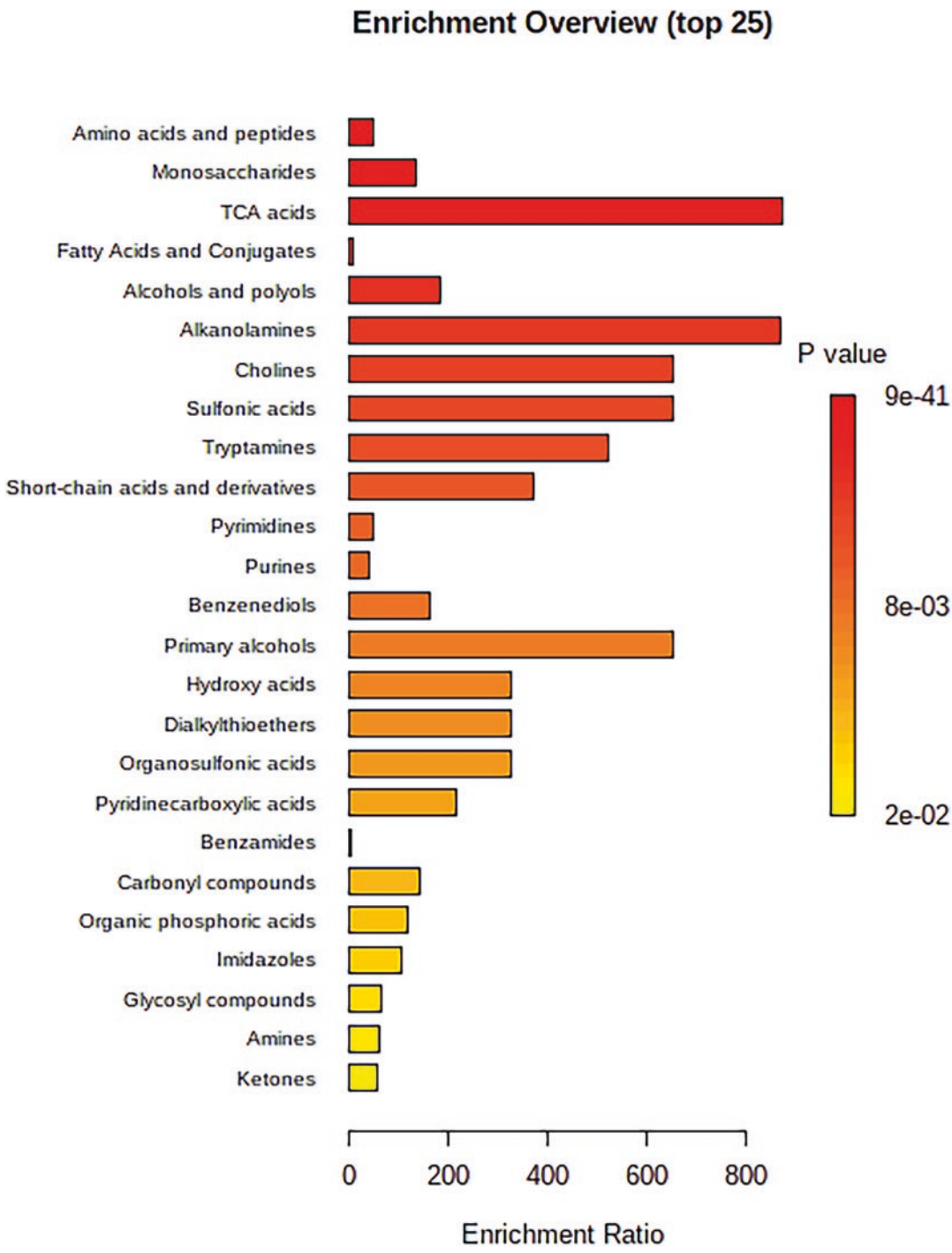

Fig. 2 Main chemical compounds from KEGG ID metabolites using MetaboAnalyst 5.0. From the list of identified metabolites for SCZ in the literature search, these were converted to KEGG ID codes at web-based platforms: Metabolites Biological Role (MBROLE: http://csbg.cnb.csic.es/mbrole/conversion.jsp), followed by enrichment analysis at MetaboAnalyst 5.0 for main chemical grouping and bar graph generation

number of identified key molecules possible, within a biological system context. An alternative to minimize the uncovered compounds, beyond the biological complexity (low abundance metabolites), and at the same time correlate its spatial resolution is using subcellular isolation protocols, such as organelles and SVs. Besides, the continuous increment in automation protocols is important to enable quality measurements of primary metabolites by metabolomics.

Looking forward, bioinformatics brings to SCZ metabolomic studies significant ways to be revisited and explored by the possibility of re-evaluating public data considering the integration of different omics data, since there is already a selection of proteins and genes with high biomarker potential, adding effectiveness to determine SCZ molecular signatures. Finally, although controversial, animal models give important clues into the global metabolism of psychiatric episodes. Based on results derived from this model, it is possible to re-evaluate diagnostic and prognostic markers, which so far lack a reliable outcome in specific SCZ diagnosis and therapeutic options.

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Modulating Specific Pathways In Vitro to Understand the Synaptic Dysfunction of Schizophrenia

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Contents

Abstract

Schizophrenia is an incurable mental disorder that affects 1% of the world population and is among the most disabling human diseases. On average, 70% of patients abandon medication due to its low efficacy and the presence of severe side effects. To change these conditions, it is necessary to understand the pathophysiology of schizophrenia at the molecular level. Besides the long-established neurodevelopmental hypothesis, works based on neuroimaging, postmortem brain proteomics, and pharmacological, genetic, and animal model studies have shown dysfunction and deficits in synaptic transmission. Currently, genetic editing has been growing, and the use of this technique has been improved in the discovery of protein functions; in addition to that, some recent studies have attributed a path to the use of genetic engineering in the treatment of diseases with a genetic nature.

Keywords

Neurodevelopmental diseases · Neural cells · Embryonic stem cells

1 Introduction

Schizophrenia is an incurable mental disorder that is associated with neurodevelopment and affects about 1% of the world population (Saha

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D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*, Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3_9

et al., 2005; Sullivan et al., 2003). This disorder represents an annual cost of over US$60 billion in the United States (Marcus & Olfson, 2008). Schizophrenia has a predicted genetic influence of 64% and induces a reduction in life expectancy of up to 20 years compared to the general population (Hegarty et al., 1994; Lichtenstein et al., 2009).

Symptoms of schizophrenia are classified into three categories: positive symptoms, such as hallucinations, delusions, and thinking disorders; negative symptoms, such as losses in social interaction, lack of motivation, and anhedonia; and cognitive deficits, such as decreased executive functions, selective attention, diminished working memory, and reduced mental flexibility (Weickert et al., 2000).

The management of symptoms is based on the use of antipsychotics, which are effective nearly exclusively in the treatment of positive symptoms. In addition to their limitations, these drugs can induce several side effects that reduce the patient's quality of life (Dieset et al., 2012; Lieberman et al., 2005; Tandon et al., 2008). Because of these side effects, about 70% of patients discontinue treatment, and further worsening the situation, 10% of patients never even show any improvement with medication (Torrey, 1995). Patients without adequate treatment cannot return to their routine activities, and each relapse builds up a toxicity in the patient's brain, leading to a progressively worsening prognosis. Furthermore, 40% of patients with schizophrenia attempt suicide at least once and succeed in 4.9% of cases (Hor & Taylor, 2010).

The pathophysiology of schizophrenia is not fully understood, with several gaps in knowledge. There are many theories on its development, the main ones being the glutamatergic, dopaminergic, serotonergic, and neurodevelopmental hypotheses. It is also believed that schizophrenia is a combination of numerous small dysregulations that lead to the spectrum of symptoms of the disorder; and studies are now showing evidence of the participation of glial cells.

Due to the impossibility of studying genetic dysregulation and the effects of environmental factors on human neurodevelopment, especially in the embryonic period, most studies that confirm the neurodevelopment hypothesis are still carried out in animal models. However, human and mouse neurodevelopment have relevant differences, especially at the molecular level. In this context, the development of human-induced pluripotency stem cells (hiPSCs), and three-dimensional cell culture models (such as organoids and spheroids), enabled the application of models more similar to the human pathological condition, becoming an alternative for the study of complex pathologies such as schizophrenia. One of the great advantages of hiPSCs is the possibility of understanding alterations resulting from the cellular pluripotency state and states subsequent to the formation of a mature cell, reflecting, for example, the stages of neurodevelopment.

hiPSCs derived from patients with schizophrenia overcome the impractical and poor accessibility of human brain cell types (Pedrosa et al., 2011) and offer the possibility to challenge and question aspects related to neurodevelopment that may underlie the development of this disease. Furthermore, current developments in human pluripotent stem cell technology and techniques for genetic editing have promoted new possibilities for the investigation of human biology in the disease, as well as in the development of therapeutic applications, especially in diseases related to the human brain. Therefore, the more models and study techniques are available, the closer we are to overcoming difficulties and challenges in schizophrenia research, such as genetic background, environmental clues, and aspects related to neurodevelopment.

2 Stem Cells

Currently, the study of the prenatally developing brain can be done in three ways: the ex vivo fetal brain investigation, the premature study, and the study of fetuses in utero. However, each of these approaches has major limitations (Thomason, 2020). Because of this, studies with stem cells have turned into a promise in the understanding of human neurodevelopment, especially at the molecular level.

Pluripotent stem cells are cells capable of possibly differentiating into all cell types of an adult organism. Because of this, they are a promise in studies of pathophysiology of diseases, in addition to being able to help in the discovery of new biomarkers, as well as in screening for the development of new drugs (Colman & Dreesen, 2009). There are two types of pluripotent stem cells: pluripotent embryonic cells (ESCs) and induced pluripotent cells (iPSCs).

Thomson et al. (Thomson, 1998) produced in vitro for clinical purposes the first culture model with human embryonic stem cells (hESCs) developed from embryos. hESCs are derived from the early stage of embryonic differentiation, where they are collected from the inner cell mass (ICM) of the human blastocyst on day 5 of the oocyte in vitro fertilization (Fig. 1) and can be propagated indefinitely at this undifferentiated stage. These cells are capable of differentiating into cells from the three different embryonic layers (endoderm, mesoderm, and ectoderm) (Pera et al., 2000). Initially, the purpose of hESC cultivation was restricted to the function of replacing cells in injured adult tissue; however, with the advancement of technologies, this cell type has become a promise in the study of molecular bases of cell differentiation (Boppart et al., 2015).

Another technique that revolutionized the field of neurodevelopment research was developed by Takahashi et al. (Takahashi et al., 2007). In this technique, adult somatic cells are reverted to the pluripotency state by reprogramming four transcription factors linked to the pluripotency state (oct-4, sox-2, Klf-4, and c-Myc); in this study, the induced pluripotent stem cells (hiPSC) were developed (Fig. 1). Like ESC, these cells can generate different cell types from the three embryonic layers (Yu & Thomson, 2008). This model holds promise in disease studies, as hiPSCs carry the genetic pattern of the donor. Both hESCs and hiPSCs have the ability to recapitulate the stages of neurodevelopment since, when treated with specific growth factors, they express genes and activate molecular pathways mimicking what occurs in vivo (Dvash et al., 2006).

3 shRNA/siRNA

The RNA interference (iRNA) technique was discovered in the late 1990s and is currently a widely used tool in regulating gene expression in mam-

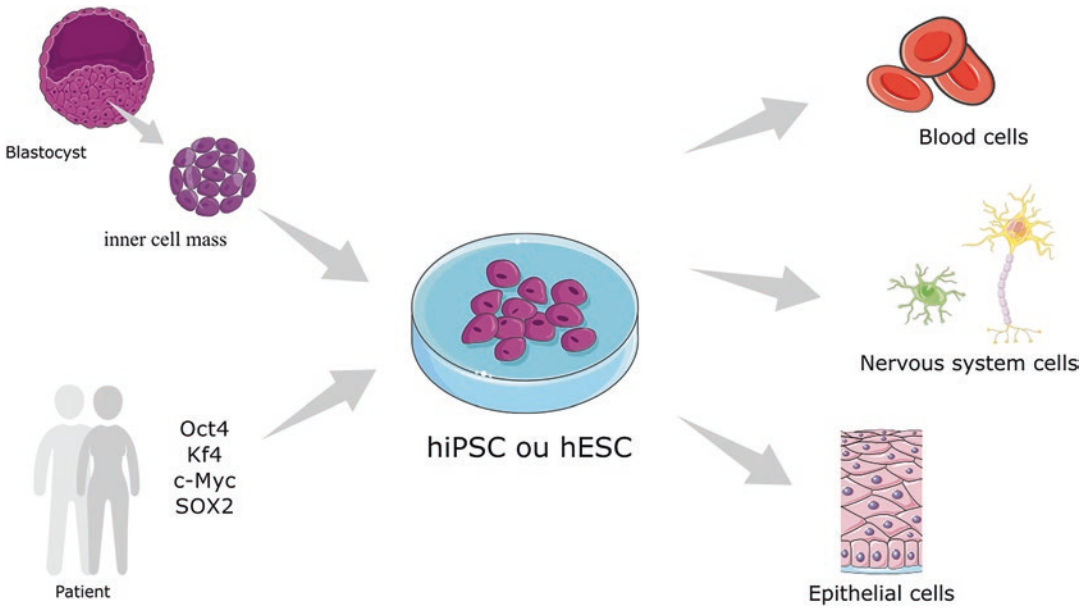

Fig. 1 Schematic of the formation of embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) culture models

mals and thus in understanding the function of these genes (Sharp, 2001). A variation of the well-known iRNA is the short hairpin RNA (shRNA); in this technique there is the stable integration of the shRNA making the long-term silencing of genes possible (Taxman et al., 2010). The structure of shRNA is composed of a sequence of 21 nucleotides (sense sequence) that is complementary to the target RNA. This sequence is linked to its antisense sequence, and between these two sequences, there is a small nucleotide sequence (loop-sequence) that is repeated in all shRNA and that is responsible for the formation of the hairpin structure.

The protein that has been modulated most when it comes to schizophrenia is disrupted in schizophrenia 1 (DISC1). This protein is located on chromosome (1; 11) (q42.1; q14.3) and the translocation of this gene is present in family members with susceptibility to various psychiatric disorders such as schizophrenia, schizoaffective disorder, and bipolar disorder (Millar et al., 2000). This protein is linked to NMDA receptor dysfunction (NMDAR), a theory widely known as one of the hypotheses for the cause of schizophrenia. Thus, a study using iRNA aiming to knock down the DISC1 protein observed that the NMDAR-mediated synaptic response in prefrontal cortical pyramidal neurons was increased (Wei et al., 2014).

Using the shRNA technique in the context of schizophrenia, Yang et al. (Yang et al., 2018) demonstrated that the knockdown of the GLT8D1 and CSNK2B proteins increases the proliferation of neural stem cells, which inhibits their ability to differentiate and even causes dysregulation in morphology and synaptic transmission of neurons.

In a study of gain and loss of genetic functions, the differentiation of astrocytes was evaluated. In this study, knockdown proteins such as SMAD4 and REST were used to reestablish functions related to astrocyte differentiation from glial progenitor cells (GPCs) derived from iPSCs of patients with schizophrenia (Liu et al., 2019). This indicates that gene modulation can also be used as a treatment for diseases related to specific genetic factors.

Problems related to mitochondrial metabolism are observed in studies of patients with schizophrenia, but little is known about the molecular bases of this dysfunction (Zuccoli et al., 2017). A recent study using this technique showed that dystrobrevin-binding protein 1 (dysbindin), which has its gene associated with schizophrenia, plays an important role in axonal mitochondrial movement. Furthermore, this study suggests that dysbindin forms a functional complex with p150glued that may affect presynaptic calcium homeostasis and thus impair mitochondrial activity (Suh et al., 2021).

4 CRISPR/CAS 9

Clustered regularly interspaced short palindromic repeats (CRISPR) associated with CAS protein (CRISPR/Cas9) are part of the adaptive immune system of bacteria and archaea responsible for promoting the immune response and recognizing and eliminating invading genetic material. CRISPR/Cas9 is a highly specific technique for gene editing, capable of knocking out or silencing genes, in addition to activating and replacing them, being a great tool for disease modeling (Torres-ruiz & Rodriguez-perales, 2017). This gene editing technique has the advantage that it is not dependent on protein engineering, as other techniques do, such as zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) (Torres-ruiz & Rodriguez-perales, 2017) (Fig. 2).

This ability to edit specific genes with high efficiency can be even more powerful when combined with other recent technologies such as stem cells (iPSCs or ESCs), especially in learning the contribution to risk genes related to a disease. The use of these new technologies is especially important in understanding multifactorial diseases such as schizophrenia.

As mentioned earlier, DISC1 is one of the most studied proteins in the field of schizophrenia. Wei et al. (2014) used the TALENs technique and generated the mutation of DISC1 in iPSCs. This study showed deficits in the release and formation of synaptic vesicles and transcriptional

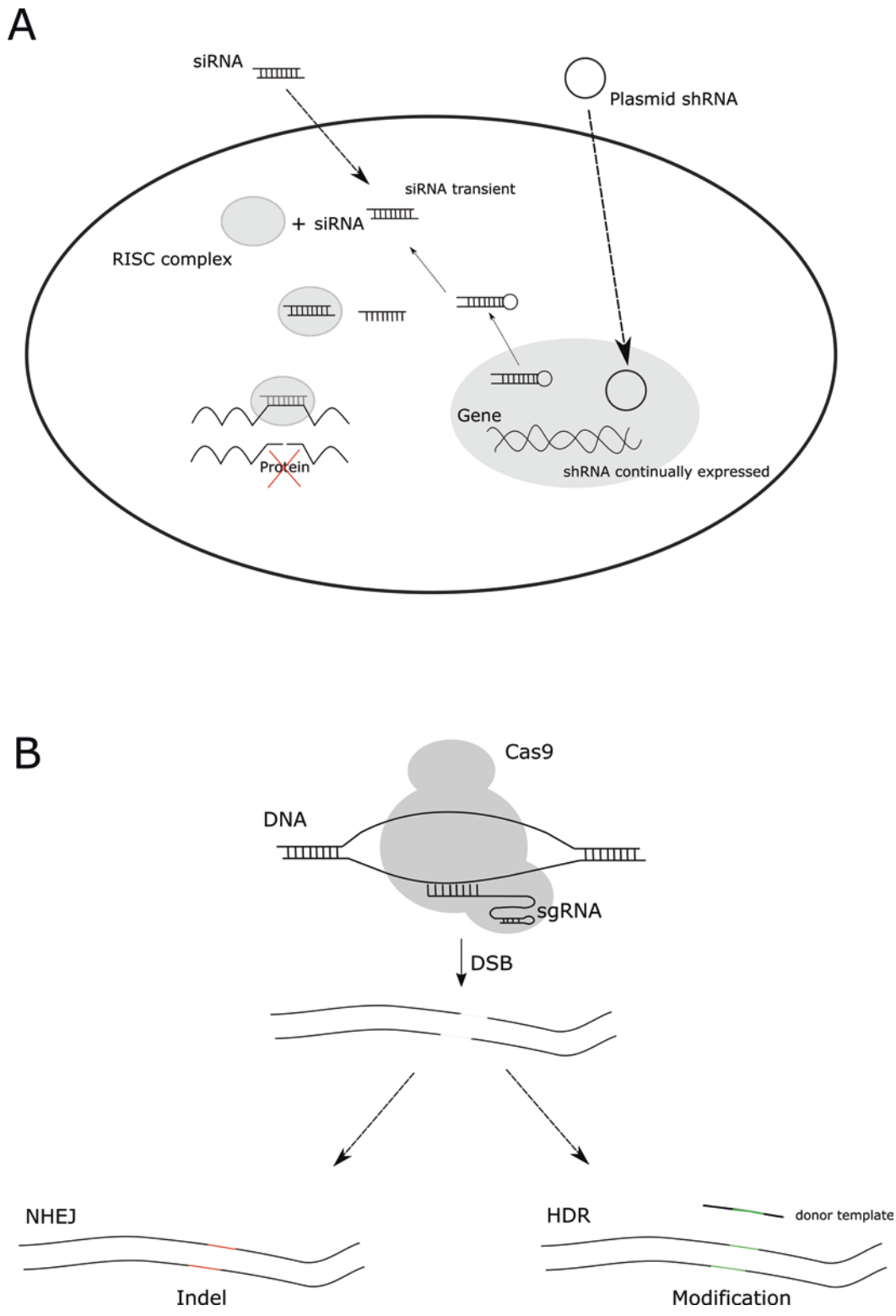

Fig. 2 (**a**) Mechanisms of shRNA/siRNA and RISC complex (Aguiar et al., 2017), (**b**) CRISPR-Cas9 system scheme (Torres-ruiz & Rodriguez-perales, 2017)

dysregulation in neurons derived from iPSCs with mutation in DISC1. Furthermore, another study used the techniques of CRISPR/Cas9 and TALENs to disrupt the DISC1 gene in iPSCs derived from control subjects. In this study, alteration in Wnt signaling and an increase in DISC1 mediated by nonsense decay of splice transcripts were observed (Srikanth et al., 2015). Also using CRISPR/CAs9, the gene-editing of the miR-137 locus, a genetic risk factor for schizophrenia, caused a reduction in the proliferation of neuronal cells, an increase in arborization of dendrites, and an increase in neurites (Hook et al., 2014).

In a recent study, Ho et al. (2017) manipulated several genes related to psychiatric disorders such as schizophrenia. This study was performed on human iPSC-derived NPCs, neurons, and astrocytes, but it focused more on establishing the technique than on related phenotypic traits and this genetic editing.

5 Final Remarks

Until now, studies of gene modulation, mainly using pluripotent stem cells, have shown promising results. However, CRISPR/CAS9, siRNA/shRNA, and iPSC techniques are still a recent field. Furthermore, these techniques have been developing each day and becoming more and more accessible, which indicates that soon we will have more studies related to schizophrenia using gene modulation.

Acknowledgments This work was supported by the São Paulo Research Foundation (FAPESP) (grant number 2016/07332-7).

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Proteomics and Schizophrenia: The Evolution of a Great Partnership

Bradley J. Smith, Victor C. Carregari, and Daniel Martins-de-Souza

Contents

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

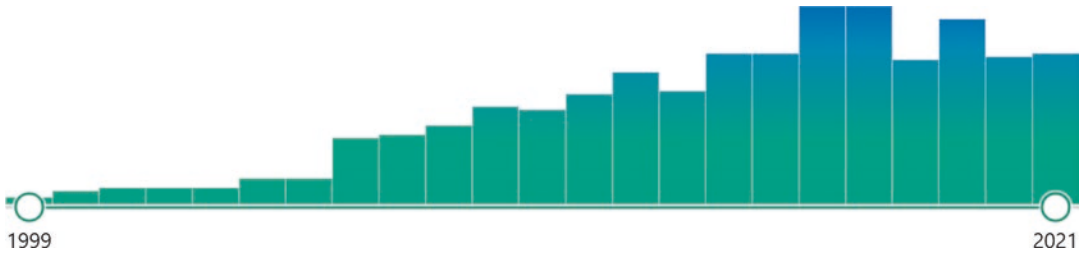

Fig. 1 PubMed search for publications involving schizophrenia and proteomics [keywords (schizophrenia) AND (proteomics)]

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

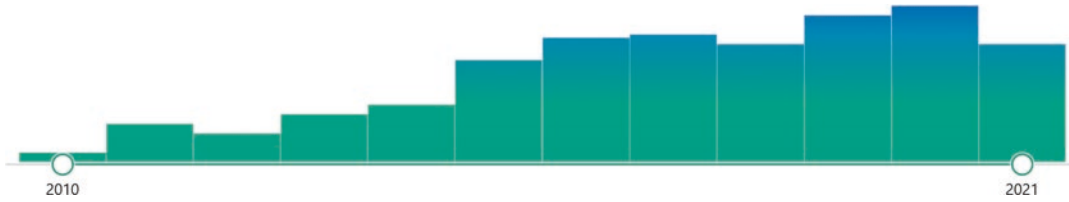

Fig. 2 PubMed search for publications involving iPSCs and schizophrenia [keywords ("induced pluripotent stem cells") AND (schizophrenia)]

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.

Acknowledgments The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES; grant number 88887.495565/2020-00) and the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP; grant number 2019/05155-9) for financial support.

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Index

D. Martins-de-Souza (ed.), *Neuroproteomics as a Tool for Understanding Schizophrenia*, Advances in Experimental Medicine and Biology 1400, https://doi.org/10.1007/978-3-030-97182-3