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Proteomics and Lipidomics in the Elucidation of Endocannabinoid Signaling in Healthy and Schizophrenia Brains

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Interest in the modulation of endocannabinoid signaling has increased since the discovery of receptors for compounds of *Cannabis sativa*. Endocannabinoids are crucial neuromodulators of many brain functions and changes in the ligands and their receptors have been associated with psychiatric disorders, such as schizophrenia. Genetic, neuroimaging, and behavioral studies have reinforced the role of endocannabinoids in the pathobiology of schizophrenia. However, molecular pathways and biological processes involved in cannabinoid effects are not totally understood. Additionally, the endocannabinoid signaling network with other non-cannabinoid targets, and the effects of phytocannabinoids increase the complexity to understand their role in schizophrenia and homeostasis conditions. Thus, proteomic studies can provide evidence about the involvement of cannabinoid receptors, as well as the metabolic and synthetic enzymes of the endocannabinoids in these disorders. Additionally, quantification of endocannabinoids in the blood serum or cerebrospinal fluid can be a useful approach to identify new biomarkers in schizophrenia, and lipidomic techniques can be used to quantify these compounds. Herein, the authors review proteomic and lipidomic studies that have been used for analysis of the endocannabinoid system in healthy and schizophrenia function. The findings may contribute to understand the involvement of endocannabinoids in the brain and in the neurobiological basis of schizophrenia.

1. The Endocannabinoid System

Cannabis sativa has been used as a recreational drug since ancient times. This plant contains more than 70 phytocannabinoids, which can present several pharmacological uses in disorders of central and peripheral nervous systems.[1] In the late 1980s and early 1990s, the cannabinoid receptors[2] and their endogenous ligands[3] were discovered. Since this time, increasing interest in modulation of the endocannabinoid system has been observed. The endocannabinoid system consists of multiple molecules (**Table 1**, **Figures 1**, **2**). The most studied of these are the endocannabinoids, anandamide, and 2-arachyidonoylglycerol (2-AG), the degradative enzymes fatty acid amide hidrolase (FAAH) and monoacylglycerol lipase (MAGL), the biosynthetic enzymes N-acyl-phosphatidylethanolamine-phospholipase (NAPE), diacylglycerol lipase alpha (DAGLα), and beta (DAGLβ), as well the type 1 (CB1) and 2 (CB2) cannabinoid receptors.[3–5]

Endocannabinoid signaling operates according to a different mechanism compared to that seen in classical neurotransmission. The classic neurotransmitters are stored at synaptic vesicles at presynaptic neurons, and once released in the synaptic cleft, act on specific receptors in postsynaptic neurons. Differently, endocannabinoids are synthesized upon demand at postsynaptic neurons (Figure 2) and, due to their lipophilic properties, they are not stored in vesicles, as classical neurotransmitters. Once released by the postsynaptic neurons, these signaling molecules act as retrograde messengers, by binding to cannabinoid receptors at presynaptic. After this, endocannabinoids are removed rapidly from synapses by transporters at neuronal membranes. In neurons, anandamide is hydrolyzed by FAAH or N-acylethanolamine-hydrolyzing acid amidase (NAAA), producing ethanolamine and arachidonic acid.[6–8] The MAGL has the greatest responsibility for degradation of 2-AG to glycerol and arachidonic acid,[9] although the serine hydrolases α,β-hydrolase domain containing protein 6 and 12 (ABHD6 and ABHD12) also mediate 2-AG degradation[10] (Figure 2).

Although the definition of endocannabinoid system as their receptors, ligands, and enzymes is widely known, this signaling presents a high complexity.[11] For instance, anandamide and 2-AG can binding to other non-CB1/BC2 receptors, such as

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G-protein coupled receptor 55 (GPR55), peroxisome proliferator-activated nuclear receptors (PPARs), and transient receptor potential vanilloid-1 (TRPV1) channel.[12–15] Moreover, the enzymes involved in biosynthetic pathways and enzymatic hydrolysis of anandamide and 2-AG can also participate in metabolic processes of other N-acyl-ethanolamines and 2-mono-acyl-glycerols. Likewise, anandamide and 2-AG can be metabolized by other non-cannabinoid enzymes, such as cyclooxygenase-2 (COX2)—an important component of inflammatory pathways.[16]

This complexity of endocannabinoid signaling can be extended to phytocannabinoids. As mentioned, *Cannabis* plant presents more than 70 compounds, among these the Δ9-tetrahydrocannabinol (Δ9-THC) seems to be the most important psychotomimetic phytocannabinoid. The main targets of Δ9-THC are the CB1 and CB2 receptors. Other phytocannabinoids, such as cannabidiol (CBD), CBD acid (CBDA), cannabidivarin (CBDV), cannabigerol (CBG), Δ9-tetrahydrocannabivarin (THCV), THCV, acid (THCVA), and CBDV acid (CBDVA) can directly act on cannabinoid receptors, or can produce effect through modulation of enzymatic process.[11]

Take this complexity into account, the investigation of endocannabinoid system, as well as the effects of phytocannabinoids or synthetic cannabinoids by several methodologies could increase the knowledge about the regulation of endocannabinoid signaling as a whole. Considering the large number of proteins and intricate protein networks involved in these functions, the implementation of proteomic and lipidomic profiling studies may help to elucidate endocannabinoid activity in basal and pathological psychiatric conditions.

2. Proteomics and Lipidomics in the Investigation of Endocannabinoid System

MS-based proteomic and lipidomic techniques are potential tools to understand the molecular mechanism involved in brain disorders. Proteomics and lipidomics can simultaneously quantify a large number of proteins and lipids in samples, under different conditions (for instance, drug treatment, substance abuse, and basal or pathological state). Associated with the proteome and lipidome data, in silico systems biology-based analysis can unravel pathways and biological process implicated in the pathobiology of diseases and physiology function, under certain condition that could not be investigated by other technical tools. Therefore, the association of these tools is crucial to better understand the role of the endocannabinoid signaling in the brain homeostasis and schizophrenia pathophysiology.

3. Schizophrenia and Endocannabinoid System

The first evidence of endocannabinoid system in schizophrenia was provide from the observation about high *Cannabis* abuse among patients.[17,18] Consistent findings have shown that cannabis abuse worsens the symptoms of schizophrenic patients,[19,20] and increases the risk for schizophrenia development in vulnerable individuals.[21] Δ9-THC seems to be the

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Table 1. Description of proteins of the endocannabinoid system.

Protein name	Gene name	Protein class	Molecular function	Localization	Protein expression
α, β- hydrolase domain containing protein 12	ABDH12	Disease related genes, Enzymes, potential drug targets, predicted intracellular proteins, and predicted membrane proteins	Acylglycerol lipase activity Lysophospholipase activity	Intracellular, Membrane	Cytoplasmic expression in cells in intestinal tract and processes in CNS.
Cannabinoid receptor 1	CNR1	G-protein coupled receptors	Cannabinoid receptor activity Drug binding	Intracellular, Membrane	CNS and immune cells
Cannabinoid receptor 2	CNR2	G-protein coupled receptors, FDA approved drug targets, Predicted membrane proteins	Cannabinoid receptor activity	Membrane	Variable levels in most tissues
Fatty-acid amide hydrolase 1	FAAH	Enzymes, Predicted membrane proteins	Acylglycerol lipase activity Anandamide amidohydrolase activity Fatty acid amide hydrolase activity	Membrane	General cytoplasmic
G-protein coupled receptor 55	GPR55	G-protein coupled receptors, Predicted membrane proteins	Cannabinoid receptor activity G-protein coupled receptor activity	Membrane	Pending normal tissue annotation
Monoacylglycerol lipase ABHD6	ABHD6	Enzymes, Predicted membrane proteins	Acylglycerol lipase activity Phospholipase activity	Membrane	Nuclear membrane and cytoplasmic expression in most tissues
Monoglyceride lipase	MGLL	Enzymes, Predicted intracellular proteins, Predicted secreted proteins	Acylglycerol lipase activity, Lysophospholipase activity, Protein homodimerization activity	Intracellular Secreted	Granular cytoplasmic expression of most tissues
N-acylethanolamine-hydrolyzing acid amidase	NAAA	Predicted intracellular proteins, Predicted secreted proteins	Hydrolase activity Transcription factor binding	Intracellular Secreted	General cytoplasmic expression.
Protein ABHD4	ABHD4	Enzymes, Predicted intracellular proteins	Hydrolase activity	Intracellular	Cytoplasmic expression in several tissues
Sn1-specific diacylglycerol lipase alpha	DAGLA	Disease related genes, Predicted membrane proteins	Acylglycerol lipase activity Metal ion binding	Membrane	Cytoplasmic expression in several tissues Abundant in CNS
Sn1-specific diacylglycerol lipase beta	DAGLB	Predicted membrane proteins	Acylglycerol lipase activity Metal ion binding	Membrane	Pending normal tissue annotation
Transient receptor potential cation channel subfamily V member 1	TRPV1	FDA approved drug targets Predicted membrane proteins Transporters Voltage-gated ion channels	Calcium channel activity Phosphatidylinositol binding Transmembrane signaling receptor activity ATP binding, and others	Membrane	Pending normal tissue annotation

main responsible for these effects. On the other hand, another phytocannabinoid—CBD—presents antipsychotic properties in patients.[8,22,23]

Studies have investigated the genetic relationship between schizophrenia and endocannabinoid system. The main findings point to CNR1 polymorphisms in this disorder,[24–27] while these polymorphisms were not confirmed by other studies.[28,29] Ho et al. found that the interaction of CNR1 genetic polymorphisms (such as rs12720071) with heavy cannabis use in schizophrenic patients resulted in decreased white matter brain volume, and cognitive impairment. This corroborates with the hypothesis that schizophrenia is mediated by genetic and environmental factors.[30] In addition, Ishiguro et al. found that variations in rs2501432 (R63Q) and rs12744386 polymorphisms in the CNR2 gene might have a role in the pathophysiology of schizophrenia.[31]

Neuroimaging studies, using positron emission tomography (PET), have been used to investigate CB1 in schizophrenia[32]

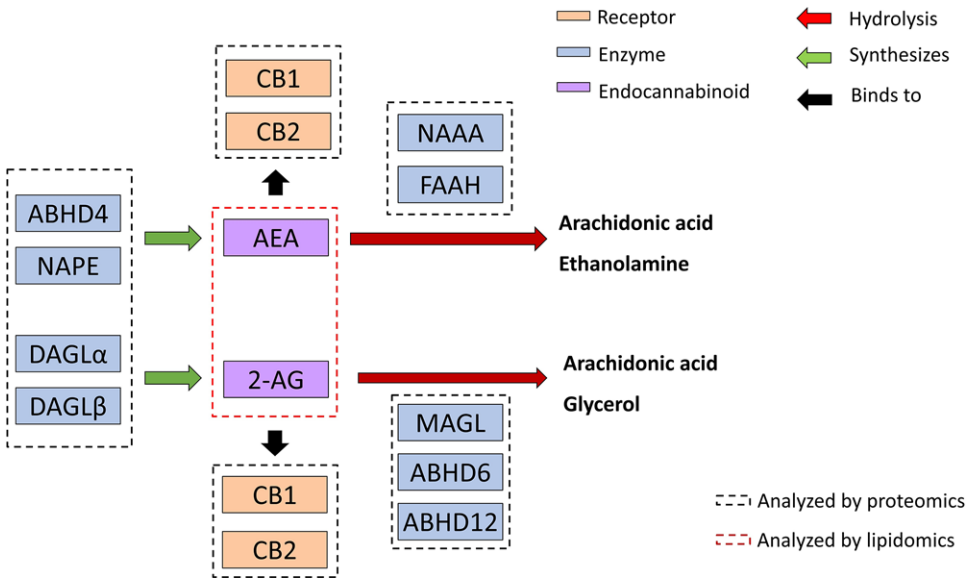

Figure 1. An overview of the components endocannabinoid system. Legend: anandamide (AEA), 2-arachyidonoylglycerol (2-AG), fatty acid amide hidrolase (FAAH), monoacylglycerol lipase (MAGL), N-acyl-phosphatidylethanolamine-phospholipase (NAPE), diacylglycerol lipase alpha (DAGLα), and beta (DAGLβ), α,β-Hydrolase-domain-containing protein 6 and 12 (ABHD6 and ABHD12), α,β-hydrolase domain-containing protein 4 (ABHD4), N-acylethanolamine-hydrolyzing acid amidase (NAAA), and type 1 (CB1) and 2 (CB2) cannabinoid receptors.

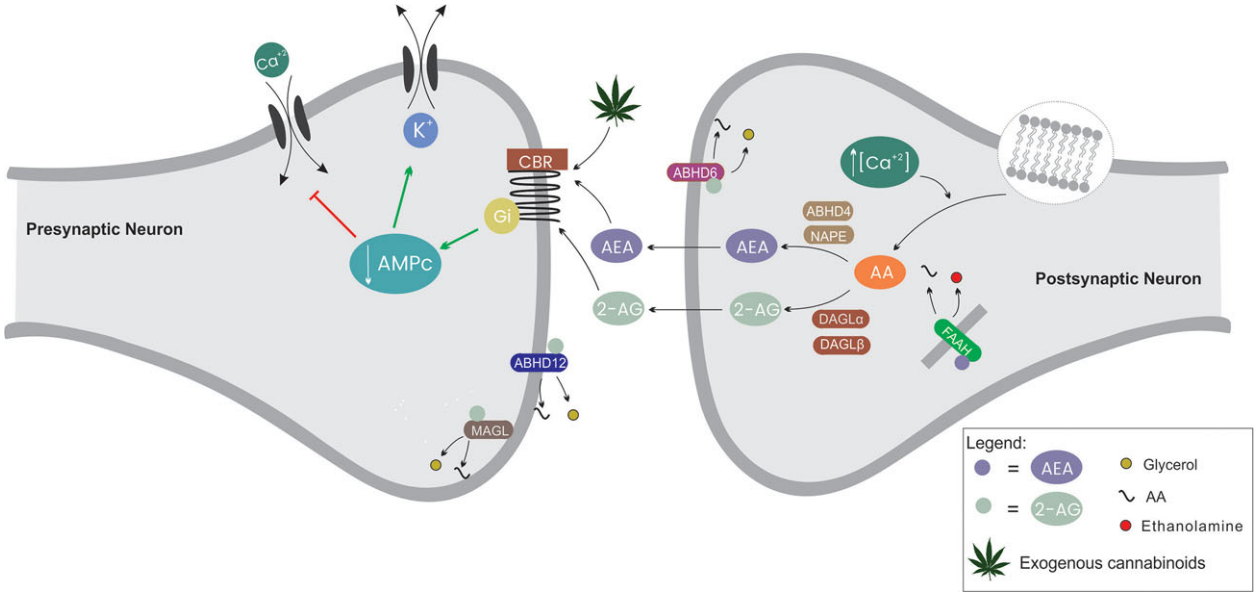

Figure 2. An overview of the endocannabinoid signaling. Endocannabinoids are synthesized upon demand at postsynaptic neurons and act as retrograde messengers. After this, the endocannabinoids are removed rapidly from synapses by transporters at neuronal membranes. On the post-synaptic side, 2-AG can be hydrolyzed into glycerol and AA by the enzyme ABHD6, embedded in the membrane. On the pre-synaptic side, 2-AG can be broken down by MAGL, loosely associated with the plasma membrane, or by ABHD12, a transmembrane protein. On the other hand, AEA is hydrolyzed by FAAH, an integral membrane enzyme, located at intracellular membranes of postsynaptic somata and dendrites.

(**Table 2**). Studies using different radioligands have shown increase of the CB1 binding in some brain areas associates with the pathophysiology of this disorder.[33–35] A postmortem study corroborated this finding.[36] In contrast, another PET investigation reported a decrease in CB1 binding in schizophrenic patients compared to controls.[37] These divergent findings might be related to the gender, sex, and age of the subjects, the different affinity and pharmacokinetics of PET traces, the disease duration, and the substance abuse.[32,38] In this context, future PET studies on CB1 in schizophrenia should be designed with a complex data set obtained in a large-scale investigation.[32] However, the PET studies are not able to elucidated pathways or biological processes in regulation of CB1 in several conditions, for instance, in *Cannabis* abuse or antipsychotic treatment conditions. Thus, proteomic-related techniques could be interesting in this field.

Table 2. The main information about PET studies that analyzed CB1R binding in schizophrenia patients.

	Ranganathan et al. (2016)	Ceccarini et al. (2013)	Wong et al. (2010)	Ceccarini et al. (2010)
Patient's Sex	Male Only	Female: • 36%: schizophrenia • 33%: Control	One female	Female: • 34.5%: schizophrenia • 33.3%: control
PET tracer	[11C]OMAR	[18F] MK-9470	[11C]OMAR	[18F] MK-9470
Symptom severity	73.9 (PANSS)	52.3 (PANSS)	34.1 (BPRS)	–
Findings Schizophrenia x Healthy Subjects	Reduction of 12% in volume of distribution, most strongly in the caudate, posterior cingulum, hypothalamus, amygdala, hippocampus, and insula.	Increased CB1R binding in the insula, mesotemporal lobe, nucleus accumbens, cingulate, inferior frontal cortex, and parietal cortex.	15–23% increase in volume of distribution in most of the brain regions assessed. However, it was significant only in the pons.	Increase of CB1R in the mesocorticolimbic circuitry, especially in the nucleus accumbens. Schizophrenia patients treated with antipsychotic monotherapy presented increased relative CB1R binding in the insula and anterior cingulate cortex (ACC).

Postmortem investigations based on radioligand binding have demonstrated alterations in CB1. Some studies measured CB1 autoradiographic density in schizophrenia, and found higher levels of this receptor in the dorsolateral prefrontal cortex (DLPFC),[39,40] and in the cingulate cortex.[41,42] On the other hand, studies using immunodetection methods detected a decreased CB1 protein expression in the DLPFC,[43–45] and no changes in CB1 levels in the cingulate cortex in schizophrenia.[46]

Moreover, increased CB1 and CB2 receptors in peripheral blood leukocytes were detected by flow cytometry in schizophrenic patients compared to controls.[47] Another group found increased levels of CB1 and CB2 mRNA in the human peripheral blood mononuclear cells (PBMCs) from patients with schizophrenia.[48] In addition, Ferretjans et al. showed that increased cannabinoid receptors expression on lymphocytes and monocytes was significantly correlated with worst outcomes in cognitive performance.[49] Together, these findings point to dysregulation of endocannabinoid signaling also in the peripheral system, and possible implications for immune responses in schizophrenia.

Animal models have also contributed to understand the pathophysiology of schizophrenia. A PET study reported increased CB1 expression in the adulthood progeny of female rats exposed to poly I:C during gestational period.[50] Another group found changes in CB1 expression in the DISC1 mutant mice.[51] The increase in CB1 was also observed in offspring from maternal malnutrition via high-fat or low-protein diets, a model of maternal disturbance.[52] Moreover, increased mRNA levels of NAPE-PLD, MAGL, CB1, DAGLβ, and DAGLα were detected in brains of socially isolated rats, especially in cortical layers, and prefrontal and thalamic regions.[53] The authors also verified a decreased FAAH mRNA expression in the caudate putamen, some prefrontal regions, and cortical layers of those animals.[53] In contrast, studies using a pharmacological model of schizophrenia, the PCP-treated rats, did not find change in CB1 expression.[54–56]

Although genetic, neuroimaging, and behavioral studies have reinforced the role of cannabinoids in the pathobiology of schizophrenia, molecular pathways and biological processes involved in cannabinoid effects are not totally understood. Moreover, the multifactorial profile of the schizophrenia etiology results in a high complexity of protein expression and molecular pathways that can be investigated using proteomic-related methods. Finally, the diagnosis and treatment of schizophrenia is only based in clinical findings. In this regard, the investigation of endocannabinoids as biomarkers for disease prognosis or treatment response could be an interest approach to assist psychiatrist.

3.1. Proteomic Contribution to Unravel the Endocannabinoid Signaling

The main proteins involved in endocannabinoid signaling can be found in several tissues, where they are involved in several biological processes (Table 1). Between these proteins, those related to enzymatic biosynthesis and metabolic play a key role in the endocannabinoid signaling pathways. Initially, it was accepted that FAAH and MAGL were the main responsible for anandamide and 2-AG hydrolyses. Although, at least for 2-AG, proteomic studies have shown the role of others enzymes in this process.

A proteomic tool used to investigate endocannabinoid enzymes is the activity-based protein profiling (ABPP).[57] This technique actively target proteins of interest, enabling the identification and visualization of active enzymes.[57,58] The ABPP probes can enrich, detect, and identify various members of a protein class, which presents conserved functional features, clarifying alterations in protein activity that are not shown in protein abundance or transcript.[59] In this regard, a study using ABPP fluorophosphonate-biotin with avidin chromatography and advanced LC–MS analysis confirmed that MAGL is the main enzyme responsible for metabolism of 2-AG in brain, although there some conversion occurs via ABHD6 and ABHD12—two previous uncharacterized enzymes in the hydrolyses of 2-AG.[60]

A recent study used the ABPP approach, coupled with high-resolution MS analysis, to quantify the activity of serine hydrolases (DAGL-α, FAAH, ABHD6, ABHD12, MAGL, and ABDH4) associated with endocannabinoid biosynthesis and degradation in the hippocampus, cerebellum, frontal cortex, and striatum.[58] Interestingly, the activity profiles of some enzymes were not found to be correlated to protein abundance, as reported in a global proteomics data set.[61] This suggests that the activity of these proteins could be regulated by other feed-forward or feed-back mechanism via alterations in posttranslational modification, such as phosphorylation. For instance, ABHD12

abundance in the hippocampus was twofold higher compared to that in other brain regions, but the activity of this enzyme was found to be the same.[58] MAGL activity was lowest in the cerebellum, while the activity of DAGL-α in that region was higher compared to the other regions, and FAAH was found to have the highest activity in the hippocampus and frontal cortex.[58] Together, these findings point to the variability in the expression of endocannabinoid enzymes across different brain regions.[1,61–63]

In this regard, these findings could be applied to understand the pathophysiology of schizophrenia. As mentioned, alterations in the endocannabinoid signaling have been reported in this disorder, but no data pinpoint the expression of endocannabinoid enzymes in schizophrenia. Thus, ABPP coupled with MS analysis can be a potential approach in this field.

Additionally, Viader et al. applied the ABPP technique combined with shotgun LC–MS to investigate the serine hydrolase activities in astrocytes, neurons, and microglia of mouse brain.[64] In this study, the brain cell proteomes were treated with a biotin-coupled fluorophosphonate (FP) probe, enriched with avidin chromatography and analyzed by Multidimensional Protein Identification Technology (MudPIT)—a chromatography-based proteomics approach that allows the large-scale shotgun analyses of complex peptide mixtures—that preset the 2D chromatographic separation coupled to tandem MS. To note, the MudPIT approach represent a potential methodology for proteomic studies because its robustness and reproducibility.[65] In this way, Viader and colleagues[64] reported that the activity of several enzymes presented a good correlation with RNA-seq data, however some enzymes were not correlated, or even anti-correlated with this data.[66] Despite these differences, the ABPP/MudPIT analysis was able to detect the differential expression in the endocannabinoid enzymes between glial cells and neurons,[64] consistent with the hypothesis that there is a cooperativity between these cells in the central nervous system.[67] In addition, Viader et al. (2016) found that serine hydrolases responsible for 2-AG metabolic process are mostly compartmentalized enzymes. The MAGL or DAGLα expression may be common in neurons, compared to microglia or astrocytes. On the other hand, ABHD12 and DAGLβ were found to be highly expressed in microglia, and DAGLα is mostly found in astrocytes, while neurons or microglia express higher levels of DAGLβ.[64]

As mentioned above, 2-AG modulates several functions, such as behavioral, mood, pain and neuroinflammation,[68,69] mainly through the effects of 2-AG on tripartite synapses and interneuronal communications.[70–73] Thus, proteomic studies may be applied to elucidate the mechanisms involved in the regulation of 2-AG levels, as well as a mean of pointing to potential novel therapeutic targets for brain disorders,[64] especially those characterized by neuroinflammation.

In vivo study combining a selective pharmacological inhibition of DAGL, with chemical proteomic/lipidomic analysis enabled the observation of rapid and extensive changes in brain lipid signaling.[74] The inhibition of enzymes by chemical compounds is a useful approach to investigate the effects of acute blocked of enzymes, and its consequences in the signaling of physiological and pathological conditions. Although the known complexity of endocannabinoid metabolic process can hamper the investigation of therapeutic potential of enzyme inhibitors, since anandamide and 2-AG share the enzymatic pathways with other lipids. In this regard, the association of pharmacological inhibition with proteomic and lipidomic techniques can provide a comprehensive overview about lipid network in brain.[74] Interestingly, this study found that the known DAGLα and DAGLβ inhibitors affected not only 2-AG content, but also anandamide, prostaglandins, arachidonic acid, and diacylglycerols levels probably through crosstalk mechanism between endocannabinoid signaling.[74]

The ABPP proteomic method also investigated the enzyme responsible for anandamide degradation (FAAH). Increasing interest in FAAH inhibitors has been shown in clinic for several disorders,[75] for instance the BIA 10–2474, an irreversible inhibitor of FAAH, has been tested in humans, with disappointed results. One volunteer died and four others were hospitalized,[76–79] with mild-to-severe neurological symptoms.[78] Therefore, van Esbroeck et al. used ABPP and MS to determine the serine hydrolase interaction landscape of BIA 10–2474, in human cells and tissues.[80] Moreover, the authors compared its selectivity with PF04457845, a highly selective FAAH inhibitor that advanced to phase 2 trials without severe adverse events.[81–83] The authors showed that, at the lowest concentration tested (0.2 mM), both drugs had good selectivity for FAAH. However, across the drug concentration range, PF04457845 maintained its selectivity, while BIA 10–2474 (and its metabolite BIA 10–2639) showed various off-targets, including xenobiotic drug-metabolizing enzymes, and lipid hydrolases (such as ABHD6 and ABHD11).[80] Most of these off-targets are substantial expressed in human brain tissue, being that many of them are involved in cellular lipid metabolism.[84,85] Therefore, the use of FAAH inhibitors must be carefully studied, since promiscuous compounds may alter cellular lipid networks in human cortical neurons and deregulates the lipid metabolism in the CNS, contributing to neurotoxicity.[80] In this context, proteomic tools, such as ABPP and MS may play a role in selectivity and toxicity studies of these compounds in humans and animal models.

Although the aforementioned studies did not use schizophrenia models or patients, they reveal interesting approaches to investigate the endocannabinoid signaling in this disorder.

3.2. Effects of Cannabinoids on Proteome: Implications for Schizophrenia

It has been shown that psychotomimetic effects of Δ9-THC occurs by CB1 activation in neurons. However, effects of Δ9-THC on proteome of brain cells are not totally understood. Similarly, the effects of CBD and others phytocannabinoids or synthetic cannabinoids on proteome are not investigated in schizophrenia pathobiology and treatment. However, some studies have used proteome-related tools to investigate the effects of Δ9-THC and CBD.

Proteomic studies can elucidate molecular fingerprints of CB1 activation by Δ9-THC or other agonists in synaptic development and axonal growth. This could lead to an increased understanding of the molecular basis of cannabis-induced psychiatric illnesses.[86] Along these lines, MALDI is an ionization technique[87] in which the sample is vaporized and ionized using an UV-absorbing chemical compound and a laser, producing multiply charged analytes[88], and electrospray ionization (ESI),[89]

was innovative since it made possible the analysis of non-volatile, relatively large biomolecules, such as peptides and proteins.[88] In this way, these techniques (nano LC–MALDI/MS/MS and nano LC–ESI/MS/MS) showed the effects of Δ9-THC on the neuronal proteome during neurodevelopment,[90] suggesting that fetal cannabis exposure may have a negative impact on establishment of synaptic connectivity in neuronal networks underpinning memory, cognition and executive skills, leading to "circuit failure" in these systems. This might account for the observed increase in incidence of certain psychiatric disorders and drug addiction in the adult offspring of those individuals who had been exposed prenatally to *Cannabis* use.

Although some studies applying genetic, image, and behavioral task methodologies, as described above (see Schizophrenia and Endocannabinoid System Section), have pinpointed a role of this system in the pathophysiology of schizophrenia,[91,92] few proteomic investigations have been carried out with the same goals in mind. In the case of preclinical studies, hippocampal samples from the neuregulin 1 transmembrane heterozygous (Nrg1 HET) mouse—a model of schizophrenia—was used in a proteome profiling analysis.[93] These animals and their wild-type (WT) littermates were treated with Δ9-THC, and the proteome analyses demonstrated that Nrg1 HET mice presented changes in the abundance of proteins involved in several biological processes (**Table 3**).

Another study investigated the effect of Δ9-THC on the hippocampal proteome of adolescent and adult Wistar rats to elucidate the role of Δ9-THC treatment on biological processes in brain.[94] This analysis found that the levels of 27 proteins involved in important biological processes were induced by Δ9-THC treatment of adolescent animals. At the same time, ten hippocampal proteins involved mainly in signaling pathways were more affected in adult rats following the same treatment. Taken together with the behavioral alterations, these proteomic findings suggest that the adolescent brain is more vulnerable to Δ9-THC exposure, in comparison to adult brains.

In order to extend our knowledge of the pivotal protein constituents involved in the endocannabinoid system and psychiatric disorders, we used the in silico systems biology tool STRING (Search Tool for Recurring Instances of Neighboring Genes; https://doi.org/10.1093/nar/gkw937). In this analysis, STRING was used to combine proteins of the endocannabinoid system (Table 1) with those modulated by Δ9-THC in Wistar, wild type, and the Nrg1 HET animals (Table 3). This highlighted the proteins NAPE-PLD and Parkinsonism associated deglycase (PARK7) as key hub proteins (**Figure 3**). NAPE-PLD is involved in the biosynthesis of several N-acylethanolamines (NAEs) in the mammalian brain,[95] including anandamide. NAPE-PLD also plays a role in inflammatory processes.[96] The levels of this enzyme increase throughout brain development, suggesting that anandamide synthesis via the NAPE-PLD pathway can be higher at maturity, compared to earlier stages of life.[97]

Moreover, NAPE-PLD-knockout mice show lower levels of anandamide and other NAEs, such as prostaglandins.[63] Thus, dysregulation of NAPE-PLD may affect the brain through inflammatory processes.[98] This is consistent with the idea that neuroinflammatory processes are implicated in the pathophysiology of schizophrenia and other psychiatric conditions. Additionally, NAPE-PLD is upregulated in adolescent Nrg1 HET mice treated with Δ9-THC, compared to a vehicle-treated group,[93] suggesting a possible mechanism of Δ9-THC in regulation of anandamide levels. Initially, it was believed that phytocannabinoids like Δ9-THC only has CB1/CB2 agonist properties, but current studies have shown the effects of these compounds in enzymatic process, and with the analyses we reported a role of Δ9-THC in NAPE-PLD (Figure 3). These findings warrant further investigation to increase our understanding of the potential role of NAPE-PLD in schizophrenia, and to explore this pathway for potential novel biomarkers and drug targets.

Another protein highlighted by our analyses was PARK7, which is involved in transcriptional regulation, protein degradation,[99] neurotransmitter homeostasis,[100–102] cell survival and proliferation,[103,104] and mitochondrial function regulation.[105] Moreover, PARK7 presents antioxidant and chaperone activity, and dysfunctions of this protein have been associated with neurodegenerative disorders, such as Parkinson disease.[99] In mammalian cells, oxidative stress induces changes in PARK7 properties.[106,107] In this context, a study reported the involvement of PARK7 in protection against oxidative stress, particularly in neurons.[108] Moreover, Meiser et al. demonstrated that loss of PARK7 decreases serine biosynthesis and glutamine influx, two pathways that provide precursors for de novo synthesis of glutathione, an important antioxidant.[109] In this regard, PARK7 levels were decreased in the hippocampus of adolescent Wistar rats treated with Δ9-THC.[94] Thus, Δ9-THC may increase the risk of oxidative stress, particularly in neurons. Moreover, the downregulation of PARK7 induced by Δ9-THC treatment may also impair neurotransmission,[110] thereby altering the expression of neurotransmitter receptors through feed-forward and feedback mechanisms.[111] However, the function of PARK7 in schizophrenia has not been completely elucidated. A postmortem study did not find changes in PARK7 mRNA levels in brain of schizophrenic patients compared to controls.[112] To note, the main effects on PARK7 aforementioned is associated with Δ9-THC that is not present in the postmortem study, since these patients were not *Cannabis* users. Thus, more efforts to understand the role of PARK7 in *Cannabis* abuse of schizophrenic patients are needed.

Another phytocannabinoid, CBD, has been implicated in schizophrenia. Unlike Δ9-THC, CBD presents antipsychotic properties in schizophrenic patients[8,22,23] and in several animal models to study schizophrenia.[113–118] Although proteomic data about CBD in schizophrenia are limited. Some studies have shown the effects of CBD on behavioral-like schizophrenia symptoms associated with changes in protein levels by western blot, immunohistochemistry, and autoradiography receptor binding[97,119,120] (see **Table 4**). These studies point to important pathways in schizophrenia pathophysiology; however, proteomic investigations could substantially increase the understanding of proteins, pathways, and mechanisms involved in antipsychotic properties of CBD.

Taken together, the proteomic findings may contribute to the understanding of the increased psychosis related to cannabis use in schizophrenia, and elucidate possible relations with genetic vulnerability in patients with this disease. Furthermore, this could lead to the identification of new biomarker candidates for monitoring disease risk, progression, or treatment response.

Table 3. Proteins differently regulated in the hippocampus of adolescent *Nrg1* HET mice, WT mice, and Wistar rats: treatment with Δ9-THC x treatment with vehicle.

Biological Process	Protein name	Animal	Regulation	Reference
NMDA receptor trafficking to the synaptic membrane	G-protein-signaling modulator 2 (GPSM2)	Nrg1 HET mice	Downregulated	[93]
Lipid raft stabilization of receptors at the synaptic membrane	Apolipoprotein A1 (APOA1)		Upregulated	[93]
	Flotillin-1 (FLOT1)		Upregulated	
Cell survival/ Cytotoxicity related proteins	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPEPLD)		Upregulated	[93]
	Programmed cell death protein 2 (PCD2)		Upregulated	
	Interleukin-2 (IL2)		Downregulated	
Proteins regulating oxidative stress	Glutathione S-transferase Mu 2 (GSTM2)	WT mice	Downregulated	[93]
	Heat shock protein (HSPA4)		Upregulated	
	Stress-70 protein, mitochondrial precursor (GRP75)	Wistar rats	Downregulated	[94]
	Glutathione S-transferase omega-1 (GSTO1)		Downregulated	
	Heat shock cognate 71 kDa protein (HSPA8)		Downregulated	
	Protein DJ-1 (PARK7)		Downregulated	
	Peroxiredoxin-6 (PRDX6)		Downregulated	
	60 kDa heat shock protein (HSPD1)		Downregulated	
Retrograde trafficking of endosomes between the Golgi apparatus and the membrane	ADP-ribosylation factor-like protein 1 (ARL1)	WT mice	Downregulated	[93]
Retention of ER resident proteins / Cotranslational protein targeting to membrane	Translocon-associated protein subunit alpha (SSR1)	Nrg1 HET mice	Downregulated	[93]
Bicarbonate transport / Interleukin-12-mediated signaling pathway	Carbonic anhydrase 3 (CA1)		Downregulated	[93]
Endosomal transport	Vacuolar protein-sorting-associated protein 25 (VPS25)		Downregulated	[93]
Sensory perception of sound	Otoraplin (OTOR)		Upregulated	[93]
Cytoskeletal	Transgelin-3 (NP25)	Wistar rats	Downregulated	[94]
	Tubulin a-2 chain (TUBA)		Downregulated	
	Tubulin b-3 chain (TUBB3)		Downregulated	
Signaling	Annexin A3 (ANXA3)		Downregulated	[94]
	14-3-3 protein zeta (YWHAZ)		Downregulated	
	14-3-3 protein gamma (YWHAG)		Downregulated	
Metabolic proteins	Phosphoglycerate mutase 1 (PGAM1)		Downregulated	[94]
	Ubiquitin-conjugating enzyme E2 variant 2 (UBE2V2)		Downregulated	
	Nucleoside diphosphate kinase B (NME2)		Downregulated	
Myelination	Myelin basic protein (MBP)		Upregulated	[94]
Intracellular membrane trafficking	Ras-related protein Rab-1A (RAB1A)		Upregulated	[94]
Control of cell cycle progression and genomic stability (among others)	NAD-dependent deacetylase sirtuin-2 (SIRT2)		Downregulated	[94]
Calcium signaling	Calcium-binding protein (CALB2)	WT mice	Downregulated	[93]

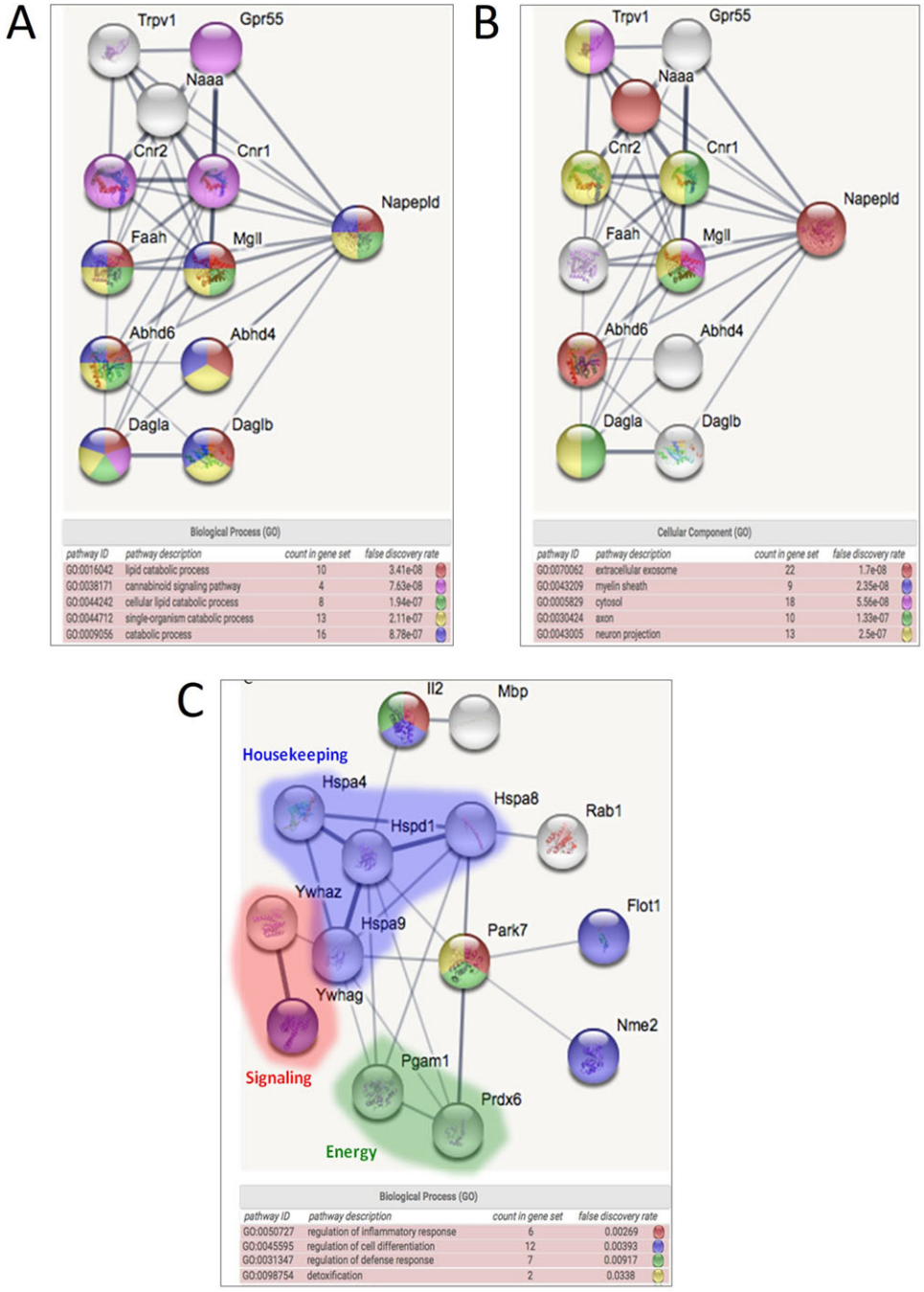

Figure 3. In silico analysis of proteins of the endocannabinoid system and those modulated by THC in normal rats and Nrg1-HET. A, B) evidence the role of NAPEPLD in the endocannabinoid system. C) THC-modulated proteins, suggesting the pivotal role of PARK7.

4. Lipidomic Studies

Lipids are comprised of compounds with long chain hydrocarbons, and they are involved in several biological processes in all tissues such as the brain, including exocytosis, ion channel regulation, membrane domain formation, localization and function of proteins in membranes, and cell signaling pathways.[121] Studies have shown that alterations in the brain lipid content may play a role in psychiatric disorders.[122,123] Thus, investigations of the lipid levels in some of these disorders may help to elucidate their role in the pathophysiology. The most characterized endocannabinoids, anandamide, and 2-AG, belong to the N-acylethanolamine and monoacylglycerol lipid classes, respectively. Therefore, the study of these endocannabinoids in healthy and pathological conditions can be achieved via lipidomic profiling techniques.

Table 4. Proteins differently regulated by CBD-treatment in animal models.

Biological Process	Protein name	Animal	CBD effects	Reference
Ion Transport (locomotor behavior, learning, memory)	NMDAR GluN1 subunit gene (GRIN1)	C57BL/6J mice treated with MK801	Upregulated	[119]
Cell metabolism, growth, proliferation and survival	Mammalian target of rapamycin (mTOR)	Sprague Dawley rats treated with Amphetamine	Upregulated	[120]
Apoptosis, cell cycle, translation regulation	Ribosomal protein S6 kinase beta-1 (p70S6K)	Sprague Dawley rats treated with Amphetamine	Upregulated	[120]
Behavior, host-virus interaction	5- hydroxytryptamine receptor 2A (5-HT_{2A} receptor)	WT mice	Reduced binding (substantia nigra)	[97]
Ion transport (regulates brain excitability)	Type A gamma-aminobutyric acid receptor ($GABA_AR$)	Nrg1 TM HET mice	Increased binding (retrosplenial granular cortex)	[97]

Lipidomics is the global study of lipids found in cells, tissues, or organisms, and the changes of its levels under different physiological conditions. Lipidomic profiling can reveal the molecular pathways involved in the biotransformation of lipids,[124] and provide data about the role of specific lipids in physiological and pathological signaling events in different tissues, such as the brain.[123] To quantify molecular species of lipids, LC or GC, combined with and ESI/MS, can be applied under atmospheric pressure conditions.[121] These platforms have allowed the acquisition of better lipid profiles, and are essential to explore the dynamics of individual and combined lipids in a signaling pathway.[122] However, these techniques are limited by chromatographic resolution for lipid separations. Additionally, the technologies required for quantification of the lipid-based endocannabinoids still requires some optimization,[125] since anandamide and 2-AG are mostly present at low concentrations (pmol g^{-1} to nmol g^{-1}) in biological samples. As mentioned in the first section, endocannabinoids are produced upon demand and are not stored in vesicles or compartments, hampering their measurements in biological samples.[126] Moreover, the quantification of 2-AG seems to be more complex, due to its isomerization into 1-arachidonoyl glycerol (1AG).[125,127] These studies suggested that LC–MS/MS methods may be limited by interference from isobaric isomers, resulting in false results about 2-AG content.

Isobaric (molecules with the same nominal mass, but differently exact mass) and isomeric (molecules with the same molecular formula, but with different chemical structure) species confer an overlap in between lipid classes, resulting in a poorly quantitative lipidomic profile in ESI/MS and LC–MS methods.[128] The isobaric/isomeric species are usually observed in lipids. In this regard, the differential mobility spectrometry (DMS), also called field-asymmetric waveform ion mobility spectrometry (FAIMS), can provide a continuous-ion monitoring, and an orthogonal ion mobility, resulting in a better separation in shotgun lipidomics.[128,129]

In DMS method the lipids are ionized by electrospray ionization (ESI), and filtered by DMS cell prior to MS analysis.[128] A high-voltage asymmetric waveform is applied across two planar electrodes in DMS cell, and the difference between the mobility during the high- and low-field portions of the waveform determines the exact trajectory taken by the ions. Thus, DMS allows the evaluation of isobaric and closely related lipids, and quantification of monitored species. Another method using stable isotope-labeled internal standards, SPE, and ultra-performance LC–MS/MS (UHPLC–ESI–QTOF–MS) was able to identify and quantify different congeners of *N*-acylethanolamides (NAEs) family, in which anandamide belongs.[126] Taken into account, these successful methods could be used in lipidome analysis from schizophrenic patients to better understand the endocannabinoid disturbances with high accuracy. Additionally, these methods could be applied to study endocannabinoids as biomarkers for treatment response of antipsychotics.

Although, DMS method is one of the most accurate for lipidome profile, some studies have reported success approaches for quantification of the lipid-based endocannabinoids. Kingsley and Marnett described that anandamide and 2-AG of mammalian tissues can be quantified using ESI–LC–MS/MS.[130] Recently, a high sensitivity microflow LC approach with detection by quadrupole MS/MS was reported to quantify the

levels of endocannabinoids.[131] Another group measured endocannabinoid levels in rat brain using LC–MS/MS with ESI and multiple reaction monitoring (also termed selective reaction monitoring).[132]

Several studies have shown changes in endocannabinoid levels in schizophrenia, employing several distinct MS-based methods (**Table 5**). A study using HPLC–MS showed that anandamide levels in plasma were higher in patients compared to healthy subjects,[133] while increased levels of anandamide and palmitylethanolamide were detected in the cerebrospinal fluid in schizophrenic patients, using LC–MS and isotope dilution GC–MS.[134] These findings have been supported by other investigations using LC–MS/MS, which confirmed the occurrence of increased anandamide levels in schizophrenia.[135–138] Interestingly, psychotic symptoms were negatively correlated with anandamide levels,[134] suggesting a protective or antipsychotic profile of this endocannabinoid.[135] Another study reported that the increase in anandamide levels in schizophrenia seems to be a homeostatic mechanism to counteract the hyper dopamine neurotransmission.[135] According to these findings, CBD attenuated the schizophrenia symptoms, and this effect was associated with increased anandamide levels.[8] Additionally, anandamide levels were found to be increased in blood samples of schizophrenic patients, and this alteration was attenuated after clinical remission with antipsychotic treatment.[139] Corroborating with these findings, a recent study reported higher levels of anandamide and palmitoylethanolamide in twin pairs discordant for schizophrenia, compared to healthy twins.[140] These studies point to anandamide as a potential biomarker for risk of developing psychosis, and for monitoring antipsychotic treatment responses in patients.

In addition to serum and cerebrospinal fluids, postmortem brain samples (cerebellum, hippocampus, and prefrontal cortex) from schizophrenic patients and control subjects have also been analyzed by lipidomic approaches.[141] This study quantified endocannabinoid levels using the LC–MS detection and the analyses showed that schizophrenic patients had higher levels of 2-AG and lower levels of anandamide in all brain regions analyzed, while docosahexaenoylethanolamine (DHEA) and dihomo-γ-linolenoylethanolamine (LEA) levels were found to be decreased in some brain regions compared to controls. Interestingly, this study found changes between antipsychotic-free and antipsychotic-treated at time of death. For instance, 2-AG levels of antipsychotic-treated patients did not differ from those of healthy individuals, suggesting a modulation of treatment in endocannabinoid signaling.[141] However, the measurement of endocannabinoids in postmortem samples can be limited. Another study highlighted the complexity of analyzing endocannabinoid levels in brain samples of variable postmortem delay.[142] Thus, this factor should be considered in the design and analysis of lipidomic data from postmortem studies, as should be the case for all molecular and structural studies of the brain and other tissues obtained in this manner.

Preclinical studies have also shown changes in the lipid-based cannabinoid levels in models of psychiatric disorders. A lipidomic profiling study detected decreased levels of anandamide and 2-AG in the ventral striatum of rodents submitted to bilateral olfactory bulbectomy, an animal model to study schizophrenia.[143] Additionally, the results of the LC–MS analyses were associated with behavioral tests, suggesting that the dysregulation mainly in 2-AG levels plays a role in the altered locomotor activity. Likewise, studies using isotope dilution-LC-atmospheric pressure chemical ionization–MS showed that the brain regions of rats treated with phencyclidine (PCP)[144] and animals submitted to the social isolation rearing have altered anandamide and 2-AG levels.[145] Moreover, the GC-chemical ionization MS approach using an isotope dilution assay detected increased or decreased anandamide levels, depending on the

Table 5. Alterations of endocannabinoid lipids in schizophrenia compared to healthy controls.

ECB	Alterations	Sample	Conditions	Reference
AEA	Increased	Blood samples	Under antipsychotic treatment	[113]
AEA PEA 2-AG	Increased Increased Not detected	Cerebrospinal fluid	First episode, antipsychotic-naive	[134]
AEA	Increased Unchanged	Cerebrospinal fluid Serum	First-episode antipsychotic-naïve schizophrenia	[138]
AEA OEA PEA	Increased Unchanged Unchanged	Cerebrospinal fluid	First episode, antipsychotic-naive	[135]
AEA	Increased	Cerebrospinal fluid	Prodromal states	[137]
AEA	IncreasedUnchanged	Cerebrospinal fluid Serum	First-episode, antipsychotic-naïve	[136]
AEA 2-AG DHEA LEA	Decreased in all brain regions Increased in all brain regions Decreased in cerebellum Decreased in cerebellum and Hippocampus	Cerebellum, hippocampus, and prefrontal cortex	Postmortem brain	[141]
AEA	Increased Decreased	Blood samples	Before antipsychotic After antipsychotic	[139]

2-AG, 2-arachidonoylglycerol; AEA, arachidonoylethanolamine (anandamide); LEA, dihomo-γ-linolenoylethanolamine; DHEA, docosahexaenoylethanolamine; PEA, palmitylethanolamide; OEA, oleoylethanolamine.

brain region analyzed, from PCP-treated rats.[55,56] Another group showed increased anandamide concentrations, and decreased or increased in 2-AG concentrations in some brain areas of Nrg1 mouse model of schizophrenia.[146] Together the lipid profiles found by these studies point to role of anandamide and 2-AG in schizophrenia-like behavior.

Although important lipidomic findings have shown the involvement of endocannabinoid changes in the pathophysiology of schizophrenia, more efforts are needed to clarify the effects of phytocannabinoids in this field as these compounds modulate enzymatic pathways of synthesis and hydrolyses of anandamide and 2-AG. For instance, a study reported that antipsychotic properties of CBD occurs mainly through the enzymatic blocking of FAAH, but the direct interaction of this phytocannabinoid with CB1, CB2, or TRPV1 receptors may not be excluded.[8] The same group used LC–MS to measure anandamide levels in CFS from first episode schizophrenic patients under low or high *Cannabis* exposure. The authors found that high frequency abuse downregulated anandamide levels,[136] suggesting that Δ9-THC can impair endocannabinoid levels. However, the molecular pathways involved are not clear in these results. Thus, proteomic-related techniques could fill these gaps.

5. Endocannabinoid System and Other Brain Disorders

Besides schizophrenia, endocannabinoids have been implicated in other multifactorial psychiatric disorders, for instance depression and anxiety. Depression is the most common psychiatric disorder and cause a severe impact on quality of life. Clinical and preclinical evidence have shown that CB1 blockade or deletion result in depression-like behavior,[147–149] while increased endocannabinoid levels[150–153] or exogenous CB1 agonists[154] are a potential antidepressant treatment. In the case of anxiety, cannabinoid agonists have both anxiolytic and anxiogenic effects depending on the dose used.[155,156] In addition, genetic or pharmacological manipulation of FAAH decreases anxiety-like behavior[157–159] and manipulation of DAGLα or MAGL activity, which results in decreased 2-AG levels, also plays a role in anxiety.[160–162]

Endocannabinoids have also been implicated in other brain disorders such as multiple sclerosis (MS) and Parkinson's disease (PD). In MS, cannabinoids provide a management of tremor as well as spasticity, neurodegenerative, and neuroinflammatory processes in preclinical studies.[163–167] Several lines of evidence also support the role of cannabinoids in the treatment of MS as reviewed by Rog.[168] With respect to PD, the endocannabinoids are closely related to control of motor activity in the brain.[169] This finding has generated new prospects about treatment of PD.[170–172]

Taken together these data reinforce the potential of endocannabinoid targets to treat not only schizophrenia, but also other brain disorders. Additionally, further investigations regarding endocannabinoid levels in serum or cerebrospinal fluid could clarify the role of these compounds in brain disorders as well as identify biomarkers for disease diagnosis and progression. Moreover, proteome investigations could fill the knowledge gap about pathways and biological processes involved in the effects of cannabinoids in these diseases. Finally, the endocannabinoid system deserves attention from omics-related studies on several conditions and not only schizophrenia as highlighted here.

6. Conclusions

There has been an increasing interest in potential dysfunctions of the endocannabinoid system in multiple brain diseases, but many aspects of endocannabinoid functions in the brain have not been completely elucidated. In this regard, the application of proteomic, lipidomic, and metabolomics studies could provide further knowledge about the role of the endocannabinoid system in healthy brain function, as well as how disturbances in this system could contribute to psychiatric disorders. These technologies have the advantage over traditional methods by proving a screening or profiling service as opposed to targeted approaches, which rely on prior knowledge. However, from all of these methods combined, the endocannabinoid enzymes and receptors have come under increased scrutiny as potential targets for improved treatment of some brain disorders. This may prove to be fruitful as it has been known for decades that some patients suffering from schizophrenia actually seek to self-medicate with *Cannabis* and other substances, and this may help to alleviate certain psychiatric symptoms and emotional distresses.[173,174] Whether or not such self-medication is truly beneficial, these observations still support the involvement and continued investigation of the endocannabinoid pathway as a potential source of biomarkers and drug targets for psychiatric diseases.

The protein constituents of the endocannabinoid pathway have been investigated by several proteomic studies that used MS and other differential display approaches. The consensus of these studies showed that not only the proteins abundance must be considered in endocannabinoid signaling, but also their functional state, cellular compartment, posttranslational regulation, and potentially their differential expression across different cell types and brain regions. MS is also widely used in lipidomic studies of anandamide and 2-AG. The direct investigation of endocannabinoid levels by several studies has confirmed a role of these compounds in the pathophysiology of these disorders. The lipidomic findings suggest that anandamide can be a potential biomarker for schizophrenia, which prove useful for improved stratification of patients, and to monitor the responses to antipsychotic treatments. In turn, this may lead to improved treatment of individuals suffering with this, and potential other, psychiatric disorders.

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Conflict of Interest

The authors declare no conflict of interest.

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