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Daniel Martins-de-Souza Campinas/Munich

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Advances in Biological Psychiatry

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Preface

Psychiatric disorders are problems of immense importance for public health, ranging from patients' welfare to health treatment systems. According to the World Health Organization (http://www.who.int), mental disorders compose 40% of the leading causes of disability in developed countries. In most of the cases there is a long delay between disease onset and clinical intervention, which can compromise treatment, leading to more severe illness. Psychiatric disorders are still neglected compared with other major health problems; it has been estimated, for instance, that in the USA proper management of depression can cost as much as that of heart diseases. Thus, in order to improve prognosis, diagnostics and current treatments, there is an urgent need to understand the molecular and biochemical bases of these disorders. These questions are still open, despite all scientific efforts.

Decipherment of the human genome pushed the development of other large-scale technologies for investigating the molecular bases of life. Proteomics and metabolomics are two of these technologies which can identify the protein and metabolite contents of a given cell, tissue or organism in a large-scale and high-throughput manner. In the case of psychiatric disorders, these technologies have been delineating a comprehensive characterization of the molecular mechanisms involved in the establishment and course of these illnesses. Moreover, proteomics and metabolomics have been unraveling biomarker candidates for prognosis, diagnosis, treatment and patient stratification.

This edition of *Advances in Biological Psychiatry* presents – apart from the basic principles of proteomics and metabolomics – all data generated by these technologies pertaining to a characterization of human samples from psychiatric patients as well as preclinical models. Eleven leading researchers in the field of schizophrenia and psychosis as well as in mood disorders contributed to this edition, reviewing the most important data generated by proteomics and metabolomics in the last decade. More than an update, this edition, entitled *Proteomics and Metabolomics in Psychiatry*, indicates the next steps to be followed in the search for biomarkers and a deeper understanding of psychiatric disorders.

Daniel Martins-de-Souza, Campinas/Munich

Proteomic Characterization of the Brain and Cerebrospinal Fluid of Schizophrenia Patients

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Abstract

As a multifactorial mental disorder, schizophrenia presents a complex combination of genetic, neurodevelopmental and environmental components. These lead to difficulties in comprehending the molecular basis of schizophrenia as well as in identifying biomarkers. These issues can be tackled by proteomics, which has been used increasingly along with genetics and other '-omics' approaches. In the present chapter, we explore some advances in proteomic studies involving brain tissue and cerebrospinal fluid collected from schizophrenic patients. We demonstrate that proteomic findings have been confirmed by other approaches, and added new information about the role of synaptic connectivity, oxidative stress, glucose metabolism and cytoskeletal alterations as core features of the disease pathophysiology. Integrative systems analysis including proteomics is a valid strategy for understanding the molecular basis of schizophrenia and may indicate the way to future clinical applications.

Schizophrenia

Schizophrenia is a severe mental disorder with a lifetime prevalence of 0.3–0.7% and presenting a heterogeneous range of symptoms [1]. It is a multifactorial disorder composed of a number of etiological factors of small effect that may be triggered by environmental components [2]. The causes of schizophrenia are strongly determined by genetics, but also by other factors such as epistatic (interaction between two or more genes controlling a single phenotype) and environmental ones (epigenetics). This is supported by the fact that 90% of individuals who develop schizophrenia have no history of schizophrenic parents or relatives. This conclusion does not exclude the heritable factor of schizophrenia, which is high (up to 80%), but reinforces the fact that environmental factors influence the onset/development of the disease [3]. Although interactions occur and are responsible for the establishment of the

disorder, it is very complicated to statistically prove such interactions once the number of environmental risks is very high, and difficult to list in long-term follow-ups and large patient cohorts, which would be crucial for genetic analysis. Among these risks, one can specify contact with pathogens (influenza, toxoplasmosis and genitourinary infections), psychological problems (depression, anxiety and stress), nutritional deficits, allergies and drug abuse [3, 4]. As a consequence, the idea that these complex disorders are in fact a dysfunction of molecular networks instead of abnormal function of certain genes is being actively explored [5]. According to association and linkage studies (genome-wide association studies), some genes such as *DISC1* (disrupted in schizophrenia 1), *DTNBP1* (dystrobrevin-binding protein 1), *NRG1* (neuregulin 1), *DRD2* (dopamine receptor D_2), *HTR2A* [5-hydroxytryptamine (serotonin) receptor 2A] and *COMT* (catechol-O-methyltransferase) are considered to be important candidate genes. Even so, the knowledge about genomes is still very limited and the collection of postmortem samples imposes some biological limitations. In addition, the correlations between phenotype and genotype are still poorly understood due to the lack of knowledge concerning gene expression network data. The molecular genetic data generated significantly increased improvements in diagnostics and therapeutics. However, differentiating between similar phenotypes and disease states is still critical to ensure that therapeutic monitoring and the prognosis for individual patients are reliable. In fact, the etiology and underlying mechanisms of most complex diseases are still poorly understood [6].

Proteomics

Considering schizophrenia as a biochemical pathway disorder, proteomics has become one of the suitable approaches to its understanding [6]. In general, proteins are the effectors of biological responses, and they are subjected to translational and degradation processes [7]. Due to this fact, characterizing the proteins present in a certain biological sample is the key to systems biology once it is possible to identify temporal variations in the proteome, as well as protein structures, its posttranslational modifications and cellular localization and interactions [8].

O'Farrell [9] and Klose [10] were the first to demonstrate that it was possible to separate proteins based on their isoelectric points and molecular weights by electrophoresis on two-dimensional polyacrylamide gel electrophoresis. So far, both visualization and identification were based on Western blotting and classical Edman sequencing, and these are relatively low-throughput methods [11]. Besides, not every protein is suitable for separation. Among these proteins, we can find those that are very basic, higher than 150 kDa or lower than 10 kDa, low-abundance proteins and highly hydrophobic ones [12]. Despite this methodological limitation, several two-dimensional proteomic analyses were already performed on different brain regions of schizophrenic patients, such as the frontal (FC), prefrontal (PFC), anterior cingulate, insular and dorsolateral prefrontal cortex (DLPFC), corpus callosum, Wernicke's area, hippocampus and thalamus. Another method of analyzing proteomic samples is called 'two-dimensional differential gel electrophoresis' (2-D DIGE) and allows up to 3 samples on the same gel where usually 2 samples and 1 internal standard are covalently labeled with 3 fluorescent dyes such as CyDye 2, CyDye 3 and CyDye 5. In addition, the amount of sample necessary for detection is very low, around 150 pg of protein. Although its high cost limits the use of this method [13], it has been widely used in studies of schizophrenia.

Trying to overcome some of the methodological issues of gel-based proteomics, direct mass spectrometry (MS)-based approaches have been developed, which are widely known as 'shotgun proteomics' [14]. Usually, in these techniques,

the proteins are enzymatically digested and produce peptides once liquid chromatography (LC)-MS analysis is more sensitive to low-molecular-weight molecules [8]. These peptides may be labeled using stable isotopes such as isotope-coded affinity tags, iTRAQ (isobaric tags for relative and absolute quantitation), isotope-coded protein labeling (ICPL) and 4-trimethylammoniumbutyryl, a deuterium-based label [15] that helps in posterior quantification. In vivo studies may also use stable isotope-labeling such as SILAC (stable isotope labeling by amino acids in cell culture) and ^{15}N metabolic labeling, although they remain high-cost experiments [7, 16, 17]. Another approach is label-free analysis, in which quantification is done directly by comparing the MS measurement of different samples once you consider that the chromatographic peak area of a given peptide corresponds to its concentration [7, 17]. The reproducibility of label-free analysis was already evaluated in blood serum samples [18].

The Importance of Studying the Brain and Cerebrospinal Fluid Proteomes

Although brain biomarkers must be translated to peripheral tissues, as brain tissue cannot be collected from living patients, they do not as a rule reflect the proteomic differences in the brain. However, the analysis of brain tissue can provide important information about biochemical pathways and the consequent cellular metabolism involved in the pathobiology of schizophrenia. Another important issue is to isolate the tissue to be analyzed, due to the high anatomical and cellular complexity of the brain. With this approach, the results happen to be more manageable and meaningful [19, 20].

Besides the brain tissue studies, another important source of biomarkers has been the cerebrospinal fluid (CSF), as it reflects the changes within the central nervous system. A major source of proteins to the CSF is blood from the cerebral circulation, despite the existence of the blood-CSF barrier. The amount of protein in CSF is around 0.3% of that found in plasma [21].

Glycolysis Pathway and Oxidative Stress

Schizophrenia has been associated with an increased risk of the metabolic syndrome related to the disease itself and antipsychotic treatment, showing impaired glucose tolerance, insulin resistance and increased intra-abdominal fat in comparison with healthy controls. Previous data demonstrated the role of glucose administration in attenuating deficits in verbal declarative memory [22] in agreement with other studies demonstrating a relation between glucose regulation and memory performance [22, 23]. Those suffering from schizophrenia have a tendency to develop the metabolic syndrome, which comprises type 2 diabetes mellitus, dyslipidemia and hypertension, all representing medical conditions that increase intra-abdominal fat deposition and are in a way also controlled by cortisol levels [24]. Evidence indicates that drug-naïve or drug-free schizophrenia patients present higher levels of plasma cortisol, which indicates an imbalance in the hypothalamic-pituitary-adrenal axis and also increased levels of corticotropin-releasing hormone in CSF samples [25]; however, some other studies do not show significant increases in cortisol levels [26].

Some proteomic evidence in the PFC of schizophrenia patients also indicated downregulation of 7 out of 10 glycolytic enzymes, increased glycogenolysis transcripts, glycogenesis genes and metabolites, indicating that there is an increased glucose demand or cellular hypoxia [27]. In agreement with these data, another proteomic study using a selected reaction monitoring assay indicates alterations in energy metabolism including glycolytic enzymes. The most consistent data include aldolase C (ALDOC), γ-enolase (ENO2), hexokinase

(HK1), phosphoglycerate mutase 1 (PGAM1), aconitate hydratase (ACO2) and triosephosphate isomerase (TPI1) [17, 28]. HK is a rate-limiting enzyme of glycolysis, and among the isozymes, HK1 is the only one that is predominantly bound to mitochondria. This attachment reduces mitochondrial reactive oxygen species (ROS) and figures as a downstream effector of the PI3K/Akt signaling pathway, which regulates mechanisms of cell survival. In postmortem parietal cortex samples of schizophrenia, bipolar disorder and unipolar depression of medicated and nonmedicated patients, a decrease in mitochondrial attachment of HK1 was found, which may lead to impairment in energy metabolism by increasing oxidative stress, and also alterations in brain growth and development [29]. ALDOC has so far been described as a brain-specific isozyme involved in fructose metabolism, and it also plays a role as a zinc-activated ribonuclease to neurofilament light polypeptide (NF-L) mRNA [30, 31]. ENO2, also known as neuron-specific enolase, has been extensively used as a marker for nervous tissue damage, especially in meningitis. It is found in neurons, tissues in the peripheral nervous system and neuroendocrine cells. Increased levels of neuron-specific enolase in serum and/or in CSF in neurological disorders such as status epilepticus, Creutzfeldt-Jakob disease and acute ischemic stroke [32] have already been reported. PGAM1 is an enzyme responsible for the conversion of 3-phosphoglycerate to 2-phosphoglycerate, acting along with glyceraldehyde-3-phosphate dehydrogenase and nucleoside diphosphate kinase A enzymes. Immunohistochemical studies demonstrated its presence in the endothelium of capillaries and arteries of the brain, liver and kidneys. This protein has been extensively described in tumor cells [33, 34] (fig. 1). In another proteomic study using two-dimensional electrophoresis gel, matrix-assisted laser desorption/ionization-time-of-flight (TOF)-MS and LC-MS/MS, key differentially expressed mitochondrial proteins such as malate dehydrogenase, ACO2, fructose bisphosphate aldolase A (ALDOA), SCOT [succinyl-coenzyme A (CoA):3-ketoacid-CoA transferase] and ATP synthase were all identified. These proteins are important in processes that involve the tricarboxylic acid (TCA) cycle (malate dehydrogenase, ACO2 and SCOT) and glycolysis (ALDOA) [35].

A well-established glycolytic enzyme that has been related to neurological disorders is TPI. This enzyme catalyzes the conversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate, and, notably, its deficiency leads to chronic hemolytic anemia associated with progressive neurological dysfunction ending in early death in childhood. There is some evidence connecting TPI with protein misfolding and formation of protein aggregates [36]. Another study demonstrated that the TPI gene is regulated by IFNG (interferon-γ), which reinforces the role of the immunological system in the development of schizophrenia (fig. 1) [37].

Following the glycolysis pathway, the TCA cycle generates energy to be used in amino acid production and also redox potential for oxidative phosphorylation (OXPHOS) [38]. Studies have already demonstrated that changes in the TCA cycle may lead to alterations in brain metabolism – such as in Alzheimer's disease (AD), in which a reduction in α-ketoglutarate dehydrogenase complex activity presents a high correlation with this dementia [39, 40]. Aconitase is one key mitochondrial enzyme of the TCA cycle catalyzing the reversible interconversion of citrate and isocitrate by the *cis*-aconitate enzyme. When aconitase is inactivated, NADPH production is interrupted, and may then be used as an oxidative stress marker [41].

Oxidative stress is another mechanism that has been described as important in the course of schizophrenia. Involvement of an impaired antioxidant defense system occurs when altered levels of peripheral glutathione peroxidase (GSHPx) activity are related to increased cortical sulcal prominence observed by CT scanning [42]. In recent years, increased evidence coming from broad analyses of antioxidant pathways, instead

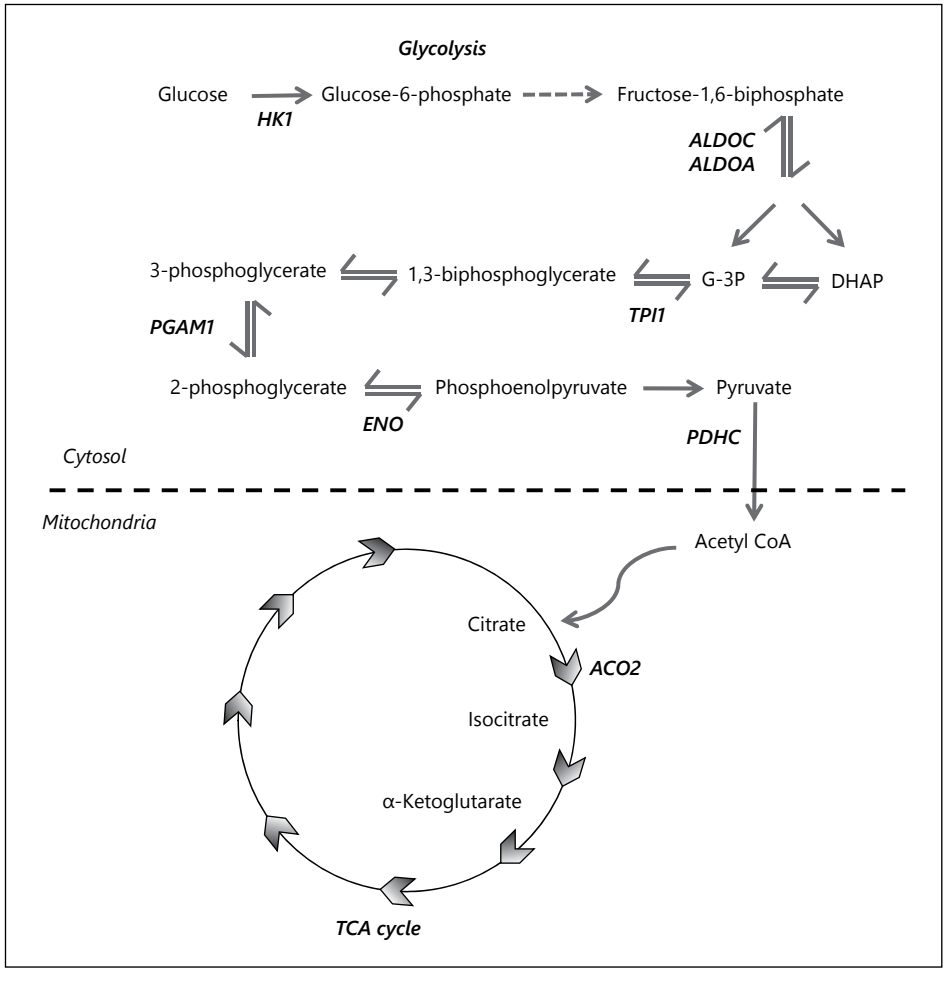

Fig. 1. Proteomic studies indicate consistent changes in glycolytic and TCA cycle enzymes. These enzymes include ALDOC, ENO2, HK1, PGAM1, ACO2 and TPI1. G-3P = Glyceraldehyde 3-phosphate; DHAP = dihydroxyacetone phosphate; PDHC = pyruvate dehydrogenase complex.

just of isolated enzymes, and an increased variety in techniques (proteomics, genomics and imaging studies) and biological material (blood, urine and brain samples) have demonstrated the important role of that mechanism in schizophrenia. These findings include reduced antioxidants in plasma, such as albumin, uric acid, ascorbic acid and vitamin E, and deficits in GSH in a nervous system investigation (CSF, PFC and caudate nucleus). The enzymes SOD, GSHPx, peroxidase and catalase were also reported, and increased evidence also indicates altered levels of nitric oxide in peripheral (blood, serum) and central (postmortem caudate nucleus) approaches [43]. Drug-naïve patients of first-episode schizophrenia and chronic schizophrenia patients presented increased plasma SOD activity in contrast to healthy controls. And after treatment with antipsychotic medication, SOD activity was even higher, indicating that the medication was probably implicated in inducing antioxidative mechanisms [44, 45]. The ROS imbalance observed in

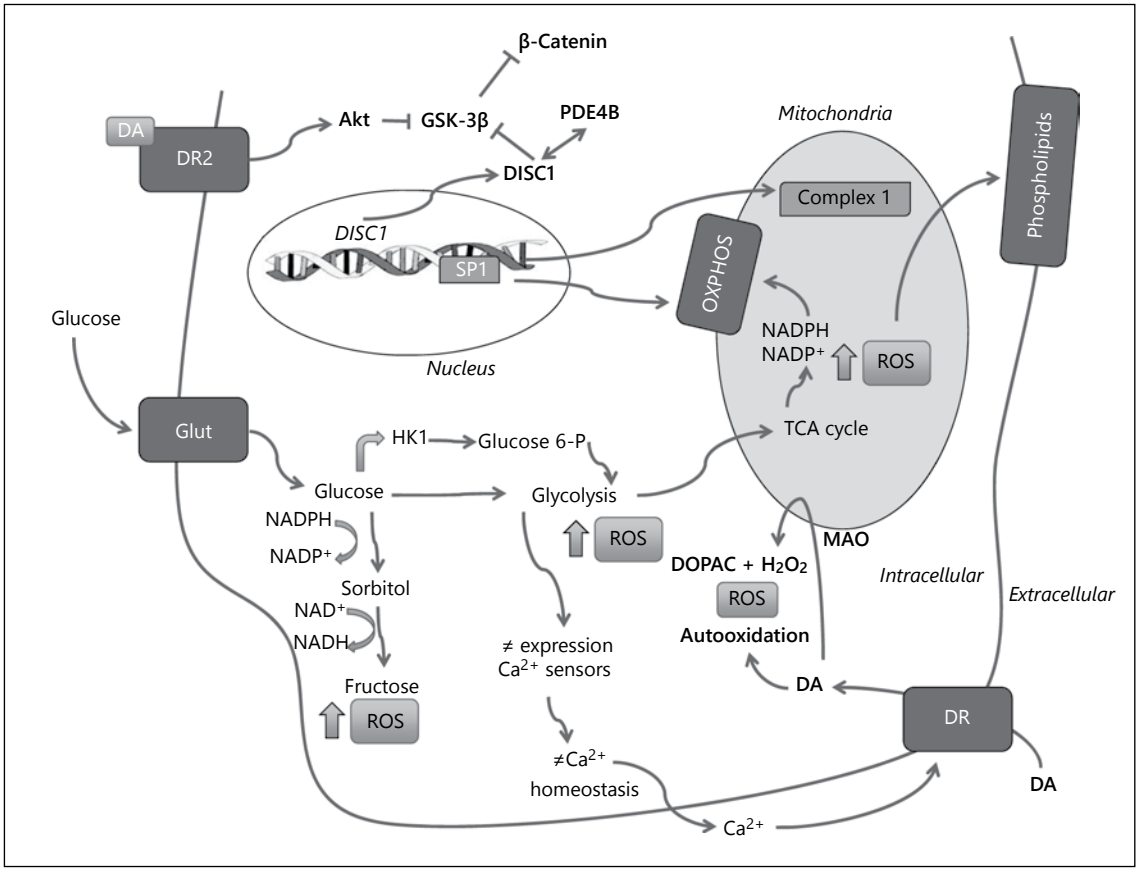

Fig. 2. Dopamine binding inhibits Akt activity in a G-protein-independent manner. DISC1 protein blocks glycogen synthase kinase 3β inhibition of β-catenin phosphorylation, which is important in neurogenesis processes. DISC1 also interacts with phosphodiesterase 4B, which can hydrolyze cAMP and regulates protein kinase A, which participates in neuronal signaling and plasticity. Dopamine may also increase ROS production through its autooxidation. In pathological conditions there is an altered specificity protein 1-dependent complex I subunit expression and activity, and modulation of OXPHOS proteins. Alterations in glycolytic processes interfere with the TCA cycle and consequently with OXPHOS. Glycolysis, the TCA cycle and OXPHOS are responsible for metabolic processes and redox potential through generation of NAD/NADH, NADP/NADPH and FAD/FADH. An imbalance in these pathways induces ROS production, establishing an oxidative-stress state, thereby altering Ca^{2+} and lipid homeostasis. Alterations in Ca^{2+} homeostasis may influence the activity of Ca^{2+}-dependent phospholipase A_2, accelerated phospholipid turnover and reduced dopaminergic activity. GSK-3β = Glycogen synthase kinase 3β; PDE4B = phosphodiesterase 4B; DA = dopamine; DR = dopamine receptor; SP1 = specificity protein 1; MAO = monoamine oxidase; DOPAC = 3,4-dihydroxyphenylacetic acid.

schizophrenia samples may lead to other consequences such as peroxidative damage to lipids affecting cellular membranes, which may happen when one of those antioxidant enzymes (SOD, GSHPx and catalase) are imbalanced without compensatory activity of others (fig. 2) [44].

The ability of the TCA cycle to form a redox potential to the OXPHOS system is tested by NADH and NADPH levels. Mitochondrial complex I is responsible for NADH dehydrogenase and oxidoreductase activities, which are involved in the transfer of electrons from NADH to the

respiratory chain. Proteomic studies demonstrated that malic enzyme 3, which converts pyruvate to malate generating NADPH, was found to be upregulated. Besides, the NADPH concentration and NADP/NADPH ratio were altered in schizophrenia [38]. Several other complex I subunits have already been described as being downregulated in the anterior temporal lobe (ATL), such as NDUFB5 [NADH dehydrogenase (ubiquinone) 1β subcomplex, 5], NDUFS1 [NADH dehydrogenase (ubiquinone) Fe-S protein 1], NDUFS3 and NDUFS6, and some controversial results (up- and downregulation) have been described for NDUFV2 [NADH dehydrogenase (ubiquinone) flavoprotein 2] and an upregulation was found in NDUFB9. Controversial data were also found for the ATP5A1 (ATP synthase α-subunit, mitochondrial; up- or downregulation) complex V subunit, and a downregulation of its other subunit, ATP5H (ATP synthase d-subunit, mitochondrial), in different schizophrenia brain regions (fig. 2) [16].

Another study using PFC tissue screened 50 proteins, among which 19 were associated with mitochondrial function, 16 with oxidative stress and another 3 with peroxisomal function. Besides that, other proteins – namely syntaxin-binding protein 1 and brain-abundant membrane-attached signal protein 1 along with others – related to cytoskeletal proteins and some associated with protein trafficking/turnover were also identified via this method [17, 27]. Other proteins were also found to be altered, identified in the DLPFC of postmortem schizophrenia samples, including some mitochondrial ones indicating impaired energy metabolism, such as dynamin-like 120-kDa protein (OPA1; upregulated), a nucleus-encoded mitochondrial protein that was already correlated to alterations in AD and Huntington's disease [43], cytochrome bc_1 (UQCRC1; upregulated), which had not previously been implicated in schizophrenia, and NDUFV2 (upregulated), which participates in nervous system development [46, 47].

The mitochondria, besides generating ATP, participate in processes involving signaling, cellular differentiation, cell death, and control of the cell cycle and of cell growth [48]. The mitochondrial OXPHOS system, also known as 'mitochondrial respiratory chain', is responsible for generating the cellular energy. There are still controversial data concerning the OXPHOS system. While some groups demonstrated changes in complex IV (increase or decrease), others have seen no difference [49].

Another key player in oxidative stress, apoptosis and inhibition of mitochondrial respiration appears to be the neurotransmitter dopamine. This neurotransmitter can induce neurotoxic effects by the formation of highly ROS, quinones and semiquinones generated by dopamine autooxidation or by its enzymatic metabolism by monoamine oxidase inducing oxidative stress [45, 48, 50]. Extensive studies demonstrated that dopamine is not related to alterations in complex I subunits [50], but instead a transcription factor known as specificity protein 1 (Sp1). This transcription factor has been related to the regulation of nuclear mitochondrial genes including OXPHOS protein-coding ones. In a comparison between controls and schizophrenia individuals there was a high correlation between Sp1 and complex I subunits in the control samples, which was not true for the schizophrenia material, increasing the evidence that Sp1 interferes with complex I activity (fig. 2) [48]. In an extensive review, it was shown that data consistently indicate increased dopamine levels to be associated with positive symptoms in mesolimbic regions, and lack of dopamine with negative symptoms in the FC [51].

Phospholipid Metabolism Disruption and Calcium Homeostasis

Postmortem studies of schizophrenia patients thalamus revealed significant differences in the phospholipid components phosphatidylcholine, sphin-

gomyelin and phosphatidylserine, and the total amount of galactocerebrosides in the left thalamus of schizophrenia patients compared with healthy control subjects. This may indicate an accelerated breakdown of membrane phospholipid, which is consistent with findings of increased phospholipase A_2 (PLA_2) activity in platelets and in the cortex of schizophrenia patients [52]. Sphingomyelin is one major component of myelin membranes and, when cleaved by sphingomyelinases, leads to the formation of ceramides, which regulate cell growth, oligodendrocyte differentiation and programmed cell death as well. A decrease in sphingomyelin levels has been related to oligodendrocyte dysfunction observed in schizophrenia patients presenting impaired myelination in the thalamus and its connections [53]. The PLA_2 activity of peroxiredoxin 6 is critical for the regulation of phospholipid turnover. A significant differential expression of peroxiredoxin 6 was found in DLPFC of schizophrenia samples, which may lead to increased phospholipid turnover and consequently to a differential dopaminergic state. Another consistent result involves alterations to glial fibrillary acidic protein (GFAP; upregulated), which can lead to compromised synaptic functioning and alterations in behavior [54]. This is corroborated by other studies, which demonstrated altered levels of GFAP as well as of α-internexin, β-actin, ATP-synthase, ALDOC and dihydropyrimidinase-related protein 2 (dihydropyrimidinase-like 2) among others, totaling 57 differentially expressed proteins in the insular cortex of schizophrenia patients mainly correlated with neuronal plasticity involving neurite outgrowth, cellular morphogenesis and synaptic function (fig. 2) [55].

ApoA1 (apolipoprotein A-I), along with other cholesterol-binding lipoproteins [ApoA4, ApoC1, ApoC2, ApoC3, ApoD, ApoE and lipoprotein(a)], has already been correlated with AD as a key component of glia/neuron cholesterol shuttles [56]. In CSF studies, using SELDI (surface-enhanced laser desorption/ionization)-TOF-MS, ApoA1 was found to be downregulated, as well as in the liver and blood samples [57]. In another study, an upregulation of this protein was also detected in CSF of schizophrenia patients treated with second-generation antipsychotics [58]. Although there is still no consensus about alterations in the ApoA1 protein, apparently the phospholipid metabolism seems to be impaired in schizophrenia. In the same study, ApoE and prostaglandin H_2 D-isomerase were also upregulated. All these data, taken together, indicate potential phospholipid and cholesterol metabolism disruption in schizophrenia, as demonstrated for other disorders such as AD [58].

Proteomic studies employing shotgun data combined with ICPL also identified altered proteins related to the calcium signaling pathway, crucial for dopamine receptor function and neurotransmitter exocytosis. Some of these proteins include neuronal protein 4.1 (upregulated), which stabilizes dopamine receptors at the neuronal plasma membrane and endophilin A1 (upregulated), which is essential for the formation of synaptic vesicles from plasma membrane. Phosphatidylethanolamine-binding protein 1 (PEBP1) is upregulated and represents a substrate for calpain, a Ca^{2+}-dependent protease that has been implicated in processes that produce persistent changes in synaptic chemistry and structure [47]. In addition, ATPase 4 protein – which is involved in maintenance of Ca^{2+} homeostasis, and regulates Ca^{2+} PLA_2 activity, phospholipid turnover and reduced dopaminergic activity – was increased in ATL samples of schizophrenia patients [59].

Signaling Pathways

Using the 2-D DIGE approach, some proteins were found to be altered in layer 2 of the insular cortex of schizophrenia patients. Some of them are 14-3-3 theta, β-synuclein, FK506-binding protein 4, eukaryotic translation initiation factor, γ-synuclein and nucleophosmin, which, in general, are related to communication/signal

transduction and protein metabolism, mainly in neuronal plasticity [55, 60]. Two-dimensional gel electrophoresis studies on the FC (Brodmann area 10) identified decreased levels of dihydropyrimidinase-related protein 2 in brains of schizophrenia patients as well as in bipolar disorder and major depressive disorder. In humans, it was shown that this protein is important in development, leading to severe neurological impairment and neurological degeneration due to delays in myelination [61].

Proteomic studies of postmortem ATL samples from schizophrenia patients and healthy controls using ICPL identified two new possible targets for the disease: PEBP1 (increased) and aggrecan core protein (decreased) [59]. PEBP1 is a calpain substrate and the precursor of hippocampal cholinergic neurostimulating peptide, which has been related to the downregulation of cholinergic neurons in AD [62]. In addition, PEBP1 seems to be involved in Raf-1 kinase pathways, and is then considered a Raf kinase inhibitor protein [63]. On the other hand, aggrecan – together with versican, neurocan and brevican – is a member of the chondroitin sulfate proteoglycan family. This protein binds to hyaluronan and is distributed in connective tissue and extracellular matrices [64], then playing a pivotal role in the cell adhesion mechanisms and neurite growth that are so important in nervous system development [65].

In another study using LC-electrospray ionization-MS/MS, an important increase in VGF23–62 peptide was demonstrated in the CSF of first-onset drug-naïve schizophrenia patients [66]. In a different approach using SELDI-TOF-MS for CSF samples, it was demonstrated that, as well as in AD, in schizophrenic elderly patients there was a reduction in amyloid β $(A\beta)_{1-42}$ levels and an increase in soluble Aβ precursor protein-α in comparison with control samples. The Aβ precursor protein has been related to neuronal plasticity/survival, nervous system development and protection against excitotoxicity. This indicates different dynamics in the aging brain, and differing participation of the same peptide in different pathologies [67].

These VGF findings were confirmed by studies undertaken in the initial prodromal state of psychosis, a phase of nonspecific symptoms that include lack of concentration and motivation, anxiety, sleep disturbances and social withdrawal, and that may be related to the onset of psychoses such as schizophrenia. This is a very vulnerable period in which environmental factors – so important in the development of this disease – may act. A proteomic study of this phase on CSF samples from drug-naïve paranoid schizophrenia patients revealed increased levels of VGF-derived peptides (VGF23–62 fragment) and also decreased levels of transthyretin protein [68]. Transthyretin protein, which has been related to thyroid hormone transport in the CSF, was also downregulated in another proteomic schizophrenia study [38].

Glia and Myelination-Related Alterations

According to findings of decreased oligodendrocyte numbers in schizophrenia [69–71], shotgun proteomic analysis of ATL samples from schizophrenia patients has shown a downregulation of proteins related to the myelin sheath and oligodendrocyte genes such as 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP), GFAP, myelin oligodendrocyte glycoprotein, myelin basic protein (MBP, the main component of the myelin sheath of oligodendrocytes and Schwann cells) and ermin (a myelinating oligodendrocyte-specific protein, found exclusively in oligodendrocytes) [72, 73]. MBP, DM20, myelin-associated glycoprotein, CNP and proteolipid protein are the major myelin proteins in the nervous system. The classic MBPs are the most abundant proteins in the nervous system and almost exclusively found in oligodendrocytes [74]. Recent studies have demonstrated that one new important role for MBP is

in calcium channels of the oligodendrocytic plasma membrane. The Golli isoforms seem to stimulate calcium influx, but in contrast to this, classic MBP isoforms seem to decrease calcium influx through voltage-operated calcium channels [75]. This calcium control function per se is extremely important once the excess of calcium influx leads to cellular death and induces activation of peptidylarginine deiminases. These enzymes regulate MBP deimination, and consequently developmental myelination, leading to a structural destabilization of adult myelin, one cause of multiple sclerosis [75].

CNP, also known as CNPase, is highly present in oligodendrocytic membrane sheaths and is responsible for generating 2′-nucleotides from 2′,3′-nucleotide hydrolysis and acts mainly in highly myelinated regions. Some studies demonstrated that a lack of this enzyme is related to axonal degradation, although its stability remains intact. CNP binds to microtubules and exhibits microtubule assembly, which is important for oligodendrocyte expansion. In addition, it associates with F-actin, which is important in processes such as the formation of filopodia and lamellipodia [76, 77]. Premyelinating oligodendrocytes present high expression of CNP during development and remain during the beginning of the myelination process and throughout life [78]. Some gene expression analyses demonstrated that reduced CNPase expression is related to the A-allele of rs2070106, which has been shown to be recurrent in schizophrenia patients [79], although there is still some controversy about it [80]. These alterations are validated by other transcriptomic assays, also of postmortem brain tissue from schizophrenia patients, in which 17 oligodendrocyte-related genes were downregulated in the DLPFC, e.g. T-cell differentiation protein, tyrosine kinase-type cell surface receptor HER3, growth-associated protein 43, glutamate decarboxylase 2, glutamic acid decarboxylase 67 kDa, G protein-coupled receptor 37, NF-L and NF-M [81].

A proteomic study of postmortem mediodorsal thalamus and CSF by using quantitative shotgun MS (iTRAQ labeling) and two-dimensional gel electrophoresis showed the differential expression of MBP and myelin oligodendrocyte glycoprotein in the mediodorsal thalamus and CSF. This evidence together with other evidence – such as decreased levels of transferrin, an essential protein in myelination – suggests oligodendrocyte alteration in schizophrenia patients [38].

Cytoskeletal Alterations

As discussed previously in the present chapter, DISC1 has been considered a genetic risk factor for schizophrenia and also for other disorders [82]. Recently, molecular studies have demonstrated that DISC1 may act as a scaffold protein for complexes involved in neurite outgrowth and function. Some examples of proteins that bind to DISC1 are glycogen synthase kinase 3β (GSK-3β) and phosphodiesterase 4 (PDE4). GSK-3β is a serine/threonine protein kinase involved in the Wnt signaling cascade, the PI3K cascade and the mitogen-activated protein kinase cascade. Microtubule-associated proteins (MAP) are phosphorylated by GSK-3β, and impairment of this cascade may lead to structural abnormalities observed in schizophrenia [82–84]. PDE4 is a cAMP-specific PDE with high affinity for cAMP and protein kinase A (PKA). The inhibition of PDE4 enhances the dopamine D_1 receptor/PKA/DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa) signaling cascade in cortical neurons. The physiological consequence is an improvement in sensorimotor gating with enhancement of cognitive function [85]. Then, DISC1 is an important player in protein-protein networks involving microtubule organization, kinesin-mediated transport of vesicles, neurite extension or the regulation of neural progenitor proliferation (fig. 2).

Cytoarchitectural and morphometric abnormalities such as decreased synaptic plasticity and decreased gene expression of cytoskeletal elements have been described in patients with schizophrenia [86, 87]. Immunohistochemical studies on elderly schizophrenia patients demonstrated that those with dementia had an increased number of GFAP-positive astrocytes in comparison with those without dementia. Cytoskeletal proteins play an important role in keeping the cellular structure, in neuritogenesis and in neurotransmission as well [88]. In schizophrenia brain tissue, proteomic studies identified altered cytoskeletal proteins such as vimentin, lamin B2, desmin, plectin 1, β-tubulin 2B polypeptide, cytoskeleton-associated protein 1 – which facilitates the dimerization of α- and β-tubulin via nitric oxide signaling – and kinesin family member 21A, which interacts with DISC1. Moreover, alterations in MAP (MAP1A, MAP2 and MAP6), which are involved in microtubule assembly, have been demonstrated to be an essential step in neurogenesis. These MAP were described to be involved in schizophrenia in other studies, and have been suggested as potential therapeutic targets [89].

Belonging to the negative symptoms, one key feature of schizophrenia is anhedonia, the inability to enjoy activities which are normally considered pleasant [90]. In a genetic study, this symptom has been related to the *DISC1* gene in a large cohort population in Finland. Besides, animal models of DISC1-related diseases displayed anhedonia-like behavior, indicated by immobility in the forced swimming test. Impairment of this gene has been related to disrupted axonal transport in neurons [91]. Another gene, CRMP1 (collapsin response mediator protein 1), which is dependent on DISC1, is also related to anhedonia. In this multidisciplinary study, it was shown that in lymphoblastoid cell lines of schizophrenia patients there was an increase in CRMP1 expression, suggesting a potential role of a blood-based diagnostic marker [91]. Another DISC1-related gene was downregulated in CSF of schizophrenia patients, namely CCDC3 (coiled-coil domain-containing protein 3 precursor) [58]. The role that CCDC3 plays in the brain, mainly in neurological disorders, is still unknown, but studies undertaken on mice demonstrated its presence in adipocytes and endothelial cells [92].

Conclusion

Proteomic profiling has produced even further evidence that schizophrenia is a multifactorial disorder, and thus that finding diagnostic and therapeutic methods is still a challenging goal. Even when we understand the molecular basis of the disorder, there is still the question of whether the altered genes or proteins figure as causes or consequences of the disorder [93]. Nevertheless, by proteomics it is possible to identify temporal variations in protein content and, more remarkably, their interactions, which is so important in defining intracellular mechanisms. The proteomics approach has then generated new hypotheses, possible biomarkers and, even more importantly, new targets to be evaluated in schizophrenia studies.

Taking this into account, research so far has identified that besides disrupted synaptic connectivity, oxidative stress, glucose metabolism and cytoskeletal alterations also play important roles in the onset and development of schizophrenia. It is also interesting to point out that many of these pathways are implicated in other neurological disorders such as AD, depression and bipolar disorder, which makes them even more complicated to treat but also sheds some light on the fact that many of these disorders, in the end, do not appear alone.

A recent extensive genomic study provided evidence that single-nucleotide polymorphism (SNPs) may be strongly associated with mental illness onset. The identified SNPs include the

ones at 4 loci, regions on chromosomes 3p21 and 10q24, and SNPs in 2 L-type voltage-gated calcium channel subunits, *CACNA1C* and *CACNB2*. The ones related to calcium channels have been confirmed by pathway analysis as being important in all 5 disorders compared, namely autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder and schizophrenia [94]. This result is supported by proteomics studies, as described in this chapter, and suggests that similar pathways may be responsible for different disorders, adding evidence to the fact that there is a co-occurrence of clinical phenotypes.

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Peripheral Biomarker Candidates in Schizophrenia

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Abstract

The identification of peripheral biomarkers for schizophrenia is of great clinical importance, with the potential to considerably improve its diagnosis, treatment and prognosis. However, despite intense research efforts and the discovery of several potential candidates for markers, no biomarker assay developed so far possesses sufficient sensitivity and specificity for clinical use. Nevertheless, with the advent of innovative technologies and methods on an analytical and statistical level, including hypothesis-free proteomic and epigenetic procedures and advanced bioinformatics, establishing biomarkers for clinical use may lay within reach. To date, the most promising candidates for biomarkers are linked to neural transmission, neural plasticity (e.g. neurotrophic factors), oxidative stress/free radicals, endocrinology, immunology, signalling pathways, gene expression regulation/activation and lipidomics.

Introduction

Historically, any research into the neurobiological basis of the phenomenon of schizophrenia has been largely futile. This is prototypically exemplified in the vain efforts of Cécile and Oskar Vogt [1], who spent decades trying to unravel clear-cut neurohistological alterations in post-mortem brains from schizophrenic patients. One fundamental problem consists in the fact that the diagnosis of schizophrenia is purely based on the presence of several psychopathological symptoms as delineated in international classification systems such as the ICD or DSM, and that any link between this clinical phenomenology and measurable neurobiological alterations is missing. Such a link might even be logically impossible assuming that similar facts observable or explorable on the behavioural, experiential, cognitive and affective levels can be caused by a plethora of very different biological pathologies. Additionally, all psychiatric conditions are the consequence of an interplay between endogenous (e.g. genetic) and environmental (e.g. traumatic) pathological factors. Thus, similar clinical pictures (i.e. schizophrenic symptoms) might be based on very divergent alterations to the brain. This is reflected by terminologies omitting the label 'schizophrenia' and replacing it by 'group of psychotic disorders' or 'schizophrenic spectrum'. Therefore, strategies have been developed which specifically focus on certain schizophrenic subtypes (e.g. catatonia) or on so-called endophenotypes which might more

likely have a 'common neurobiological basis' or are at least less heterogeneous from a neurobiological and neuropathological perspective.

However, from a clinical point of view, schizophrenia remains one of the most devastating and destructive neurobiological disorders, with grave consequences for the patients concerned and their families. It is therefore still mandatory for researchers to intensify their efforts, aiming to elucidate and better understand the underlying pathological changes on molecular and cellular levels in order to improve the diagnosis, treatment and prognosis of schizophrenia.

While classical research into the histopathology and genetics of schizophrenia – despite producing some interesting scientific results – has been widely illusive and disappointing regarding its applicability to daily clinical practice, present-day proteomic and metabolomic approaches using state-of-the art methodologies are classificatorily, statistically and technically promising as, finally, they are possibly overcoming the shortcomings that have traditionally hampered schizophrenia research [2, 3].

Search for Biomarker Candidates: Methods and Challenges

In principle, there are two ways of identifying possible candidates which might be used as biomarkers: a hypothesis-driven approach would focus on theories developed so far regarding possible neurobiological alterations underlying schizophrenia (e.g. certain neurotransmitters) and thus deduct parameters possibly linked to the condition in question, whereas a hypothesis-free approach would, without any a priori assumptions, make use of screening technologies developed for the identification of specific markers out of a large number of molecules (ideally all molecules) which quantitatively or qualitatively significantly differ between index and control groups, i.e. patients suffering from schizophrenia and individuals without neuropsychiatric disorders. Any clinically useful markers for routine application also need to be identifiable in easily accessible patient samples – that is, ideally, in serum or plasma. Fulfilling this important requirement, however, can render the search for such markers even more difficult because subtle biochemical changes in the central nervous system (CNS) might not be reflected in the peripheral blood, although interesting empirical data have been generated demonstrating that potential peripheral biomarkers consisting of inflammatory, endocrinological and growth factor parameters might be linked to CNS processes via regulatory and neuroendocrine proteins [4].

On the other hand, it is not mandatory for the biomarkers themselves to be directly involved in the neuropathology of schizophrenia; a stable and discriminatory correlation disregarding any aetiopathogenetic causality would be sufficient for the purpose of just marking (i.e. identifying) patients. While such biomarker tests are principally well conceivable, reliability, specificity and sensitivity are crucial for their clinical application, and so far no test for serum or plasma has achieved a sufficient level of quality regarding these key criteria.

Nevertheless, hypothesis-free screening methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry are presently used in order to identify possible markers which might – perhaps in combination with each other – reach sufficient sensitivity and specificity to be of use for clinical testing [5]. After the identification of reliable markers, the techniques used for quantifying them might be considerably simplified and automated.

Another way of identifying possible peripheral biomarkers is based on genome-wide expression analyses of genes via the microarray technology and of non-coding but regulatory microRNA in blood in order to detect possible expression profiles specific for schizophrenia [6, 7].

Promising markers might be related to specific neurotransmitters and their metabolites, to

molecules involved in neural plasticity such as neurotrophic factors, to immunological and endocrinological processes, to signalling pathways and gene expression, to certain genes or to lipid metabolism.

Markers for Neural Transmission

Neurotransmitter hypotheses are among the earliest and most widely investigated neurobiological theories of neuropsychiatric disorders including schizophrenia [8]. While mechanistic conjectures about the role of single neurotransmitters in the pathophysiology of schizophrenia are now largely recognized as being too simplistic, it remains intriguing to consider these processes as at least one part of the complex aetiopathogenetic cascades ultimately leading to the clinical picture of this disorder. Accordingly, the expression pattern of dopaminergic receptors by T cells analysed via flow cytometry has been proposed as a possible peripheral biomarker [9]: while CD4 and CD8, subtypes of T cells, significantly differed between schizophrenic patients and controls regarding their expression of D_4 receptors, the expression pattern of serotonergic receptors in the periphery did not. Interestingly, there was also a positive relation between these biological phenomena and standard clinical assessment tools for psychiatric symptoms (e.g. the Brief Psychiatric Rating Scale and the Positive and Negative Syndrome Scale). In a quantitative RT-PCR-based study focusing on the mRNA level of D_3 receptors expressed in peripheral blood cells, a role for this receptor subtype could not be established; however, the authors were able to report an increased expression of mitochondrial complex I in schizophrenic patients [10]. An altered peripheral mRNA expression of the 75-kDa subunit of mitochondrial complex I might also be a potential biomarker for early-onset schizophrenia [11], a finding supported by research efforts of others [12].

Using a similar approach together with microarray analysis, both D_2 receptor and $K_{ir}2.3$ (inwardly rectifying potassium channel) were reported to be overexpressed in drug-naïve patients suffering from schizophrenia [13]. Apart from their potential diagnostic use, such neurotransmitter markers are also interesting candidates with regard to predicting treatment responses to various antipsychotic drugs, given characteristic affinity profiles for specific receptor systems.

Neurotrophic Factors

The neurotrophin hypothesis of schizophrenia has gained considerable momentum during the last 15 years [14–20]. Among other things, this is probably due to the fact that schizophrenia has been accepted as a so-called neurodevelopmental disorder, and given the crucial role of neurotrophic factors in the maintenance of neural plasticity, they represent ideal candidates for peripheral biomarkers.

In line with these theoretical considerations, a meta-analysis including 16 studies on brain-derived neurotrophic factor (BDNF) levels in the peripheral blood of schizophrenic patients provided evidence for lower concentrations in the patient groups independently of medication effects [21]. However, despite sufficient precision, the individual studies exhibited considerable, yet unexplainable, heterogeneity regarding their results, thus necessitating further research and improvement in study quality.

While most studies suggest a decreased neurotrophin level in schizophrenia, in some studies a higher level of BDNF was described for a subset of patients specifically suffering from paranoid symptoms, and in some studies an association of low BDNF levels with reduced volume of the hippocampus (for a review see Martinotti et al. [22]). Negative symptoms within patient groups seem to be positively correlated with

BDNF in serum, although a major caveat of all studies based on the ELISA technology is their need for differentiating between precursor and mature BDNF, which might not be possible when commercial kits are used with antibodies lacking specificity [19]. Another possible confounding factor is the impact of hypocaloric diets on peripheral BDNF levels [18]. Interestingly, matrix metalloproteinase 9, which converts pro-BDNF into mature BDNF, might also be implicated in the pathophysiology of schizophrenia and therefore represents an additional biomarker candidate deserving further investigation [23]. These findings are supported by a proteome study including 79 analytes which points to the possibility that peripheral profiles of a specific set of molecules including BDNF, epidermal growth factor and several chemokines represent promising candidate biomarker signatures of schizophrenia as well as of other neuropsychiatric disorders [24].

While neurotrophic factors remain exciting peripheral biomarker candidates, it would be desirable if future studies widened the focus from BDNF to other neurotrophins (e.g. NT-3 and NT-4) and molecules involved in neural and synaptic plasticity (e.g. synaptic vesicle proteins and cell adhesion proteins) [25, 26].

Markers for Oxidative Stress

Oxidative stress has been implicated in the pathophysiology of neurodegenerative as well as neurodevelopmental disorders. By some scientists, schizophrenia is also seen as a condition in which 'neurodegeneration-like' processes occur, and interestingly, Kraepelin coined the historic term 'dementia praecox', which was only later renamed 'schizophrenia' by Bleuler. Thus, molecules involved in oxidative stress regulation have been investigated as potential markers for schizophrenia by several groups (for a review see Ciobica et al. [27]).

As thioredoxin (TRX) acts as a redox-regulating molecule, it represents an oxidative stress indicator which has been investigated for its possible use as a biomarker in schizophrenia. In an initial publication, one group reported significantly increased TRX levels in first-episode and acute-stage patients with schizophrenia compared with controls and chronic-stage patients on antipsychotic drugs, respectively [28], but was later unable to confirm generally significant differences in TRX levels between patients and controls while still reporting a differential association between the cognitive performance of patients and TRX, at least suggesting a partially impaired oxidative stress status in patients suffering from schizophrenia [29].

An increase in plasma thiobarbituric acid reactive substance (TBARS) levels, another marker of oxidative stress, was described in schizophrenia patients but not in patients with bipolar disorder, which underscores that this marker might be relatively specific for certain neuropsychiatric conditions, thus rendering it a potential biomarker worth further investigation [12, 30].

An interesting and potentially clinically important aspect was investigated in a study focusing on the interaction between oxidative stress markers and thyroid hormones in schizophrenia. Apart from increased levels of thyroxine and triiodothyronine, increased concentrations of malondialdehyde, a prototypical marker for lipid oxidation, and of total plasma peroxides were found in the peripheral blood of patients [31]. Thus, a combination of endocrine and oxidative-stress-representing molecules can be considered a further possible peripheral biomarker candidate for schizophrenia.

Endocrine Markers

Biochemical parameters involved in the hormone system are also interesting biomarker candidates, specifically because they can easily be analysed in

peripheral blood samples. In line with this, significant differences in serum prolactin concentrations were reported for unmedicated patients with various subtypes of schizophrenia; the lowest levels were found in patients of the paranoid, intermediate and schizoaffective types, whereas so-called 'disorganized' patients exhibited the highest levels [32]. Because the levels were generally close to 'normal' levels, this parameter might rather be a subtype and/or prognostic marker than a diagnostic marker per se, differentiating between individuals with and those without schizophrenia.

There has been at least one report which, by using a steroid metabolome approach, found that a sensitivity of 95–100% can be achieved for identifying schizophrenia solely by laboratory analysis; however, no specificity was given by the authors [33], and low specificity levels strongly relativize the usefulness of such biomarker tests for daily clinical practice. According to other work published by the same group, male patients with schizophrenia exhibit significantly reduced levels of dehydroepiandrosterone sulphate and of 7α-hydroxy-dehydroepiandrosterone, while female patients exhibit higher cortisol levels in their peripheral blood, both findings being independent of the patients' medication status [34]. Given that 7α-hydroxy-dehydroepiandrosterone possesses neuroprotective and immunomodulatory properties in the CNS, these findings also link this potential neuroendocrine marker to neurodegenerative and inflammatory hypotheses of psychotic disorders.

Immunological Parameters as Possible Markers

Following the hypothesis that changes to neural and synaptic plasticity in schizophrenia might have a downstream impact on immunological and inflammatory processes, cytokines – specifically interleukins (IL) as important mediators of immune response – have been proposed as possible peripheral markers. Indeed, significantly increased IL-6 levels were described in schizophrenic patients compared with controls, as well as decreased IL-10 levels in late-stage patients [30]. Similarly, receptors for tumour necrosis factor (TNF) – i.e. soluble TNF receptor (sTNFR)1 and sTNFR2, circulating cytokines mainly expressed by macrophages and crucially involved in immune response – have recently been found to be elevated in schizophrenic patients compared with controls without neuropsychiatric disorders, and to exhibit a negative correlation with global functioning; furthermore, sTNFR1 seemed to be elevated in treatment-resistant patients when compared with responders and controls, leading to the conclusion that inflammatory biomarkers might be indicatory of the severity of the clinical course and/or treatment resistance [35].

However, no correlation of inflammatory markers with symptom severity was found by another group, which nevertheless also reported increased sTNFR1 and sTNFR2 levels in patients suffering from schizophrenia when compared with these levels in controls; at the same time, TNF-α levels did not differ between the two groups [36]. Furthermore, the results of a twin-based study suggest that antimicrobial peptides (α-defensins) might be early markers for the risk of developing schizophrenic symptoms later in life [37]. Another study found that the acute-phase proteins C3 and C4 as well as ceruloplasmin, which interestingly also possess antimicrobial properties, correlate with negative symptoms in acute paranoid schizophrenia [38]. Consequently, the role of bacterial translocation markers and C-reactive protein in schizophrenia was examined in a recent study pointing to complex alterations to the immune state in patients suffering from this condition, which could help to reveal further biomarkers [39].

Signalling Pathways, Gene Expression Processes and Epigenetic Markers

Beyond neurotransmitter-receptor interaction, intracellular signalling cascades leading to changes in gene expression are crucial processes of signal transduction. Accordingly, in recent years, the focus has shifted from mechanisms exclusively located in the synaptic cleft to transsynaptic phenomena occurring in the postsynaptic neuron. In order to identify possible peripheral markers, in this context, the phosphoinositide signalling system in platelets has been investigated, with results suggesting a possible alteration in schizophrenic patients without being influenced by treatment [40]. Studies focusing on gene expression changes subsequent to the activation of signalling systems have so far generated data pointing to several possible peripheral markers, including pathways of immune response and regulation, but they did not lead to the identification of distinct biomarkers that could be used in daily clinical routine (for a review see Mamdani et al. [41]).

Whereas signalling cascades such as the phosphoinositide pathway involving second and third messengers regulate short-term gene expression, long-term gene activation is controlled by epigenetic processes. Epigenetic phenomena have also been proposed in order to explain non-Mendelian characteristics of schizophrenic heritability and the intricate interaction between genes and the environment [42]. Interestingly, recent studies have suggested a dysregulated epigenome in schizophrenia, a notion which might lead to the discovery of biomarker candidates as DNA methylation signatures and modifications to histone variants are detectable in peripheral blood and peripheral blood cell preparation, respectively [43–45]. Furthermore, antipsychotic drugs might influence epigenetic processes; hence, markers for DNA methylation might also be useful as predictors of therapy response [46].

Lipidomics

Cholesterol and associated proteins such as apolipoproteins might play an important role in the pathophysiology of schizophrenia, since they are – among others – crucially involved in brain development and maintenance of neural functioning. Proteomic methods for biomarker discovery can be successfully used in order to detect potential schizophrenia-specific signatures of the lipidome as part of the metabolome (for a review see Woods et al. [47]). In fact, a recent global metabolic profiling study resulted in the identification of ketone bodies and multiple fatty acids as possible schizophrenia markers detectable in the serum of patients [48].

Lipidomics research has also shown that phospholipid n–3 fatty acid deficits might represent markers for early, perhaps even preclinical, stages of schizophrenia, indicating a 'metabolic vulnerability' that could at least partly be 'corrected' by treatment with antipsychotic medication resulting in a 'normalization' of the respective peripheral lipidome markers [49].

Discussion

Recent research has generated a plethora of data suggesting a large number of possible biomarker candidates which can be identified in the peripheral blood (table 1). However, so far these research efforts have not led to the development of any markers that could be applied in daily clinical practice. Due to specific problems inherent in the concept of 'schizophrenia', such as the fact that its diagnosis is solely based on clinical phenomenology (i.e. patients' report of symptoms and observable behavioural alterations) and that these phenomena may have very heterogeneous neurobiological bases, it is unlikely that a simple blood test will ever be developed in the future. However, by focusing on specific schizophrenic subtypes and endophenotypes and by combining several bio-

Table 1. Overview of biomarker candidates in schizophrenia

Biomarker	Study group	Methods	Reference
Neurotransmitter markers			
Increased expression of D_4 receptors on CD4 and CD8 T lymphocytes	40 male patients with chronic schizophrenia	Flow cytometry	[9]
Increased mRNA expression of the mitochondrial complex I 75-kDa subunit in whole blood cells	10 neuroleptic-naïve patients with acute first episode of early-onset schizophrenia	Quantitative real-time PCR	[10, 11]
Overexpression of D_2 receptor and $K_{if}2.3$ genes in peripheral blood lymphocytes	13 drug-naïve patients	Microarray analysis	[13]
Decrease in mitochondrial complex I activity in PBMC	18 chronically medicated patients in stable period of schizophrenia	Spectrophotometry	[12]
Neurotrophic factors			
Increased plasma level of MMP-9	22 patients with treatment-resistant schizophrenia treated with clozapine	ELISA	[23]
Increased levels of BDNF and EGF in plasma	229 patients with schizophrenia	Quantitative multiplexed immunoassay	[24]
Alternative splicing of Nrx3 at SS4	Knock-in mice with alternatively SS4 of Nrx3	Quantitative RT-PCR assay	[25]
Markers for oxidative stress			
Increased levels of thioredoxin	60 first-episode and acute-stage patients	ELISA	[28]
Increased levels of TBARS	61 patients with chronic schizophrenia (22 at early stage, 39 at late stage)	TBARS assay	[30]
	18 chronically medicated patients in stable period of schizophrenia	TBARS assay	[12]
Increased concentrations of MDA and TPP	30 first-episode patients with acute schizophrenia	Spectrophotometry	[31]
Endocrine markers			
Serum prolactin levels; lowest levels: paranoid subtype; intermediate levels: schizoaffective subtypes; highest levels: disorganized patients	48 first-episode and 38 recurrent unmedicated schizophrenia patients	Microparticle enzyme immunoassay	[32]
Increased levels of T_4 and T_3	30 first-episode patients with acute schizophrenia	ELISA	[31]
Decreased levels of dehydroepiandrosterone sulphate and 7α-hydroxy-dehydroepiandrosterone	13 male patients with schizophrenia	Radioimmunoassay	[34]
Increased cortisol levels in peripheral blood	9 female patients with schizophrenia		
Immunological markers			
Increased IL-6 levels; decreased IL-10 levels in late-stage patients	61 patients with chronic schizophrenia (22 at early stage, 39 at late stage)	ELISA	[30]
Increased sTNFR1 and sTNFR2 levels	40 male patients with schizophrenia; 54 chronically medicated patients with schizophrenia	ELISA	[36] [35]

Table 1. Continued

Biomarker	Study group	Methods	Reference
Increased α-defensin expression in T cells	6 minimally medicated patients with schizophrenia	ELISA	[37]
Levels of ceruloplasmin, C3 and C4 in blood correlated with negative symptoms in acute paranoid schizophrenia	15 patients with acute paranoid schizophrenia	Nephelometry	[38]
Increased sCD14 and LBP levels in plasma	2 cohorts: (1) 141 medicated schizophrenia patients; (2) 78 antipsychotic-naïve and 38 medicated first-episode schizophrenia patients	ELISA	[39]
Signalling pathways, gene expression processes and epigenetic markers			
Increased level of IP_3 in platelets	10 drug-free and 26 neuroleptic-treated schizophrenic patients	Receptor binding assay	[40]
Aberrant DNA damage response signalling in dividing lymphoblasts	28 schizophrenia patients	Flow cytometry	[43]
Hypermethylated region of S-COMT	177 patients with schizophrenia	Luminometric methylation assay	[45]
Altered DNA-methylation patterns of C-phosphate-G dinucleotides	98 patients with schizophrenia	Luminometric methylation assay	[44]
Chronic olanzapine treatment alters methylation in genes encoding for dopamine receptors, transporter, synthesis and metabolism	2 olanzapine-treated rats	Rat-specific methylation arrays	[46]
Lipidomic markers			
Increased levels of multiple fatty acids and ketone bodies in serum and urine	112 schizophrenic patients	GC-TOF mass spectrometry, ^{1}H-NMR	[48]
Decreased level of n–3 class PUFA in drug-naïve patients with a first episode of schizophrenia	20 drug-naïve patients with a first episode of schizophrenia; 20 patients with chronic schizophrenia non-adherent to prescribed medications	Chromatography	[49]
Higher levels of pregnenolone sulphate, sulphated 5α- and 5β-saturated metabolites of C21 steroids; lowered levels of 5β-reduced metabolites of C19 steroids	22 drug-naïve patients with a first episode of schizophrenia	GC-mass spectrometry	[33]

PBMC = Peripheral blood mononuclear cell(s); TBARS = thiobarbituric acid reactive substance(s); MMP-9 = matrix metalloproteinase 9; EGF = epidermal growth factor; Nrx3 = neurexin-3; SS4 = spliced sequence 4; MDA = malondialdehyde; TPP = total plasma peroxides; T_4 = thyroxine; T_3 = triiodothyronine; sCD14 = soluble CD14; LBP = lipopolysaccharide-binding protein; IP_3 = inositol 1,4,5-trisphosphate; S-COMT = soluble catechol-O-methyltransferase; GC = gas chromatography; TOF = time-of-flight; NMR = nuclear magnetic resonance; PUFA = polyunsaturated fatty acids.

markers, the development of assays with sufficient sensitivity and specificity for clinical use is conceivable. Although, for the patients concerned, individual psychiatric-clinical care will remain the basis of the management of schizophrenia, these 'marker assays' might, in the near future, prove to be of considerable help with diagnostic and prognostic processes, not only from a research perspective but also from a clinical point of view. While not replacing clinical judgement in diagnosing patients, they might indicate individuals at increased risk, thus contributing to prevention and early intervention, help to identify subgroups of patients which might respond to a specific treatment scheme and possibly allow a better prognosis in individual cases. While the history of schizophrenia research is full of disappointed hopes, it remains important to try to apply ever more sophisticated methods to this relatively frequent and very grave psychiatric condition that is still not fully understood and still cannot be satisfactorily cured in many cases.

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Schizophrenia: Blood-Serum-Plasma Metabolomics

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Abstract

Schizophrenia is a severe mental disorder characterized by heterogeneous presentation. Owing to its intricate spectrum of symptoms, the current diagnosis of schizophrenia remains subjective, thus representing a serious burden to the healthcare system. Despite recent progress in understanding the pathophysiology of schizophrenia and the mechanisms of antipsychotic drug action, the development of biomarkers for diagnosis and therapeutic monitoring of schizophrenia is still challenging. In this connection, global profiling approaches such as unbiased metabolomics-based analysis of body fluids are expected to be able to translate discriminating molecules into clinical biomarkers. Metabolomics shows the potential to scrutinize candidate markers that will improve the diagnosis of schizophrenia and, therefore, facilitate the development of novel therapeutic strategies. The aim of the present review is to depict the most significant studies that have defined signatures of metabolic markers for the diagnosis of schizophrenia. These analyses were performed on various categories of samples including cerebrospinal fluid, blood (i.e. plasma/serum), erythrocytes, urine and postmortem brain tissue. Advances in innovative methods based on nuclear magnetic resonance spectroscopy and mass spectrometry, all capable of accurately assessing hundreds/thousands of small molecules in biological samples, will enable the discovery of biomarkers and allow elucidation of metabolic disruptions linking schizophrenia, biochemical pathways and treatment effect-response in order to provide new insights into disease pathophysiology and novel therapeutic strategies.

Introduction

Schizophrenia, a severe brain disorder with symptomatic onset in early adulthood persisting during the entire course of life [1], affects approximately 0.5–1% of the world's population [2] with high heritability [3]. Owing to its early onset,

schizophrenia leads to long-term disability, and therefore is associated with high costs of caretaking [4]. Genetic factors and gene-environment interactions account for the development of the disease [5]. Indeed, some genome-wide association studies [6–14] have demonstrated that the disease risk is amplified by the presence of common variations [6, 7, 10, 11] and individually rare structural variants [12–14]. Moreover, environmental factors including childhood trauma, living environment and drug abuse [15] play a role in schizophrenia pathogenesis.

The presentation of the disease is heterogeneous, with clinical signs at different levels of importance across patients and time [16]. Because of a lack of consistent analytical methods, diagnosis of schizophrenia currently merely relies on subjective analysis of symptoms; furthermore, the molecular mechanisms of the disease have yet to be explicated. The absence of disease biomarkers to support unbiased laboratory tests still constitutes a problem for the clinical assessment of schizophrenia. A high prevalence of comorbid medical disorders is associated with schizophrenia. For instance, diabetes is 2–4 times more widespread in schizophrenic patients than in the general population [17, 18]. Various disorders such as the metabolic syndrome, obesity, diabetes and cardiovascular disease display alterations in lipid metabolism. It is not clear whether patients with schizophrenia are inclined to have metabolic disorders or whether such disorders are due to a treatment effect. In light of this, numerous investigations have focused on patients with first episodes of schizophrenia to examine whether patients with no drug exposure were more likely to have developed a metabolic disorder than healthy individuals. However, those studies produced inconsistent data [19, 20].

It has been suggested that early intervention slows progression of the pathology and improves therapeutic outcomes [21, 22]. The discovery of biomarkers will support early disease prevention and facilitate prognosis. Global profiling strategies such as unbiased proteomics or metabolomics of body fluids are believed to have a major potential for translating discriminating molecules into clinical biomarkers [23, 24].

Overview of Metabolomics and Metabolome

Major advances in analytical chemistry have led to the novel area of metabolomics. Metabolomics, the most recent of the '-omics' disciplines, offers potent tools for outlining perturbations in metabolic pathways/networks characterizing human diseases [25–27]. In contrast to classical biochemical methodologies strictly focusing on single metabolites, distinct metabolic reactions and definite sets of linked reactions and cycles, metabolomics accumulates quantitative data on a larger series of metabolites in an attempt to paint a complete picture of the metabolism and/or metabolic fluctuations linked to the relevant pathological condition [26].

Presently, metabolomics provides analytical tools that can simultaneously measure thousands of elements contained in a biological sample. This analytical feature must then be combined with mathematical tools that can recognize a molecular signal of a disease among millions of data [28]. Ideally, metabolomics in the end will produce a comprehensive map of the modulation of metabolic pathways, and therefore of the interaction of proteins with environmental factors, including drug exposure.

In light of this, the metabolome indicates a 'state' function for an individual at a particular point in time or after exposure to a definite environmental stimulus (such as a specific drug or a mood state). The metabolome represents a metabolic condition as modulated by net interactions between genetic and environmental influences and helps bridge the gap between genotype and phenotype [24]. Metabolites are small molecules that are chemically altered during metabolism; as a result they offer a functional pattern of the cel-

lular state. In contrast to genes and proteins – the activities of which are affected by epigenetic modulation and posttranslational modifications, respectively – metabolites act as direct signatures of biochemical activity and are easier to correlate with a phenotype. In this context, metabolite profiling – i.e. metabolomics – has become an influential strategy that has been accepted for clinical diagnostics [29].

Presently, one of the most popular applications of metabolomics has been achieved in functional genomics, involving the understanding of gene functions in different organisms such as yeast [30], plants [31] and mice [32], where phenotypes associated with given mutants have been distinguished. Remarkably, one has to emphasize that minor changes in the concentration of enzymes have only minor effects on metabolic fluxes but can promote significant modifications in the amount of metabolic intermediates. Because the metabolome is a 'downstream' of both the transcriptome and the proteome, it has been reported to be amplified, theoretically and experimentally [33]. Thus, it represents a more subtle level of organization than the transcriptome and the proteome to elucidate a complex biological outcome such as the one represented by neurodegenerative diseases [34].

Metabolomics in Clinical Disciplines

The central application of metabolomics in clinical and pharmacological areas is represented by biomarker discovery. In this scenario, biomarkers refer to changes in endogenous metabolites in the form of a variation in concentration (or biosynthetic flux) that is associated with a specific phenotype when compared with a control phenotype. These biomarkers might be employed for the diagnosis, selection of therapy, effect assessment of a given treatment, and monitoring of the evolution of the disease. In drug development practice, biomarkers can lead to a point in a pathway which might be the cause and/or effect of a specific pathology, and thus find new targets for drug therapy. Metabolomics can be employed in preclinical trials to detect biomarkers of toxicity and efficacy, which will then be scrutinized in clinical trials [35].

With reference to cost assessment, metabolomic approaches are reasonably cheap on a per-sample basis, and consequently, they are extensively exploited in toxicology screening [36]. Although the number of reports focusing on metabolomics as a diagnostic tool both in biological fluids [37] and human beings in vivo [38, 39] is growing, this still is an area requiring to be further developed. Notably, given that the metabolism is conserved during evolution (e.g. metabolic networks are basically comparable between rodents and humans), an additional benefit of metabolomics over other '-omics' is that it is transferable from one species to another. As a result, metabolomics is expected to become a perfect tool for translational research. Metabolic patterns related to pathology or therapeutic responses in animal models are supposed to be directly transferrable to the clinical setting [34].

Potential Role in Diagnosis

Metabolic markers in diagnosis are thought to be one of the most fascinating categories of biomarkers. Since a biomarker should be detected and measured in a sample obtained using noninvasive procedures, body fluids including plasma/serum, urine, saliva and, in some measure, cerebrospinal fluid (CSF) are thought to be ideal sources for biomarker monitoring [35].

Nevertheless, some possible drawbacks should be considered. First of all, age, sex, nutritional status and time of sampling might affect the metabolite composition of the selected fluid. Fluctuations in these physiologically normal metabolic patterns could theoretically mask molecular alterations due to pathology [40, 41]. In addition, potential markers could be diluted in the moderately large volumes of body fluids. Finally, the heterogeneity of several diseases may generate some problems. For example, the metabolic pro-

files of CSF from multiple sclerosis patients have disclosed some divergence among different studies [42]; this is most likely due to variations between patient cohorts.

In summary, aspects such as nutritional/physiological status, the nature of the sample, cohort size and heterogeneity as well as analytical sensitivity should be prudently evaluated before starting a metabolomics study intended to investigate clinically relevant biomarkers [35].

Metabolic Signatures

Several pathologies have been shown to result in disruption in metabolic pathways. Therefore, they can produce long-term metabolic alterations that can be observed in terms of metabolic signatures. Metabolomic profiling can be quite easily performed on peripheral tissues as well as biofluids including CSF or plasma/serum, thus making this strategy important for clinical application. Initial metabolomic signatures have already been described for many disease states such as schizophrenia [43–46], depression [47], motor neuron disease [48], Alzheimer's disease [49] and Huntington's disease [50]. These signatures consist of tens of metabolites that are dysregulated, with altered levels in the state of disease or following drug exposure. Thus, examination of these signatures and their constituents may hypothetically offer valuable information concerning disease pathophysiology. Metabolic signatures of central nervous system disorders could lead to the identification of biomarkers for a disease, as well as its progression and response to therapy [24].

Selection of Metabolomic Platforms

Metabolomics implicates the examination of the repertoire of small molecules – i.e. metabolites – detected in cells, tissues, organs or biological fluids. In this scenario, small molecules encompass: (1) endogenous compounds – including glucose, biogenic amine neurotransmitters, cholesterol and signaling lipids – that originate from or participate in primary and intermediary metabolism, and (2) exogenous compounds including drugs. An assortment of methods is available for separating and quantitating the constituents of a metabolome. By selecting the proper platforms, these molecules can be investigated in relation to their individual properties [25]. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the technologies utilized by most studies. At present, no single analytical methodology is able to capture all metabolomic information in a sample. It should be taken into account that each platform has benefits and limitations, since the classes of metabolites that are identified by every approach are different. As a result, a combination of these systems is required, especially in the area of biomarker discovery [51].

Notably, owing to progress in instrumentation, it has become realistic to simultaneously measure thousands of metabolites from only very low volumes of sample [52]. Interestingly, recent advances in technology, bioinformatic tools and software have made possible the complete exploration of cellular metabolites without bias. The application of these analytical methods has unveiled system-wide changes in unpredicted metabolic pathways associated with phenotypic perturbations. Moreover, most of the molecules discovered are presently not incorporated in databases and metabolite repositories, therefore indicating that the depiction of cellular metabolism is still incomplete [53, 54].

MS-Based Methods for Metabolomics

MS involves the initial separation of metabolites, usually by gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE). This step is followed by ionization of me-

tabolites and resolution based on the mass-to-charge ratio [55].

GC separates molecules by exploiting the molecular interactions between the carrier gas and the column. Components need to be volatile and able to tolerate high temperatures. Hence, large (500- to 1,000-Da) thermolabile metabolites, as intact molecules, are not identified [35]. GC in conjunction with MS (GC-MS) is a highly attractive analytical platform, owing to its high separation efficiency, reproducible retention time and available compound libraries, to detect metabolites according to both retention time in the column and the fragmentation pattern generated by the mass spectrometer [56]. The use of GC-MS allows the monitoring of 200–400 metabolites [35]. GC-MS is frequently employed in the examination of lipid subsets [45, 57, 58].

LC separates molecules by exploiting the interactions between the carrier liquid and the column. Thus, LC seems to be appropriate for studying labile macromolecules and nonvolatile polar and nonpolar metabolites in their native form. LC embraces a variety of technologies such as high-performance LC (HPLC) and ultrahigh-performance LC (UPLC). HPLC coupled with MS (HPLC-MS) has gained growing approval as a system for metabolomic analyses, due to its high throughput and good coverage of metabolites as well as its sensitivity and quantitative reproducibility [59]. Similarly, UPLC permits better separation and resolution of molecules by reducing the column size and using higher chromatography pressures [60]. In particular, UPLC employing sub-2-μm particles has been demonstrated to be helpful in terms of detecting more metabolites, thanks to the better resolution, which results in higher peak numbers [61–63]. It was shown, for instance, that UPLC has been able to detect about 20% more metabolite features than HPLC in the same sample, using an equivalent run time [62]. LC followed by coulometric array detection has been applied to disclose signatures of amyotrophic lateral sclerosis [48] and, more recently, of Parkinson's disease [64]. It is excellent for charting neurotransmitter pathways [65] and those implicated in oxidative stress.

The use of CE is thought to be especially suitable to separate polar and charged substances, given that molecules are separated on the basis of their charge-to-mass ratio. Separations based on CE can be efficiently accomplished without prolonged sample pretreatment. The benefits of CE also encompass the small amount of other reagents needed, and the use of simple fused silica capillaries rather than expensive LC columns. A limitation is represented by its poor concentration sensitivity, which is attributable to the restricted sample volume (nanoliters) that can be introduced into a capillary. However, its sensitivity can be increased by combining CE with MS. In this regard, since MS can provide selective recognition of and structural information about unknown metabolites, CE-MS has been acknowledged as a powerful metabolomic platform. It provides data on the metabolomic composition of biological specimens complementarily to LC-MS. Therefore, the use of both methods would result in wide coverage of the metabolome [66].

Many studies emphasize the need for MS-based metabolomics to utilize multiple ionization/analysis systems in such a way that the amount of metabolites experimentally scrutinized is increased [67, 68]. In this connection, it has been reported that by examining the same human plasma sample in both positive and negative ion mode using the electrospray ionization modality, the number of unique metabolite features was doubled compared with using only the positive or the negative electrospray ionization ion mode [67]. High-resolution mass spectrometers including Orbitrap™ LC/MS technology (Thermo Fisher Scientific Inc., Waltham, Mass., USA) and Fourier transform-ion cyclotron resonance-MS are very captivating for metabolomics, since their high resolution provides the

chance to determine more masses because of diminished mass overlap, and also offers improved tools for isolating unknown compounds with their high mass accuracy for elemental composition determination. In this regard, Han et al. [69] documented the efficiency of direct-infusion Fourier transform-ion cyclotron resonance-MS for high-throughput metabolomics when they found the elemental composition for 250 out of 570 metabolite features and positively detected 100 metabolites quantified in a 3-min infusion analysis.

NMR Spectroscopy-Based Metabolomics

In contrast to the structural information provided by MRI, NMR spectroscopy allows metabolites to be measured within the brain. NMR is conceptually dependent on the phenomenon of chemical shift in order to differentiate among several cerebral metabolites, whereby the ^{1}H signals from the metabolites display slightly different resonant frequencies based on their specific chemical environment. NMR is sensitive to within-individual alterations in the concentration of metabolites over time on the order of 1 mmol/l, enabling a volume of interest between 1 and 8 cm^3 [70].

NMR provides spectra in which each peak indicates a metabolite (or a group of metabolites), its position being distinctive for the resonance frequency of its constituent nuclei, and is expressed in parts per million of the resonance frequency of a reference metabolite. The signal intensity, represented by the peak's surface, is proportional to the metabolite concentration. Molecular groups produce definite resonance patterns on the spectrum, either as single peaks, doublets or more complex spectra. As the magnetic field strength is amplified, separation of the peaks is enhanced. Careful selection of pulse sequence and echo time is needed as each can influence the variety in biochemical substances measured in the NMR scan. Long echo times (120–300 ms) lead to plots with a flat baseline, on which only creatine (Cr), choline (Cho), N-acetylaspartate (NAA), lactate and lipids are discernible. Short echo times (20–40 ms) lead to spectra showing contributions from a large number of metabolites, whose separation might be problematic [71]. Given the existence of many confounding measurement factors, consistent absolute quantification is often hard to attain and requires the use of a reference signal. A simple but commonly effective approach is represented by exploration of the metabolite profile in disease and comparing this with a known physiological metabolite profile [72]. Furthermore, in diffuse or general brain pathologies, a ratio of one key metabolite to another is often measured (e.g. NAA/Cr, Cho/Cr or NAA/Cho) and then compared with known values for these ratios for normal brains determined in equal conditions [73].

Physiological Role of Brain Metabolites Measured by NMR Spectroscopy

NAA is an amino acid derivative produced in the mitochondria of neural cells, and its amount is related to oxygen consumption. Since NAA participates in the synthesis of myelin, it is a specific marker for viable neurons, axons and dendrites. The diagnostic value of NAA is linked to its capacity to measure the degree of neuronal injury/depletion on a regional basis; as a result, it is largely employed as a marker of neuronal density and as an in vivo marker of neurometabolic fitness. Decreased amounts have been observed for a number of disorders involving cognitive decay and may reflect a combination of depletion of neurons, axonal loss, reduced neural metabolism and myelination as well as depletion of dendritic structures [74].

The glutamine-glutamate complex is a mixture of amino acids, amines and derivatives. The glutamate neurotransmitter participates in motor, cognitive and emotional activities. Glutamine is a precursor both for synthesis of glutamate and for the inhibitory neurotransmitter γ-aminobutyric acid. It normally plays a role in

the detoxification and modulation of glutamate within the astrocyte body [75].

Cho is used as a term for several soluble components of brain myelin and fluid-cell membranes. The Cho peak is a measure of the total level of mobile Cho. It is a rate-limiting precursor in the production of acetylcholine, and a precursor of cell membrane phosphatidylcholine (PC). For this reason, it is considered a marker of cellular density and membrane turnover.

myo-Inositol, a sugar alcohol, is employed as a marker of glial cell number. Lipids are characterized by a small peak in the spectrum in healthy brain tissue. The presence of lipids can be of diagnostic significance in the evaluation of brain tumors, where it indicates necrosis. Lactate, a product of anaerobic glycolysis, is evident in brain pathologies in which hypoxia is predominant, including stroke and encephalopathy. It can also act as a nonspecific marker of intracranial masses, for example of cancer and abscesses [76].

Development of Metabolic Biomarkers for Schizophrenia

Over the last half-century, neurochemists have identified numerous molecules in the brain which fulfill essential messenger functions, including dopamine, epinephrine, norepinephrine, serotonin, acetylcholine, γ-aminobutyric acid and many more. These are molecules largely assumed by most as separate molecules. In contrast, metabolomics makes available novel potent strategies for investigating, simultaneously and quantitatively, hundreds of these crucial molecules [26], and allows exploring metabolites within significant pathways in order to clarify their involvement in central nervous system disorders and to more clearly represent mechanisms of action of drugs targeting such pathways. Psychiatric diseases have been discovered to be associated with metabolic pathway disturbances [24, 77] which could well be reproduced in metabolomic profiles. Particularly in schizophrenia, various abnormalities concerning membrane composition, neurotransmitters, signal transduction, the antioxidant system and immune activities have been reported among other alterations [78]. Evidence exists that phospholipids, which contribute to the structure and function of membranes, are compromised in schizophrenia [78–80]. Lipids and their constituent fatty acids which provide scaffolding for several key functional systems – such as neurotransmitter receptor binding, signal transduction, transmembrane ion channels, prostanoid synthesis and mitochondrial electron transport – could induce the pathogenesis of schizophrenia [81].

Presently, several techniques using MS are accessible for qualitative and quantitative examination of the major lipids in complex samples, including tissues and cell extracts, and from many biological species [82]. The first reports of MS-based study of complex lipid mixtures via ionization-based methods date back to the 1990s [83, 84]. MS supported the development of lipidomics, a specific branch of metabolomics where the focus is on detecting and quantifying a wide range of polar and nonpolar lipid metabolites in order to obtain a comprehensive picture of human lipid biochemical pathways [82].

In 2004, the metabolomic signature of postmortem brain tissue by NMR spectroscopy was employed as a complement to genomics and proteomics, supporting the mitochondrial dysfunction hypothesis on schizophrenia [85]. The occurrence of diminished concentrations of various polyunsaturated fatty acids (PUFA) and augmented breakdown of phospholipids in both central and peripheral tissues was constantly documented in schizophrenia patients. Since membrane phospholipids were suggested as the biochemical basis of the neurodevelopmental hypothesis on schizophrenia [79], lipid metabolism became the focus of early metabolomic research.

Furthermore, significant changes in levels of free fatty acids and PC in gray and white matter were observed in schizophrenia by using UPLC-MS. In addition, ceramide levels were found to be considerably augmented in white matter in this neuropsychiatric disorder [86]. Interestingly, a metabolomic study on postmortem tissue from the prefrontal cortex supports the idea that anomalies in glutamatergic neurotransmission and myelin synthesis may be involved in schizophrenia [87].

As brain tissue represents a rare source, CSF – reflecting brain metabolic status – is a valid alternative which can provide samples for the detection of modified compounds directly linked to neuropsychiatric diseases [88]. In this regard, Holmes et al. [43] examined the use of ^{1}H NMR spectroscopy with CSF specimens from first-onset, drug-naïve patients with schizophrenia, and from patients on antipsychotic medication therapy. They disclosed an anomalous biochemical CSF profile in schizophrenia patients as compared with healthy controls (HC), displaying modified levels of glucose, acetate, alanine and glutamine [43]. The therapy with antipsychotic medications led to reversion to normality of the abnormal metabolic signature in half of the patients. Thus, early management of a first psychotic episode seems to contribute to restoring the metabolic state. Impairment of glucose and acetate metabolism indicates a potential disruption to pathways of energy metabolism and lipid biosynthesis in schizophrenia [43]. The same group of researchers also found that these fluctuations in CSF can be traced in the initial prodromal state of psychosis. However, since the biochemical changes detected in the initial prodromal state of psychosis were not significantly associated with the risk of developing schizophrenia, these alterations are not considered satisfactory to predict clinical outcome. In this study, ^{1}H NMR spectroscopy and surface-enhanced laser desorption/ionization-time-of-flight (TOF)-MS allowed the analysis of the metabolic and proteomic CSF profiles, respectively [89].

Given that CSF is only clinically accessible via complex and invasive procedures including lumbar puncture, the use of peripheral blood has soon been integrated in metabolomic analyses. Tsang et al. [90] – after evaluating the ^{1}H NMR spectra of plasma from a cohort of 21 pairs of monozygotic twins discordant for schizophrenia and 8 pairs of age-matched healthy twins – observed variations in lipid signatures of both affected and unaffected twins' populations. However, as the affected twins were under treatment with antipsychotics, the exact role of the drugs in modifying lipid profiles is not clear [90].

In a study by Kaddurah-Daouk et al. [45], a dedicated lipidomics platform (Lipomics Technologies Inc., West Sacramento, Calif., USA [91]) was employed to quantify about 300 polar and nonpolar lipid metabolites, mainly structural and energetic lipids, across 7 lipid sets to assess lipid alterations in schizophrenia before and after therapy with three atypical antipsychotics, i.e. olanzapine, risperidone and aripiprazole. The depiction of lipid signatures was obtained from plasma samples of 50 patients with schizophrenia before and after 2–3 weeks of treatment with the abovementioned antipsychotics [45]. At baseline and before drug administration, the most important alterations were reported for two phospholipid groups: PC and phosphatidylethanolamine (PE). This finding indicates that phospholipids implicated in membrane structure/function appear to be compromised in schizophrenia. In particular, biochemical defects were observed within the ω–3 and ω–6 subgroups in PC and PE. Furthermore, shifts between saturated fatty acids and PUFA were described [45]. The effects of the antipsychotic medicines on lipid pathways were then assessed by comparing metabolic profiles at baseline with those after treatment. Notably, each drug exhibited a distinctive signature. The amount of PE, which was lowered at baseline in

schizophrenia patients, was increased after therapy with all three drugs. However, olanzapine and risperidone had an impact on a broader range of lipids than did aripiprazole, with nearly 50 lipids elevated after treatment with these medicines, but not after aripiprazole administration. The alterations generated in the lipidome due to aripiprazole were negligible; these data are in line with its restricted metabolic side effects. In addition, elevated levels of triacylglycerol and reduced amounts of free fatty acids were revealed following olanzapine and risperidone but not aripiprazole treatment. These data indicate the existence of peripheral effects potentially linked to the metabolic side effects reported for these drugs and emphasize hepatic lipases as probable pharmacological targets. Finally, baseline lipid changes were found to be associated with acute treatment response [45]. Intriguingly, these data raise the possibility that a more complete, randomized long-term study of these medicines might produce biomarkers associated with therapeutic response monitoring. Furthermore, they show the way in which metabolomics might improve the interpretation of drug response phenotypes.

The previously mentioned specialized lipidomics platform [91] was utilized to determine individual lipid classes in 20 medication-naïve patients showing a first episode of schizophrenia, 20 showing chronic schizophrenia and not following the approved treatment (recurrent-episode patients), and 29 race-matched HC. Lipid metabolic signatures were assessed and compared between the study groups and within the groups before and after atypical antipsychotic therapy, i.e. with risperidone and aripiprazole [92]. Compared with the HC, first-episode patients displayed a substantial downregulation of many ω–3 PUFA within the main phospholipid classes PC and PE. However, levels of ω–6 phospholipids were comparable between the first-episode schizophrenia patients and HC. Thus, these results suggest an early occurrence of changes in ω–3 lipid metabolism in the pathology. In contrast, the recurrent-episode patients did not have any major variation in ω–3 or ω–6 phospholipids compared with the HC. This suggests that either disease advancement or previous drug therapy enhanced the early modifications in lipid metabolic pathways connected to schizophrenia [92].

Another significant discovery was that in first-episode patients, antipsychotics caused important alterations (before vs. after treatment) in both ω–3 and ω–6 lipid levels. However, in the recurrent-episode group, drug administration caused marginal fluctuations in phospholipids. As a result, the effects of risperidone and aripiprazole on lipid metabolism were changed, either by disease progression or by previous pharmacological therapy [92].

The results highlighting that the medication-naïve first-episode patients presented deviations in their ω–3 and ω–6 pathways were consistent with prior studies on lipid-metabolic changes linked to schizophrenia [93]. Data according to which variations in lipid metabolism arise early in schizophrenia are in line with the hypothesis by Horrobin and Huang [94], with former analyses of red blood cells [95] and with two meta-analyses [96, 97]. Earlier investigations have revealed modified lipid metabolism in schizophrenia, mainly in the PC and PE included in erythrocytes [98], postmortem brains [99] as well as living brains [100].

Interestingly, ω–3 and ω–6 fatty acids participate in the stability of anti- and proinflammatory mechanisms. In this regard, arachidonic acid (ω–6), accounting for the synthesis of inflammatory cytokines, competes for the same enzymes with eicosapentaenoic acid (ω–3), inhibiting cytokine production. This confirms that schizophrenia is associated with amplified inflammation [101].

The role of metabolomics in finding diagnostic/therapeutic biomarkers by examining global alterations in the subject's metabolic profile in response to pharmacological intervention has been

further investigated. A GC-MS-based metabolomic system was exploited by Xuan et al. [102] to define global changes in metabolic signatures of serum samples from untreated Han Chinese patients with schizophrenia before and after an 8-week risperidone monotherapy. The aim was the identification of possible biomarkers linked to schizophrenia and risperidone therapy. Twenty-two metabolic markers contributing to the differentiation between schizophrenic patients and HC were detected. Citrate, palmitic acid, *myo*-inositol and allantoin showed the highest discriminatory power. Twenty markers accounting for the discrimination between pretreatment and posttreatment patients were found, with *myo*-inositol, uric acid and tryptophan exhibiting the highest discriminatory power. Overall, the detected markers indicated disruption at the level of energy metabolism, antioxidant system, neurotransmitter metabolism, fatty acid biosynthesis and phospholipid metabolism in schizophrenic patients, which could in some measure be regularized after risperidone treatment. Notably, the assessment of metabolic variations in responders and nonresponders led to the observation of a major influence of risperidone therapy on overall metabolism. Thus, metabolites that become significantly changed in responders are probably related to the therapeutic action of risperidone and might be employed as biomarkers for monitoring therapeutic response [102].

A metabolomics-based strategy exhibiting wide analytical coverage was exploited by Orešič et al. [104] to serum specimens from a well-characterized population cohort [103] to define metabolic profiles that correlate with dissimilar psychotic disorders. These were mainly divided into schizophrenia, affective psychoses and other nonaffective psychoses [104]. In particular, two analytical systems were utilized: (1) a global lipidomics platform, UPLC-MS, covering phospholipids, sphingolipids and neutral lipids, and (2) a platform for small polar metabolites based on two-dimensional GC coupled to TOF-MS (GC × GC-TOF-MS), exploring amino acids, free fatty acids, various other organic acids, sterols and sugars. After grouping both the lipidomic and metabolomic data into subsets, the schizophrenia patients were found to have significantly higher metabolite concentrations in 6 lipid clusters – primarily including saturated and longer-chain triglycerides – and in 2 small-molecule clusters, mostly represented by: (1) branched-chain amino acids, phenylalanine and tyrosine, and (2) proline, and glutamic, lactic and pyruvic acids [104].

Lipids detected in lipid clusters are common in liver-produced very-low-density lipoproteins and are linked to insulin resistance [105]. Consistent with these findings, schizophrenia patients were insulin resistant and exhibited increased fasting serum insulin amounts [104]. Therefore, schizophrenia is expected to present insulin resistance, improved hepatic very-low-density lipoprotein biosynthesis [106] and augmented serum levels of specific triglycerides. These results are strengthened by data reporting anomalous insulin release and response [107–109], irregular glucose tolerance and risk of diabetes [110] already in first-episode, drug-naïve patients with schizophrenia. Further data demonstrated high rates of diabetes in unaffected first-degree relatives of people with schizophrenia (19–30 vs. 1.2–6.3% in the general population) [111]. Finally, genes associated with the risk of both schizophrenia and diabetes have been discovered [112].

Regarding molecules included in small-molecule clusters, serum glutamate levels were higher in all psychoses compared with HC [104], leading to speculate that glutamate-related metabolic aberrations are part of a common pathway across psychoses [113]. Serum proline upregulation was considered typical of schizophrenia; in this connection, polymorphisms in the *PRODH* gene, encoding a mitochondrial proline dehydrogenase involved in proline catabolism, correlate with schizophrenia risk [114, 115]. *PRODH* gene

functional variants, responsible for decrease in proline oxidase activity and hyperprolinemia, are related to amplified risk of schizophrenia and alterations in frontostriatal structure/function [115, 116].

In summary, the data found emphasize the presence of two dissimilar specific metabolic abnormalities – associated with glucoregulatory mechanisms and proline metabolism – that are strictly connected with schizophrenia and, therefore, should indicate the existence of two different disease-associated metabolic pathways [104].

Of note, the same authors detected the lipidomic profiles in twin pairs discordant for schizophrenia [117]. By using UPLC-TOF-MS, they discovered higher serum levels of triglycerides and greater insulin resistance in schizophrenic twins than in age- and gender-matched healthy twins. The integration of the lipidomic results in MRI data unveiled a major correlation between reduced gray matter density and augmented triglycerides [117].

In an analysis by Yang et al. [118], spectra of serum metabolites from 112 schizophrenia patients and 110 HC were generated, employing GC-TOF-MS. This technology was also applied together with ^{1}H NMR for the examination of urine samples to isolate additional schizophrenia markers. Among the 22 discovered metabolites responsible for the differentiation of schizophrenia from healthy status, a serum biomarker panel including 5 molecules – glycerate, eicosenoic acid, β-hydroxybutyrate, pyruvate and cystine – was characterized. Such a diagnostic profile was a good classifier for the identification of schizophrenia. Intriguingly, the incorporation of urine β-hydroxybutyrate to the serum signature resulted in a panel characterized by a more satisfactory accuracy [118].

The link between dysregulated pathways associated with energy metabolism and schizophrenia has been demonstrated based on indications from proteins, transcripts and metabolites in the human brain [77]. Increased amounts of pyruvate, a significant intermediate of glucose metabolism connecting glycolysis and the Krebs cycle, suggests a grown energy demand in schizophrenia. An elevated energy demand was due to inefficiency in brain circuitry [119]. In schizophrenia, brain energy supply is limited as a result of mitochondrial dysfunction and, therefore, of inhibited glucose metabolism. For this reason, the brain is supposed to modify its energy demand in some measure from glucose toward ketone bodies as an unconventional energy source. Hence, fatty acids in the liver are mobilized and catabolized to release the required ketone bodies. Thus, multiple fatty acids and ketone bodies were considerably elevated in both patients' serum and urine, highlighting an upregulated fatty acid catabolism [118].

The antioxidant glutathione (GSH) is critical for the cellular detoxification of reactive oxygen species in brain cells [120]. Cystine is the preferred form of cysteine for the synthesis of GSH in cells participating in immune activities. The decrease in serum levels of cystine might suggest a disrupted GSH system in the brain under elevated oxidative stress in neurological disorders [118].

In a recent original study by He et al. [46], a metabolomic platform based on flow injection analysis (FIA)-tandem MS (FIA-MS/MS) was employed to measure 103 metabolites – acylcarnitines, amino acids, glycerophospholipids, sphingolipids and hexose – in plasma samples from 265 schizophrenic patients (categorized into 'neuroleptics taken' and 'neuroleptics-free') and 216 HC and to detect substantial alterations in metabolite concentrations between the groups [46]. Specifically, 5 metabolites – consisting of lower levels of arginine, glutamine, histidine and PC ae C38:6 and higher amounts of ornithine in schizophrenia – differed between the neuroleptics-free cases and HC and were not affected by antipsychotic treatment [46].

Notably, the use of a schizophrenia-specific molecular network revealed associations between

the 5 significantly altered metabolic markers and 13 schizophrenia risk genes. Such a metabolite-gene/protein strategy led to elucidation of the anomalous signaling pathways linked to the altered metabolites. In particular, arginine, ornithine, glutamine, histidine and their related genes/proteins were largely implicated in nitrogen molecule biosynthetic activities as well as arginine/glutamine metabolic pathways. PC ae C38:6, together with its associated genes/proteins, were primarily involved in immune-related mechanisms and neurotrophin-based signaling pathways [46].

There are enough indications to propose a contribution of abnormal neurotransmission in schizophrenia pathogenesis. Defective dopaminergic, serotonergic and glutamatergic systems have been observed [121]. Moreover, participation of histamine and acetylcholine neurotransmitter systems has been suggested [122]. Nevertheless, it is uncertain as to what extent these data reflect primary rather than secondary pathology and compensatory mechanisms. In addition, the interactions of these pathways in the genesis of schizophrenia need to be elucidated.

The purine pathway has been suggested to be a factor in the homeostatic response of mitochondria to oxidant stress and might be critically implicated in inhibiting progressive mitochondrial dysfunction by producing the antioxidant agent uric acid [123]. Uric acid, the assumed final product of purine catabolism in man, contributes to about 60% of the free radical scavenging process in human blood [124].

The use of an electrochemistry-based metabolomic approach, based on HPLC coupled with electrochemical coulometric array detection (LCECA) [65], allowed the quantitative detection of different purine metabolites and their simultaneous comparison in plasma samples from first-episode neuroleptic-naïve patients with schizophrenia (FENNS) and HC. Of note, a shift in favor of xanthosine biosynthesis from xanthine with a subsequent reduction in uric acid formation and in its plasma levels was reported for the purine pathway in FENNS [125]. These results are consistent with former data showing a major decline in plasma uric acid concentrations in FENNS [126] and in patients with chronic schizophrenia [127]. These conclusions support the idea of a dysregulation at the level of the antioxidant defense system in the pathology. In summary, this strategy revealed a significant homeostatic disproportion in purine catabolism early in the course of schizophrenia [125], which is in line with free radical-mediated neurotoxicity and neuropathological alterations observed in schizophrenia [128, 129].

The LCECA platform was also exploited to compare metabolic profiles represented by 13 tryptophan metabolites from the same patient groups. Plasma levels of N-acetylserotonin were higher in the FENNS than in the HC [130]. Thus, the conversion of serotonin to N-acetylserotonin might have been upregulated in the FENNS. Given the role of N-acetylserotonin as a powerful antioxidant, such increases in N-acetylserotonin concentration might represent a compensatory mechanism for the amplified oxidative stress. Furthermore, some metabolite interactions within the plasma tryptophan pathway were thought to be changed and involved in the development of schizophrenia [130].

Conclusions

Progress in the area of biomedical research has emphasized that the understanding of a disease relies on the capability to characterize a pathological condition using phenotypic analysis, molecular diagnostic strategies and decoding of the mechanisms responsible for the aberrant and disrupted molecular processes that cause the disease. In order to accomplish this aim, data originating from gene, protein and metabolite profiling are needed [131, 132].

Metabolomics, the screening of the complete repertoire of small molecules in biological fluids as well as in cells, tissues and organs, is considered an important and rapidly growing element of the 'new biology' [88]. The development of cutting-edge technologies including NMR spectroscopy, GC-MS, HPLC-MS (or UPLC-MS), CE-MS, FIA-MS/MS and LCECA, all capable of precisely measuring hundreds/thousands of small molecules in biological samples, is expected to significantly improve our understanding of disease pathophysiology and to make possible the discovery of biomarkers for various disorders.

Use of all the abovementioned methods enables metabolites to be separated and quantified from different tissues. Studies on neuropsychiatric disorders such as schizophrenia have employed CSF, plasma/serum, urine, erythrocytes or postmortem brain tissues to define panels of metabolic markers discriminating patients from HC. Although postmortem brain and CSF samples are certainly preferred, more accessible tissues such as plasma and serum are typically utilized in clinical practice.

The application of metabolomics-based systems will permit simultaneous measurement of several metabolites in crucial interacting pathways in schizophrenia. As a result, the novel emerging biomarkers might provide relevant clinical data. The understanding of metabolic disruptions linking schizophrenia, biochemical pathways and treatment effect-response should provide new insights into schizophrenia pathophysiology and novel strategies for therapeutic monitoring and outcome [88].

Such an approach is at the basis of systems biology, combining different sources of biological data to attain a deep knowledge of interrelated biological mechanisms [131, 132] for diagnosing, monitoring and treating the pathology [133]. Since a complex disease like schizophrenia is likely to be characterized by disturbances at the system level, the use of '-omics' platforms, namely genomics/epigenomics, proteomics and metabolomics (including lipidomics), might offer a fuller and more appropriate picture for the identification of biomarkers.

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Proteomic and Metabolomic Evidence for Glial Alterations in Schizophrenia

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Abstract

It has become clear that glial cells play a major role in the pathophysiology of schizophrenia. There are three main glial cell populations in the brain, (1) astrocytes, (2) oligodendrocytes and (3) microglia, and all three glial populations have been implicated in this disorder. Abnormalities in glial function may be further elucidated by quantification and characterization of proteins and other molecules expressed by these cell populations. In this review we evaluate evidence for changes in expression of oligodendrocyte-, astrocyte- and microglia-associated proteins and metabolites in cortical grey and white matter in schizophrenia.

Role of Glial Cells in the Pathophysiology of Schizophrenia

While the majority of studies investigating the cellular and molecular basis of schizophrenia have focused on neurons, it has become clear that glial cells also play a major role in the pathophysiology of this disorder [1]. Cytoarchitectural investigations have observed reduced glial density in several regions including prefrontal grey matter [2] and temporal white matter [3]; however, it is not known whether this deficit is restricted to any individual glial population (i.e. oligodendrocytes, astrocytes and microglia). Oligodendrocytes are the major myelin-forming cells of the central nervous system (CNS) [4]. Astrocytes have been implicated in many essential CNS processes, including neurotransmission, glucose metabolism, blood flow regulation, ion homoeostasis, neuroprotection and inflammatory responses [5]. Microglia are essential players in the brain's response to injury and may also be involved in CNS plasticity [6]. Given the divergent roles of these cell types, it is important to parse out which glial populations are affected in schizophrenia, and how their functions are affected by disease.

Oligodendrocyte and myelin dysfunction have been implicated in the pathophysiology of schizophrenia. Reduced oligodendrocyte density has been noted in prefrontal grey matter [7–9]. However, in white matter, where the majority of these glial cells are located, evidence for decreased oli-

godendrocyte density [7, 10] is outweighed by studies finding no change [8, 11–13]. Theories that schizophrenia is associated with progressive brain changes [14] and an exaggerated inflammatory response [15] suggest that astrocytes and microglia also play a role in this disorder. However, cytoarchitectonic studies of astrocyte and microglial density have produced inconsistent results [12, 16–22].

Overall, it is likely that glial pathophysiology in schizophrenia is more complex than simple alterations in cell density and might be further elucidated by quantification of proteins expressed by these cell populations. Furthermore, non-hypothesis-driven approaches may identify novel glial anomalies and point to new avenues for research. In this review we will discuss proteomic and metabolomic evidence for glial alterations in schizophrenia. While glial populations express many hundreds of proteins, most of these proteins are present in multiple cell types. In order to focus on the most promising candidates, we compiled a list of the top astrocyte-enriched and oligodendrocyte-enriched genes from transcriptomic studies by Cahoy et al. [23] and Lein et al. [24]. We then compared this with lists of schizophrenia-associated proteins from 14 previous proteomic studies of cortical grey and white matter [25–38]. The intersection of these two datasets is summarized in table 1 and is the main focus of discussion.

Proteomic Evidence for Oligodendrocyte Alterations in Grey Matter in Schizophrenia

Proteomic studies have identified several oligodendrocyte-associated proteins in grey matter in schizophrenia. The findings are consistent, with decreased carbonic anhydrase 2, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP), ermin, gelsolin, hyaluronan and proteoglycan link protein 2 (HAPLN2), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and transferrin reported in prefrontal and temporal brain regions, using several methods (table 1). Reductions in CNP, MBP and transferrin have been noted in more than one study.

While some of these proteins are present in oligodendrocyte cell bodies, others are located in non-compact or compact myelin, suggesting that alterations extend to both oligodendrocytes and the myelin sheath. Carbonic anhydrase 2 is present mainly in oligodendrocyte processes and has been implicated in the regulation of intracellular pH and myelin maintenance [39]. Transferrin is located in oligodendrocyte cell bodies, and is involved in the transport of iron. This protein has also been implicated in oligodendrocyte maturation and myelinogenesis, as overexpression in a mouse model stimulates oligodendrocyte differentiation [40]. CNP, ermin, gelsolin and HAPLN2 are present in non-compact myelin and have been associated with the oligodendrocyte cytoskeleton. The enzyme CNP associates with cytoskeletal elements including microtubules and actin microfilaments, and may thereby play a role in the outgrowth of oligodendrocyte processes and myelin sheath maintenance. CNP has also been implicated in axon-glial cell signalling, oligodendrocyte differentiation and RNA metabolism [4]. The novel actin-binding protein ermin has been linked with the regulation of the oligodendrocyte cytoskeleton [41], while gelsolin is involved in the depolymerisation of actin filaments, and may contribute to oligodendrocyte process outgrowth [42]. While the specific function of HAPLN2 (also known as BRAL1) in oligodendrocytes remains unclear, this protein can form complexes with extracellular matrix proteins at nodes of Ranvier [43]. MAG and MOG are also present in non-compact myelin and have been implicated in axon-glial signalling [4]. MBP plays a major role in the development and maintenance of compact myelin, where it binds the cytosolic surfaces of oligodendrocyte membranes together to restrict protein diffusion [44].

Table 1. List of glial-associated proteins implicated in schizophrenia

Protein	Direction of change	Brain region	Method used	Reference No.
Grey matter				
Oligodendrocyte				
2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP)	decrease	DLPFC	shotgun-MS	25
		DLPFC	2-D DIGE	26
Carbonic anhydrase 2 (CA2)	decrease	anterior temporal	shotgun-MS	27
Ermin (ERMN)	decrease	anterior temporal	shotgun-MS	27
Gelsolin (GSN)	decrease	DLPFC	2-D DIGE	26
Hyaluronan and proteoglycan link protein 2 (HAPLN2)	decrease	anterior temporal	shotgun-MS	27
Myelin-associated glycoprotein (MAG)	decrease	DLPFC	LC-MS	28
Myelin basic protein (MBP)	decrease	anterior temporal	shotgun-MS	27
		DLPFC	2-DE	29
Myelin oligodendrocyte glycoprotein (MOG)	decrease	DLPFC	shotgun-MS	25
		anterior temporal	shotgun-MS	27
Transferrin (TRF)	decrease	DLPFC	2-D DIGE	26
		DLPFC	2-DE	29
		ACC	2-DE	30
		DLPFC	2-DE	31
Astrocyte				
Apolipoprotein E (ApoE)	increase	DLPFC	shotgun-MS	25
Aquaporin 4 (AQP4)	increase	DLPFC	LC-MS	28
Fructose-bisphosphate aldolase C (ALDOC)	increase	DLPFC	2-DE	29
		PFC (BA 10)	2-DE	32
		insula	2-D DIGE	33
	decrease	ACC	2-DE	30
		Wernicke's area	2-DE	34
		DLPFC	SELDI-TOF	35
		DLPFC	2-D DIGE	26
Glial fibrillary acidic protein (GFAP)	increase	DLPFC	2-DE	29
		DLPFC	2-DE	31
		insula	2-D DIGE	33
		Wernicke's area	2-DE	34
	decrease	anterior temporal	shotgun-MS	27
		ACC	2-DE	30
		PFC (BA 10)	2-DE	32
Glutamine synthetase (GLUL)	decrease	DLPFC	2-D DIGE	26
		DLPFC	SELDI-TOF	35
Peroxiredoxin 6 (PRDX6)	increase	DLPFC	shotgun-MS	25
		Wernicke's area	2-DE	34
	decrease	DLPFC	LC-MS	28
Solute carrier family 9, subfamily A, member 3, regulator 1 (SLC9A3R1)	increase	DLPFC	shotgun-MS	25
White matter				
Oligodendrocyte				
CNP	decrease	DLPFC	2-D DIGE	26
GSN	decrease	DLPFC	2-D DIGE	26
Proteolipid protein (PLP)	increase	DLPFC	2-D DIGE	36
Transferrin (TRF)	increase	DLPFC	2-D DIGE	26

Table 1. Continued

Protein	Direction of change	Brain region	Method used	Reference No.
Astrocyte				
ALDOC	increase	DLPFC	2-D DIGE	36
	decrease	DLPFC	2-D DIGE	26
		DLPFC	2-D DIGE	36
		ACC	2-DE	37
GFAP	increase	corpus callosum	2-DE	38
	decrease	corpus callosum	2-DE	38

Overlap between lists of schizophrenia-associated proteins from 14 previous proteomic studies of cortical grey and white matter [25–38] and top astrocyte-enriched and oligodendrocyte-enriched genes identified by transcriptomic studies [23, 24]. 2-DE = Two-dimensional electrophoresis; 2-D DIGE = two-dimensional difference gel electrophoresis; ACC = anterior cingulate cortex; BA = Brodmann area; DLPFC = dorsolateral prefrontal cortex; LC = liquid chromatography; MS = mass spectrometry; PFC = prefrontal cortex; SELDI-TOF = surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry.

Proteomic findings of oligodendrocyte protein abnormalities are consistent with microarray studies, which have detected decreases in CNP [45, 46], gelsolin [46], MAG [45–48], MOG [46, 47] and transferrin [45–47] gene expression, although these findings were not replicated by in situ hybridization or quantitative PCR [49, 50]. Overall, the available data suggest an oligodendrocyte deficit in grey matter in schizophrenia. An alternative explanation is downregulation of specific genes/proteins. The RNA-binding protein quaking regulates expression of several oligodendrocyte-related genes and is decreased in schizophrenia [51]. In addition, polymorphisms in MOG, MAG, proteolipid protein 1 (PLP1), transferrin and CNP have been associated with schizophrenia [50], which could potentially affect gene expression.

Proteomic Evidence for Oligodendrocyte Alterations in White Matter in Schizophrenia

While the majority of oligodendrocytes reside in white matter, proteomic studies have failed to identify consistent changes in oligodendrocyte-associated proteins in white matter in schizophrenia. Decreased CNP and gelsolin, but increased transferrin and PLP, a major constituent of compact myelin [4], have been reported in prefrontal white matter (table 1). Studies of mRNA expression in white matter have not provided clarification, with decreased expression of CNP and transferrin mRNA, but no change in gelsolin noted in cingulate white matter [49], and unaltered CNP mRNA in the prefrontal region [50].

To examine the relationship between protein levels and oligodendrocyte density, we performed Spearman's rank correlation analysis between protein spots identified as significantly altered in prefrontal white matter in schizophrenia in a two-dimensional gel electrophoresis study by English et al. [36] and oligodendrocyte density [unpubl. data] in the same samples. While we found no association between oligodendrocyte density and levels of the myelin-associated protein PLP, we did observe a significant positive correlation between oligodendrocyte density and two cytoskeletal proteins: neurofilament heavy ($r = 0.388$, $p = 0.006$) and stathmin ($r = 0.306$, $p = 0.027$). Neurofilament heavy is an intermediate filament implicated in the regulation of myelin-

ated axonal diameter and axonal transport [52], while stathmin modulates the stability of microtubules by inhibiting tubulin polymerization and is present at high levels in oligodendrocyte precursor cells [53].

Proteomic Evidence for Astrocyte Alterations in Grey Matter in Schizophrenia

While several astrocyte-associated proteins have been identified in grey matter proteomic studies, changes are inconsistent, with some proteins upregulated, others downregulated and some altered in both directions. Increased apolipoprotein E (ApoE), aquaporin 4 (AQP4) and SLC9A3R1 (solute carrier family 9, subfamily A, member 3, regulator 1) and decreased glutamine synthetase (GLUL) have been reported. In addition, multiple studies identified increased and decreased levels of peroxiredoxin 6 (PRDX6), fructose-bisphosphate aldolase C (ALDOC) and glial fibrillary acidic protein (GFAP; table 1).

ApoE is the major apolipoprotein in the CNS, and is produced mainly by astrocytes. While its primary role is in cholesterol transport, ApoE synthesis is upregulated following CNS damage, suggesting that this protein may be involved in neuronal injury response [54]. AQP4, a water channel protein found in astrocytic end-feet, has been implicated in the regulation of extracellular volume, neuroinflammation and reactive astrocyte migration [55]. SLC9A3R1 (also known as NHERF1) is thought to act as a scaffolding protein, interacting with GFAP to anchor proteins such as glutamate transporters to the plasma membrane [56]. GLUL is localized to astrocytic processes, where it is involved in glutamate-glutamine cycling (fig. 1) [57]. The bifunctional enzyme PRDX6 possesses both antioxidant and phospholipase activities and is thought to play an important role in the oxidative stress response [58]. ALDOC is the brain-specific isoform of fructose-bisphosphate aldolase, part of the glycolytic pathway (fig. 1) [59]. The intermediate filament GFAP is a key component of the astrocyte cytoskeleton. While GFAP is commonly used as an astrocyte marker, expression varies between astrocytes and many mature grey matter astrocytes do not express detectable levels of GFAP. GFAP synthesis is upregulated following CNS insult and during ageing [5].

Findings from proteomic studies are mostly consistent with those from targeted protein investigations of astrocytes. Increased ApoE levels have been noted in grey matter in schizophrenia [60, 61] using Western blotting. ApoE levels are associated with APOE genotype [62], and the ε4 allele has been associated with increased schizophrenia risk [63]. Western blotting studies, like proteomic approaches, also found reduced GLUL levels in schizophrenia [64, 65]. However, conversely, a recent targeted study found significantly reduced AQP4 mRNA expression in the anterior cingulate cortex [66], in contrast to the increase in protein shown by Martins-de-Souza et al. [25] in the prefrontal cortex. GFAP, ALDOC and PRDX6 are all present as multiple spots on two-dimensional electrophoresis gels [32, 36, 58]; therefore, discrepancies in the direction of change between and within studies could reflect abnormalities in post-translational modifications [32]. Altered ALDOC and PRDX6 levels in schizophrenia have only been identified by proteomic studies. GFAP, however, has been investigated both as a marker of astrocyte density in immunocytochemical studies and through targeted protein/gene expression studies, with inconsistent findings. While we, and others, have reported increased GFAP protein levels in the dorsolateral prefrontal cortex in schizophrenia [67–69], decreased GFAP protein [65] and unchanged GFAP mRNA expression [66] have been noted in cingulate grey matter.

Proteomic studies have also implicated two additional proteins, not present on our list of astrocyte-enriched proteins but previously associated with astrocyte activation: vimentin and bystin. Vimentin is an intermediate filament expressed in

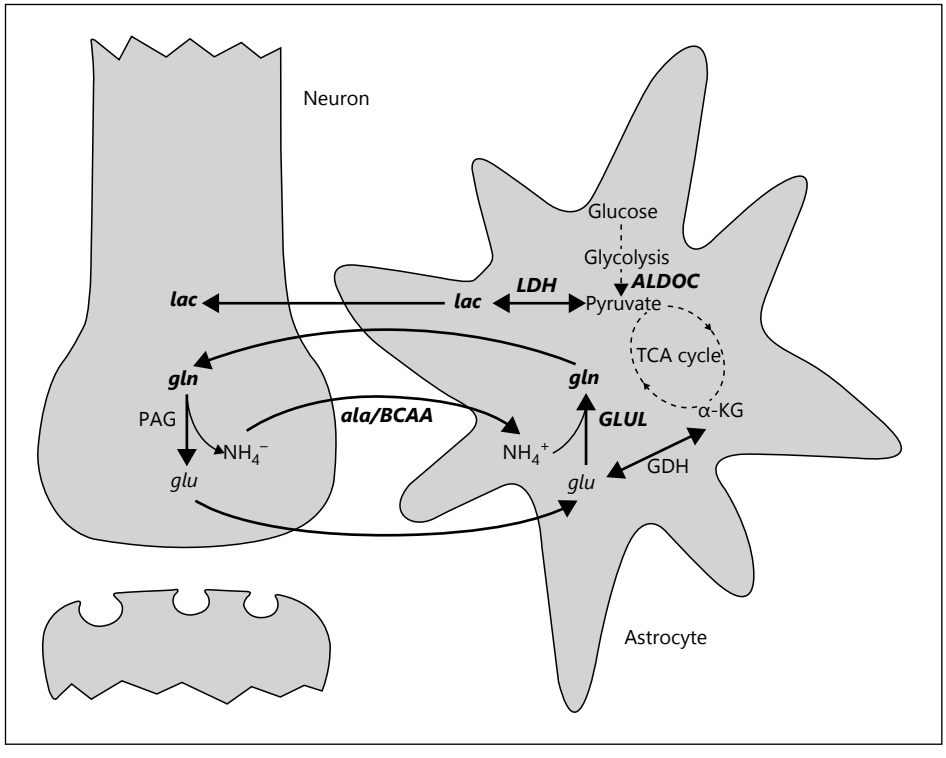

Fig. 1. Simplified schematic showing astrocyte-neuron coupling of energy metabolism and the glutamate-glutamine cycle (for further details see reviews [60, 78]). Metabolites and astrocyte-enriched proteins associated with schizophrenia according to metabolomic or proteomic studies are highlighted. Note that glycolysis and the TCA cycle are not specific to astrocytes. Furthermore, while altered levels of additional enzymes involved in glycolysis, the TCA cycle and the glutamate-glutamine cycle have been reported in proteomic studies in schizophrenia (e.g. GDH), these proteins are not enriched in astrocytes, and as such are not discussed in this review. α-KG = α-Ketoglutarate; ala = alanine (decreased in schizophrenia [28, 76]); ALDOC (increased/decreased in schizophrenia [26, 29, 30, 32–37]); BCAA = branched-chain amino acid (decreased in schizophrenia [28]); GDH = glutamate dehydrogenase; GLUL (decreased in schizophrenia [26, 35]); gln = glutamine (increased in schizophrenia [26, 28]); glu = glutamate; lac = lactate (increased/decreased in schizophrenia [26, 73]); LDH = lactate dehydrogenase (decreased in schizophrenia [37]); PAG = phosphate-activated glutaminase; TCA = tricarboxylic acid.

immature and in some mature astrocytes that, similar to GFAP, is markedly upregulated after CNS insult [70]. Vimentin expression was increased in prefrontal grey matter in schizophrenia [25]. Conversely, expression of bystin was decreased in this region [35]. While the exact function of bystin in the CNS is unknown, this protein is upregulated in astrocytes following CNS injury [71].

Proteomic Evidence for Astrocyte Alterations in White Matter in Schizophrenia

While astrocytes are abundant in white matter, few proteomic studies have found changes in astrocyte-associated proteins in white matter regions in schizophrenia. As in grey matter, both increased and decreased expression of ALDOC

and GFAP have been reported in white matter (table 1). While increased GFAP mRNA expression has previously been observed in the internal capsule in schizophrenia [72], others found no change in protein [73] or mRNA [66].

Proteomic studies have also documented increased vimentin in white matter in schizophrenia [36]. In addition, decreased expression of aldehyde dehydrogenase 1A1, aldehyde dehydrogenase 7A1 and lactate dehydrogenase B, described by Cahoy et al. [23] as astrocyte metabolic pathway enzymes, was reported in white matter in this disorder [26, 37, 38]. Aldehyde dehydrogenases catalyse the oxidation of aldehydes [74], while lactate dehydrogenase interconverts pyruvate, the final product of glycolysis, to lactate (fig. 1) [75]. These data may implicate dysregulation of neuron-glial metabolic interactions in schizophrenia.

Proteomic Evidence for Microglial Alterations in Schizophrenia

Recent genome-wide findings associating schizophrenia with the human major histocompatibility complex locus [76] have reignited interest in the potential role of inflammation, and therefore microglia, in this disorder. Proteomic studies, however, have found no specific microglial proteins – and only a few proteins associated with inflammatory response and immune function – to be altered in this disorder. One example is annexin A1, which was increased in anterior cingulate grey matter in schizophrenia [30]. Annexin A1 is present in microglia, as well as in other cell types, and has been implicated in the regulation of neutrophil migration and phagocytosis [77]. Lack of microglial-associated findings may be consistent with suggestions that inflammation is only present in a subpopulation of patients [22], although difficulty detecting microglial proteins by proteomic methods remains a possibility.

Impact of Clinical and Demographic Factors on Proteomic Data

The neuroproteomics of schizophrenia is complicated by confounding demographic and clinical variables such as age, sex, postmortem interval, brain pH, patient comorbidities and use of medication, alcohol, nicotine and illicit drugs. These factors should be considered when interpreting studies utilizing human postmortem tissue. For example, sex-specific differences in glial proteins have been reported in schizophrenia, with changes in ALDOC found in male patients and changes in GLUL in female patients [78]. Associations between ALDOC and age [33], ALDOC and lifetime antipsychotic exposure [33, 36] and GFAP and brain pH [33] have also been observed in previous proteomic studies.

Technical Limitations

Many of the studies discussed in this review analysed brain tissue using two-dimensional gel electrophoresis (2-DE) or difference gel electrophoresis (DIGE). These techniques are advantageous as they allow for simultaneous visualization of hundreds of protein spots, quantification of their levels, and examination of protein isoforms, degradation products and posttranslational modifications. However, gel-based methods can be biased as they cannot easily visualize low-abundance proteins, membrane-associated proteins, exceptionally small or large proteins (below 10 kDa or above 150 kDa) and proteins that are highly hydrophobic or basic. The last is a particular confound when analysing the myelin proteome, but can be mitigated by optimized experimental protocols [79]. To further overcome some of these limitations, gel-free techniques, referred to as 'shotgun proteomics', have emerged. In these approaches, gel separation of intact proteins is omitted and the sample subjected to proteolytic digestion

followed by chromatography. Shotgun methods have detected alterations in the myelin proteins MOG [25, 27] and MAG [28] in schizophrenia, which had not been found using gel-based techniques.

Metabolomic Evidence for Glial Alterations in Schizophrenia

Metabolomics is the global study of small molecule metabolites within a sample, which can include a range of biochemicals including amino and organic acids, sugars and lipids [80]. Experimental techniques used to study the metabolome include mass spectrometry (MS)-based methods such as gas chromatography-MS and liquid chromatography-MS, as well as nuclear magnetic resonance (NMR) spectroscopy.

To date, metabolomic techniques have mainly been implemented in the study of cerebrospinal fluid and blood samples in schizophrenia. A small number of NMR-spectroscopic and MS-lipidomic investigations, however, have been performed on human postmortem tissue. While changes in lipid composition have been reported in schizophrenia [81], unlike the proteins discussed above, there are no glia-specific lipids, and available lipidomic data provide few clues to glial pathophysiology in this disorder. NMR studies, however, may be more informative. NMR spectroscopy has been performed on cortical grey and white matter samples in schizophrenia [26, 28, 73, 82], with data demonstrating differences in metabolite profiles between disease and non-disease states. Although these metabolites are not unique to glial cells, several have been associated with astrocyte function, and are discussed below.

A number of metabolites found to be affected in schizophrenia, including alanine, acetate, branched-chain amino acids, lactate and glutamine, have been implicated in astrocyte-neuronal coupling of amino acid and energy metabolism, complementing findings from proteomic studies (fig. 1). Astrocytes play a key role in neuroenergetics and glutamate transmission, as discussed in previous reviews [57, 75]. Briefly, pyruvate is produced by astrocytes during glycolysis and either enters the TCA (tricarboxylic acid) cycle or is converted to lactate. Lactate may then be transferred to neurons, where it can be utilized as an alternate fuel source. In addition, α-ketoglutarate, a TCA cycle intermediate, serves as a precursor of glutamate synthesis. Astrocytic glutamate is converted into glutamine and is transported into neurons, where it is converted back to glutamate as part of the glutamate-glutamine cycle. Concurrently, nitrogen is transported from neurons to astrocytes via an amino acid shuttle involving the transfer of alanine or a branched-chain amino acid. Decreased alanine and branched-chain amino acid and increased glutamine concentrations have been reported in grey matter in schizophrenia [28], which may be suggestive of abnormal glutamate cycling. However, two other studies found no significant change in metabolites in grey matter in this disorder [26, 82]. Decreased alanine and increased glutamine levels have also been reported in white matter, along with altered lactate levels [26, 73], suggesting that disruption to astrocyte-neuronal metabolic coupling is not confined to the cortex. Reduced concentrations of acetate, a specific marker of astrocyte metabolism [83], have also been noted in white matter in schizophrenia [26]. Finally, levels of adenosine, recently implicated in the astrocytic control of sleep homoeostasis [84], are also lower in schizophrenia [26].

Conclusions

Proteomic studies have provided good evidence for oligodendrocyte deficits in cortical grey matter in schizophrenia, in keeping with published cytoarchitectural and transcriptomic data. Oli-

godendrocyte-associated changes in white matter are less convincing, but may suggest abnormal axon-oligodendrocyte interactions. Evidence for alterations in astrocyte-associated proteins in schizophrenia is also inconsistent, although overall proteomic and metabolomic data may be indicative of mild activation of astrocytes, or dysregulation of neuron-astrocyte metabolic interactions. Proteomic studies have provided no substantial evidence for microglial involvement in this disorder.

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Insights from Proteomic Studies on Schizophrenia Preclinical Models: What Can We Learn for Drug Discovery?

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Abstract

Proteomics offers the ability to monitor and identify changes in protein expression and/or signalling pathways with high precision, accuracy and reproducibility. Proteomic techniques, including bioinformatics software, are steadily advancing, and technical improvements to the methods currently employed for protein separation and protein identification will dramatically increase the number of proteomics-based schizophrenia research studies. The advent of high-throughput techniques allows scientists to quantify complex biological mixtures, such as postmortem brain, and to identify and validate new biomarkers. Animal models have contributed much to our understanding of disease mechanisms and are often utilised in research because of their abundant supply and ease of manipulation. Different theories regarding the aetiology of schizophrenia make the design of a single schizophrenia animal model impossible. Therefore, there are many preclinical animal models for schizophrenia research, each of which has relevance to different aspects of the neurobiology of the disorder and its treatment. Preclinical animal models in schizophrenia research are vital for biomarker discovery and in the exploration of disease neurobiology. Furthermore they are central for the testing of new drugs. Animal models in this aspect have the advantage that they enable the investigation of the neurobiology of the phenomena of interest using invasive techniques that cannot be used in humans. Proteomics-based research of these preclinical models can greatly increase our understanding of schizophrenia and shape the direction of future research. Current proteomic studies have so far been insightful and point to a role for mitochondrial dysfunction, alteration in cell signalling and cytoskeletal functioning in preclinical models. Proteomic studies of antipsychotics in preclinical models are an indispensable tool for investigating underlying schizophrenia neurobiology. Current investigations have found alterations in core mitochondrial proteins and in protein synthesis and cell signalling pathways in preclinical models of schizophrenia undergoing antipsychotic treatment. Research is encouraging, and advancement with preclinical models of schizophrenia can be made with more reliable quantitative proteomic methods. Proteomic studies on preclinical models of schizophrenia are, to date, somewhat sparse, but will hopefully multiply with the establishment of better quantitative approaches.

Introduction

The definitions and boundaries of schizophrenia have varied over the past century. This changing concept of schizophrenia has been influenced by available diagnostic tools and treatment, by related conditions from which it most needs to be distinguished and by scientific paradigms [1]. Furthermore there is significant heterogeneity in the aetiopathology, symptomatology and course of the disease. Additionally, the severity of positive, negative and cognitive symptoms varies across patients. Although the therapeutic armamentarium for the treatment of schizophrenia has grown, current treatments for schizophrenia are inconsistent, with their success and their benefits varying from patient to patient. Furthermore, certain side effects can still have an impact on patient health and quality of life, giving rise to relapses in medication and worsening of symptoms [2]. The hope for further progress relies upon variation affecting drug and interindividual differences in pharmacoresponse [3]. Current strategies, most often guided by various disease hypotheses, often focus on the identification of novel biomarkers to aid in the development of more effective therapies. The utilisation of preclinical models is pivotal in this endeavour.

Schizophrenia: Epidemiology and Clinical Presentation

Schizophrenia is a debilitating neuropsychiatric disease that disturbs perception, cognition, thinking and social behaviour. It is estimated that the risk of developing the disease over a lifetime ranges from 0.3 to 2% with an average of 0.7% [4]. Incidence studies based on service provision may grossly underestimate incidence rates as some affected individuals may not seek professional treatment. However, numerous meta-analyses have confirmed longstanding suggestions of a strong link between urbanicity, migration and male gender with an increased risk of developing schizophrenia. Several candidates have been proposed to explain what risk factor may be linked to urbanicity, and among these, differences in substance misuse, prenatal health, poverty and social stress have been proposed [5]. It was originally believed that the prevalence and risk of developing schizophrenia was similar among males and females, but more recent studies have revealed that compared with females, males have a 4-fold increased risk of the disease [6, 7]. Having an affected family member greatly increases the risk of developing schizophrenia. This risk increases substantially as the degree of genetic affinity with the affected family member increases, a concept pioneered by Kallman in 1946 with his assessment of schizophrenic twins [8]. The genetic theory of schizophrenia was strengthened by investigating adopted-away twins, where the risk of schizophrenia in the twin was related to the presence of the disease in the biological parent, and not in the adoptive [9, 10]. One of the most replicated findings in schizophrenia research is that offspring born in the winter and spring months have a 10% increased risk of developing schizophrenia compared with offspring born at other times of the year [11–13] and maternal nutrition and mineral deficiency pose significant risks to the developing fetus [14]. It is well established that obstetric complications, those that occur prenatally and perinatally, increase the risk of schizophrenia in the offspring. Among these adversities are included bleeding during pregnancy and emergency caesarean section [15, 16].

Schizophrenia: Clinical Presentation

Schizophrenia is recognised by a diverse set of signs and symptoms which ultimately include distortion of thought and perception, avolition, apathy and cognitive impairments. As a result of these disabilities, patients experience increased feelings of isolation and day-to-day living becomes extremely difficult. These symptoms or abnormali-

ties are classified into positive, negative and cognitive disorganisation [17]. Delusion, hallucinations and several other reality distortions comprise the positive symptoms. The onset of positive symptoms generally occurs in late adolescence or early adulthood, where reality distortion marks the formal onset of the disease [18]. Hyperactivity of the dopaminergic mesolimbic pathway appears to underlie the positive symptoms, and these are the most responsive to antipsychotics [19, 20]. Negative symptoms involve a loss of affective and conative functions. Patients exhibit a loss of motivation, alogia, avolition (loss of initiative) and a general loss of social drive [21, 22]. 'Extrinsic' factors such as neuroleptic treatment and depression are common causes of so-called secondary negative symptoms [23]. The cognitive abnormalities associated with schizophrenia include deficits in episodic memory, sustained attention, working memory, processing speed and reasoning/problem solving [24]. Cognitive deficits persist over the long-term course of schizophrenia and modest improvements are only observed with antipsychotic treatment [25]. The therapeutic armamentarium comprises the earlier first-generation, so-called 'typical', antipsychotics such as chlorpromazine and haloperidol, and the second-generation, so-called 'atypical', antipsychotics such as olanzapine, quetiapine and risperidone [26]. Although typical antipsychotics are successful in tackling the positive symptoms of the disease, they are minimally effective against the negative and cognitive symptoms, which are responsible for much of the disease-related disability [1]. Although antipsychotics ameliorate a range of symptoms and prevent a risk of relapse, the extent to which they improve psychosocial function in patients is less clear [27]. The molecular basis for the effects of antipsychotic drugs is not fully understood, which makes the design of further agents a challenging process [26]. There is an unmet need for a revolution in the development of pharmacological treatments which can only be addressed with better knowledge of the disease origin and pathophysiology.

What Is a Preclinical Model?

Purpose

The term 'preclinical' is defined as the period of research prior to clinical trials in the general population. This period of a drug trial is of extreme importance, as it is during this time that iterative testing and drug safety data are collected. A model is defined as any experimental preparation developed for the purpose of studying a disease.

The model is composed of both the independent variable (i.e. inducing manipulation) and the dependent variable (i.e. the measure used to assess the effects of the manipulation, e.g. behavioural testing) [28]. The choice of preclinical model for a study depends on the aetiology of the disorder of interest, such as schizophrenia. If the aetiology of the disease is known, pathologies with strong homology to the human case can be induced in animals more readily. In schizophrenia, this remains challenging as there are several hypotheses regarding the aetiology of the disease, and the pathology at the protein, pathway and genetic levels is not clearly defined. Preclinical models can involve either animal- or human-derived preparations. The advantage with utilising non-human models is that it enables the investigation of the neurobiology of the phenomena of interest using invasive techniques that cannot be used in humans [29]. If standardised and used correctly, non-human preclinical models can save costs hugely during drug development. However, as will be discussed later on, patient-derived human samples, such as stem cells, are becoming increasingly important.

Criteria for a Preclinical Model

In choosing and developing a preclinical research model it is imperative to consider the purpose intended for the model. There are several criteria required, as displayed in figure 1.

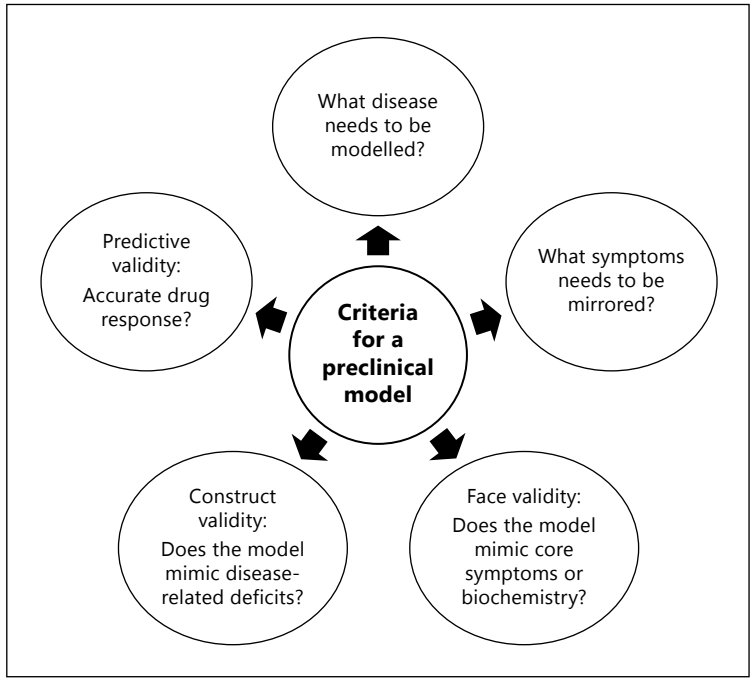

Fig. 1. Criteria to be considered in choosing a preclinical model [30–35].

Preclinical Models of Schizophrenia

Preclinical models of schizophrenia are indispensable tools for testing disease hypotheses which cannot be directly addressed in human subjects for technical and ethical reasons. However, even though they are limited in their capability to mimic the complete clinical presentation of a schizophrenic patient, they are effective in testing specific causative or mechanistic hypotheses regarding schizophrenia [36]. Currently, available preclinical models of schizophrenia, predominantly rodent, fit into four different induction categories: (1) developmental, (2) drug-induced, (3) lesion or (4) genetic manipulation; they are displayed in figure 2.

More recently, preclinical models of schizophrenia have moved to an in vitro setting, utilising patient-derived neural stem cells such as those from the olfactory bulb [58]. An emerging report by Sawa and co-workers [59] shows olfactory cells to reflect the developing brain in gene expression profiles and, more surprisingly, to have an expression profile closer to those of stem cells over blood cells. Investigations have also identified dysregulation in core cellular pathways responsible for cell growth and differentiation [60]. Stem cells have the potential to become a powerful tool for researchers engaged in the pathological study of neuronal disorders such as schizophrenia. This advent of human induced pluripotent stem cells makes it possible to study schizophrenia-derived tissue in vitro, where there is already a genetic predisposition towards the disease state [61]. A big advantage of utilising olfactory neural tissue is that it is accessible in human adults [62]. Induced pluripotent stem cells can recapitulate in vitro aspects of patients' neuronal development and thus help to clarify early pathological processes in the disease [58, 60, 61, 63].

Proteomics

Proteomics holds the promise of unravelling pathological mechanisms at the molecular level. Mass spectrometry (MS) and gel-based approach-

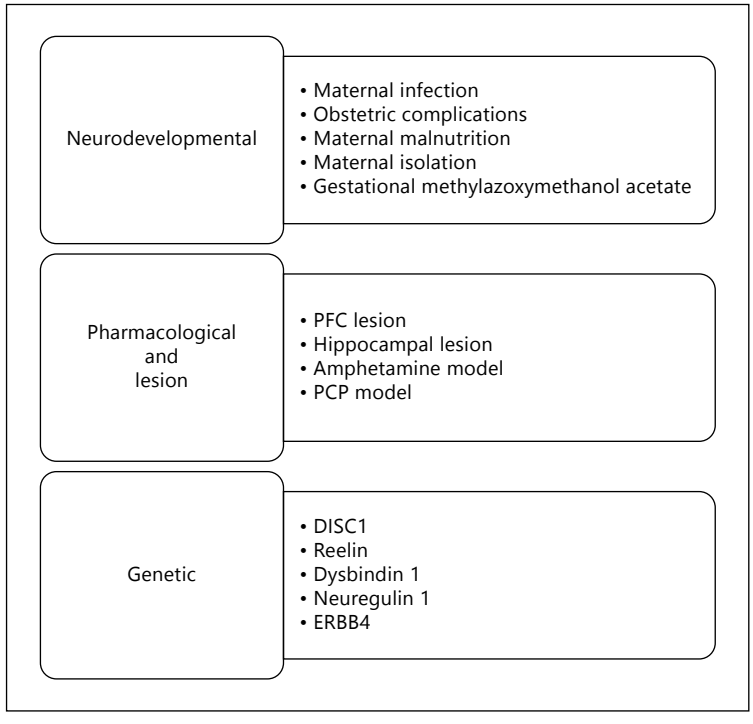

Fig. 2. Summary of well-known preclinical models of schizophrenia [14, 20, 32, 37–57]. PFC = Prefrontal cortex; PCP = phencyclidine; DISC1 = disrupted in schizophrenia 1; ERBB4 = receptor tyrosine-protein kinase erbB-4.

es such as two-dimensional difference gel electrophoresis (2-D DIGE) allow in-depth analyses of entire proteomes. Furthermore their use is becoming increasingly appreciated in the field of biomarker discovery [64]. MS-based proteomics is increasingly used to address basic and clinical questions in biomedical research through studies of differential protein expression, protein-protein interactions and posttranslational modifications. The field of proteomics is undergoing rapid development in a number of different areas including improvements in mass spectrometric platforms, peptide identification algorithms and bioinformatics. In particular, new and/or improved approaches have established robust methods that allow not only for in-depth and accurate peptide and protein identification and modification, but also for sensitive measurement of relative or absolute quantitation [65]. In terms of biomarker investigations, proteomic methods have been utilised in the clinical field and applied to a variety of samples [66]. Proteomic methods can be categorised into those that are gel based and those which are not.

2-D DIGE is a powerful technology for protein abundance studies. Originally described by O'Farrell, it is based on separation of the proteins according to their charge in the first dimension by isoelectric focusing and relative molecular mass in the second dimension by SDS-PAGE [67]. It is often coupled to MS for protein identification, resulting in protein 'maps'. Their resolution, however, is not sufficient relative to the enormous diversity of cellular proteins in terms of size, and problems are especially prominent for poorly water-soluble (hydrophobic) proteins such as membrane and nuclear proteins [68]. Traditional gel-based proteomic quantitation approaches are being overcome by the simplicity of MS-based approaches, which address issues of reproducibility and allow for high precision over traditional methods [69, 70]. Non-gel-based

methods are coming to the fore and their success is owed to their ability to analyse more complex samples and to generate data faster in a high-throughput manner [71]. These approaches combine liquid chromatography with MS. In label-free approaches, complex samples are digested to peptides with an enzyme such as trypsin, separated by high-performance liquid chromatography, and their mass analysed within the mass spectrometer. Methods involving stable isotope labelling have been advantageous in investigating the differential quantitative expression of proteins in samples. In vivo labelling, for example, employs biosynthetic incorporation of isotopically labelled nutrients or amino acids into proteins, and in vitro labelling is achieved postbiosynthetically at a specific site in proteins or peptides [72]. SILAC (stable isotope labelling by amino acids in cell culture) is an example of such in vivo labelling. SILAC exhibits the capability to quantify both the proteome and its modifications in response to stimuli and perturbations and has also made advances in quantitative phosphoproteomics (for a recent review see Mann [73]). Protein labelling with isotope-coded affinity tags followed by tandem MS allows sequence identification and accurate quantification of proteins in complex mixtures [74]. Pioneered by Gygi et al. [75], side chains of lysine and cysteine are primarily used to incorporate an isotope-coded mass tag. Isotope-coded affinity tags can significantly reduce the complexity of the peptide mixture, which can be advantageous when analysing highly complex samples [76]. Another stable isotope labelling technique uses amine-specific isobaric tags (iTRAQ). The design of the iTRAQ reagents allows quantitative comparison of 4 (or more) protein pools simultaneously. The method is conceptually elegant as peptides are labelled at the N-terminus and at the ε side chain of lysines. Furthermore, iTRAQ differs by deriving quantification at the MS/MS rather than MS level to result in a high signal/noise ratio [77]; for a recent review on the current iTRAQ literature, see Evans et al. [78]. Another non-gel-based method for the multidimensional separation of proteins is the novel analytical technique referred to as 'surface-enhanced laser desorption/ionisation'. It is a high-throughput-array-based technology and useful for detection of disease-associated proteins for biomarker discovery [79]. Label-free 'shotgun' proteomics can estimate protein abundance more accurately than gel-based methods, and can observe the differential abundance of proteins over a larger dynamic range than labelling techniques [80]. It is hoped that advances in instrumentation and bioinformatics software will overcome obstacles associated with quantitative reproducibility and will increase proteome coverage.

Proteomics in Schizophrenia

The field of neuroproteomics is undergoing developments in various areas such as improving mass spectrometric platforms, algorithm building and bioinformatics for analysis of large data sets from clinical samples. However, the area remains challenging due to the high degree of complexity, such as in the postmortem brain with its high heterogeneity of cell types. Therefore there is a need for optimal sample preparation, sensitive instrumentation and high-throughput validation [81]. Schizophrenia biomarker discovery heavily relies on MS-based proteomics to unravel the complexity of biological samples such as brain tissue, blood, urine, cerebrospinal fluid (CSF) and plasma. The advancement in this area is made evident by the surge of research which is focusing on the application of MS in neuropsychiatry [82–86]. This move from gel-based methods is a result of improved MS instrument design, which allows in-depth analysis of tissues of high complexity such as brain.

Proteomic brain studies in schizophrenia have reported changes within both the white and grey matter of the brain for proteins such as al-

dolase C, creatine kinase, enolase, septin 3 and dynamin 1. These changes are related to metabolic, cytoskeletal and synaptic function, respectively, and have been identified in the prefrontal cortex, anterior cingulate cortex, corpus callosum and insular cortex [87–92]. These findings provide the support for a view of altered cytoskeletal and mitochondrial function as core components of the neuropathology of the disease [91, 93, 94]. Myelin protein changes have also been reported, further reinforcing the hypothesis of disrupted connectivity in schizophrenia [95, 96]. Clathrin-mediated endocytosis, a protein trafficking process, has recently been hypothesised to be a core pathological feature of schizophrenia [97]. Neuroproteomic studies of schizophrenia are not exclusive to brain tissue, and many have utilised CSF and, in particular, blood serum to report disease-related protein changes [84, 85, 98]. Ongoing proteomic studies reinforce the interest in particular pathways and also encourage the search for new potential biomarkers [99, 100]. There has yet to be a major assessment of current proteomic studies on preclinical models of schizophrenia, therefore this current review tries to fill in that gap. Proteomics will lead to an improved understanding of schizophrenia pathophysiology and thus facilitate novel drug discovery.

Proteomic Studies on Preclinical Models of Schizophrenia

Using PubMed as a search tool for identifying existing studies, it is clear that this area of research is still in its infancy. At the time this chapter was being put together (December 2013), with the terms 'proteomics' and 'schizophrenia', 152 results were retrieved, with fewer than 20 studies implementing the use of preclinical models. Some of these studies will be discussed in terms of their findings, of how they may contribute to our further understanding of the disease and also of how they may aid in the development of novel therapeutics. Below, we summarise the proteomic studies according to their model types, which are predominantly based on developmental stressors and genetic manipulation.

Developmental Stressors

Phencyclidine

Phencyclidine (PCP) induces a broad range of schizophrenia-like symptoms, a finding that has contributed to the hypoglutamatergic hypothesis of schizophrenia [101]. In terms of its role as a preclinical model of schizophrenia, its chronic intermittent low dosage produces a pattern of metabolic and neurochemical changes in the rodent brain that mirrors those observed in the brains of schizophrenic patients with remarkable precision. This makes it an encouraging model in the search for newer effective antipsychotic drugs [102]. In 2012, Ernst et al. [103] analysed changes in brain and serum molecular profiles of PCP-treated rats and compared these changes with those observed in first- and recent-onset antipsychotic-free schizophrenia patients. The authors identified similar behavioural and molecular alterations in PCP in rat and human schizophrenia, such as altered insulin signalling. Overall, this study proposes that the overlapping changes may have potential as biomarkers to facilitate the development of novel drugs.

Earlier in 2013, Pickering et al. [104] published a research article showing the effect of chronic PCP on synaptic proteins in the rodent prefrontal cortex. Firstly, in validation of their model, PCP-treated animals made more incorrect alternations than did controls in the Y-maze test, indicating and confirming cognitive dysfunction and, in other words, dysfunction at the level of the prefrontal cortex. In agreement with previous reviews of PCP effects [105] suggesting that this drug changes synaptic transmission and plasticity and also affects glycolysis and gluconeogenesis, the authors, utilising 2-D DIGE, found dysregulation of proteins, mainly of those with synaptic,

mitochondrial and cytoskeletal functions. In sum, this study reported protein changes similar to those evident in human postmortem schizophrenic brains, predominantly related to mitochondrial function. Not only does it support the use of PCP as a valid preclinical model of schizophrenia, but it also points to its role in translational research in the search for new, more targeted antipsychotic drugs. A more recent study by Wesseling et al. or the Bahn group [106] combined a proteomic approach with metabonomics to compare human schizophrenic brain pathology with that of the frontal cortex of rodents exposed to PCP. With this approach they identified proteomic alterations in mitochondrial function, glutamate-mediated calcium signalling and cytoskeletal remodelling. Changes in the calcium signalling system and calcium-related protein kinases have previously been implicated in the aetiology of schizophrenia [107, 108], reinforcing the dysregulation of cognition and long-term potentiation. Additionally, metabonomic profiling of the PCP-treated animals revealed changes in levels of pyruvate, glutamine, glycine and glutamate, which agrees with evidence of mitochondrial dysfunction and impaired brain energy metabolism in human schizophrenia [109]. Martins-de-Souza et al. [110] established a selective reaction monitoring assay for multiplex analysis of enzymes of the glycolysis pathway which are already known to be affected in human schizophrenia and the PCP model. The results showed altered levels of several core glycolytic enzymes in PCP rats compared with controls. This study not only supports the PCP preclinical model, but also that selective reaction monitoring MS could be used in the development of multiplex classification tools for schizophrenia.

Maternal Separation

A study by Marais et al. [111] in 2009 explicitly used the preclinical model of maternal separation for a study into depression, but considering that depression is often a core negative symptom of schizophrenia, it will be included in this review. Proteins that were significantly changed by maternal separation included molecular chaperones, proteins related to energy metabolism, neuroplasticity, oxidative stress regulation and protein metabolism. Treatment with escitalopram, a selective serotonin reuptake inhibitor, induced changes in a different group of proteins, except for a few involved in energy metabolism and neuroprotective pathways. This model highly correlates with previous postmortem proteomic studies of schizophrenia [88, 105, 112–114]. In 2012, Daniels et al. [115] performed iTRAQ proteomic analyses of protein expression to determine whether exercise counteracts the detrimental effects of early-life adversity in the form of maternal separation on protein expression in the brain. Proteins involved in neuronal structure, metabolism, signalling, antioxidative stress and neurotransmission were affected by maternal separation. Remarkably, many of these proteins were restored to normal by subsequent exposure to voluntary exercise in adolescence.

Dimatelis et al. [116] quantified methamphetamine-induced protein expression in the shell and core of the nucleus accumbens in animals exposed to maternal separation using iTRAQ with MS. The most revealing outcome was that, compared with maternal separation or methamphetamine alone, the maternal separation/methamphetamine combination altered more proteins involved in cellular growth and energy metabolism. This study confirmed that both early-life stress and drug exposure behave in a 'two-hit' manner and result in more proteins being affected than each by treatment/adversity alone [116].

Exercise has been associated with improvement in mood and anxiety in animals and in human studies [117, 118], but the underlying molecular mechanisms had yet to be elucidated. This is a novel use of the preclinical model of maternal separation utilising proteomics to convey a possible alternative to antipsychotic intervention in

adolescence. Kitteringham et al. [119], with label-free MS and multiple reaction monitoring, show that maternal separation from early weaning leads to dysregulation of markers of mature oligodendrocytes and genes involved in protein translation and other categories, an apparent downward biasing of translation, and to methylation changes in the promoter regions of selected dysregulated genes. These findings are likely to prove useful to our understanding of the mechanism by which early-life neglect affects brain structure, cognition and behaviour. Additionally, a strength of this study was that multiple reaction monitoring proteomics methods were used to provide further confirmation of the effects of maternal separation on myelin-related proteins [119]. Isolation rearing from weaning/maternal separation captures the neurobiological relationship between environmental stress and schizophrenia [120]. It causes changes reminiscent of negative and cognitive impairments probably as a consequence of alterations in frontal corticolimbic circuits. The advantage of this neurodevelopmental preclinical model over pharmacological models such as that of PCP is its ability to allow proteomic investigations in the absence of confounding drug interventions. Isolation-reared rodents show impaired prepulse inhibition of acoustic startle compared with group-housed controls, which is thought to reflect sensorimotor gating deficits. Proteomic studies utilising this model, however, are sparse. Roncada et al. [121], utilising 2-D DIGE and matrix-assisted laser desorption/ionisation (MALDI)-time-of-flight (TOF)-MS, correlated gating deficits in isolation-reared rats with proteomic alterations in the nucleus accumbens. Notably, α-synuclein and 14-3-3 proteins were increased in the isolation-reared rats. Considering the role of these proteins in synaptic trafficking, the findings provide more clues to the foundations of the psychosis-like behaviours in isolation-reared rats [122]. This investigation possibly offers the possibility of novel drug targets [121].

Prenatal Psychosocial Stress

It has been hypothesised that prenatal exposure to maternal stress increases the risk of subsequently developing schizophrenia, so therefore animal models of chronic stress may offer insights into pathways operating in the disease [123]. Proteomic studies in this regard are sparse, and just three to date have utilised proteomic techniques to identify the protein changes induced by repeated exposure to psychosocial stress. Investigations utilising the maternal restraint stress paradigm have reported – by way of 2-D DIGE and MS – changes in proteins involved in the regulation of signal transduction, synaptic vesicles, cytoskeleton dynamics and energy metabolism in the offspring born to these mothers [Föcking et al., under review; 124]. An earlier study of the hippocampus employing a social defeat model found changes in offspring relating to protein folding, signal transduction, synaptic plasticity, cytoskeleton regulation and energy metabolism with 2-D DIGE and MS [125]. It is clear from these studies thus far that there is a common effect of psychosocial stress on the hippocampus of offspring born to these mothers. Future proteomic investigations should further examine these particular pathways as they could provide novel targets for drug discovery.

Pharmacological/Lesion Models

Methylazoxymethanol acetate is a DNA-alkylating agent that, when administered during gestation to the fetus, can produce behavioural and anatomical brain abnormalities in adulthood which model some aspects of schizophrenia. Hradetzky et al. [126] reported hippocampus-specific effects of methylazoxymethanol acetate in the rat, using label-free MS. These proteomic alterations mainly concerned those proteins related to synaptic plasticity, such as AMPA1, a major player in synaptic plasticity events [127]. This result in particular agrees with previous literature implicating AMPA in the pathophysiology of schizophrenia [128].

NMDA dysfunction is hypothesised to play a role in schizophrenia aetiology, which can be mimicked in the MK-801 rat model. MK-801 can selectively block NMDA-mediated neurotransmission in rodents, who then display behavioural abnormalities akin to human schizophrenia [129]. The few studies which have approached this model using proteomic methods have identified alterations in the cortex and thalamus of MK-801-treated rats relating to mitochondrial and skeletal function [130, 131]. Proteomics has been indispensable in the critical analysis of MK-801 as a valid schizophrenia model. Paulson et al. [132] used 2-D DIGE coupled with MS to reveal that different treatment times yield different biochemical results. Therefore it is important to use the same treatment time for comparative studies.

Smalla et al. [133] in 2008 utilised 2-D DIGE with MS to compare the synaptic proteomes in human schizophrenia with the cortex of ketamine-treated rodents, another pharmacological model of NMDA dysfunction [134]. Consistent changes were identified between the two, but the overlap was limited and only one protein was upregulated in both [133]. This study paved the way for future studies to further examine the synaptosome in models of NMDA dysfunction. In order to investigate the MK-801 model of NMDA dysfunction at the synaptic level for contributing pathways, Zhou et al. [135] isolated the synaptosome from MK-801-treated rodents and, utilising MALDI-TOF-MS, reported energy metabolism and semaphorin signalling to be the most affected pathways, further emphasising the importance of synaptic function in schizophrenia.

An insult to the brain during a critical period of development can produce subtle anatomical changes sufficient to predate behavioural changes in adulthood. Such a risk has been investigated in the neonatal ventral hippocampal lesion model as it mimics a spectrum of neurobiological and behavioural features of schizophrenia [136]. Vercauteren et al. [137] performed a study with 2-D DIGE coupled with MS on the prefrontal cortex of postpubertal, neonatally ventral hippocampal-lesioned rats identifying dysregulation of core synaptic proteins such syntaxin-binding protein. This outcome provides vital clues as to what protein changes are behind the schizophrenia-like behavioural changes [137]. Additional proteomic studies with developmental lesion models will be indispensable to identify early protein changes that precede the schizophrenia phenotype, therefore aiding in the development of early intervention therapies.

Prenatal Nutritional Deficiency Models

In utero exposure to nutrient deficiency is a well-known risk factor for developing schizophrenia [138–142], and proteomic studies of this model are underinvestigated, with only few studies having been undertaken. A rat model of prenatal vitamin D deficiency assessed protein changes in the prefrontal cortex and hippocampus with 2-D DIGE coupled with MS. The authors reported changes in proteins relating to core metabolic pathways including oxidative phosphorylation, synaptic plasticity and neurotransmission, and, using computational analysis, discovered that the impaired synaptic network may be a consequence of mitochondrial dysfunction, providing further insight into the pathological effects of this nutrient deficiency [143]. A study by McGrath et al. [144] in 2008 used the same model to show, via MALDI-TOF-MS, disruptions in proteins related to calcium binding and mitochondrial function. These protein changes may also underlie some of the behavioural features associated with animal models of developmental vitamin D deficiency [46, 145], and even further studies can validate these putative pathways. Guest et al. [146] completed molecular profiling of blood serum, the frontal cortex and hypothalamus from adult offspring prenatally exposed to a low-protein diet. This model influences impaired insulin signalling, which is found to be altered in first-

onset schizophrenia [147]. The analysis revealed alterations in proteins relating to synaptic and glutamatergic transmission and cytoskeletal function, recapitulating pathophysiological attributes seen in schizophrenia. Drug discovery can benefit from the results of this study in identifying therapeutics that can modulate these systems.

Patton et al. [148] utilised the iTRAQ method to quantitatively assess proteins in the CSF of prenatal iron-deficient anaemic monkeys. Again, the proteins identified to be altered are involved in neurite outgrowth, migration or synapse formation. These aberrations may account for the behavioural deficits observed in iron-deficient children [149, 150].

The latest report by English et al. [151] identified the protein pathways disrupted as a consequence of chronic ω–3 deficiency in the hippocampus of mice. The findings pointed towards disturbed synaptic function, neuritogenesis and mitochondrial function, furthering our understanding of the molecular mechanism by which ω–3 deficiency impairs normal brain function.

Prenatal Maternal Infection

Prenatal maternal infection has also been explored as a preclinical model by proteomic methods. Lipopolysaccharides and polyinosinic:polycytidylic acid are both compounds which result in production of fever and induction of proinflammatory cytokines that affect neurodevelopment and predate behavioural abnormalities akin to human schizophrenia in animal models [43, 152–155]. Proteomic studies utilising lipopolysaccharides in the brain have yet to be reported; however, polyinosinic:polycytidylic acid associated with viral infection has been relatively underinvestigated. To our knowledge, three studies have used proteomics to investigate this preclinical model of schizophrenia. So far, metabolic processes have been reported to be altered, such as the cellular immune response, glucose and lipid metabolism, and together they have implications for understanding the causality of prenatal infection and may offer potential biomarkers and novel treatment targets for new treatments [156–158].

Genetic Manipulation

Proteomic characterisation of core molecular pathways in genetic preclinical models would be a fundamental advancement in understanding schizophrenia, and may have equally significant therapeutic implications. Studies of this kind are few and insufficiently investigated. Bader et al. [159] identified disease-specific epitopes of collapsin response mediator protein 1 in postmortem brain samples of schizophrenia patients. Strikingly, further genetic association analysis found that collapsin response mediator protein 1 functionally interacts with disrupted in schizophrenia 1, suggesting it as a possible gene worth further investigating and incorporating it into a preclinical knockout model of schizophrenia. Dysbindin, a candidate schizophrenia gene, is involved in synaptic trafficking, but its molecular function is largely unknown. To investigate this, Hikita et al. [160] performed MALDI-TOF-MS and liquid chromatography-MS/MS on isolated dysbindin-interacting molecules from rat brain lysate. Proteins identified included Munc18-1, helping to shed light on the molecular function of dysbindin, which had been largely unknown. Reelin, a glycoprotein secreted by GABAergic interneurons, is substantially decreased post mortem in schizophrenia, making it a suitable candidate gene for the disease [161]. Martins-de-Souza and co-workers [162] elegantly compared proteome changes caused by reelin heterozygosis with those found in their earlier proteome analyses of schizophrenia human brain tissue and discovered similar findings in terms of energy metabolism and cytoskeletal function. These findings support the heterozygous reelin mouse as a preclinical model, considering its strong overlap with the authors' previous findings on humans, and reveal new pathways involved in reelin dysfunction, therefore providing new targets for the development of drug therapies.

Table 1. Summary of the 9 proteomic studies to date of rodent brain/cells under antipsychotic treatment

Study	Drug	Proteomic method	Findings
Bro et al. [166], 2003*	Lithium	2-D DIGE	1. 450 proteins identified in the yeast proteome 2. 27 were downregulated 2-fold with lithium, 21 proteins were upregulated 3. Protein transcription and nucleotide metabolism were downregulated 4. Stress response and monosaccharide metabolism were upregulated
O'Brien et al. [167], 2006	Risperidone	2-D DIGE, MALDI-TOF-MS	1. 30 protein spots related to drug treatment in the rat striatum 2. Drug-related changes in cellular metabolism, cell signalling, transport, protein metabolism, chaperone activity, DNA binding and cell cycle function
La et al. [168], 2006	Chlorpromazine, Clozapine	2-D DIGE MALDI-TOF-MS	1. Drug-related changes in malate dehydrogenase, peroxiredoxin 3, ATP synthase subunit and MAPK1 in the rat hippocampus
Chen and Chen [169], 2007	Clozapine	2-D DIGE ESI-MS/MS	1. Drug related up-regulation of transthyretin in the rat hippocampus
Kashem et al. [170], 2009	Haloperidol, Risperidone	2-D DIGE	1. Drug-related protein changes related to the cytoskeleton, calcium regulation, metabolism, signal transduction and oxidative stress in rat neural stem cells 2. Risperidone expressed more proteins than haloperidol
Ji et al. [171], 2009	Chlorpromazine, Clozapine, Quetiapine	2-D DIGE	1. Drug-related protein changes in the respiratory electron transport chain and oxidative phosphorylation were found in rat mitochondria
Ma et al. [172], 2009	Haloperidol, Olanzapine	LC-MS/MS	1. Haloperidol and olanzapine altered proteins associated with cellular assembly/organization and nervous system development/function in the rat frontal cortex 2. Olanzapine induced more protein changes related to glycolysis/gluconeogenesis
Martins-de-Souza et al. [173], 2011	Clozapine	2-D DIGE, LC-MS/MS	1. Drug-related changes in cellular metabolism, cell signalling, cell growth and chaperone activity were found in cultured rat astrocytes
Ahmed et al. [174], 2012	Haloperidol, Risperidone	2-D DIGE MALDI-TOF-MS	1. Protein changes unique to haloperidol treatment related to the cytoskeleton, calcium regulation, oxidative stress and apoptosis in rat neural stem cells 2. Protein changes unique to risperidone treatment related to cellular metabolism

* First proteomic paper to utilise a schizophrenia-related medication. Lithium is commonly used as an adjunct to antipsychotics [163]. ESI = Electrospray ionisation; LC = liquid chromatography; MAPK1 = mitogen-activated protein kinase 1.

Proteomic Studies of Preclinical Animal Models Utilising Antipsychotics

Preclinical models are vital to understand the molecular mechanisms behind antipsychotics in order to advance the development of more novel therapeutics. As the precise mechanisms of action of antipsychotic medications are not fully known, these quantitative proteomic studies of the effects of antipsychotic drugs on the brain are warranted. The obstacle to our discovery of novel treatments lies in our lack of understanding of the disease-related biological mechanisms. Few studies have been undertaken utilising antipsychotics with proteomic studies in preclinical schizophrenia models. Using the search terms 'antipsychotics and proteomics' in PubMed, 45 studies were reported. However, of these, only 9 were performed on rodent brain or stem cells. Considering the focus of this review, only these studies have been summarised in table 1. These current proteomic studies of preclinical models utilising antipsychotics have predominantly employed the less sensitive gel-based methods but have thus far been insightful in relation to drug-related protein changes. From the proteomic studies identified (table 1), antipsychotics are seen to act through a variety of pathways, primarily relating to cellular metabolism, cytoskeletal function and oxidative stress, mechanisms previously found to be altered post mortem in schizophrenia [105]. These types of investigation are essential for elucidating the mechanism of antipsychotics and are crucial for novel therapeutic developments.

Challenges Currently Faced and Concluding Remarks

Clearly, many preclinical animal models exist for research into schizophrenia neuropathology and drug development. The variety arises because an ideal preclinical animal model of schizophrenia does not exist and each animal model focuses on a particular spectrum of the schizophrenia disorder. As with all animal research, the three R's underpinning their humane use must be mentioned: upon the discovery of an adequate preclinical model, experiments must be 'refined', numbers must be kept to a minimum for studies, or 'reduced', and, ultimately, animals must be 'replaced' [164]. This last principle is becoming more promising with the use of human-derived neural stem cells [58, 165], and further research utilising this avenue will hopefully obviate the necessity for animals in schizophrenia research to a good extent. Although research involving rodent models is relatively economical, and an abundance of information exists on their genomes, the aetiology of schizophrenia contains a large genetic component, and patient-derived cell models have the superiority in that they do not require genetic manipulations to mirror this aspect of the disease [58].

There is no doubt that current proteomic studies of preclinical animal models have advanced our understanding of schizophrenia neurobiology by providing candidates that, once observed to be differentially expressed in the brain, can now be tested in the serum of schizophrenia patients [English et al., in press]. They have also accelerated the search for reliable biomarkers, and progress in proteomic methods, such as more quantitative approaches of validation and targeted methods, will help in this respect. Until then, we must remain cautious in the interpretation of results, pick the best validated/predictive biomarkers for drug efficacy studies and, most importantly, report contradicting results from current proteomic studies. Without a doubt, research in this area will have a striking impact on the future of schizophrenia treatment.

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Metabolomics to Study Psychotic Disorders and Their Metabolic Comorbidities

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Abstract

Primary obesity and psychotic disorders are similar with respect to the associated changes in energy balance and comorbidities, including the metabolic syndrome. The specific underlying mechanisms linking the expansion of adipose tissue to these comorbidities are unknown. Such similarities do not necessarily demonstrate causal links, but instead suggest that specific causes of and metabolic disturbances associated with obesity play a pathogenic role in the development of psychotic disorders. Both brain and peripheral metabolic organs use metabolites, particularly lipids, as components of their integrated homeostatic system to control energy balance as well as to regulate peripheral insulin sensitivity. Given the intrinsic complexity and widespread role of metabolism, a detailed metabolic characterization is essential to identify the molecular factors contributing to psychotic disorders. Knowledge of common and specific mechanisms may help in the etiopathogenic understanding, early disease detection as well as identification of subjects who may benefit from specific treatments for psychotic disorders or who may be especially vulnerable to metabolic side effects, as well as in discovery of unexpected novel therapeutic avenues.

Introduction

Psychotic disorders are mental disorders characterized by impaired reality testing or reality distortion. Psychotic symptoms can appear in many psychiatric disorders such as schizophrenia or psychotic episodes of affective disorders (bipolar and unipolar depression). Psychotic symptoms are typically observed as delusions, hallucinations, disorganized speech, and bizarre or catatonic behavior. The incidence of psychotic disorders peaks in young adulthood [1], a period of development when significant changes in fatty acid composition occur in the cerebral cortex due to axonal myelination [2], and their lifetime prevalence is about 3.5%, the most common being schizophrenia with approximately 1% lifetime prevalence [3].

Unhealthy lifestyle and pharmacological side effects have been suggested as major causes of excess morbidity and mortality of patients with psychotic disorders. Among these patients, those with negative or deficit symptoms are more prone to be overweight and to have higher rates of metabolic syndrome (MetS). These patients

with deficit schizophrenia (e.g. negative symptoms) have less healthy and more sedentary lifestyles, which may in turn induce increased cardiovascular morbidity [4]. On the other hand, the use of antipsychotic drugs, especially second-generation ones, has been consistently associated with weight gain, insulin resistance and the development of MetS [5–7], which seems to be more marked in younger people [8]. After only 6 months of treatment with some second-generation antipsychotics, the percentage of previously drug-naïve first-episode adolescent patients at risk of developing MetS rises from 17 to 40% [9]. This evidence suggests that these psychotropic drugs target central nervous system neurons that also regulate mechanisms controlling energy balance and associated metabolic alterations.

Data on schizophrenia patients from the pre-antipsychotic era already showed that the prevalence of diabetes mellitus or glucose intolerance was higher in patients than in controls [10]. Abnormal glucose tolerance, hyperinsulinemia and accumulation of visceral fat are already detected during the first episode in drug-naïve patients, prior to antipsychotic treatment and independently of obesity [11]. Furthermore, unaffected first-degree relatives of people with schizophrenia also have high rates of diabetes mellitus (19–30%, as compared with 1.2–6.3% in the general population) [12]. Of relevance, recent genetic studies have detected genes that increase the risk of both schizophrenia and type 2 diabetes [13]. Taken together, these observations suggest that metabolic disturbances associated with obesity may contribute to the etiopathogenesis of psychotic diseases. Along those lines, analysis of data from the worldwide cross-national World Health Organization World Health Survey found an increased probability of having diabetes as the number of psychotic symptoms increased [14]. Type 2 diabetes, in turn, is among the major determinants of excess mortality of people with psychotic disorder [15].

Metabolome in Health and Disease

Despite the undeniable, strong genetic component in many complex diseases, with heritability estimated at 40% or higher in the MetS [16] or in the order of 65% or higher in schizophrenia [17], it is becoming increasingly evident that current approaches studying genetic associations with disease traits can explain only a fraction of the known disease heritability [18].

According to the systems biology view, most of the genetic component of complex disease susceptibility is not to be found in individual genes, but in their interactions with other genes as well as with the environment [19]. In this context, the measurement of traits that are modulated but not encoded by the DNA sequence – commonly referred to as 'intermediate phenotypes' [20] – is of particular interest. Changes in the concentration of specific groups of metabolites are sensitive and specific to pathogenically relevant factors such as genetic variation [21], diet [22, 23], development [24], age [25], immune system status [26, 27] or gut microbiota [28, 29].

Metabolomics has emerged as a powerful tool for the characterization of complex phenotypes as well as for the development of biomarkers for specific physiological responses [30]. The metabolome is sensitive to genetic as well as environmental factors, which makes metabolomics a powerful phenotyping tool needed for predictive, preventive, personalized and participatory medicine [31].

Lipids are a diverse group of essential metabolites that exert many key biological functions, such as structural components of cell membranes, energy storage sources and intermediates in signaling pathways. Lipids are under tight homeostatic control [32] and exhibit spatial and dynamic complexity at multiple levels [33]. It is thus not surprising that altered lipid metabolism has a global reach as a pathogenic mechanism and is involved, for instance, in diabetes and lipotoxicity-induced insulin resistance [34, 35], Alzheim-

er's disease [36, 37], schizophrenia [38–40], autism [41], cancer [42, 43] and atherosclerosis [44]. Until recently, sensitive platforms for global and quantitative studies of lipids from the cellular to organism levels have been lacking. Lipidomics emerged as a subdiscipline of metabolomics which is dedicated to the global study of lipidomes, including pathways and networks of cellular lipids in biological systems [32].

Techniques of Metabolomics

For a broad coverage of the metabolome, applications of multiple analytical platforms are still needed [45–47]. Analytical technologies based on liquid chromatography coupled to mass spectrometry (MS), gas chromatography coupled to MS, capillary electrophoresis coupled to MS as well as nuclear magnetic resonance have most commonly been applied [46–48].

The analytical strategies for metabolomics are commonly divided into nontargeted (i.e. global) and targeted. The targeted methods aim to quantitatively cover metabolites of a specific, biologically relevant functional class which are difficult to cover by global approaches, such as eicosanoids [49], bile acids [50] or metabolites of central carbon metabolism [51]. Recent technological advances have also enabled the development of methods which can quantitatively cover over 100 metabolites across multiple functional classes [52, 53].

The nontargeted strategies aim to cover the metabolome as broadly as possible while at least semiquantitatively determining the concentrations of metabolites across the samples measured. While possibly being less accurate than targeted approaches, the nontargeted strategies may afford detection of previously unknown or poorly characterized metabolites. The selection of the analytical approach thus ultimately depends on the aims of a study – for instance, whether it is a discovery-, hypothesis- or validation-driven study. Specialized software packages have been developed for nontargeted metabolomic approaches, including the open-source packages MZmine [54–56] and XCMS [57, 58] as well as several commercially available software programs.

Metabolomics to Study Psychotic Disorders

A recent study applied metabolomics to produce metabolic profiles associated with schizophrenia, other nonaffective psychosis or affective psychosis [59]. The analysis indicated that schizophrenia is associated with elevated serum levels of specific triglycerides (particularly those with a low double bond and carbon count, such as in nonalcoholic fatty liver disease) [60], with hyperinsulinemia and also with upregulation of serum proline, an amino acid. Interestingly, serum glutamate was elevated in all psychoses studied [59]. Using a network approach, the metabolic profiles were then combined with other clinical and lifestyle data (fig. 1a) to create a diagnostic model which discriminated schizophrenia from other psychoses (fig. 1b). This study demonstrated how network analysis and metabolomics can be powerful tools for dissecting complex disease-related metabolic pathways and for identifying candidate diagnostic and prognostic markers in psychiatric research.

Other recent studies on metabolite markers in schizophrenia and in first-episode psychosis have also highlighted the significance of glucoregulatory processes [61, 62] and lipid abnormalities [63–65] in psychotic disorders, particularly in schizophrenia. Interestingly, some of the disturbances of glucoregulatory processes in first-episode psychosis seem to improve after initiation of antipsychotic medication [62]. Lipid abnormalities in the brain in schizophrenia include alterations in free fatty acids and phosphatidylcholine in gray and white matter, and an increase in ceramides in white matter [64]. Orešič et al. [38] studied plasma lipidomic profiles in twin pairs discordant for schizophrenia and found that the

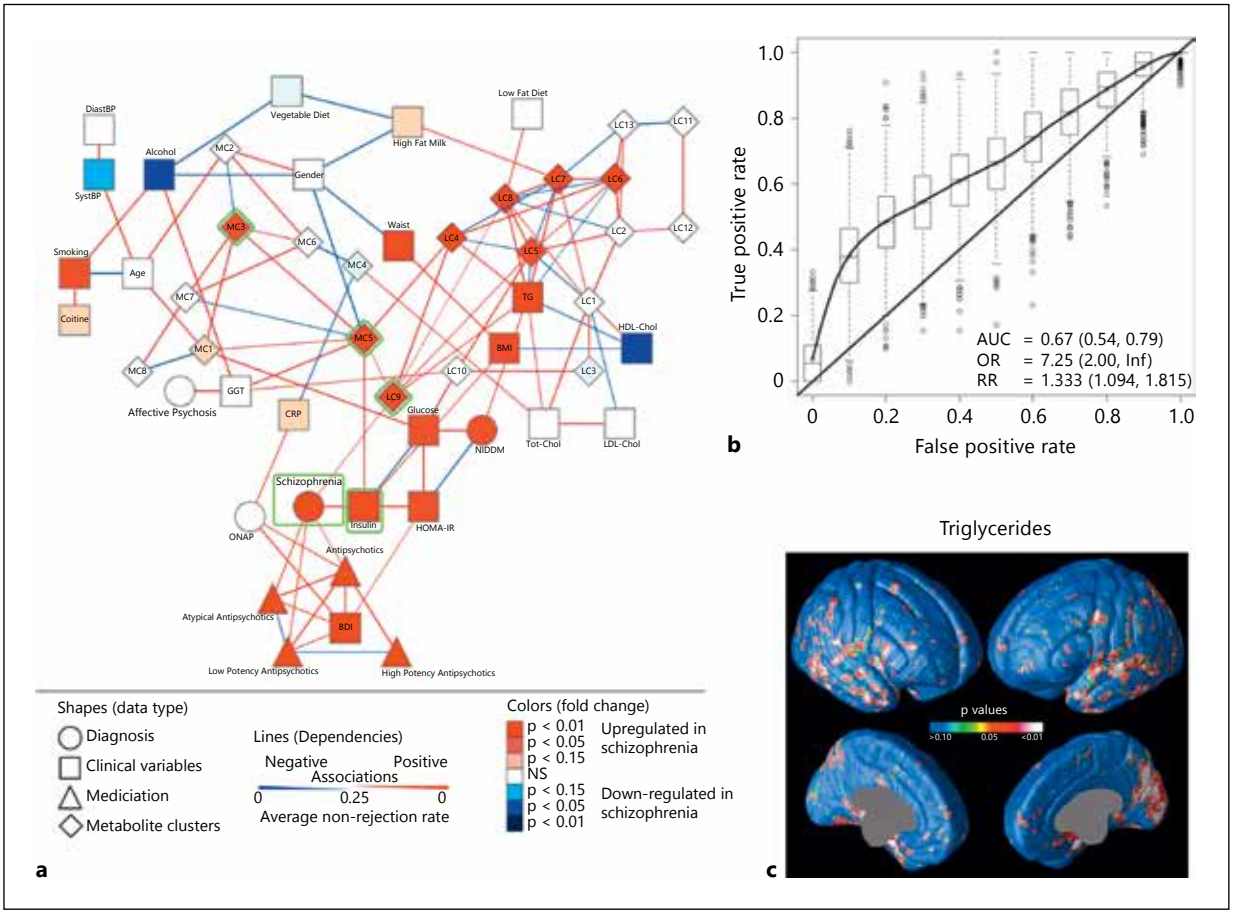

Fig. 1. Metabolomic approach to studying psychosis. **a** Dependency network in schizophrenia and other psychotic disorders, in the context of other environmental, metabolic and drug use data. Reproduced with permission [59]. **b** Diagnostic model of schizophrenia, separating schizophrenia from other psychoses, based on proline and triglyceride TG(18:1/18:0/18:1) concentrations. Reproduced with permission [59]. **c** Significant correlations between serum triglyceride levels, as obtained from lipidomics, and cortical gray matter density, based on integrative analysis of magnetic resonance images and plasma lipidomics in monozygous twin pairs discordant for schizophrenia. Reproduced with permission [38].

patients were more insulin resistant and had higher triglyceride levels than their co-twins. Furthermore, integrative analysis of magnetic resonance imaging and lipidomics data revealed significant associations of decreased gray matter density with elevated triglycerides in plasma (fig. 1c). Recently, Yang et al. [65] found multiple fatty acids and ketone bodies to be elevated in the serum and urine of patients with schizophrenia. The changes were similar in first-episode and more chronic patients. Kaddurah-Daouk et al. [39] examined the effects of antipsychotic medication on serum lipidome, and found significant changes in lipid metabolism already after 2–3 weeks of medication use. In line with these findings, gene expression studies have detected that antipsychotics strongly activate genes involved in lipid homeostasis [66, 67].

A recent large, targeted metabolomics study on 216 healthy controls and 265 schizophrenic patients (including 52 cases that did not take antipsychotic medication), integrated with genetic analyses, implicated aberrations in biosynthetic pathways linked to glutamine and arginine metabolism and associated signaling pathways as genetic risk factors, which may contribute to pathomechanisms and memory deficits associated with schizophrenia [40].

Conclusions

One can conclude that the few metabolomics studies having investigated psychotic disorders so far are consistent particularly concerning the lipid metabolism, but less so with regard to polar metabolites. Investigations of psychotic disorders are inherently challenging due to the ambiguities associated with disease diagnosis because of many confounding factors such as antipsychotic medication use and the associated comorbidities, and the lack of adequate disease models.

In clinical practice, biomarkers are urgently needed for the identification of persons at very high risk of developing psychosis and developing efficient preventive treatment for them, for early prediction of treatment response instead of the current practice of waiting 4–6 weeks before switching to another antipsychotic medication. The metabolomic studies so far suggest that metabolites have diagnostic potential in psychiatry, both as markers sensitive to disease progression and outcomes and for predicting treatment response. Metabolic disease biomarkers such as for nonalcoholic fatty liver disease [60] may also be useful for the prediction and monitoring of metabolic comorbidities associated with psychotic disorders. Given the unmet diagnostic needs in psychiatry, molecular biomarkers such as those derived from metabolomics have a potential to make an immediate impact on clinical practice.

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Peripheral Biomarkers for Depression by Plasma and Serum Proteomics

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Abstract

Blood has been the object of a search for biomarkers for psychiatric disorders since long before the advent of proteomics. A series of investigations have been conducted on serum or plasma, either by focusing on protein candidates such as cytokines or brain-derived neurotrophic factor (BDNF) or by testing multiple candidates simultaneously. Notwithstanding the amount of suggestive evidence for depression, no real biomarker application has made an impact on clinical practice so far. Relatively consistent findings have been obtained for a few candidate proteins, such as BDNF – which, however, appears to be nonspecifically dysregulated across many neuropsychiatric conditions. Combination of biomarkers and the broader picture represented by proteomic profiles are providing a better chance to divide the disorder into subtypes and gain specificity versus other diseases. Most efforts have focused on the identification of proteomic signatures able to discriminate patients from controls, even leading to the proposal of diagnostic tests for mental disorders; however, no major attempts have been made to fulfill the clinical need for signatures able to predict response to antidepressants. In this review, the most interesting findings obtained by searching serum or plasma by proteomic approaches are put into the context of their potential clinical application, highlighting potential pitfalls and opportunities. New directions in proteomic biomarker efforts beyond cross-sectional studies in case-control collections as defined by diagnosis are advocated, and the need for an effective integration of biomarkers at different levels is emphasized. Capturing the contribution of genetic variability to protein expression, and ultimately integrating this information with imaging measures of brain structure and function, will open new avenues for the discovery of mechanisms and circuits involved in disease pathophysiology and drug response.

Introduction

Blood has been the object of a search for biomarkers for mental disorders since the 19th century, with the original work by William Lauder Lindsay [1], who, with the help of a microscope, was trying to identify structural elements in the blood of psychiatric patients that may correlate with their level of insanity [2]. Based on the abovementioned work, MacPhail [3] developed the 'corpuscular richness paradigm' at the end of the century, claiming a difference in the relative proportion of red and white cells as a correlate of

different stages of madness, and establishing a putative connection between the 'quality of the blood' and mental state. Two centuries later, thanks to the availability of sophisticated tools which allow us to define the exact nature of the constituents of cells, organs and tissues, and to quantify their levels, biological psychiatric research has produced a wealth of data on blood and other peripheral tissues. Notwithstanding an amount of suggestive evidence for depression biomarkers produced by many investigators, no real application has made an impact on clinical practice so far. In this chapter I will try to review some of the most interesting findings obtained by searching serum or plasma via proteomic approaches, and put them into the context of their potential clinical application, highlighting potential pitfalls and opportunities.

Blood and the Need for Noninvasive Biomarkers for Depression

Major depressive disorders (MDD) are diagnosed based on the description of groups of symptoms assessed by psychiatrists via patient interviews. The criteria are captured by the Diagnostic and Statistical Manual of Mental Disorders (DSM), a constantly revised classification system developed by the American Psychiatric Association with the aim of positioning the reliability of psychiatric diagnoses at the level of diagnoses of other complex medical conditions. The newest version of the DSM is the DSM-5, released in May 2013 by the American Psychiatric Association 13 years after the preceding DSM-IV [4]. The DSM-5 was expected to capture the need for a pathophysiologically based classification and to develop a system based on dimensional measures [5], but it still appears to be centered solely on diagnostic categories [6].

Patients with mental disorders deserve better – according to Tom Insel, National Institute of Mental Health Director [7], who is supporting a new system based on dimensions of observable behavior and neurobiological measures to drive future diagnosis and treatment [8]. Biomarkers could represent the objective laboratory measure that helps the transformation of psychiatry into evidence-based medicine. Neurophysiological and neuroimaging approaches hold real promise as biomarkers that are able to reduce the phenotypic complexity of psychiatric disorders, and neuroimaging correlates of treatment response have been suggested [9, 10]. But is it conceivable that a 'simpler' biological test run on blood could still inform our diagnosis or help stratifying patients into biologically more homogeneous subtypes? Plasma and serum are probably the richest biofluids; they are readily accessible, standard operating procedures for their collection exist [11], and a large amount of data on their elements in many disease contexts is available. Intuitively, studying cerebrospinal fluid (CSF) would appear to make more sense for psychiatry biomarkers. However, besides the complication of lumbar puncture, only 20% of the proteins in CSF appear to derive from the brain, whilst the remaining part is derived from blood via the blood-CSF barrier [12]. A recent CSF proteome investigation based on high-sensitivity and -resolution liquid chromatography-mass spectrometry (MS) has actually shown that about 56% of the proteins detected are CSF specific, i.e. they are not present in analogous plasma protein databases [13]. However, a comparison between proteome-derived studies of brain postsynaptic density (PSD) tissue, plasma and CSF has revealed an interesting pattern of overlap among the three [14]. According to the data reported, the plasma proteome contains about 16 and 12% of the CSF and PSD proteins, respectively, whilst the CSF in turn is represented by 40% of plasma and 14% of PSD proteins. Interestingly, both plasma and CSF proteomes contain specific PSD proteins that are not detected in their counterpart. Both fluids present technical challenges due to the extremely wide dynamic range of concen-

trations of their proteomes and the presence of highly abundant proteins, which calls for depletion approaches to unmask disease biomarkers [15]. Finally, looking at the blood for depression biomarkers is justified by a large body of evidence on multiple interactions between the brain and the peripheral system via neuroendocrine and inflammatory markers [16]. Accordingly, a recent investigation comparing the results of multiple analyte profiling in blood and postmortem brain tissue from schizophrenic patients, as well as mRNA data, confirms a common alteration in proteins related to the inflammatory and neuroendocrine system [17]. Based on these data, the authors also suggest a model in which the brain and periphery are coordinated through hormones and additional regulatory molecules that are released into the blood via the diffuse neuroendocrine system.

One Step at a Time or the Whole Nine Yards

Until late 1990s, the search for peripheral biomarkers of depression has been mainly driven by the monoaminergic, the immune-inflammatory and the neuroendocrine biological hypotheses [16]. Candidate proteins have been investigated in cross-sectional studies including patients and controls, or in longitudinal studies on depressive individuals in different states, leading to a number of suggested biomarkers, none of them fully validated so far. More recently, the hypothesis of a dysregulation of the neurotrophin system in depression has prompted a large number of studies on brain-derived neurotrophic factor (BDNF). BDNF is a prototypical example of candidate protein biomarker research in psychiatry. The first paper on peripheral BDNF in depressed patients appeared in 2003, suggesting a decreased serum level in untreated patients versus controls or treated patients, as well as a negative correlation between disease severity and protein level [18]. Since then, more than 300 studies have investigated BDNF as a putative plasma or serum biomarker for depression, according to PubMed (a search for human plasma or serum BDNF in depression resulted in about 324 papers at the time of writing of this chapter). Overall, there is agreement on the notion of a reduction in peripheral BDNF in depression according to meta-analyses [19–22]; however, it is not clear yet whether BDNF could be considered as a state or a trait marker, and to which extent it correlates with response to antidepressants [23]. In addition, similar findings for other psychiatric and nonpsychiatric conditions suggest that BDNF should be viewed as a nonspecific biomarker related to neuropsychiatric disorders characterized by neurodegenerative changes [24].

Indeed it is not to be expected that a single biomarker could be able to provide specific information on complex and heterogeneous disorders such as depression. The chance for BDNF to capture at least one specific facet or symptom of depression seems to have been negated by a recent study, where an attempt to correlate BDNF levels with individual symptoms failed [25]. Combination of biomarkers or the broader picture represented by proteomic profiles may provide a better chance to divide the disorder into subtypes, or to gain specificity versus other diseases.

One of the first 'modern' proteomic approaches to plasma of depressed patients was described as early as 1988 [26]. By two-dimensional electrophoresis, the authors were able to identify 'minor qualitative and quantitative differences between the proteins in pooled samples from depressed and normal subjects'. However, the same paper anticipated some of the main issues of proteomics in depression. The authors acknowledged that the abnormalities detected could be unrelated to depression, and the ones of interest could be at undetectable concentrations or masked by the heterogeneity of the patient group, and they finally concluded that the differences observed were most likely artifacts.

Probably because of the complexity of the task, efforts in measuring peripheral changes in depression by large-scale proteomic approaches have been limited until very recently. Most of the papers have focused on MS-based applications for the identification of disease biomarkers; additional two-dimensional gel-based studies on humans have not been reported. A preliminary study was reported in 2009 by Lo et al. [27], who analyzed serum samples from major depression patients and from healthy donors by acid hydrolysis followed by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight-MS analysis. The study mainly focused on candidate proteins, resulting in the identification of the transferrin/fibrinogen intensity peak ratio as a putative biomarker. The same group more recently reported an optimized MALDI-MS method for the analysis of acid-hydrolyzed serum samples, resulting in 80% assay sensitivity and specificity for discrimination between patients and controls based on the transferrin/fibrinogen intensity ratio [28]. In the same paper, they reported a principal component analysis of acid hydrolysis-based serum peptide profiling data, pointing out additional unknown peaks contributing to the classification, the identity of which could not be established. In 2012, Xu et al. [29] reported a quantitative proteomic approach based on isobaric tags followed by multidimensional liquid chromatography-MS/MS, which they claimed to be the first proteomic study investigating peripheral biomarkers for MDD. The study, based on pooling samples from first-onset treatment-naïve patients or healthy controls, followed by high-abundance protein depletion and iTRAQ (isobaric tags for relative and absolute quantitation) labeling, resulted in the identification of differentially expressed proteins mainly involved in lipid metabolism and immunoregulation. However, some of the results were not consistent with previously published findings and could not be replicated in individual samples when analyzed by immunoblotting methods.

Another recent application of MALDI-MS-based protein/peptide profiling in the analysis of serum samples from depressed patients led to the identification of three peptide signals of unidentified origin that differ significantly between cases and controls [30]. Although principal component analysis of the entire peptide profile did not allow for distinct clustering of the two groups, a receiver operating characteristic curve built with the combined use of all three candidates resulted in a sensitivity and a specificity of 100 and 86.7%, respectively. However, the combined set identified by post hoc analysis was not subjected to validation in an independent cohort and would deserve further assessment to establish its robustness and putative clinical utility.

Multiplexing: Finding the 'Dirty Dozen'

Like for genome-wide investigation, hypothesis-free open proteomic studies would probably need large collections and replication in independent samples in order to produce robust biomarker data which could really open new research directions in the neurobiology of depression. On the other hand, as mentioned before, it is unlikely that keeping the focus on single biomarkers linked to preexisting hypotheses will significantly increase our understanding of depressive disorders or revolutionize therapeutic approaches. Because of the heterogeneity of depression both in terms of symptoms and its underlying etiology (see Hasler et al. [31] and the concept of endophenotypes), a combination of biomarkers belonging to different pathways appears more promising. Encouraging attempts to integrate biomarker candidates derived from distinct hypotheses have been reported; see, for example, the integration of inflammatory biomarkers with neurotransmitter-related markers [32] and with neuroendocrine markers [33], or the integration between BDNF and neuroendocrine markers [34].

Within the immune-inflammatory hypothesis, a number of single biomarker investigations have contributed to the development of a 'cytokine hypothesis of depression' [35–37]. A detailed analysis of the cytokine network in serum was conducted in 2007 by Simon et al. [38], who exploited the availability of multiplex assays to simultaneously assess 20 cytokines in MDD patients and gender-matched controls. The study revealed the dysregulation of a number of cytokines and chemokines not previously implicated in depression, showing the potential of protein profiling by multiplex immunoassays. In 2006, we reported preliminary data from a large biomarker investigation based on protein profiling of a wider range of cytokines, chemokines and neurotrophins in both depression and schizophrenia [39]. The study, based on multianalyte profiling conducted with a panel developed by Rules Based Medicine Inc (RBM), is probably still the largest serum or plasma proteomic study performed on depressed patients so far. By profiling plasma samples from ca. 730 subjects (including 245 with MDD, 229 with schizophrenia and 254 healthy controls), we identified an informative set of analytes, including a number of cytokines, chemokines and neurotrophins potentially involved in the pathophysiological mechanisms of the two disorders [40]. The derived markers also permitted the discrimination of patient groups from controls with high specificity and sensitivity, illustrating the potential of peripheral protein profiling in large psychiatric collections. More recently, researchers from the ADNI (Alzheimer's Disease Neuroimaging Initiative) have exploited the availability of plasma biomarker data obtained by an extended multiplex immunoassay panel from RBM to look for correlations with depressive symptoms in the ADNI cohort [41]. The analysis of 146 plasma analytes from 566 participants resulted in the identification of a subset of proteins discriminating between subjects with and those without depressive symptoms among older adults with 80% accuracy, generating additional candidate biomarkers for depression.

The abovementioned studies have probably stimulated further exploration of protein profiling as a tool for identifying diagnostic signatures of psychiatric disorders. A series of studies have been conducted by Bahn and co-workers [42, 43], particularly on schizophrenia, resulting in the identification of a proteomic signature in serum which allows classification of schizophrenia versus other disorders or healthy controls. The signature, developed by using extended RBM panels such as DiscoveryMAP, led to the development of a 'laboratory-based diagnostic' for schizophrenia which was commercialized in the USA as VeriPsych™, currently under revision [44]. In depression, similar efforts to develop diagnostic biomarker signatures based on protein profiling have been reported. In 2013 Papakostas et al. [45] published their results from a multiassay, serum-based, biological biomarker diagnostic test conducted on two sets of samples with a total of 70 patients and 43 controls. The study confirmed the possibility of deriving a putative peripheral signature of depression by multiplex protein analyte assays of blood-derived material. The biomarker results were combined via an algorithm to generate the 'MDDScore', defining the probability of an individual to have MDD. The results are at the basis of the development of a laboratory blood test for MDD now commercialized in the USA by a neurodiagnostics company [46]. The identified panel is based on a small set of analytes (n = 9) selected among the pathways putatively involved in depression pathophysiology, resulting in a similar sensitivity to that of the 10 plasma marker subsets previously described [40]. Looking for consistency between the two sets of results, 5 out of the 10 analytes proposed by Papakostas et al. [45] were also investigated in plasma by Domenici et al. [40]. Only 2 of them showed consistent results in the two studies, whilst the remaining 3 markers (one of them BDNF) showed dysregulation in MDD

versus controls in an opposite direction. For BDNF, upregulation in the MDDScore panel is increasing the chance of having MDD – in contrast with evidence of a decreased BDNF suggested by most studies and meta-analyses published so far.

Do We Need Diagnostic Tests?

The above-described work addresses an important need in psychiatry, that is, the availability of objective measures to help in a better diagnosis of depression, and, as a potential follow-up, the use of biomarkers to assist clinicians in their selection of effective therapeutic treatments. The studies described also illustrate how challenging the task of identifying a set of robust biomarkers for MDD with potential diagnostic or clinical utility is. Differences in assay technology, sampling procedures as well patient cohorts – in terms of collection size, absence or presence of treatment, and accuracy of clinical diagnosis – are certainly playing a role in generating inconsistencies across different studies. However, it is probably the heterogeneous nature and course of depression that still makes the identification of reliable diagnostic biomarkers a chimera. Identifying putative diagnostic tests from case-control studies is not free of potential pitfalls. First of all, there is a high chance of stratification effects if patients and controls are recruited from different hospitals or centers, or if studies are dealing with inpatients versus externally recruited healthy controls – which may, for instance, explain at least in part the recurrent findings on inflammatory markers. Making no effort to control or account for diet and sampling time may lead to identification of markers simply resulting from abnormal food and sleep behaviors displayed by depressed patients, and not of disease markers directly related to pathophysiological mechanisms or associated with disease risk. Citing a little tale reported in a recent review on biomarkers for treatment-resistant depression [47], a policeman investigating a car collision occurring on a bright sunny day, and finding the cars' headlights broken, should not claim the destroyed headlights to be the cause of the accident.

Lastly, and more importantly, the 'diagnostic' value of peripheral biomarkers for depression cannot be confronted with objective diagnoses based on histological or instrumental assessment, as can be done in other therapeutic areas (not only with cancer, as the classical example, but also with other central nervous system disorders such as neurodegenerative ones). Indeed, developing diagnostic biomarkers starting from case-control studies defined by clinical diagnostic tools such as the DSM will inevitably result in tests with sensitivity and specificity inferior to those of a structured clinical interview. Claims that there are blood tests for diagnosing psychiatric disorders, supported by relatively small studies still in the process of validation, may have a potentially negative effect also in the clinical context, opening the door to false classification of healthy individuals and, possibly, overdiagnosis. But do we really need laboratory diagnostic tests for depression? Indeed, some of the tests described above reach a sensitivity and specificity of 80–90%, but similar or higher levels are actually achieved also by self-administered clinical questionnaires such as the Patient Health Questionnaire, which has both a specificity and sensitivity of 88%, and has been widely validated on thousands of patients [48].

Nevertheless, since peripheral biomarkers may potentially reflect underlying pathophysiology, they may find some application beyond simple diagnosis. As an example, molecular alterations revealed by protein profiling before the clinical manifestation of a disorder may help early therapeutic intervention [49]. Additionally, it has been shown that multiple analyte profiling can divide schizophrenia patients into distinct subgroups based on their predominant molecular ab-

normalities, and open the prospect of tailored therapeutic approaches targeting specific molecular mechanisms [50]. Similar application of proteomic profiling methods may, in the long run, facilitate the development of personalized medical approaches or more rational treatment selection in schizophrenia [51].

Reading the Coffee Grounds: Peripheral Biomarkers of Antidepressant Response

Perhaps one of the applications of peripheral proteomics with the highest potential for having an impact on clinical practice is the identification of signatures or biomarkers which may predict response to treatment [52]. This is an area where few or no foretelling tools are at hand for clinicians when choosing a therapeutic approach. At the same time, identifying more homogenous subsets of patients who better respond to a particular treatment would greatly facilitate the development of novel antidepressants by pharmaceutical companies.

Encouraging data come from schizophrenia research, where a multiple analyte panel-based signature from serum was shown to be able to provide information regarding treatment outcome, both in terms of response to antipsychotic medication and time to relapse [53]. For antidepressants, discovery efforts based on plasma or serum proteomics have been quite limited so far. Evidence that peripheral signatures of antidepressant response can be derived by large-scale approaches is mainly derived from gene expression profiling [54]. However, clinical networks have now been established to facilitate both pharmacogenomic and pharmacoproteomic discovery of biomarkers to predict antidepressant response predictors, such as the Canadian Biomarker Integration Network in Depression [55], the Munich Antidepressant Response Signature project [56, 57], the Early Medication Change trial [58] and the Genome-Based Therapeutic Drugs for Depression project [59]. The last is probably the first systematic approach combining genome-wide association, gene expression profiling and proteomic investigation of antidepressant response in a translational context. Results from the proteomic arm of the clinical investigation are still pending, but interesting results pointing to putative baseline predictors based on blood biomarkers have been reported [60, 61].

Future Peripheral Biomarker Research in Depression: A Menu Advice

We have provided a number of examples illustrating how protein profiling or proteome-wide approaches are opening up novel opportunities for the discovery of depression biomarkers. However, any real impact on drug development or clinical practice has yet to be gauged [62], since most investigations so far have focused on the search for markers which differentiate cases from controls or are associated with symptom severity, resulting in mere statistical associations between biological signatures and clinical diagnostic parameters.

In our opinion, efforts in finding peripheral biomarkers for depression would greatly benefit if they were pushed beyond cross-sectional studies in case-control collections as defined by diagnosis, in particular by focusing on the following objectives:

(1) Biomarkers for the classification of patients affected by depression into subtypes, potentially linked to endophenotypes [31]. In theory, the markers could be the basis for a novel 'molecular taxonomy' of depression, suggesting subtypes independent of symptoms. These biological markers or signatures could supplement clinical observations and help reducing the heterogeneity of disorders, with a potential impact on clinical practice,

should longitudinal studies reveal differential outcomes among subsets of patients

(2) Biomarkers predictive of response (or early response markers). Biological markers or signatures that, when measured at baseline or after a short treatment period, are associated with improved disease outcome using a particular therapeutic approach. They should not necessarily reflect disease severity but rather provide information about the trajectory of response to a therapeutic treatment ahead of improvement in clinical symptoms, opening up the opportunity for more rationalized or personalized therapies

(3) Biomarkers associated with the severity of (one of) the disease symptoms, which could offer a more objective evaluation of disease progression in drug development. Similarly to predictive biomarkers, they should be supported by mechanistic approaches based on the modulation of the drug target pathway. In perspective, they could also help in clinical practice if displaying sufficient sensitivity and 'stability' to supplement clinical symptom assessment with biological observation.

Still, the next revolution will arise from multidisciplinary approaches enabling an effective integration of biomarkers at different levels, from genetic markers over molecular ('-omic' and non'-omic' markers) to neurophysiological and instrumental parameters. The pathophysiology of neuropsychiatric disorders is likely due to dysregulation of complex biological pathways involving multiple, interacting gene products and their interaction with environmental factors. Therefore, integrative approaches linking the genetic make-up with intermediate phenotypes or endophenotypes (from gene to protein expression, neuroanatomical and neurophysiological data, and ultimately behavioral phenotypes) are the next step in generating novel biomarkers able to break down the disease and provide information regarding the effectiveness of therapeutic approaches. In a simple model, protein biomarker data could be integrated with genetic information to take into account the effect of genetic variation in protein expression or protein quantitative trait loci [63]. This approach has been used for BDNF in order to understand genetic control of its concentration in serum as an example [64], but genome-wide data based on multiple protein panels or proteomics have shown that common genetic variation influences levels of many clinically relevant proteins in human serum and plasma [65–68]. Likewise, the interaction between genetic and epigenetic variation (or epigenetic genotype-dependent effects) could further modulate disease marker expression, as has also been shown for serum BDNF [69]. These results are calling for the implementation of integrative approaches able to capture the contribution of genetic variability to protein levels and to uncover disease-associated mechanisms underlying depression biomarkers. Finally, combination of 'distal biomarkers', such as peripheral signatures, with more 'proximal biomarkers', such as imaging measures of brain structure and function, will be fundamental to elucidate the circuits involved in disease pathophysiology and drug response [70]. Neuroimaging biomarkers have already shown their potential as response predictors in depression [71, 72], and they can provide the putative endophenotypic measure on the causal path of a biological response to antidepressant therapy, connecting peripheral biomarkers with clinical outcome.

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Proteomics of Preclinical Models of Depression

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Abstract

Major depressive disorder (MDD) is a widespread and disabling disease whose aetiology and pathophysiological basis are still incompletely understood. In this review, hypothesis-free proteomic analyses carried out on animal models of MDD to study the neurobiological correlates of MDD are described and discussed. Most proteomic investigations rely on the induction of depression-like behaviours via exposure to stressful experiences, similarly to known mechanisms of MDD occurrence. Animal models included exposure to chronic corticosterone treatment, prenatal restraint stress, early-life stress, chronic mild stress, restraint stress and social defeat, as well as the induction of learned helplessness and analysis of genetically selected strains. In other instances, models of anxiety behaviours were examined, since anxiety disorders are often associated with MDD. Although proteomic analyses made use of different technologies, in most instances protein separation was achieved by means of gel electrophoresis. The majority of the studies were performed on brain regions allegedly affected in MDD patients, such as the hippocampus, prefrontal cortex, amygdala and hypothalamus. Proteins connected to different biochemical functions were modulated in the distinct animal models and brain regions, while, in some cases, the comprehensive impact on signalling pathways was evaluated as well. Globally, a large number of different proteins were identified in the proteomic experiments, although a methodological bias for water-soluble abundant proteins should be appreciated. Subsequent approaches should be adopted to validate, interpret and exploit more extensively the vast amount of information produced. The results summarised in this review provide potential new avenues for the investigation of the molecular neurobiology of MDD.

Introduction

Major depressive disorder (MDD) is a widespread, severe and disabling disease exerting a heavy toll of morbidity and mortality [1, 2]. Its main symptoms include depressed mood, diminished interest or pleasure in most activities, modifications in appetite and sleeping, psychomotor agitation or retardation, fatigue or diminished ability to concentrate, feelings of worthlessness, recurrent thoughts of death or suicide. Anxiety disorders and MDD are frequently comorbid [2]. The disease was recognised since antiquity, but the aetiology and pathophysiological changes

triggering MDD are still incompletely understood. Several hypotheses about the molecular mechanisms underlying MDD have been proposed based on clinical and preclinical investigations, but none of them were conclusively demonstrated [3, 4]. Accumulating evidence suggests that both genetic and environmental components are needed to bring about MDD episodes, with the genetic contribution relying on many genes. Among environmental components, stress is believed to be a major player [2, 5, 6]. Comprehension of the neurobiological correlates of MDD would greatly increase our understanding of the physiology of brain functioning and the likelihood of discovering new therapies; for this reason, system-based approaches were implemented to provide a contribution.

Animal Models of MDD

Animal models of MDD are thus investigated to study the molecular neurobiological correlates of the disease; to this aim, they need to be endowed with construct validity. Moreover, animal models are expected to aid the development of more effective antidepressants (AD); to reach this objective, they should be endowed with predictive validity. In particular, proteomic studies of animal models of MDD adopt a hypothesis-free approach to gain more understanding of the molecular mechanism of MDD. Due to the poor understanding of the disease pathophysiology, animal models of MDD are not completely satisfying [7, 8]. Nevertheless, they successfully reproduce at least some MDD symptoms such as states of social withdrawal, anhedonia and dysfunctions of the hypothalamic-pituitary-adrenal (HPA) axis and circadian rhythm. Some of these changes are amenable to responding to AD treatment, providing confidence that available animal models may offer useful, albeit incomplete, information about the neurobiological basis of MDD.

Animal Models Investigated in Proteomic Studies

A list of studies that investigated animal models of MDD by proteomic approaches is displayed in table 1. The animal models here considered mainly rely on the induction of depression-like behaviours. In most instances, the behaviours are brought about by exposure to a stressful experience, in analogy to known mechanisms of MDD occurrence [6]. In other cases, models of anxiety behaviours were investigated, since anxiety disorders are often associated with MDD. Specifically, models based on chronic corticosterone treatment aim at reproducing the hyperactivity of the HPA axis displayed by a large number of depressive patients, to investigate the correlated molecular adaptations in the brain that may play a role in MDD pathophysiology [34]. Linked to the long-term effects of inappropriate activation of the HPA axis is the prenatal restraint stress model. Several long-lasting abnormalities observed in rats exposed to prenatal restraint stress parallel those found in MDD patients, possibly because they reproduce environmental factors implicated in the aetiology of the disease. It is supposed that the permanent consequences observed in this model are due to corticosterone effects on hippocampal plasticity in offspring and altered maternal behaviours contributing via epigenetic modifications [35].

Compelling evidence originating from many clinical investigations suggests that early-life stress constitutes a major risk factor for the development of MDD in adult life [5]. Therefore, investigators devised different protocols of reduced maternal care that model this environmental challenge in experimental animals, inducing long-lasting behavioural effects in analogy with human experience [36, 37]. Proteomic analyses were carried out to gain insight into the molecular correlates associated with long-term effects of early-life inadequate care. With the objective of reproducing the combination between genetic and envi-

Table 1. Proteomic studies of animal models of MDD

Species	Sex	Model	Brain region	Method	Reference No.
CD1 mice	male	chronic corticosterone treatment	hypothalamus, hippocampus, cortex	DIGE	9
Sprague-Dawley rats	male	prenatal stress	hippocampus	2-DE	10
C57BL/6J mice	male	early-life neglect	prefrontal cortex (medial)	label-free proteomics	11
Sprague-Dawley rats	male	maternal separation	hippocampus (ventral)	2-DE	12
Flinders Sensitive Line rats	male	maternal separation	hippocampus, prefrontal cortex	2-DE	13
C57BL/6J, FVB/NJ mice	n.a.	maternal separation, chronic mild stress	hippocampus	2-DE, TMT	14
Wistar rats	male	chronic mild stress	hippocampus (ventral)	DIGE	15
Wistar rats	male	chronic mild stress	hippocampus (ventral)	DIGE	16
Wistar rats	male	chronic mild stress	hippocampus (CA1, CA3)	iTRAQ	17
Wistar rats	male	chronic mild stress	hippocampus (DG)	2-DE	18
Sprague-Dawley rats	male	chronic mild stress	hippocampus	2-DE	19
Sprague-Dawley rats	male	chronic mild stress	prefrontal cortex	2-DE	20
Sprague-Dawley rats	male	chronic mild stress	hippocampus (Syn)	2-DE	21
BALB/c mice	male	chronic mild stress	whole brain	DIGE	22
Zebrafish	both	chronic mild stress	whole brain	2-DE	23
Sprague-Dawley rats	male	restraint stress	whole brain	DIGE	24
Sprague-Dawley rats	male	learned helplessness	hippocampus, prefrontal cortex (Syn)	2-DE	25
Sprague-Dawley rats	male	social defeat	hippocampus	2-DE	26
Inbred mouse strains	male	AX vs. NAX (anxiety behaviours)	whole brain	DIGE	27
CD1 mice	male	HAB vs. LAB (anxiety behaviours)	hypothalamus, amygdala, motor cortex	2-DE	28, 29
CD1 mice	male	HAB vs. NAB; LAB vs. NAB (anxiety behaviours)	cingulate cortex (Syn)	^{15}N metabolic labelling	30
CD1 mice	male	HAB vs. NAB; LAB vs. NAB (anxiety behaviours)	hippocampus (cytosol, microsomes), plasma	^{15}N metabolic labelling	31
CD1 mice	male	^{15}N HAB vs. ^{14}N HAB (depressive behaviours)	cingulate cortex (Syn), hippocampus, cerebrum	^{15}N metabolic labelling	32
C57BL/6N mice	male	Mg-restricted diet	amygdala-hypothalamus	2-DE	33

n.a. = Information not available; AX/NAX = high/low anxiety; HAB/LAB = high-/low-anxiety-related behaviour; NAB = normal anxiety-related behaviour; CA = cornu ammonis; DG = dentate gyrus; Syn = synaptosomes; DIGE = difference gel electrophoresis; 2-DE = two-dimensional gel electrophoresis; TMT = tandem mass tagging; iTRAQ = isobaric tags for relative and absolute quantitation.

ronmental challenges, the stress of maternal separation (MS) was applied to a genetically selected rat strain which displays behavioural and neurochemical features similar to those of depressive patients, the Flinders Sensitive Line (FSL) rat [38]. In addition, animal models based on the repeated exposure to mild but unpredictable stressful events (chronic mild stress, CMS) during adult life intend to induce anhedonia, a loss of responsiveness to pleasant events that is a core symptom of MDD, measured in animals as reduction in drinking of palatable sweet solutions [39]. The repeated application of restraint stress intends to reach the same objective of overstimulating the animal stress response, causing an anhedonic response [34]. In the learned helplessness model, exposure to uncontrolled and unpredictable adverse events (usually mild electroshocks) causes an escape deficit in a subset of susceptible animals. This model is endowed with construct, face and predictive validity as it derives from the cognitive theory of MDD, postulating inappropriate adaptations to life experiences, it decreases sensitivity and responsiveness to rewards, and it responds to chronic but not acute AD administration [40]. The stress applied in the social defeat model strives to reproduce the finding that the majority of stress stimuli that lead to psychopathology in humans are of a social nature, thus increasing construct validity. In this model, male experimental animals are introduced into the territory of an aggressive male conspecific which attacks and defeats them. Social defeat exposure induces long-lasting behavioural and endocrine effects [41].

Since anxiety disorders are highly comorbid with MDD, the investigation of the neurobiological basis for anxiety-like behaviours may bear relevance to the study of the molecular correlates of MDD. High- (HAB) and low-anxiety-behaviour (LAB) mice were generated by selective breeding of the animals displaying the more extreme phenotypes of the specific trait [42].

Other proteomic studies employed treatments with clinically efficacious AD to gain insight into the molecular pathways that they affect, reasonably assuming that these pathways may play a role in the pathophysiology of MDD. Apart from those that also include an MDD model and are therefore included in the studies quoted in table 1, they will not be further discussed in this review, but they were summarised by Carboni et al. [43], with more recent additions [44–51].

When investigations were not performed on the whole brain, possibly to maintain – as much as possible – an open view, they focussed on regions that are reported to be affected in MDD patients or are supposed to play a role in the disease or its associated symptoms, such as the hippocampus, prefrontal cortex, amygdala and hypothalamus (table 1). Some authors specifically focussed on subcellular fractioning, especially regarding synaptic terminals, or examined specific subregions considered more relevant to the disease (table 1).

Proteomic Methods Used in MDD Model Investigations

Proteomic technologies share the objective of analysing a large number of proteins at the same time. Expression proteomics experiments compare the level of protein expression in two (or more) experimental groups to identify the modulations induced by a treatment. Different methods are adopted to overcome the many technological hurdles due to the distinct biochemical properties of proteins, further modified by posttranslational modifications, and wide concentration spans. In general, proteomic analyses avail of gel-based or chromatography-based techniques to separate individual proteins and may or may not utilise labelling approaches to aid comparisons among groups. Each technology displays specific weaknesses and strengths, which are discussed by Filiou et al. [52, 53], Drabik et al. [54] and Craft et al. [55] in neuroscience applications. In MDD model investigations, protein separation is achieved in most instances by means of gel electrophoresis

(table 1). In two-dimensional gel electrophoresis [56–58], proteins are separated by isoelectric point in the first dimension and by molecular mass in the second dimension. Gels are stained and protein amounts are compared by image analysis. This classical method allows a good resolution of complex protein mixtures, although it is biased for hydrophilic abundant proteins and exhibits high technical variability. Difference gel electrophoresis [59, 60] avails of the same basic principle, but sample group comparisons are eased by reducing the inherent variability of two-dimensional gel electrophoresis via differential labelling with fluorescent dyes, thus allowing comparisons within the same sample and eliminating the need for subsequent staining. In both cases, protein identification is achieved by mass-spectrometric techniques, usually by peptide mass fingerprinting after proteolytic digestion. Gel-free methods rely on mass-spectrometric quantifications after chromatographic separations. In label-free quantitative proteomic analysis, sample quantification is based on the principle that protein concentration is proportional to peak areas of derived peptides. In contrast, the iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tagging) methodologies are based on differential sample labelling that generates reporter ions of unique masses upon tandem mass spectrometry fragmentation to be used for quantification. Approaches based on labelling strategies are more accurate in than label-free methods, but they are more expensive and complex [60]. The labelling can also be carried out in vivo, thereby minimising the introduction of biased experimental error, although at the expense of increased experimental complexity [61].

Stress-Related Models

Chronic Corticosterone Treatment

Skynner et al. [9] modelled the effect of the high glucocorticoid hormone levels experienced by many MDD patients by chronic corticosterone administration to mice. In addition to reduced hippocampal neurogenesis, the authors observed altered expression of 150 protein spots in the hypothalamus, hippocampus and cerebral cortex, some of which the brain regions had in common. Pathway analysis was carried out on identified proteins to map which biochemical functions were significantly affected by the modulation. Three major pathways were modified by corticosterone treatment across the three brain regions, including cell death, cellular assembly and organisation and cell morphology. Among canonical signalling pathways, glycolysis, gluconeogenesis, citrate cycle, phenylalanine metabolism, glutamate metabolism and nitrogen metabolism were affected by treatment. Interaction networks built with the Ingenuity Pathways Knowledge Base suggested that modulated proteins differently affected cellular functions in each brain region [9].

Prenatal Stress Exposure

Long-lasting effects on hippocampal protein levels induced by prenatal exposure to restraint stress were investigated by Mairesse et al. [10]. Indeed, prenatal stress altered the hippocampal proteome of adult animals, and the densities of 24 spots were significantly different from those in control rats, mostly in the direction of downregulation. After identification, the proteins were sorted into groups based on their biological function, with energetic metabolism being the most populated, followed by cytoskeleton dynamics, protein synthesis, synaptic vesicles and signal transduction [10].

Early-Life Stress

Since early-life stress is associated with increased risk of developing MDD, several investigators exploited MS protocols to evaluate the molecular changes occurring as a consequence in adult life. Bordner et al. [11] discovered that most protein level alterations observed in murine prefrontal cortex were in the direction of downregulation (37

out of 46), differently from mRNA changes. Changed proteins mainly belonged to signalling pathways, metabolism, histones and neuronal markers. Among the modulated proteins, the authors highlighted the identification of myelin basic protein, since dysregulations of myelin-related pathways emerged also from transcriptomic analysis and was confirmed in multiple reaction monitoring proteomic analyses [11]. Marais et al. [12] analysed rat ventral hippocampi after exposure to MS, finding 37 modulated proteins, quite evenly split between up- and downregulated ones. They chiefly belonged to heat-shock proteins, nucleotide metabolism, carboxylic acid metabolism and glycolysis-gluconeogenesis. Proteins altered by subsequent treatment with the AD escitalopram did not overlap, suggesting that its therapeutic effects are achieved by modulating different targets [12]. MS as an environmental challenge was also employed by Piubelli et al. [13], who applied this procedure to FSL rats, a genetically selected strain displaying depression-like behaviours, to investigate molecular correlates of gene-environment interactions. FSL rats were firstly compared with the respective controls belonging to the Flinders Resistant Line: 43 differently expressed proteins were detected in the hippocampus, and 145 in the prefrontal cortex, mainly influencing proteins involved in energy and macromolecule metabolism, establishment of cellular localisation, intracellular transport, cytoskeleton organisation and apoptosis. The exposure to MS stress modulated a different number of proteins depending on the strain and brain region; the identification confirmed that non-overlapping changes were induced depending on the genetic background, in particular with cytoskeleton organisation and regulation of neuron morphogenesis modulated in FSL, and energy metabolism, cellular localisation, neuron differentiation and intracellular transport in Flinders Resistant Line rats [13]. MS together with CMS were utilised as MDD models, followed by AD treatment, in an effort to identify biomarkers for drug response to support individualised prescription of AD [14]. In stressed versus control groups, multivariate statistical analyses identified 11 proteins whose levels were more heavily affected by MS exposure. Among them, the prevalent functions were related to protein phosphorylation and monosaccharide metabolism [14].

Chronic Mild Stress

The CMS model was studied with proteomic technologies by several investigators. Bisgaard et al. [15, 16] analysed this model per se and after AD treatment to identify proteins involved in susceptibility to stress-induced anhedonia and in AD response. In the first study [15], 5 proteins identified as stress resilience markers were classified as axon guidance proteins, cytoskeleton proteins, neurotransmitter release proteins and molecular chaperones. In a subsequent study [16], proteins associated with the anhedonia-like response were successfully identified and attributed to cytoskeleton, mitochondria, immune and vesicle-mediated transport proteins. In the same model, a specific analysis of the cornu ammonis hippocampal subregion performed with a different technique [17] revealed that 22 proteins were differentially regulated between the control and anhedonic rats; 32 proteins were modulated in the comparison between resilient and anhedonic rats, and 19 proteins were altered in the comparison between resilient and control rats. The authors propose that sodium channel SCN9A upregulation in stress-susceptible rats may be a novel biomarker of MDD, and that the conservation of vesicular functioning is central for stress coping strategies [17]. The same MDD model was analysed by Kedracka-Krok et al. [18] with the objective of examining the mechanism of action of AD by comparing different modes of administration. In the comparison between a control and a CMS groups, 12 proteins had detectable changes in their levels, belonging to: neuronal vesicular cell trafficking and synaptic plasticity; signalling; regulation of transcription and translation; and protein degradation processes [18]. In a different

rat strain, an analysis of hippocampal proteins of animals exposed to CMS revealed that 15 proteins were differentially expressed [19]. The proteins identified belong to the functional categories of neurogenesis, oxidative metabolism, transcription and signal transduction [19]. In a study performed on prefrontal cortices [20], 29 differential proteins were identified between stressed and control rats, and they were analysed for enrichment in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways to obtain functional insights into the differences between proteomes. The energy-metabolic pathways were the pathways represented most often: glycolysis/gluconeogenesis, tricarboxylic acid cycle, oxidative phosphorylation and pyruvate metabolism. Glycine, serine and threonine metabolism pathways were also represented, together with the Alzheimer's disease pathway [20]. To gather specific information on the impact of CMS on synaptic proteins, Hu et al. [21] analysed proteomic differences in hippocampal synaptosomes. Nineteen differentially expressed proteins in the CMS group, as compared with the control group, were successfully identified. Among them, 5 were upregulated; 11 proteins localised to the presynaptic terminal and 17 to the postsynaptic terminal, showing a high degree of overlap. Regarding their molecular functions, the modulated proteins were classified into groups of synaptic exocytosis, synaptic endocytosis, molecular chaperoning, cytoskeleton and energy metabolism [21].

In mice, proteomic analysis of whole brains was carried out on 7 different end points to gain insight into the development of depression-like behaviours [22]. There were 25, 31, 26, 26, 24, 29 and 27 significantly changed spots at days 4 and 10 and weeks 3, 4, 6, 8 and 10, respectively. It was observed that an abnormal expression of proteins involved in energy metabolism was firstly witnessed and ran throughout the whole experimental period, possibly reflecting abnormal energy mobilisation coping with the stressful situation. Several proteins related to stress and antioxidants were consistently increased, whereas cytoskeleton-related proteins were continuously declined [22]. Finally, a protocol of CMS was established in zebrafish and a proteomic approach was used to uncover brain molecular markers [23]. A total of 18 differentially regulated proteins were identified in zebrafish brain tissue. The modulated proteins are associated with small molecule metabolism, phosphocreatine metabolism and nucleotide metabolism; most of them are regulators of mitochondrial function, glycolysis and oxidative stress [23].

A proteomic analysis performed on whole brains showed that exposure to chronic restraint stress triggered significant modulation of 5 protein spots, including the hippocampal cholinergic neurostimulating peptide precursor and the α_7-nicotinic receptor [24].

Learned Helplessness

Mallei et al. [25] investigated the learned helplessness model of MDD by comparison with respective non-susceptible controls after subcellular fractionation to obtain synaptic terminal enrichment. AD treatment was also carried out. In the comparison between learned helplessness and non-learned helplessness rats, 11 and 5 differentially expressed proteins were identified in the hippocampus and prefrontal cortex, respectively, belonging to the pathways of lipid metabolism, nucleic acid metabolism, small molecule biochemistry, cellular assembly and organisation, and cellular function and maintenance [25].

Social Defeat

The exposure to single versus repeated social defeats differently affected the hippocampal proteome: 8 versus 21 modulated spots were identified after single versus repeated exposure [26]. Proteins modulated by repeated exposure to social stress are associated with the cellular functions of protein folding, signal transduction, synaptic plasticity, cytoskeleton regulation and energy metabolism, which were not activated after single stress exposure [26].

Anxiety Models

Szego et al. [27] analysed an inbred anxiety mouse model by comparing the brain proteome with that of normal, non-anxious mice. In the anxiety strain, 82 proteins showed significantly different concentrations; the modulated proteins belonged to different functional groups, including synaptic transmission, carbohydrate metabolism, amino acid metabolism and proteolysis, nucleotide metabolism, haem and lipid metabolism, protein biosynthesis and folding, cytoskeletal proteins, brain development and neurogenesis, oxidative stress and signal transduction [27].

In the comparison between HAB and LAB mice, proteomic investigations carried out with gel-based technologies allowed the identification of protein markers of anxiety including glyoxalase I and enolase phosphatase [28, 29]. In subsequent experiments carried out with in vivo metabolic labelling, an analysis performed on synaptic terminals of the cingulate cortex demonstrated that 264 proteins were differentially expressed at least 1.3-fold between the HAB and LAB groups [30]. Oxidative phosphorylation, metabolic processes and fatty acid metabolism were overrepresented among the proteins with increased changes in the HAB mice, whereas overrepresentation of pathways involved in antioxidant defence was observed among the proteins with increased changes in the LAB mice [30]. In a study on hippocampi [31], 312 and 206 proteins were found to be differentially expressed in the cytosolic and microsome fractions, respectively. Investigation of the pathway enrichments showed that proteins in the phosphatidylinositol signalling system, ubiquitin-specific protease activity, proteins relevant to long-term potentiation, oxidative stress proteins and energy metabolic proteins were differentially expressed in the HAB and LAB mice [31].

Surprisingly, in vivo labelling with ^{15}N induced an AD-like effect on the behavioural phenotype of HAB mice, prompting the investigation of its molecular correlates by proteomic technologies [32]. Altered proteins mainly belonged to major metabolic pathways such as the tricarboxylic acid cycle, oxidative phosphorylation, and glyoxylate and bicarboxylate metabolism [32].

Other Models

Whittle et al. [33] investigated the molecular alterations associated with exposure to an Mg^{2+}-restricted diet, since experimental reduction in Mg^{2+} levels is known to elicit depression-like behaviours in rodents. AD treatment was also performed. In a brain area including both the amygdala and the hypothalamus, exposure to Mg-restricted diet induced the alteration of 4 proteins involved in nitric oxide signalling, oxidative stress and energy metabolisms [33].

Concluding Remarks

Although several different experimental models were adopted to investigate the molecular correlates of MDD by proteomic approaches, it emerges that the majority of them rely on stress exposure, a well-recognised factor triggering MDD. Globally, a large number of different proteins were identified in the proteomic experiments, although a methodological bias for water-soluble abundant proteins should be appreciated. The subsequent steps should be to take new approaches to interpreting and exploiting more extensively the vast amount of information produced in large-scale experiments. The results summarised in this review provide potential new avenues to be explored in investigating the molecular mechanisms of MDD which will need validation in different animal models and, ultimately, in MDD patients.

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Antidepressant Response Metabonomics

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Abstract

Depression is a serious common mental disease and complex psychiatric illness often associated with stressful events. Metabonomics, which has been applied in evaluation of mechanisms of depression and therapeutic effects of antidepressants, is a systematic study of the endogenous, small-molecule metabolites involved in specific biological processes that provides an assessment of the physiological status of an organism. Biomarkers, surrogate endpoints that are intended to substitute a clinical endpoint, can aid in staging and classification of the extent of a disease, allowing for disease stratification, which is the basis of personalized medicine. During the past several years, a deeper understanding of the metabonomics of depression has been achieved. Studies on the classification and finding of biomarkers were performed with urine, plasma and tissues, using gas chromatography-mass spectrometry (MS), liquid chromatography-MS and nuclear magnetic resonance. Recent research results have suggested that several classes of physiological biomarkers, including amino acids, fatty acid and glycometabolism, might be useful for predicting depression and the response to antidepressants. Once such biomarkers will be validated, they could form the basis of new paradigms for antidepressant treatment selection.

Depression

Depression is a complex epidemiological psychiatric illness which is often associated with stressful events and leads to low morale, weight loss and anhedonia. It is a major cause of disability, suicide and physical disorders [1]. It amounts to 12.3% of the global burden of disease, predicted to rise up to 15% by 2020 [2]. The etiology of depression is not fully understood so far, and may involve changes in the nervous, immunological and endocrine systems. It has been considered a systemic disorder caused by impairment of different biochemical pathways such as serotonin (5-HT), kynurenine (KYN), tricarboxylic acid (TCA), etc [3]. The etiology of the condition leads to a difficulty in the diagnosis of depression and in evaluating antidepressant effects of diverse drugs. In order to study clinical depression better, metabonomics has been widely used in depression research.

Antidepressants

Antidepressants are drugs used for the treatment of major depressive disorder and other conditions. There are many chemical medicines such as

venlafaxine hydrochloride and sertraline which are widely used for the treatment of depression. However, many synthetic chemical antidepressants are not satisfactory as there is often a variety of side effects. Therefore, more and more new products for the treatment of depression which are derived from traditional Chinese medicine (TCM) are drawing ever-increasing attention worldwide, and several TCM are used for depression. In the following, we list several commonly available chemical antidepressants and TCM.

Chemical Antidepressants

The general classes of antidepressant medicines are selective serotonin reuptake inhibitors (SSRI), serotonin-norepinephrine reuptake inhibitors (SNRI), tricyclic antidepressants, monoamine oxidase inhibitors and other drugs. SSRI and SNRI are the most commonly prescribed antidepressant medications for the treatment of depression [4–6]. The following are the details of several antidepressants.

Venlafaxine Hydrochloride

Venlafaxine hydrochloride is an antidepressant which is a selective SNRI and slightly inhibits reuptake of dopamine, without significant affinity for muscarinic, histaminergic or adrenergic receptors. It is the first inhibitor that inhibits the reuptake of both 5-HT and norepinephrine. Because of its unique pharmacological properties, venlafaxine hydrochloride provides a positive clinical efficacy that is comparable with or superior to that of tricyclic antidepressants and SSRI.

Sertraline

Sertraline is an antidepressant of the SSRI class. Sertraline is primarily used to treat major depression in adult outpatients as well as obsessive-compulsive, panic and social anxiety disorders in both adults and children. The efficacy of sertraline in depression is similar to that of older antidepressants, but its side effects are much less pronounced.

Fluoxetine Hydrochloride

Fluoxetine hydrochloride (Prozac) is an SSRI used to treat depression, obsessive-compulsive disorder, etc. It works by increasing the amount of serotonin, a natural substance in the brain that helps maintain mental balance. It was used to relieve the symptoms of premenstrual dysphoric disorder, including mood swings, irritability and bloating.

Traditional Chinese Medicine

TCM is a holistic approach to health that attempts to bring the body, mind and spirit into harmony. Recently, many Chinese herbs such as *Hypericum perforatum*, radix bupleurum and morinda have been found by modern pharmacological studies to have good antidepressant effects [7]. In addition, there are several TCM which have been commonly recognized as safe and effective prescriptions in the treatment of various depressive disorders.

Xiao Yao San

Xiao yao san (XYS) is a famous TCM prescription with a long history of use in clinical settings, containing the following 8 herbal medicines: radix bupleuri, radix angelicae sinensis, atractylodes macrocephala, radix paeoniae albae, poria cocos, radix glycyrrhizae, menthae haplocalycis herba and rhizoma zingiberis recens. From TCM experience, XYS exerts various actions, including soothing the liver, improving the circulation of qi to relieve depression. In China, it has been commonly recognized as a safe and effective prescription in the treatment of depressive disorder. Studies have reported that XYS showed significant antidepressant effects in decreasing immobility in the tail suspension and forced swimming tests [8].

Dang Gui Shao Yao San

Dang gui shao yao san (DSS), a famous Chinese compound prescription, was recorded for the first time in *Jin Kui Yao Lue* (a famous ancient Chinese medicine book, published early in the 3rd century AD). It consists of 6 Chinese herbs:

radix angelicae sinensis, radix paeoniae albae, rhizoma chuanxiong, rhizoma atractylodis macrocephalae, poria and rhizoma alismatis. DSS has been widely used in China in the treatment of various kinds of diseases such as to improve hemorrheology, inhibit platelet aggregation, regulate the hypothalamic-pituitary-endocrine system and affect immune-neuroendocrine functions [9, 10]. Previous studies proved that DSS could enhance the memory of normal mice and improve the memory deficits in a mouse model by chemical substances, ovariectomy or ischemia-reperfusion [11]. In recent years there have been many reports about its effect on depression.

Chai Hu Shu Gan San

Chai hu shu gan san (CSGS), which contains 7 Chinese herbs, is one of the most widely used TCM formulas for treating depression clinically in China [12]. A metabonomics study suggested that the antidepressant effect of CSGS could involve regulating dysfunctions of multiple metabolic pathways [13]. Though these antidepressants are common drugs for depression, better drugs need to be found, because the effects of those drugs are not satisfactory to patients. What is more, choosing and evaluating effects of diverse antidepressants is still difficult, even with the help of models of depression [14, 15].

Models of Depression

Behavioral Tests

Behavioral tests can be used as an effective way to evaluate the validity of a model. Generally, the open field test and the sucrose preference test are conducted in the field of depression.

Open Field Test

The open field test was performed as usual to measure spontaneous activity in rats. Briefly, an apparatus consisting of a square arena sized 100 × 100 × 40 cm was divided into equal 25 × 25 cm squares on the floor of the arena. A single rat was placed in the center of the cage, and after 30 s of adaptation, the number of locomotor activities, the number of rears and the frequency of grooming were counted manually for 5 min [16].

Sucrose Preference Test

Rats underwent the sucrose preference test to evaluate their activity. The rats were trained to adapt to 1% sucrose solution (w/v) before the test; after adaptation, they were deprived of water and food for 24 h, followed by the sucrose preference test, in which rats housed in individual cages for 24 h had free access to 2 bottles, with 1 containing 1% sucrose and the other tap water. The bottles were counterbalanced across the left and right sides of the cages throughout the experiment. After 24 h, sucrose and water consumption was measured (in milliliters) and sucrose preference was calculated as sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption) × 100%.

Animal Models

Animal models are often used to study the pathogenesis of depression, which in animals expresses symptoms similar to those in humans.

Chronic Unpredictable Mild Stress

Chronic unpredictable mild stress (CUMS) is one of the most common antidepressant screening models based on its mimicking of several symptoms of human depression. Many behavioral and biochemical changes induced by CUMS are reversible by antidepressant treatments. Meanwhile, the CUMS model of depression has good face validity, construct validity and predictive validity, which makes it suitable for investigating the pathophysiology of depression and the antidepressant effects of diverse drugs [17]. Animals in a CUMS model group were individually housed and repeatedly exposed to a set of CUM stressors, as follows: cage tilting and damp sawdust; noises; swimming in 4°C cold water; exposure to an ex-

perimental room at 50°C; food and water deprivation; tail clamp; unpredictable shocks; and restricted movement. Gao et al. [18] investigated the antidepressant effects of XYS in the CUMS depression model, and the results showed that the CUMS model is reliable, stabile and repeatable.

Chronic Variable Stress

The chronic variable stress (CVS) model works relatively well as a depression model and it is widely used preclinically for evaluation of antidepressant activity; the series of variable stimuli includes immobilization, swimming, withholding food, thermal stimuli, withholding water intake, electric shock to the pelma and noise stimuli. Su et al [13] used the CVS model to investigate the antidepressive effect of CSGS; the results suggested that the therapeutic effect of CSGS on depression may involve regulating dysfunctions of energy metabolism and tryptophan metabolism, bone loss and liver detoxification.

There are many other models, such as the tail suspension test, forced swimming test (FST), used for the study of depression in addition to the models above.

Metabonomics and Methods

Metabonomics

Metabonomics is concerned with both targeted and nontargeted analysis of endogenous and exogenous small-molecule metabolites and presents a promising tool for biomarker discovery. It has been used in assessing responses to environmental stress, comparing mutants, drug discovery, toxicology and nutrition, studying global effects of genetic manipulation, cancer, comparing different growth stages, diabetes and natural product discovery. It is a global metabolic profiling framework which utilizes high-resolution analytics to derive an integrated picture of both endogenous and xenobiotic metabolism [19]. Metabolites are biological characteristics that are objectively measured and evaluated as indicators of normal biological and pathological processes or pharmacological responses to a therapeutic intervention, widely used in clinical practice for clinical diagnosis [20]. Metabonomics is mainly used on the level of small-molecule biomarkers including peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids and inorganic species, which represent the functional phenotype in a cell, tissue or organism. Separation and identification of these small molecules is made possible by the technological advances in metabolomics. These innovational technologies, including accurate measurement by high-resolution mass spectrometry (MS), nuclear magnetic resonance (NMR), capillary electrophoresis, high-performance liquid chromatography (HPLC) and ultra-performance LC (UPLC) technology, can accomplish detection of metabolites within a few minutes. Measuring such low-molecular-weight metabolites could offer deeper insights into mechanisms of influence by lifestyle and dietary factors with regard to specific diseases.

Methods

Nuclear Magnetic Resonance

As one of the most common spectroscopic analytical techniques, NMR can uniquely identify and simultaneously quantify a wide range of organic compounds in the micromolar range. NMR has been introduced to the emerging field of metabonomics where it can provide unbiased information about metabolite profiles. Conventionally, within the field of metabolomics of biofluids, NMR has been the technique of choice, due to its ability to measure intact biomaterials nondestructively as well as the rich structural information that can be obtained. Hence, extensive research and significant improvements have been carried out using NMR to measure populations of low-molecular-weight metabolites in biological samples. A number of biofluids such as blood, urine, cerebrospinal fluid, cell culture media and many others can be obtained at a high sampling

frequency with minimal invasion, permitting detailed characterization of dynamic metabolic events [21]. NMR can provide detailed information regarding the structural transformation of a compound as a consequence of metabolism in drug discovery and development [22].

Generally, plasma, urine or tissue homogenate samples are collected in heparinized tubes and centrifuged at low speed, such as 3,500 rpm, for 10 min. Then, the samples are mixed with D_2O, CD_3OD, etc. and centrifuged at high speed, such as 14,000 rpm, for 10 min. The supernatants are transferred to an NMR tube for analysis.

Recently, there has been much interest in the use of high-throughput NMR techniques for the detection of biomarkers [23–25]. NMR has been thoroughly tested, and applied to identify plasma and urine biomarkers for XYS; samples were analyzed by NMR-based metabonomics combined with multivariate statistics.

Gas Chromatography-Mass Spectrometry

The goal of metabonomic analysis is systematic understanding of all metabolites in biological samples. Many useful platforms have been developed to achieve this goal. Currently, as a core analytical method for metabonomics, gas chromatography (GC)-MS has been used as a platform in nontargeted analysis, especially for hydrophilic metabolites [26]. Generally, GC-MS-based metabonomics requires a high-throughput technology to handle a large volume of samples and accurate peak identification through the standard retention times and mass spectra. GC-MS has been widely used for metabonomics and can provide efficient and reproducible analyses [18, 27–29]. A plasma metabonomics method based on GC-MS was developed, and 9 key metabolites were considered potential biomarkers [18].

For separation on the GC column, GC-MS requires a derivatization reaction to create volatile compounds. Nonvolatile compounds are not derivatized and will not be detected in a GC-MS analysis. Using this approach, the volatile metabolites can be directly separated and quantified by GC-MS, and it is also possible to simultaneously profile several hundreds of compounds. Resum samples are collected by centrifugation at 5,000 rpm for 10 min to remove residues. When samples are analyzed by GC-MS, they are first centrifuged to precipitate protein by using methanol or other reagents, and then chemically derivatized to increase the volatility and thermal stability; several common derivatization reactions such as silylation, alkylation, acylation and condensation are commonly used. Finally, samples are dried for analysis.

Liquid Chromatography-Mass Spectrometry

MS and HPLC are commonly used for compound characterization and obtaining structural information; in the field of metabolomics, these two analytical techniques are often combined to characterize unknown endogenous or exogenous metabolites present in complex biological samples. With HPLC coupled to MS there is no need to derivatize compounds prior to analysis. HPLC separations are better suited for the analysis of volatile and nonvolatile polar and nonpolar compounds in their native form. Recently, LC-MS techniques have been developed which employ a soft ionization approach, making MS more robust for daily use. Furthermore, it should be noted that LC-MS can provide a list of m/z values, retention times and an estimation of relative abundances of identified metabolites that are not actually identified. Overall, high-resolution and reproducible LC-MS measurement sets up the basis for subsequent data processing and multivariate data analysis. Large-scale metabolomic technologies based on LC-MS are increasingly gaining attention for their use in the diagnosis of human disease [30]. An LC-MS metabolomics-based diagnostic provides an essential tool and has the potential to monitor the progression of onchocerciasis [31–33].

UPLC-MS technology is a powerful technique in biomolecular research and can also be used to

quantify the activity of signaling and metabolic pathways in a multiplex and comprehensive manner. The recent introduction of UPLC, employing porous particles with internal diameters smaller than 2 μm, in conjunction with MS results in higher peak capacity and enhanced specificity and high-throughput capabilities compared with conventional HPLC columns, therefore making it even more suitable for a metabolomic approach. Quadrupole time-of-flight (Q/TOF)-MS is coupled with UPLC for the analysis and identification of trace components in complex mixtures, as a powerful means of making accurate mass measurement at levels of less than 5 ppm with effective resolution. The UPLC-MS technique represents a promising hyphenated microseparation platform in metabonomics, since the majority of primary metabolites intrinsically are polar compounds. Recently, LC-MS techniques have been used for the detection of metabolites of depression. A metabonomic analysis based on UPLC-quadrupole TOF-MS was used to profile the metabolic fingerprints of urine obtained from CVS-induced depression rat models with and without CSGS treatment [13]. Samples are commonly thawed at room temperature and centrifuged at 10,000 rpm for 10 min; then the supernatant liquid is mixed with a suitable reagent and filtered through a 0.22-μm membrane filter for LC-MS analysis. These techniques provide a method of qualitative and quantitative analysis for a lot of small molecules, but the techniques still have their limitations. Therefore, more advanced equipment and technology needs to be developed.

Biomarkers

Biomarkers can be used as surrogate endpoints that are intended to substitute a clinical endpoint. The development of biomarkers to guide treatment decision-making in depression would offer significant advantages. Many putative biomarkers have been identified that provide information about the general prognosis for recovery from depression and, in some instances, about whether a specific treatment may lead to remission. Recently, to determine biomarkers for depression and antidepressants, a lot of researchers have done much research on these. The researches on the metabonomics of depression and effects of antidepressants are listed in table 1. This research provides many biomarkers, and several metabolic pathways, main biomarkers and their classification are summarized in table 2. An overview is shown in figure 1.

Pathways

At present, a variety of endogenous biochemical metabolites are known, some of them considered potential biomarkers for antidepressant response.

Amino Acid Metabolism

Tryptophan Metabolism

Tryptophan and its metabolites, kynurenic acid (KA) and xanthurenic acid (XA), were significantly changed in the urine of CUMS-treated rats. Tryptophan is an essential amino acid and has two main metabolic pathways: the 5-HT pathway and the KYN pathway. Indoleamine 2,3-dioxygenase (IDO) is the first enzyme in the KYN pathway, which converts tryptophan to kynurenine; it has been reported that depression is associated with increased proinflammatory cytokines, which activate IDO [34–36]. The activated IDO may produce an effect on depression by two aspects. On the one hand, promotion of the KYN pathway through the activated IDO leads to a decreased concentration of tryptophan. The lower urine tryptophan level in CUMS-treated rats compared with controls observed in the study was consistent with less tryptophan in urine of patients with endogenous depression than in that of control subjects [37]. Tryptophan is the precursor of the neurotrans-

Table 1. Summary of research on depression

No.	Model	Sample	Analysis method	Intervention drug	Ref. No.
1	CUMS	urine	GC-MS	XYS	25
2	CUMS	urine	UPLC-MS	–	29
3	CUMS	plasma	NMR	XYS	23
4	CUMS	urine	NMR	–	24
5	CUMS	urine	GC-MS	–	26
6	CUMS	plasma	GC-MS	XYS, venlafaxine	18
7	CUMS	plasma	GC-MS	–	27
8	CUMS, FST	plasma	NMR	–	25
9	CVS	urine	UPLC-MS	CSGS	16
10	CVS	plasma	UPLC-MS/MS	CSGS	30
		hippocampal	UPLC-MS/MS	CSGS	
11	CUMS	urine	UPLC-MS	CSGS	14
12	–	hippocampal	LC-MS/MS	paroxetine	4
13	CUMS	urine	GC-MS	venlafaxine, fluoxetine	5
14	patients	serum	LCECA, GC-TOF-MS	sertraline	6
15	CVS	plasma	behaviors	*Glycyrrhiza uralensis*	15
16	CUMS	plasma	^{1}H NMR, UPLC-MS	–	31
17	patients	serum	GC-TOF-MS	sertraline	19
18	all	all	all	–	20

LCECA = LC electrochemical array detection.

mitter 5-HT; its low level possibly results in declined 5-HT biosynthesis. Decreased 5-HT is related to the monoamine hypothesis of depression, which has been refined in a way that depressive illness may arise specifically from decreased brain 5-HT function [38]. It has been reported that depressed patients showed significantly lower tryptophan and ratios of tryptophan to large neutral amino acids in plasma [39, 40]. Clinical experimentation indicated the concentrations of 5-HT and 5-hydroxyindoleacetic acid in postmortem brain tissue of depressed patients were lower than in control subjects [41, 42]. These findings indicated that the neurotransmitter 5-HT and tryptophan abnormalities were associated with the pathogenesis of depression. The lower tryptophan in urine of depressed rats in this study provided evidence to support a deficiency in serotonergic neurotransmission as the pathophysiology of depression from the perspective of the urine metabolite. On the other hand, an activated KYN pathway makes the

Table 2. Summary of potential biomarkers

Pathways	Biomarkers for depression	Urine	Plasma	Tissue	Effect of antidepressant
Amino acid metabolism	kynurenic acid, xanthurenic acid	–	↑	–	√
	phenylalanine	↑	↑	↓	√
	glycine	↑	↑	↓	√
	glutamine	↑	↑	–	√
	alanine	↓	↑	–	–
	tyrosine	↑	↓	–	–
	tryptophan	↓	↑	↓	√
	isoleucine	↓	↓	–	–
	valine	↓	↓	–	–
	glutamate	↓	↑	↑	√
Energy metabolism	pyruvate	↑	–	–	√
	α-ketoglutarate	↓	–	–	–
	citrate	↓	–	–	√
	creatinine	↓	–	↓	–
	linoleate	–	↓	–	–
	octadecanoic acid	–	↓	–	√
	hexadecanoic acid	↓	↓	↓	√
	lipid	↓	↑	–	–
	lactate	–	↓	–	–
	fructose	–	↑	–	√
	glucose	–	↑	–	√
Others	acetoacetic acid	↓	↑	↓	–
	3-hydroxybutyrate	↓	↓	–	–
	indoxyl sulfate	↓	↓	–	–
	indoleacrylic acid	↓	↓	↓	–
	indole-3-acetate	↓	–	–	–
	hippuric acid	↑	↑	–	√
	phenylacetyl glycine	↑	–	–	–

↑ = Increased in model group compared with control group; ↓ = decreased in model group compared with control group; – = no difference was found.

downstream metabolites KA and XA increase in the depression model. It was reported that 3-hydroxykynurenine produced oxidative stress and caused neuronal cell death, which was frequently associated with depression. Considerable clinical and experimental data supported the existence of an association between cytokines and depression [43]. Cytokine therapy was accompanied by development of depressive symptoms in a significant proportion of patients. Meanwhile, major depression was accompanied by increased production of proinflammatory cytokines. Activation of IDO could represent an important link between the immune system and the pathogenesis of depression. It has been reported that major depressive patients exhibit a significant increase in plasma concentration of KYN and in KYN/tryptophan ratio during interferon-α therapy. CUMS-treated rats excreted more KYN metabolites (KA and XA) in urine, indicating an activation of IDO and the KYN metabolic pathway. This activation resulted in a decreased concen-

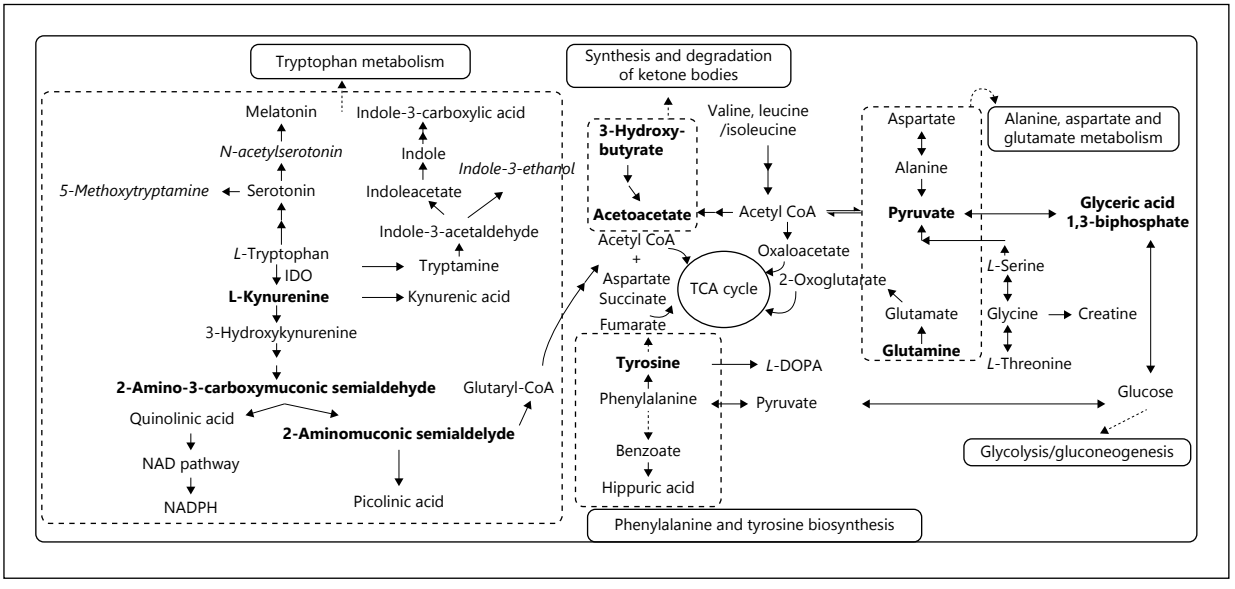

Fig. 1. Potential biomarkers for depression and its pathways [14].

tration of tryptophan, reducing the biosynthesis of the neurotransmitter 5-HT, which is important in the pathology of depression [44].

Phenylalanine Metabolism

Phenylalanine in urine was significantly increased in a model group compared with that in a control group. Phenylalanine is an essential amino acid and its hydroxylation by phenylalanine hydroxylase to tyrosine is the major metabolic pathway for phenylalanine. Some studies showed that the plasma phenylalanine-to-tyrosine ratio was increased in depressed patients, suggestive of lower phenylalanine hydroxylase activity [45]. Tetrahydrobiopterin (BH_4) is the essential cofactor for phenylalanine hydroxylase. It has been found that the level of BH_4 was decreased in serum of depressive patients [46, 47]. Decreased BH_4 could lead to phenylalanine hydroxylase deficiency and prevent the transformation of phenylalanine to tyrosine, which was possibly associated with the lower level of tyrosine metabolites (norepinephrine, epinephrine and dopamine) in the state of depression [48].

Tyrosine was significantly decreased in the plasma of a model group compared with a control group. Tyrosine was produced by phenylalanine hydroxylation from phenylalanine, and its decreased level suggested lower phenylalanine hydroxylase activity, which is in agreement with a previous report that the plasma phenylalanine-to-tyrosine ratio was increased in depressed patients [19].

Glutamate Metabolism and Others

Glutamic acid, the excitatory neurotransmitter in the mammalian nervous system [49], was significantly decreased in a plasma model group; glutamine in the plasma of the model group was significantly increased, suggesting that glutamic acid and glutamine biosynthesis was affected after CUMS treatment [18]. In addition, a significant decrease in alanine was also observed after CUMS treatment. This decrease may have been due to either decreased synthesis or increased degradation of alanine [50]. It is hypothesized that glutamate is metabolized to α-ketoglutaric

acid by alanine aminotransferase [51]. This is accompanied by the production of alanine, and alanine is then changed to pyruvic acid by the same enzyme, accompanied by the production of glutamate [52]. The decrease may also be explained by metabolism of glutamate, an important excitatory neurotransmitter, which was decreased in depressive rats [49, 53, 54]. Thus, as an excitatory neurotransmitter, metabolism of insufficient glutamate may have reduced the synthesis of alanine.

Energy Metabolism

Fatty Acids

Fatty acids can be decomposed by β-oxidation to acetyl coenzyme A (CoA) to participate in the energy supply of the body. It was reported that hexadecanoic acid, linoleate and octadecanoic acid were lowered considerably in the plasma of a model group compared with that of a control group in a study, suggesting dysfunction of fatty acid metabolism. The decrease in fatty acid levels detected may be caused by fatigue due to resistance to physical stressors, which is one of the most frequently represented depressive symptoms in major depressive disorder [55].

TCA Cycle

Citrate, 2-oxoglutarate and succinate, key intermediate products of the TCA cycle, are associated with energy metabolism. Pyruvate can be converted by the pyruvate dehydrogenase complex to acetyl CoA, which further enters the TCA cycle. Pantothenic acid is a precursor of CoA, which is derived from pantothenic acid by type I pantothenate kinase and plays an important role in heme synthesis, in lipid metabolism or as a prosthetic group in the TCA cycle. Proline, the catabolite of peptide degradation by proline iminopeptidase, is a precursor of pyruvate. Pyruvate can be converted into acetyl CoA, which is the main input for a series of reactions known as the TCA cycle. The TCA cycle is an important biological metabolic pathway in the body; it involves not only glucose aerobic oxidation but also the major pathways for fat and amino acid metabolism [22]. The lower levels of citrate, 2-oxoglutarate and succinate and the high level of pyruvate in urine of CUMS-treated rats were indicative of TCA cycle dysfunction [24]. Energy deficiency, one of the most commonly represented depressive symptoms, is connected with reduced activity and curiosity in some way [56]. Acetate is produced from acetyl CoA through acetyl phosphate, and the decreased urinary level also revealed a disorder of energy metabolism [57].

Glycometabolism

The concentration of fructose and glucose in a model group were increased significantly. It was reported that depression was associated with glucose metabolism in its biological mechanisms, and that glucose metabolism might be affected by the abnormal secretion of depression-related hormones [58]. It is believed that the increased content of sugar is related to a lack of norepinephrine and 5-HT in the brain [59]. A study found that the concentration of adrenocortical hormone or its metabolites was increased in patients with depression. In addition, the concentration of blood sugar can be further increased by the increased secretion of adrenocortical hormone [60, 61]. Lactate is the end product of glucose metabolism under anaerobic conditions. Glutamate may produce proline after decarboxylation, thus the increased glutamate concentration and reduced proline concentration could be linked with the lower level of decarboxylation, indicating a disruption to glutamate metabolism.

Ketone Metabolism

The decrease in β-hydroxybutyrate, a known ketone body, in serum reflected a reduction in energy production through fatty acid oxidation, suggesting that disorders of carbohydrate and energy metabolism may have been present in mild-stress group animals. It has been shown that the

production of ketone has a positive influence on the production of GABA, which is illustrated by reduced levels in the plasma of depressed patients [25].

Monoamine Neurotransmitter Metabolism

Norepinephrine and dopamine are two important neurotransmitters like 5-HT, which is derived from tryptophan metabolism. 3-*O*-Methyldopa is one of the main biochemical markers for aromatic L-amino acid decarboxylase deficiency, which affects norepinephrine and dopamine biosynthesis. The concentration of 3-*O*-methyldopa in a CVS group increased significantly compared with drug-naïve rats, which is consistent with an increased level of 3-*O*-methyldopa in aromatic L-amino acid decarboxylase deficiency in urine [62]. Indole-3-carboxylic acid, KA and XA are the metabolites of tryptophan, the precursor of the neurotransmitter 5-HT. 5-HT showed significantly lower levels in depressed patients [56]. Up-regulation of indole-3-carboxylic acid and down-regulation of KA and XA may be induced by excessive activation of tryptophanase in the metabolic pathway producing indole and inhibition of tryptophan 2,3-dioxygenase in the metabolic pathway generating kynurenine in the CVS process, which will cause the abnormality of tryptophan metabolism directly [13].

Conclusions and Perspectives

From these studies, we found some potential biomarkers of depression and their possible metabolic pathway, and this method can be a useful tool for predicting depression and the response to antidepressants. However, there are still several questions regarding metabolites. Firstly, the compounds characterized by the commonly used methods of NMR, LC-MS and GC-MS in metabolomics are only part of the complete set. Generally, potential biomarkers obtained by different analyses are supplemental, so a combination of measures should be developed. Secondly, most potential biomarkers are detected only semi-quantitatively; there is no precise absolute quantification. In addition, the signal of trace amounts of a potential biomarker is too weak to be detected by analysis; thus some biomarkers of great significance occurring only in trace amounts may be ignored or not recognized. Thirdly, the majority of potential biomarkers are lipid compounds, they cannot be separated very well in detection; thus their qualitative and quantitative assessment provided a challenge to us. Finally, metabonomics should be used by combing it with other technologies such as proteomics, genomics and so on, to elaborate the mechanism of depression and the effect of antidepressants.

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Proteomics and Metabolomics of Bipolar Disorder

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Abstract

Bipolar disorder (BD) is a chronic psychiatric illness characterised by transitions between manic and depressive episodes. Diagnosis of BD is based on subjective clinical evaluation and, consequently, quite difficult, due to the oscillating character of the disease. Increasing accuracy regarding BD diagnosis can improve the mental health and treatment of patients. A path that can lead to a more reliable medical diagnostic of BD and also possibly to an optimisation of treatment for each patient is the identification of specific biological markers. Recently, proteomics and metabolomics have become the most applied advanced analytical tools allowing the discovery of protein and metabolite biomarkers for diseases and/or treatments. The present chapter reviews studies described in the literature that employ proteomic and metabolomic strategies to investigate potential biomarkers for BD and also for lithium therapy, which is the most widely used drug treatment of this illness. The main results from investigations using human samples are presented, as well as hypotheses concerning the biological processes and molecular mechanisms involved in BD pathophysiology and treatment, as demonstrated by proteomic and metabolomic analyses.

Introduction

Bipolar disorder (BD), formerly known as 'manic-depressive illness', affects about 4% of the adult population worldwide, independently of race, ethnicity or gender [1]. BD is characterised by recurrent mood disturbances that include periods of depression, mania, hypomania and mixed states. The absence of depressive or manic episodes is named 'euthymia' [2]. BD is further categorised into subtypes that include bipolar I (one or more episodes of mania with or without major depressive episodes) and bipolar II (one or more episodes of hypomania and at least one major depressive episode) [3]. Diagnosing BD is challenging due to the heterogeneity of the clinical presentation of the disease, the unclear boundaries regarding other mental illnesses, and the late occurrence of the first episode of mania/hypomania after recurrent episodes of depression. The factors that originate BD are still unknown, although a variety of biochemical, genetic and environmental elements seem to be involved in both causing and triggering bipolar episodes. Until this moment there has been no independent exami-

nation to confirm the disorder, and diagnosis is based only on clinical assessments. Very often, BD patients are misdiagnosed as unipolar depressive (because the first hypomanic or manic episode may only show later onset, after one or several depressive episodes), leading to inadequate treatment and outcome, besides the fact that treatment with antidepressant drugs for patients with bipolar depression may provoke a switch into hypomania or mania. Therefore, increasing accuracy in BD diagnosis is the key to improving the mental health and treatment of individuals with the disorder, and this can be attained by the identification of differential biomarkers that reflect pathophysiologic processes in the presence of the illness [4–6].

Medications normally used in the treatment for BD include mood stabilisers (lithium, valproic acid and carbamazepine), second-generation antipsychotics (olanzapine, risperidone and quetiapine), antidepressants (especially selective serotonin reuptake inhibitor drugs such as fluoxetine, paroxetine, sertraline and citalopram) and anxiolytics (clonazepam and diazepam) [2]. Lithium is the most widely used drug in many cases for BD treatment, both to treat existing episodes and to prevent further ones. For this reason, lithium is the drug therapy focused on in this chapter. Several theories on the mechanism of lithium action have been proposed, from alterations in ionic transport to modulation of gene expression [5, 7]. However, the precise neurobiological mechanisms by which lithium exerts its clinical effects are unclear, and some results found in the literature are contradictory. In this case, the detection of biological markers that indicate pharmacological responses to lithium treatment appears to be an important way to improve BD treatment, and perhaps, in the future, it may become a tool for treatment optimisation for each patient in the context of personalised medicine.

The search for disease or treatment biomarkers nowadays is mainly performed by means of proteomics or metabolomics methodologies. The results of research studies exploring potential biomarkers for BD and lithium treatment revealed by proteomics and metabolomics are discussed in the following sections. Only studies using human samples (brain, red blood cells, serum, etc.) were considered as there is no definitive animal model of BD.

Proteomics and BD

Proteomics consists in the study of protein properties (expression level, posttranslational modifications, etc.) on a large scale in order to obtain a global vision of biological disturbances such as disease or drug treatment [8]. Although very challenging, a number of proteomics studies involving psychiatric disorders have been described in the literature, mostly in schizophrenia research [9]. Studies concerning BD and its treatment with lithium are described in this section. A summary of the differential proteins related to BD discovered by proteomics is shown in table 1. Figure 1 presents a schematic connecting the main BD differential proteins to their major molecular functions, which will be discussed along this section.

The first published work describing a proteomic study on BD was performed by Johnston-Wilson et al. [10] in the year 2000. The authors analysed postmortem brain tissues (frontal cortex) from 23 BD patients and compared them with 23 controls (schizophrenia and major depressive patients were also studied, but this is not the focus of the present chapter). The proteins were separated by two-dimensional polyacrylamide gel electrophoresis, and the differential protein spots (determined by multivariate analysis) were characterised by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS). An increase in the level (upregulation) of aldolase C and a decrease in the level (downregulation) of dihydropyrimidinase-related protein 2 and glial fibrillary acidic protein were observed for BD patients in comparison with the healthy controls.

Table 1. Potential BD protein biomarkers described in the literature and determined by proteomics analyses

Protein(s)[a]	Source	Analytical technique(s)	BD regulation	Reference No.
Aldolase C (ALDOC, P09972)	Brain	2-D PAGE and ESI-MS/MS	Upregulation	10
Dihydropyrimidinase-related protein 2 (DRP, Q16555); glial fibrillary acidic protein (GFAP, P14136)	Brain	2-D PAGE and ESI-MS/MS	Downregulation	10
Aldolase C (ALDOC, P09972); ankyrin repeat domain protein 12 (ANK12, Q6UB98); bystin (BYS, Q13895); CCAAT-box-binding transcription factor 2 (CBF, Q03701); Dickkopf homolog 2 (DKK2, Q9UBU2); myelin basic protein 18590 (MBP, P02686); peroxisomal 2,4-dienoyl-CoA reductase 2 (PDCR, Q9NUI1)	Dorsolateral prefrontal cortex	SELDI-TOF-MS and MALDI-TOF-PSD-MS	Upregulation	11
Brain acid soluble protein 1 (BASP, P80723); guanine nucleotide-binding protein G(I) (GNB, P62873); limbic system-associated membrane protein (LAMP, Q13449); syntaxin-binding protein 1 (SB1, P61764); tubulin β-chain (TBC, P07437)	Dorsolateral prefrontal cortex	2-D DIGE and MALDI-TOF-MS, SDS-PAGE and LC-MS/MS	Upregulation	12
β-Actin (BAC, P60709); voltage-dependent anion-selective channel protein 1 (VDAC1, P21796)	Dorsolateral prefrontal cortex	2-D DIGE and MALDI-TOF-MS, SDS-PAGE and LC-MS/MS	Downregulation	12
Apolipoprotein A-I (ApoA1, P02647)	Serum	2-D DIGE and MALDI-TOF-MS/MS, SELDI-TOF-MS, immunoturbidimetry, LC-MS/MS	Downregulation (euthymia)	13, 22
Tumour necrosis factor-α (TNF-α, P01375)	Serum	ELISA, LC-MS/MS	Upregulation (manic and depressive episodes) and downregulation (euthymia)	22, 27, 28
C-C motif chemokine 16 (CC16, O15467); CD40 ligand (CD40L, P29965); connective tissue growth factor (CTGF, P29279); endothelin 1 (EN1, P05305); Fas ligand (FASL, Q0VHD7); glutathione S-transferase (GST1, P08263); insulin-like growth factor-binding protein 2 (IGF2, P18065); lutropin subunit-β (LSHB, P01229); lymphotactin (LTN, P47992); macrophage migration inhibitory factor (MIF, P14174); pro-epidermal growth factor (EGF, P01133); tumour necrosis factor receptor superfamily member 5 (TNFR5, P25942)	Serum	LC-MS/MS	Upregulation (euthymia)	22
Apolipoprotein C-III (ApoC3, P02656); C-C motif chemokine 26 (CC26, Q9Y258); interleukin-13 (IL-13, P35225); stem cell factor (SCF, P21583)	Serum	LC-MS/MS	Downregulation (euthymia)	22

Table 1. Continued

Protein(s)[a]	Source	Analytical technique(s)	BD regulation	Reference No.
Aconitate hydratase (ACO, Q99798); actin-interacting protein (AIP, O75083); clathrin heavy chain 1 (CLH17, Q00610); cyclophilin A (PPIaseA, P62937); cytoskeleton-associated protein 5 (Ch-TOG, Q14008); cytosolic non-specific dipeptidase (CNDP, Q96KP4); endoplasmin (ENPL, P14625); eukaryotic translation initiation factor 3 subunit A (eIF3a, Q14152); genetic suppressor element 1 (GSE1, Q14687); heat shock 70-kDa protein 8 (HSP8, P11142); heat shock protein HSP 90-β (HSP90, P08238); HLA-B-associated transcript 1 (HLA, Q13838); kinesin-like protein KIF7 (KIF7, Q2M1P5); leucine zipper protein 1 (LZP1, Q86V48); L-lactate dehydrogenase A chain (LDH-A, P00338); myomesin-1 (MYO1, P52179); myosin-13 (MYH13, Q9UKX3); myosin-2 (MYH2, Q9UKX2); myosin-7 (MYH7, P12883); sterile α-motif domain-containing protein 9-like (SAM9, Q8IVG5); tubulin α-1A chain (TBA1A, Q71U36); ubiquitin-activating enzyme (UBA1, P22314); uncharacterized protein KIAA1529 (KIAA, Q9P1Z9); valosin-containing protein (VCP, P55072)	Peripheral blood mononuclear cells	LC-MS/MS	Upregulation (euthymia)	22
Annexin A2 (AN2, P07355); desmuslin (DMN, O15061); myosin-10 (MYH10, P35580); myosin-11 (MYH11, P35749); myosin-4 (MYH4, Q9Y623); myosin-Va (MYHVa, Q9Y4I1); nuclear mitotic apparatus protein (NUMA, Q14980); Ran-binding protein (RBP, Q99666); Rho guanine nucleotide exchange factor 17 (RHO, Q96PE2); structural maintenance of chromosomes protein 2 (SMC2, O95347); zinc finger CCCH domain-containing protein 13 (ZF13, Q5T200)	Peripheral blood mononuclear cells	LC-MS/MS	Downregulation (euthymia)	22

2-D PAGE = Two-dimensional polyacrylamide gel electrophoresis; ESI = electrospray ionisation; MS = mass spectrometry; SELDI = surface-enhanced laser desorption/ionisation; TOF = time-of-flight; MALDI = matrix-assisted laser desorption/ionisation; PSD = postsource decay; 2-D DIGE = two-dimensional difference in gel electrophoresis; SDS = sodium dodecyl sulphate; LC = liquid chromatography; ELISA = enzyme-linked immunosorbent assay.

[a] Abbreviations and accession numbers in parentheses.

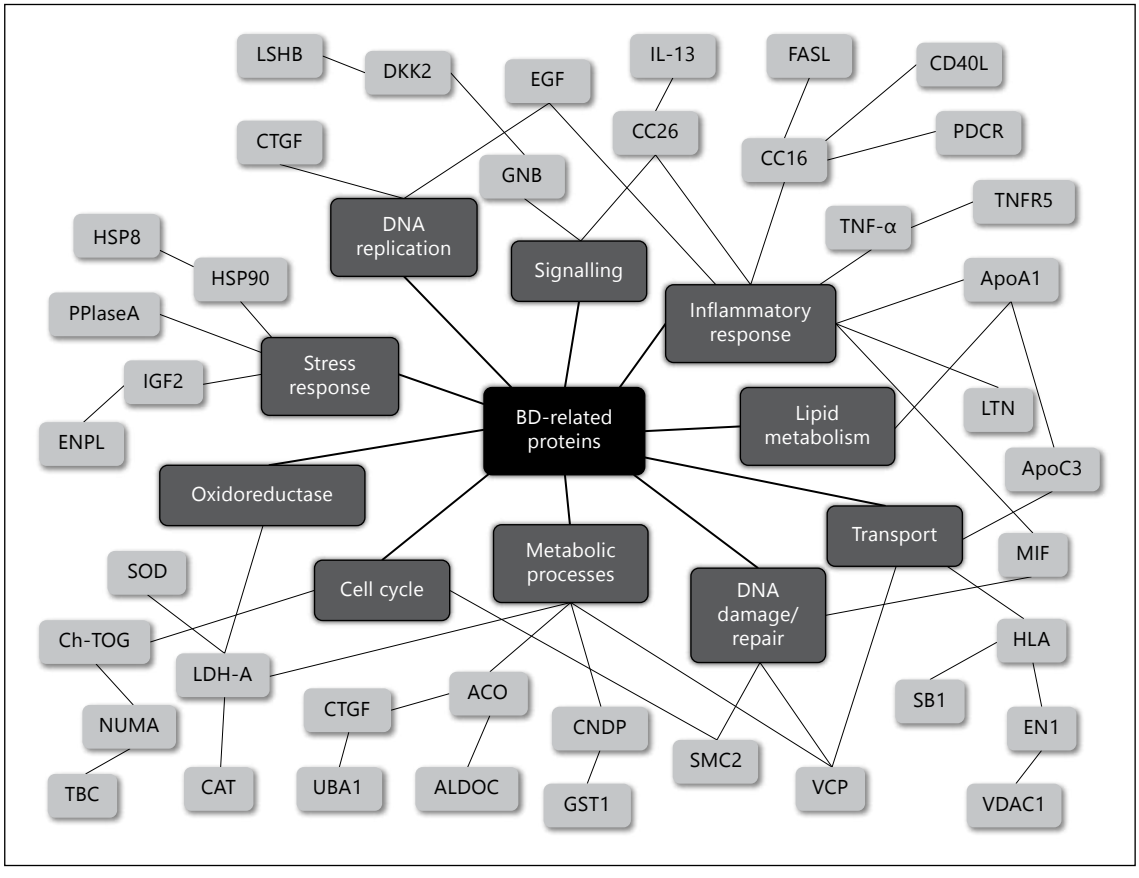

Fig. 1. Main differential proteins related to BD and their corresponding key molecular functions. Abbreviations are explained in table 1.

However, the results of this study were inconclusive when considering potential BD protein biomarkers, since the 3 differential proteins described for BD were also observed with the same expression behaviour in schizophrenic and major depressive patients.

Another proteome profiling study of postmortem brain samples (dorsolateral prefrontal cortex) of BD (and also schizophrenia) patients was described by Novikova et al. [11] in 2006. The proteins were separated and analysed by surface-enhanced laser desorption/ionisation-time-of-flight-MS (SELDI-TOF-MS), and the potential biomarkers indicated by statistical analyses were identified by matrix-assisted laser desorption/ionisation-TOF-postsource decay-MS (MALDI-TOF-PSD-MS). The authors identified a heterogeneous group of 7 proteins (table 1) to be upregulated in BD, including aldolase C, which had previously been identified by Johnston-Wilson et al. [10]. Only 2 of the 7 differential proteins were found with an altered expression exclusively in BD (Dickkopf homolog 2 and myelin basic protein 18590). The findings shown in this study suggest that BD might be associated with complex multifactorial molecular changes involving cell signalling, lipid and glucose metabolism and other intracellular processes.

In 2009, Behan et al. [12] analysed dorsolateral prefrontal cortical samples from BD (and schizophrenia) patients using two proteomics approaches: (1) protein separation by two-dimensional difference in gel electrophoresis (2-D DIGE) followed by MALDI-TOF-MS identification, and (2) protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by liquid chromatography-tandem MS (LC-MS/MS) separation/identification. The authors found 5 proteins to be upregulated and 2 downregulated in BD patients (table 1). β-Actin was the only protein among the potential biomarkers that was shown to be altered in BD and not in schizophrenia. Three proteins with roles in synaptic function (brain acid soluble protein 1, limbic system-associated membrane protein and syntaxin-binding protein 1) were further validated by Western blotting as the authors hypothesised that glutamatergic neurotransmission and synaptic functional abnormalities are implicated in BD pathogenesis. Nevertheless, the results of this study were uncertain, since the potential protein biomarkers showed an upregulation only in one of the two brain series tested.

Until this point, proteomic studies involved the analysis of postmortem brain samples only, and BD patients were not distinguished according to their treatment. The proteome analysis of biofluids is an important strategy for studying the biological bases of diseases and their treatments as they usually reflect the state of health, thus representing a qualified source of material for the identification of potential biomarkers. In 2011, Sussulini et al. [13] published a blood serum proteomic study of BD patients separated into two groups: patients treated with lithium and patients treated with drugs other than lithium, comparing them with healthy controls. Two different proteomic approaches were applied: (1) SELDI-TOF-MS and (2) 2-D DIGE combined with MALDI-TOF-MS/MS. The main result described was that serum levels of apolipoprotein A-I are decreased in BD patients, but restored to control levels under lithium treatment. Such a finding was further validated by immunoturbidimetric analysis, which is a routine clinical assay that indicates the risk of coronary artery disease [14].

Apolipoprotein A-I plays roles in cholesterol transport, neuronal and glial metabolism [15, 16], and regulation of inflammation [17]. Apolipoproteins in general have also been related to psychiatric disorders such as BD and schizophrenia, since apolipoproteins D, E and L have been found to be upregulated in the brains of subjects with neuropsychiatric disorders [15] and apolipoprotein A-I has been found at decreased levels in subjects with Alzheimer's disease, Parkinson's disease and Down syndrome [17]. A recent review describing the relation of the cholesterol system and associated proteins to psychiatric disorders can be found in Woods et al. [18]. As apolipoproteins act in lipid metabolism, an imbalance of lipid homoeostasis appears to be a good hypothesis for the pathogenesis of BD and other psychiatric illnesses.

Considering BD treatment, a decrease in apolipoprotein E levels was observed in the plasma of BD patients not medicated, but an increase in the levels of this protein in patients treated with mood stabilisers (valproate or lithium) was described by Dean et al. [16]. The same behaviour was described for apolipoprotein A-I [13], i.e. upregulation in BD patients treated with lithium and downregulation in patients not treated with this drug. Hence, it is possible to conclude that lipid homoeostasis is also affected by the use of mood stabilisers, and this can be a starting point for further studies focusing on the mechanism of action of these drugs.

Other studies using proteome profiling approaches were applied to samples obtained from the cerebrospinal fluid, brain and peripheral tissues of schizophrenia patients [19], and to serum [20] from first-onset and drug-naïve patients. In these studies, apolipoprotein A-I was established as a biomarker for schizophrenia based on its lower levels in the diseased state. Since the same

profile was also observed for BD [13], it was concluded that apolipoprotein A-I cannot be used alone to distinguish BD from schizophrenia. However, such findings point to an equivalent deregulation for both psychiatric diseases, supporting the notion that schizophrenic and bipolar disorders are similar (but not at the same level) in some epidemiologic aspects such as age at onset, lifetime risk, course of illness, worldwide distribution, risk of suicide, gender influence and genetic susceptibility [21].

Apolipoprotein A-I was also described to be downregulated in the serum of BD patients by Herberth et al. [22]. Proteomic analyses were performed by LC-MS/MS on serum and peripheral blood mononuclear cells (PBMC). As observed in table 1, the authors found 13 upregulated proteins in serum and 24 in PBMC, as well as 5 downregulated proteins in serum and 11 in PBMC. The majority of the differential proteins found in this study are involved in cell death/survival pathways. In PBMC, this was manifested in the form of cytoskeletal and stress response-associated proteins, while in serum most proteins were associated with inflammatory processes.

Taking inflammatory processes into consideration, apolipoprotein A-I is described as an acute-phase protein (APP). The acute-phase response is characterised by two types of APP: positively regulated proteins that appear at higher concentrations and negatively regulated proteins that appear at lower concentrations in response to inflammatory processes which are related to the phenomenology, pathophysiology, comorbidity and treatment of BD [23]. The serum proteomic profiling study that compared BD patients treated with those not treated with lithium [13] reported 5 differential APP besides apolipoprotein A-I: α-1-antitrypsin, antithrombin III, Ig γ-1, transthyretin and vitronectin. All of them were downregulated in BD patients under lithium therapy in comparison with patients treated with different drugs. Yang et al. [24] described apolipoprotein A-I and transthyretin as being negatively regulated and α-1-antitrypsin as being positively regulated in the plasma of schizophrenia patients. Transthyretin, a negatively regulated APP, is also described as being downregulated in the cerebrospinal fluid of schizophrenia [25] and depression [26] patients. However, as described by Sussulini et al. [13], this protein was positively regulated in BD patients not treated with lithium, showing an upregulated APP behaviour instead of that described for schizophrenia (downregulated APP). Based on such an observation, it is possible to formulate a premise that this protein may be a marker differentiating between schizophrenia and BD patients, also suggesting a point to be considered in further studies evaluating the efficiency of lithium in BD treatment.

Serum proteomic analyses also showed that tumour necrosis factor-α (TNF-α) was downregulated in euthymic BD patients [22]. Nevertheless, current evidence suggests that this protein is upregulated during manic and depressive episodes [27]. An independent study focusing on TNF-α level measurement by enzyme-linked immunosorbent assay (ELISA) showed a higher level of this protein in patients with poor lithium response compared with those with good response [28]. Together, these results indicate that this protein, related to inflammation, like apolipoprotein A-I, might be a marker for lithium treatment response and also a confirmation that inflammatory processes might be related to BD pathophysiology.

There is also evidence that oxidative processes are involved in the pathogenesis of BD. Through proteomic analyses, some oxidative stress response enzymes (cyclophilin A and L-lactate dehydrogenase A chain) were found to be upregulated in BD [22]. Other studies, not exactly using proteomic methodologies, corroborate the oxidative stress hypothesis. A recent study determined glutathione levels and antioxidant enzyme activities in plasma samples of BD patients [29]. The authors observed decreased catalase activity and (total and reduced) glutathione levels in BD pa-

tients when compared with controls. Another study [30] shows that catalase and superoxide dismutase present lower levels in red blood cells of BD patients in relation to control subjects, although a more recent study using ELISA reported a higher level of superoxide dismutase in serum of BD patients when compared with controls [31]. Additionally, it has been proven that the oxidation of apolipoprotein A-I leads to dysfunctional cholesterol processing, deregulation of inflammatory processes and neurodegeneration [17]. Apolipoprotein A-I oxidation leads to elevation of peripheral levels of TNF-α that can cross the blood-brain barrier, causing a signalling cascade which can contribute to neuronal damage.

The proteomic studies described in this section indicate a combination of biological processes that might be implicated in BD pathophysiology (lipid metabolism, inflammation and oxidative stress), as well as 2 proteins (apolipoprotein A-I and TNF-α) that are involved in these biological processes and have been pointed out as potential BD and lithium treatment biomarkers by distinct research groups.

Metabolomics and BD

Metabolomics consists in the analysis of the multitude of small molecules (metabolites) present in a biological system. Metabonomics, a subset of metabolomics, is defined as the measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification [32–35]. As the focus of this chapter is on the study of metabolites that present altered levels resulting from a disease process (BD) or drug treatment (lithium), the term metabonomics will be used from now on. Latent biochemical information obtained from metabonomic studies may be used for diagnostic or prognostic purposes and reflects actual biological events rather than the potential for disease which gene expression data provide [35]. Different analytical platforms, mainly based on MS or nuclear magnetic resonance (NMR) spectroscopy, are currently used for such studies. For BD investigations, primarily in vivo hydrogen magnetic resonance spectroscopy (^{1}H MRS) [36], hydrogen magnetic resonance imaging (^{1}H MRI) [37] and ^{1}H NMR spectroscopy have been applied to metabonomics studies [38]. Table 2 presents a summary with the major differential metabolites related to BD discovered by metabonomics.

A metabonomic approach was employed to evaluate the metabolic profile of blood serum from BD patients treated with lithium or other drugs [39]. BD patients were distinguished from control subjects and BD patients under treatment with lithium were differentiated from those not treated with this drug by comparing ^{1}H NMR metabolic profiles and using chemometric tools for data evaluation. This comparison allowed the identification of potential biomarkers for BD and lithium treatment, mostly lipids (glycoprotein, mono- and polyunsaturated) and lipid-metabolism-related molecules (table 2). The lipid-metabolism-related molecules found were acetate, glutamate, choline and *myo*-inositol. Acetate is formed in the body by the metabolism of certain substances, particularly in the liver during the oxidation of lipids. Glutamate is the most common precursor of the brain neurotransmitter γ-aminobutyric acid. Choline is a natural amine found in the lipids that make up cell membranes and is the precursor of the neurotransmitter acetylcholine, which acts in cholinergic neurotransmission [40]. Choline, lithium and BD are linked by interactions at many levels. Clinically, there is evidence that the choline precursor lecithin (phosphatidylcholine) is moderately effective in some patients presenting mania. Furthermore, lithium exerts a potent and specific inhibitory effect on human choline transport [41]; *myo*-inositol is a sugar involved in the regulation of neuronal osmolarity, in the metabolism of membrane-bound phospholipids and in the phosphoinositide

Table 2. Potential BD metabolite biomarkers described in the literature and determined by metabonomic strategies

Metabolite(s)	Source	Analytical technique(s)	Reference No.
α-Linoleic acid; DHA; EPA	Red blood cells	GC-FID	30
Creatine	Brain	^{1}H MRS	36
N-acetylaspartate	Brain	^{1}H MRI	37
γ-Aminobutyric acid; creatine; glutamate; *myo*-inositol	Brain	^{1}H NMR	38
Lipids; lipid metabolism-related molecules (acetate, choline, glutamate, *myo*-inositol); amino acids (arginine, asparagine, glutamine, lysine, proline, valine)	Serum	^{1}H NMR	39
N-acetylaspartate; choline; creatine; glutamate/glutamine; *myo*-inositol	Brain	^{1}H MRS	42
α-Hydroxybutyrate; choline; isobutyrate; N-methylnicotinamide	Urine	^{1}H NMR	44
Free fatty acids; phosphatidylcholine	Grey and white matter and red blood cells	LC-MS	47

DHA = Docosahexaenoic acid; EPA = eicosapentaenoic acid; GC = gas chromatography; FID = flame ionisation detector.

secondary messenger pathway [42]. Changes in *myo*-inositol levels may reflect increased inositol monophosphatase activity, which would lead to an increase in the levels of *myo*-inositol containing compounds in patients treated with lithium [43]. Differential amino acid (proline, glutamine, valine, asparagine, arginine and lysine) levels were also found when comparing control subjects with BD patients under the different treatments. This suggests an alteration in the amino acid metabolism of BD patients.

Also using ^{1}H NMR spectroscopy-based metabonomic analyses, Lan et al. [38] identified molecular changes in postmortem brain samples of BD patients and in rat brain samples after chronic treatment with lithium or valproate. Glutamate levels were increased in the postmortem brains of BD patients (independent of treatment), while the glutamate/glutamine ratio was decreased following valproate treatment, and γ-aminobutyric acid levels were increased after lithium treatment. Creatine and *myo*-inositol levels were increased in the postmortem human brain, but decreased in the treated rat brains. Here it is possible to notice a discrepancy between *myo*-inositol levels found in human serum samples (increased) [39] and in animal brain samples (decreased) [38] when taking into account the lithium treatment effects.

The metabolites N-acetylaspartate, choline, *myo*-inositol, glutamate/glutamine and creatine separately were also reported for euthymic, manic, depressed adult and child/adolescent BD patients by ^{1}H MRS analyses in specific cerebral regions [42].

A unique approach was used by Zheng et al. [44] by employing ^{1}H NMR to characterise the urinary metabolic profile of BD patients and healthy controls. The authors detected 4 potential biomarkers (table 2) including choline, which had previously been identified in serum [39] and brain [42].

Lipid level changes associated with BD have previously been reported in the literature by analytical strategies (ELISA and spectrophotometry) other than metabonomics. As an example, Atmaca et al. [45] observed decreased serum cholesterol and leptin levels in BD patients with manic episodes and in patients with BD I in full remission. More recently, Ozbulut et al. [46] evaluated cholesterol, leptin and ghrelin levels in euthymic BD patients that received lithium maintenance monotherapy and found that decreased serum ghrelin and increased total cholesterol levels in the patients under lithium treatment were detected when compared with the controls. The authors suggest that ghrelin and total cholesterol might be associated with lithium treatment and lithium-induced improvement in symptoms, such as food intake and sleep-wake regulation, but not with weight gain.

Schwarz et al. [47] used a high-throughput MS approach (LC-MS) to analyse samples of grey and white matter and red blood cells to compare schizophrenia and BD patients with control subjects. Significant alterations in the levels of free fatty acids and phosphatidylcholine were detected. Such differences suggest that lipid abnormalities may be an intrinsic feature of both schizophrenia and BD that is reflected by significant changes in the central nervous system, as well as in peripheral tissues. Therefore, these studies support the hypothesis that lipids can be pointed out as potential biomarkers for BD.

In some studies, the use of ω–3 fatty acid supplementation for BD symptoms reduction has been proposed [48, 49]. The primary constituents in ω–3 fatty acid, docosahexaenoic acid and eicosapentaenoic acid, exert an inhibitory effect on cell signalling pathways. This is similar to the proposed mechanism of action of commonly used mood stabilisers such as lithium and valproate.

Lower levels of α-linoleic acid, eicosapentaenoic acid and docosahexaenoic acid were found in red blood cells of BD patients [29] by gas chromatography coupled to a flame ionisation detector. Such oxidative stress-related lipids indicate that this mechanism might be involved in BD pathogenesis, and also that antioxidant and essential fatty acid supplementation might affect clinical outcome. Moreover, a higher level of α-hydroxybutyrate detected in the urine of BD patients [44] also suggests increased oxidative stress in BD patients. As mentioned in the previous section, there is a suggestion that oxidative stress contributes to the pathogenesis of BD [28], and metabonomic findings are further evidence for this hypothesis, including alterations in lipid metabolism, which are also related to lithium therapy.

Conclusions and Perspectives

BD is a complex mental disorder and the exact molecular mechanisms involved in its pathophysiology are still not fully understood. Proteomic and metabonomic studies have been performed in order to achieve a better understanding of the causes of the disease, as well as of the pharmacological mechanisms involved in its treatment. Lithium and other mood stabilisers are commonly used for BD treatment, since they positively influence both mania and depression, enhance the effect of other antidepressants and reduce suicide rates significantly. Until now, however, it is also unknown how these drugs act in the organism. As revealed by proteomic and metabonomic investigations together, the biological processes that appear to be involved in BD pathogenesis and lithium treatment effects are lipid metabolism and oxidative stress. Inflammation and amino acid metabolism were further indicated by proteomics and metabonomics, respectively, among the important biological processes.

In the near future, additional studies should be performed in order to validate the existing ones (employing different analytical techniques and using higher numbers of samples) and also to discover new molecular targets related to BD

and its treatment. The research studies discussed along this chapter should be considered as starting points for upcoming studies on the molecular mechanisms underlying BD pathogenesis and lithium action, as well as in the establishment of biomarkers which can be useful for more accurate diagnosis of BD and for observation of individual pharmacological responses to lithium treatment.

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Use of Metabolomics and Proteomics to Reveal Pathophysiological Pathways in Anxiety Disorders

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Abstract

With a frequent occurrence of approximately 10.6% in adult populations, anxiety disorders are among the most common mental health problems worldwide. Although anxiety disorders are rather prevalent, their underlying biochemical mechanisms remain unclear. As a functional endpoint of all biological events, the metabolome represents the most precise and direct molecular expression of a phenotype. Combining metabolic information with proteome data, systems biology can draw an even more comprehensive picture of the biological processes. Here, we provide a review summarizing the results from human as well as animal studies analyzing metabolic and proteomic traits in different tissues for associations with anxiety. In addition, we give an overview of animal studies that applied a systems biology approach using metabolic as well as proteomic data to identify anxiety-related pathways.

Introduction

Anxiety disorders rank above mood and substance abuse disorders worldwide and occur in approximately 10.6% of adult populations, according to a World Health Organization survey from 14 countries, with 12-month prevalence rates ranging from 2.4 to 18.2% [1]. In the 10th International Classification of Disease for mental and behavioral disorders, anxiety is considered among the following afflictions that are classified under 'Neurotic stress-related and somatoform disorders: F40–F48': obsessive-compulsive disorder (OCD), posttraumatic stress disorder, panic disorder, phobias and generalized anxiety disorder (GAD). GAD is described as 'a period of at least six months with prominent tension, worry and feelings of apprehension, about every-day events and problems'. In the newly released 5th edition of the Diagnostic and Statistical Manual

of Mental Disorders, the first two disorders, OCD and posttraumatic stress disorder, are each classified separately from anxiety disorders. In any case, human studies of anxiety that will be discussed here deal predominantly with GAD and OCD, while preclinical, experimental models often use animal strains that are prone to anxiety-like behavior, or are induced to have anxiety-like behavior, by unpredictable chronic mild stress, sleep deprivation or inflammation [2, 3].

Anxiety encompasses overwhelming feelings of worry and fear. While fear is an evolutionarily hard-wired and life-saving response to a perceived threat, anxiety disorders result from overreaction to a stimulus that is subjectively perceived as menacing [4, 5]. Phobias are triggered by a specific external stimulus (object or situation), while in GAD and OCD there is not necessarily an external trigger but rather persevering memories of threatening situations. Many anxiety disorders begin in childhood, and these afflicted young adolescents carry a 2- to 3-fold increased risk of suffering from either major depressive disorder or anxiety later in their adult lives [6]. Anxiety and depression are often coexpressed [7, 8], and anxiety – independent of cognitive complaints, self-perceived health and depression – predicts recurrent depressive symptomatology [9]. While depression is associated with anhedonia (low positive affectivity), anxiety is more commonly linked to hyperarousal [10].

Both anxiety and depression are associated with a facilitated memory response that reinforces fear and negative affect, respectively. Persevering emotional memories typical of depression are often related to personal failures and shortcomings, while anxious individuals ruminate over worrisome or fearful experiences [11]. Thus, an underlying neurological basis for anxiety behavior is a conditioned fear response [12] which involves abnormal functioning of the prefrontal cortex in threat perception [13] and of the amygdalae and hippocampus, part of the limbic system that forms emotional memory and fear (fight-or-flight) responses. Adolescent and adult patients who suffer from anxiety disorders have impaired hypoactivation of threat-safety discrimination [14]. This atypical emotional memory functioning involves three major neural systems of amygdala function (pertaining to sensory perception and allocation of attention) and neuroendocrine function, and the interactive effects of these systems with mood [11]. Other biophysiological pathways hypothesized to contribute to anxiety and depressive disorders involve chronic inflammation [15], oxidative and nitrosative stress, mitochondrial dysfunction and epigenetic effects [16, 17].

Genomic studies of anxiety disorders show associations with the serotonin receptor and other neurotransmitter systems [18, 19], circadian clock gene variants [20], stress response circuits involving oxytocin [21] as well as novel transmembrane proteins [22]. Functional genomic studies have identified shared pathways involving responses to environmental stimuli in both anxiety and psychiatric disorders that suggest a new overlapping schizo-anxiety domain [23]. In any event, behavioral, environmental and genetic interactions all influence the pathogenesis and severity of anxiety disorders, as demonstrated by gene-environment interaction studies [24]. Despite our understanding of molecular underpinnings in anxiety disorders that support targeted drug therapeutics such as serotonin reuptake inhibitors, benzodiazepine sedatives or pregabalin, which acts on voltage-dependent calcium channels to limit neurotransmitters (i.e. glutamate and adrenaline), a meta-analysis demonstrated that while pharmacologic treatment reduces anxiety, cognitive behavioral therapy is more effective in the long run [25]. Thus, further efforts to reveal mediators of this disabling mental health disease are required. The study of metabolomics, which may be viewed as a momentary read-out of an organism's genetic potential expressed under specific environmental circumstances, may offer new insights for novel therapeutic approaches [26].

Metabolomics

Metabolomics, one of many '-omics' disciplines, aims at the identification, quantification and analysis of the metabolome of a biological system. The metabolome comprises the entirety of all metabolites (molecules with low molecular weight) that are synthesized at a specific state and time point by the biological system [27–30].

For a long time, in molecular biology, the dogma of linear communication in only one direction from genes to transcripts to proteins was favored. Proteins with enzymatic function were thought to singularly influence metabolic processes, leading to the phenotype of an organism. This concept was outdated by the insight that cellular processes are a dense interactive network with posttranscriptional and posttranslational modifications as well as feedback loops. For example, as cofactors or inhibitors for enzymes, and as transcription factors, metabolites influence the transcriptome as well as the proteome and subsequently the metabolome itself [31–33].

In addition to individual genetic predisposition, the effect of environmental factors such as medication, physical activity and nutrition often become apparent in altered expression patterns of the metabolome. Thus, the metabolome can be described as a functional endpoint of all biological events in a respective biological system. Consequently, as a functional endpoint, changes in the metabolome are potentiated, compared to the transcriptome and proteome [34, 35]. In contrast to the transcriptome or proteome, which generally point to a possible cause of a special phenotype, the metabolome represents the most precise and direct molecular expression of a phenotype. When combining information from the transcriptome and/or the proteome with metabolic data, systems biology can draw a comprehensive picture of biological processes in the cell.

However, the influence of environmental factors on the metabolome has the consequence that a metabolome is highly individualistic and a complete, generic metabolome cannot be defined for an organism, nor can it be fixed on a chip (unlike the transcriptome) [31, 36]. For the measurement of metabolites, several analytical methods are available. Here, we will briefly introduce some of the most frequently reported methods.

Nuclear magnetic resonance spectroscopy determines the resonance frequencies for each atomic nucleus of a molecule by using a magnetic field. The frequency of an atomic nucleus depends on the chemical surroundings, so that nuclear magnetic resonance spectroscopy can be used for determination of the structure of molecules. Other important methods for the analysis of metabolites are provided by mass spectrometry (MS). Fourier transform ion cyclotron resonance MS belongs to ion trap MS and is based on identification of the mass-dependent circular path and rotational frequency in the cyclotron that are characteristic for every ion and that can be detected by applying a homogeneous magnetic field. This method allows an extreme mass resolution and a high mass correctness. Time-of-flight MS uses the principle that particles with different masses but the same kinetic energy need different amounts of flying time to reach the detector. Finally, tandem MS (MS/MS) couples multiple steps of mass separation in a row. This enables its usage for screening large amounts of samples by providing high sample throughput as well as low sample consumption.

The MS methods described can also be combined with chromatographic techniques. A combination with gas or liquid chromatography (GC-MS, LC-MS) is commonly used, while combinations with capillary electrophoresis and ion mobility spectrometry are less frequently used.

Metabolite Studies and Anxiety

Metabolite association studies of anxiety in animal models and clinical cohorts have identified altered levels of a number of metabolites (table 1). Several

Table 1. Overview of the main tissue-specific associations of metabolites in human studies of mood disorders and animal models of anxiety

Metabolite	Model	Mood disorder	Tissue	Study
Choline	–	GAD	↓ centrum semiovale, subcortical white matter	Coplan et al. [59, 61], 2006, 2012
Creatine	–	GAD	↓ centrum semiovale	Coplan et al. [59], 2006
	–	GAD	↓ left dorsolateral prefrontal cortex	Yue et al. [60], 2012
Glutamate	Sleep deprivation-induced anxiety, rats	–	↑ thalamus and hippocampus	Cortese et al. [54], 2010
	High anxiety behavior, mice	–	↑ plasma	Zhang et al. [51], 2011
Glutamate/creatine	–	OCD	Cortex (medial temporal lobe)	Bédard and Chantal [53], 2011
Kynurenic acid	UCMS mice	–	↓ amygdala	Laugeray et al. [48], 2011
Kynurenine	–	Endogenous anxiety	↑ plasma	Orlikov et al. [46], 1994; Lapin [44, 45], 1989, 1996
	–	GAD, depression	↑ plasma	Altmaier et al. [65], 2013
	–	Type D personality	↓ plasma	Altmaier et al. [65], 2013
	Inflammation-induced, rats	–	Hippocampus	Gibney et al. [49], 2013
	$Tdo^{-/-}$ mice	–	Liver, plasma	Kanai et al. [47], 2009
	UCMS mice	–	Lung	Laugeray et al. [48], 2011
	Naïve mice, administration of kynurenine	–	–	Salazar et al. [50], 2012
Kynurenine/tryptophan	Inflammation-induced anxiety, mice	–	Frontal cortex and hippocampus	Gibney et al. [49], 2013
	UCMS mice	–	Cingulate cortex	Laugeray et al. [48], 2011
Serotonin (5-HT)	UCMS mice	–	Cingulate cortex	Laugeray et al. [48], 2011
	$Tdo^{-/-}$ mice	–	Hippocampus and midbrain	Kanai et al. [47], 2009
5-HIAA	UCMS mice	–	Cingulate cortex	Laugeray et al. [48], 2011
	Inflammation-induced, rats	–	Frontal cortex, hippocampus	Gibney et al. [49], 2013
	$Tdo^{-/-}$ mice	–	Hippocampus and midbrain, plasma	Kanai et al. [47], 2009
Xanthurenic acid	–	Anxiety and depression	↑ urine	Hoes [41], 1979

$Tdo^{-/-}$ = Tryptophan dioxygenase knock-out; UCMS = unpredictable chronic mild stress; 5-HIAA = 5-hydroxyindoleacetic acid.

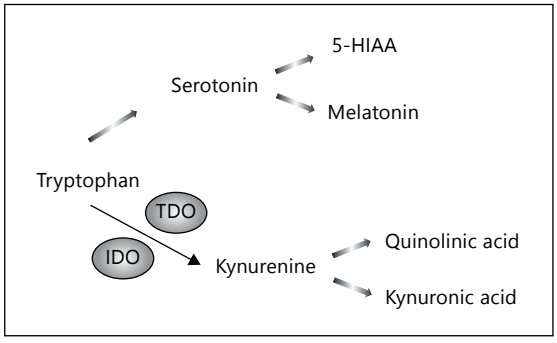

Fig. 1. Tryptophan degradation via the kynurenine and the serotonin pathways. 5-HIAA = 5-Hydroxyindoleacetic acid; TDO = tryptophan dioxygenase; IDO = indoleamine 2,3-dioxygenase.

studies have found associations for altered tryptophan metabolism – involving both the kynurenine as well as the serotonin pathways – in anxiety behavior (fig. 1). Kynurenine is the first stable metabolite formed along the tryptophan degradation pathway. The enzymes mainly involved in the synthesis of kynurenine are tryptophan dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). Altered concentrations of TRYCAT (tryptophan catabolites along the IDO pathway), including kynurenine, kynurenic acid, xanthurenic acid and quinolinic acid, are implicated in several mental health diseases [17, 37–39]. While quinolinic acid aggravates inflammation, the other three metabolites have been shown to downregulate inflammation [40]. It has long been know that increased excretion of xanthurenic acid in urine is characteristic of anxious but not depressive patients [41].

Another pathway in the degradation of tryptophan leads to serotonin and 5-hydroxyindoleacetic acid (5-HIAA), which are also associated with mood and anxiety disorder. Serotonin can also be metabolized to melatonin, which of course is critical in regulating circadian rhythm and is thus also a relevant factor contributing to mood and anxiety disorder [42]. In fact, a synthetic melatonin, agomelatine, reduced anxiety symptoms in patients with GAD [43].

One of the first groups to draw attention to the association between the metabolite kynurenine and anxiety disorder was under the direction of Professor Izyaslav P. Lapin [44, 45]. Based on these findings, Orlikov et al. [46] examined 16 patients with endogenous anxiety and 15 healthy controls. Their results showed that the concentration of kynurenine was increased in the plasma of anxious patients. In addition, a significant positive correlation between kynurenine concentration and severity of anxiety was observed.

Studies by Kanai et al. [47] examined tryptophan metabolism and the production of kynurenine. Since TDO is a rate-limiting enzyme in this pathway, mice deficient in TDO ($Tdo^{-/-}$) were examined. Behavioral tests with the elevated plus maze test and the open field test indicated that deficiency in TDO in TDO KO mice induced anxiolytic effects. Increased levels of tryptophan as well as a 2-fold higher concentration of kynurenine were observed in $Tdo^{-/-}$ mice compared with wild-type mice. However, liver lysates of $Tdo^{-/-}$ mice had decreased conversion of tryptophan to kynurenine. The authors suggested that compensatory mechanisms may explain the increased kynurenine levels in plasma, and that these mechanisms may only occur in extrahepatic tissues. Further analyses of tryptophan metabolism down the serotonin pathway indicated increased serotonin and 5-HIAA levels in the hippocampus and midbrain of $Tdo^{-/-}$ mice, while an increase only in 5-HIAA could be observed in blood plasma.

The tryptophan catabolic pathway was also the subject of the study by Laugeray et al. [48] in which mice were subjected to the unpredictable chronic mild stress procedure for a period of 6 weeks and thus showed anxiety-like behavior. In the peripheral tissue (lung) of these mice, a higher kynurenine/tryptophan ratio indicated increased kynurenine pathway activity. In contrast, the concentration of kynurenic acid in the amygdala was decreased in the unpredictable chronic mild stress mice compared with wild-type mice. The increased kynurenine/tryptophan ratio in

the lung was positively correlated to the level of serotonin and 5-HIAA in the cingulate cortex, but not in the amygdala.

Increased kynurenine pathway activity in the brain was examined by Gibney et al. [49] as well, but with an inflammation-induced model of anxiety used on rats via administration of the immunostimulant polyinosinic:polycytidylic acid, a synthetic analog of viral RNA. As expected, these rats showed anxiety-like behavior in the open field test. Hippocampal levels of kynurenine and tryptophan were both increased, although not equally, resulting in a higher kynurenine/tryptophan ratio that may indicate increased kynurenine pathway activity. While no changes in serotonin levels were observed, 5-HIAA levels were higher in the frontal cortex and the hippocampus of rats treated with polyinosinic:polycytidylic acid.

Another inflammation-induced anxiety mouse model has recently been investigated by Salazar et al. [50], in which pretreatment with an IDO inhibitor ameliorated the anxiety-inducing effects of *Escherichia coli* lipopolysaccharide administration. Furthermore, the systemic administration of kynurenine to naïve mice precipitated a dose-dependent anxiogenic effect similar to that seen with lipopolysaccharides, which demonstrates the key role of IDO and its enzymatic product in a preclinical model of anxiety behavior. These studies and their results suggest that tryptophan metabolism is involved in the underlying pathogenesis of anxiety. Due to the complexity of metabolic processes, the detailed interactions remain unclear.

Another anxiety-related metabolite of interest is glutamate, an essential amino acid and the most abundant neurotransmitter in the human nervous system. High concentrations of glutamate and subsequent overactivation of glutamate receptors (NMDA and AMPA) can be toxic to cells. This excitotoxicity results in the influx of too much calcium into cells and the subsequent activation of calcium-dependent enzymes that contribute to cell damage. A recent metabolomic approach that identified high levels of glutamate to be associated with anxiety was used by the group of Christian W. Turck [51], in which mouse strains with low versus high anxiety behavior (LAB vs. HAB) were analyzed. Behavioral analysis was assessed by ultrasonic vocalization, the elevated plus maze test and the tail suspension test. Using GC-MS, this group measured 265 plasma metabolites, of which 86 could be identified. The metabolite with the strongest positive association with HAB was glutamate.

In neurons, a protective measure against glutamate toxicity is a high concentration of the non-essential nutrient creatine, which can be synthesized from several amino acids (arginine, glycine and methionine) and provides a substantial energy reserve to buffer the toxicity of glutamate in neurons [52]. The ratio of glutamate-glutamine to creatine was analyzed in the study by Bédard and Chantal [53]. The brain activity of patients with OCD was examined using proton magnetic resonance spectroscopy, and compared with healthy controls. The OCD patients had higher anxiety than the controls, as measured by the Beck Anxiety Inventory, as well as a decreased ratio of glutamate-glutamine to creatine in the right as well as in the left medial temporal lobe.

In animal models studied by Cortese et al. [54], rats were deprived of sleep, which induced an increased risk-taking and reduced fear-like behavior observed in elevated plus maze test performance. Sleep deprivation is associated with reduced fear behavior in anxious animal models [55, 56]. Glutamate levels in both the thalamus and hippocampus were significantly elevated in sleep-deprived rats. These studies show that the purported important role of glutamate in anxiety [57] can be supported by metabolic measurements.

Besides its relation to glutamate discussed above, and because of its importance in energy metabolism, creatine is also critical for brain development and performance [58]. Patients with GAD were examined in a study by Coplan et al. [59], in which metabolites in the centrum semiovale (cerebral white matter) were analyzed. Us-

ing proton magnetic resonance spectroscopy, a decreased concentration of creatine was observed in GAD patients compared with healthy controls. Such a decrease in creatine was also observed by Yue et al. [60] in the left dorsolateral prefrontal cortex of patients with social anxiety disorder. Thus, a decreased creatine level combined with an increased glutamate level in anxiety may worsen the toxic effect of glutamate.

Another metabolite that is associated with anxiety is choline, which serves as a precursor of the common neurotransmitter acetylcholine. In the study on 15 patients with GAD by Coplan et al. [59] mentioned above, a decrease in the concentration of choline-containing compounds was also observed in the centrum semiovale. In 2012 the same group conducted a study on 26 GAD patients [61] and confirmed their result of a decreased level of choline-containing compounds in subcortical white matter. Interestingly, worry was positively correlated with IQ in GAD patients, but in healthy controls this correlation was negative.

The study by Zhang et al. [51] previously mentioned in the context of glutamate used a metabolomic approach including more than 200 metabolites. Additional metabolites that showed a significant association with anxiety in mice were *allo*-inositol, *myo*-inositol, fumarate, malate, glycine and γ-aminobutyric acid.

In recent years, Type D personality has emerged as an independent risk factor for cardiovascular disease [62, 63] that is also strongly associated with social anxiety in the general population [64]. Type D personality is a combined phenotype of negative affect and social isolation [62] that is prevalent in approximately 25% of the population. Results from a recent population-based study that applied a metabolomic approach examining the metabolite profiles of individuals with Type D personality, anxiety and depression will be discussed.

The KORA (Cooperative Health Research in the Region of Augsburg) study consists of population-based surveys and subsequent follow-up studies conducted in the region of Augsburg in southern Germany. A metabolomics approach was applied to the F4 survey that was conducted from 2006 to 2008. Among a great amount of other information, 1,502 participants provided information in a self-administered questionnaire used to determine Type D personality as well as anxiety and depression. The Type D (DS14) scale, the GAD-7 and the 9-item Public Health Questionnaire were used to identify Type D personality, anxiety and depression, respectively. For each study participant, the serum metabolic profile containing 517 metabolites was measured by Metabolon Inc., using LC-MS/MS and GC-MS. The aim of the study was to identify those metabolites that were most strongly associated with Type D personality, anxiety and/or depression [65]. The metabolite with the strongest association with Type D personality that was still significant after multiple testing correction was kynurenine. Its concentration in serum was lower in participants classified as Type D than in the control group. This was, as far as we know, the first time that this association between kynurenine and Type D personality was detected in a human population study.

As previously stated, kynurenine was the only metabolite significantly associated with any phenotype after multiple testing correction. Multiple testing correction is used when more than one hypothesis is tested, since there is a large probability that true hypotheses are rejected [66]. Testing more than 500 metabolites – and thus more than 500 hypotheses – needs this correction.

The analysis of participants with GAD and depression, respectively, did not yield a significant association after multiple testing correction. However, having a look at the only nominally significant results for kynurenine, it is noteworthy that for anxiety as well as for depression the association is positive, while it is negative for Type D personality (table 2). Overall, these analyses strengthen the theory that the kynurenine pathway plays an important role in the pathophysiology of mood and anxiety disorders [67, 68].

Table 2. Results for associations with kynurenine

	Direction of association, β	p
Anxiety	0.004	0.002
Depression	0.007	0.002
Type D	–0.021	7.08×10^{-05}

A linear regression analysis was applied with the confounders age, gender, HDL, LDL, cholesterol, triglycerides, hypertension, BMI, diabetes and the intake of antidepressive medications; p values are not corrected for multiple testing; only for Type D personality, the association is significant after multiple testing correction.

Metabolomics Combined with Proteomics

Proteomics is the global analysis of proteins using reduction in sample complexity and different separation techniques combined with MS methods [69]. Proteomic approaches have been applied to psychiatric models for over a decade and were reviewed recently [70, 71]. Proteomic analyses of anxiety models have revealed many proteins possibly involved in the pathology of this disorder. For example, using a mouse model of anxiety Szego et al. [72] found changes in 82 proteins in the total brain proteome. This result lead the authors to suggest that changes in carbohydrate metabolism, redox regulation, synaptic docking and serotonin receptor-associated proteins contribute to anxiety. Ditzen et al. [73] and Krömer et al. [74] analyzed HAB and LAB mice and identified increased expression in LAB versus HAB of glyoxalase I, an enzyme involved in detoxification of the cytotoxic metabolite methylglyoxal. Ditzen et al. [73] also identified alterations in enolase phosphatase, an enzyme involved in maintaining intracellular levels of the essential amino acid methionine.

Since stress is involved in the pathology of many mental disorders, Carboni et al. [75] analyzed rat models of chronic stress and identified 21 proteins with altered levels in the hippocampus. The proteins identified are involved in signal transduction, energy metabolism, protein folding, cytoskeleton regulation and synaptic plasticity. Another proteomic analysis of the hippocampus from adult rats submitted to prenatal restraint stress demonstrated changes in the expression profiles of proteins involved in developmental programing triggered by early-life stress, which also included proteins that regulate the aforementioned functions identified in the Carboni study [76]. Most recently, a proteomic study was undertaken with a chronic unpredictable stress-induced anxiety model on zebrafish which confirmed findings of differently regulated mitochondrial proteins that have been well documented in rodent and clinical studies [77].

Linking metabolomics with proteomics provides information not only about substrates and products but also about the enzymes catalyzing the reactions. This may give a more detailed insight into which reactions and pathways are involved in the formation of the phenotype examined. The previously mentioned mouse anxiety studies by the Turck group conducted not only metabolic but also proteomic profiling of these anxious mice from plasma [51] as well as samples from the cingulate cortex [78].

In plasma, several pathways have been identified that may be involved in the etiology of anxiety. The examination of metabolite as well as protein levels showed that the phosphatidylinositol signaling system was impaired in mice with high anxiety-related behavior, especially the binding and signaling of phosphatidylinositol as well as the metabolism of inositol phosphate. Combined analysis also yielded an altered energy metabolism in anxiety including changes in the tricarboxylic acid cycle. The authors argue that this is in accordance with findings from their own proteomic analysis as well as from other studies, implicating a role of oxidative stress in anxiety. Oxidative stress, in turn, is caused by altered mitochondrial energy pathways.

A subnetwork analysis revealed another important result of the analysis which demonstrated that proteins and metabolites interacting with dexamethasone maintained different concentrations in mice with high anxiety-related behavior compared with those with low anxiety-related behavior. Dexamethasone is a synthetic glucocorticoid that affects the hypothalamic-pituitary-adrenal axis, a dynamic system of interactions between the hypothalamus, the hypophysis and the adrenal glands. Dysregulation of this axis contributes to depression among a myriad of other stress-associated states, including anxiety. Further associations with anxiety resulting from this study concern proteasome-ubiquitin-mediated proteolysis, neurotransmission and the Ras/Raf/MEK/ERK pathway.

In a second publication, the Turck group reported the combined analysis of proteins and metabolites in the cingulate cortex of HAB, normal anxiety-related behavior and LAB mice [78]. A network analysis of these data mainly showed alterations between HAB and LAB mice in glycolysis, synaptic neurotransmission and mitochondrial function. Regarding mitochondrial function, especially the citric acid cycle/oxidative phosphorylation, oxidative stress/antioxidant defense, and transport into and within mitochondria were affected.

The current review on metabolomic studies of anxiety has employed different analytic strategies to identify contributing pathogenic factors. Whether targeted pathways or screening strategies were employed, both approaches provide useful and complementary results that improve our understanding of key underlying mechanisms of this very common and debilitating mental health disorder. Future studies that explore overlapping information about metabolomics and proteomics will likely improve this knowledge base and offer promising new therapeutic targets.

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