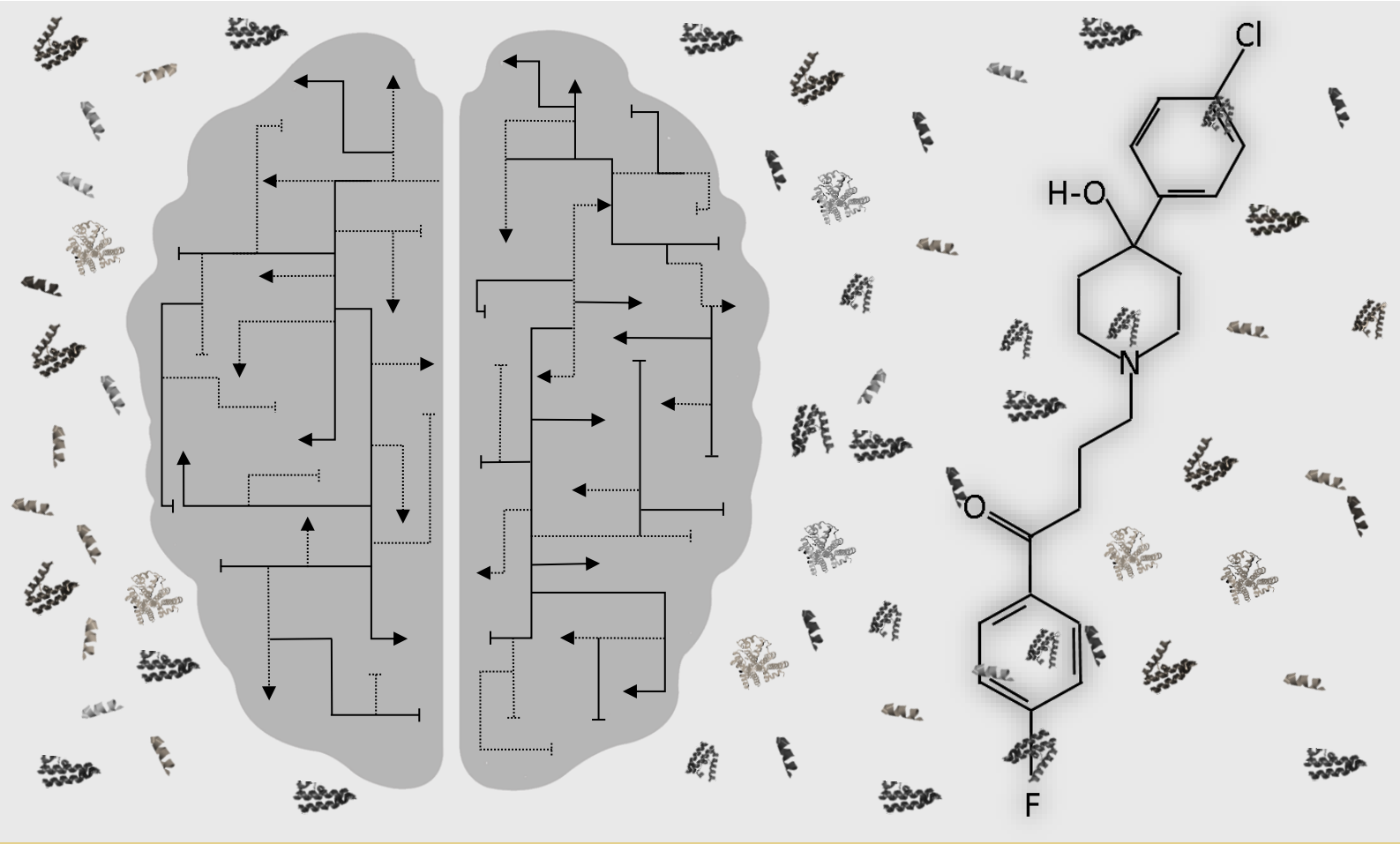

Joana Francisca dos Santos da Costa Lopes Coelho

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Dissertação de Mestrado em Investigação Biomédica, orientada pelo Doutor Bruno Manadas (Centro de Neurociências e Biologia Celular) e pela Prof. Doutora Joana Barbosa de Melo (Universidade de Coimbra e apresentada à Faculdade de Medicina da Universidade de Coimbra

Maio/2017

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UNIVERSIDADE DE COIMBRA

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Este projecto foi realizado no grupo Life Sciences Mass Spectrometry do Centro de Neurociências e Biologia Celular (Universidade de Coimbra, Portugal), orientado pelo Doutor Bruno Manadas.

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Prefácio

A presente dissertação resultou da colaboração entre a Faculdade de Medicina da Universidade de Coimbra e o Centro de Neurociências de Coimbra. Pretendia-se elucidar os efeitos moleculares a longo prazo do Haloperidol no estriado de ratinho. Resultou igualmente da colaboração um artigo de revisão que teve como objetivo avaliar os estudos, elaborados até ao presente ano, de biomarcadores circulantes na Esquizofrenia através da espectrometria de massa.

Circulating biomarkers in schizophrenia – a proteomics perspective. Manadas, B; Santa, C; Coelho F, J. Em submissão para o International Journal of Clinical Neurosciences and Mental Health

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Resumo

O Haloperidol é um dos antipsicóticos mais usados no tratamento da esquizofrenia. A esquizofrenia é uma doença mental severa, caracterizada pela combinação de efeitos positivos, negativos e cognitivos. Pensa-se que o Haloperidol é capaz de aliviar os sintomas positivos pelo antagonismo dos recetores dopaminérgicos D2 expressos no estriado. O que não é tão claro são os efeitos moleculares a longo prazo desta medicação. Uma grande quantidade de estudos tentaram perceber os efeitos moleculares induzidos por medicação antipsicótica em pacientes esquizofrénicos, no entanto, a maior parte das amostras estudadas foram obtidas através de pacientes medicados, ou em alguns casos, pacientes que em alguma altura da sua vida foram medicados. Consequentemente, estes estudos não são capazes de assegurar que as mudanças observadas são relacionadas com a doença em si, ou se foram induzidas pela medicação ou até mesmo se não são uma consequência da medicação crónica.

O objetivo principal deste estudo é avaliar os efeitos moleculares da terapia crónica com Haloperidol no estriado de ratinho. As amostras foram analisadas através da cromatografia líquida acoplada à espectrometria de massa (LC-MS/MS). Aquisição dependente da informação (IDA) e aquisição sequencial de todos os espectros teóricos de iões fragmentados (SWATH) foram os métodos de aquisição utilizados neste estudo. No total 3311 proteínas foram identificadas. Das proteínas quantificadas com grande confiança através do SWATH, 1366 passaram os critérios definidos. No entanto, esta lista foi reduzida para 459 proteínas com valor de $p < 0.05$ e/ou uma variação de 20%. A grande maioria das proteínas alteradas estão envolvidas em três grandes vias: via metabólica, sinalização de cálcio e apoptose. Perturbações na cadeia respiratória mitocondrial, particularmente na fosforilação oxidativa e também na inibição do complexo I foram observadas. A neurotransmissão também se verificou estar afetada, as sinapses GABAérgicas, glutamatérgicas e dopaminérgicas foram sugeridas como sendo alvo de modulação por parte do Haloperidol. Mudanças em proteínas relacionadas com a calmodulina e também nas de extrusão de Ca^{2+} foram observadas, sugerindo algumas alterações na via de sinalização do cálcio. Relativamente aos processos apoptóticos, é possível que o Haloperidol consiga interferir com a morte celular, mas também com a sinalização de sobrevivência celular.

Todas estas descobertas realçam algumas das vias afetadas pelo tratamento crónico com Haloperidol que estão envolvidas nos efeitos moleculares desta medicação. Este estudo elucida também novas direções para o conhecimento e diferenciação das alterações relacionadas com a doença ou com a medicação.

Abstract

Haloperidol is one of the most frequently used antipsychotic in the treatment of Schizophrenia (Scz). SCZ is a severe mental disorder characterized by a combination of positive, negative and cognitive symptoms. Haloperidol is thought to alleviate the positive symptoms of SCZ by antagonizing dopamine D_2 receptors expressed in the striatum. What is less clear are the long-term molecular effects of this medication. A large amount of studies have accessed the molecular alterations induced by antipsychotic medication in schizophrenic patients, however, most of the samples obtained are from medicated patients, or in some cases, patients that underwent treatment at some stage in their lives. Consequently, reports from these studies are not able to assure if those changes observed are disease related and not drug related or even a consequence of chronic impairment.

The main aim of this study is to evaluate the molecular effects of chronic Haloperidol in mice striatum. Mice striatum samples were analyzed by liquid chromatography-coupled to tandem mass spectrometry (LC-MS/MS). Information-dependent acquisition (IDA) and Sequential Window acquisition of all theoretical fragment ion spectra (SWATH) acquisition methods were used in this study. In total, 3311 were identified. From the proteins quantified by SWATH-MS analysis with high confidence, 1366 proteins passed defined criteria. However, this list was reduced to 459 proteins with p-value<0.05 and/or 20% variation. The majority of the altered proteins were involved in three main pathways: metabolic, calcium signaling and apoptosis. Disturbances in the mitochondrial respiratory chain, particularly in oxidative phosphorylation were observed, as well as inhibition of complex I. Neurotransmission was also affected, GABAergic, glutamatergic and dopaminergic synapses were suggested as being modulated by Haloperidol. Changes in several CaM-related proteins and Ca^{2+} extrusion proteins were also detected, suggesting disturbances in calcium signaling pathway. Relatively to apoptotic processes, it is possible that Haloperidol may interfere with cell death along with cellular surviving signaling.

Altogether, these findings highlight several pathways affected by Haloperidol chronic treatment that are involved in the molecular effects induced by this medication. This study also elucidates new directions for recognizing and differentiate disease related or medication related changes.

Table of Contents

List of abbreviations

2DE - Two-dimensional gel electrophoresis

5-HT - Serotonin 5-hydroxytryptamine receptor

AKT – Protein kinase B

APCI - Atmospheric pressure chemical ionization

Arg - Arginine

ARMS - Ankyrin repeat-rich membrane-spanning protein

Asp - Aspartic acid

cAMP - Cyclic adenosine monophosphate

CBT – Cognitive behavioral therapy

CID - Collision-induced dissociation

CNVs – Copy Number Variants

CSF – Cerebrospinal fluid

D2R – Dopamine D2 Receptor

DA – Dopamine

DDA - Data-Dependent Acquisition

DIA - Data-Independent Acquisition

DISC 1 - Disrupted in Schizophrenia 1 gene

EI – Electron ionization

EPS – Extrapyramidal symptoms

ERK - Extracellular signal-regulated kinase

ESI – Electrospray ionization

FDA – Food and drug administration

FGA – First generation antipsychotic

GABA - Gamma-Aminobutyric acid

GSK-3 - Glycogen synthase kinase-3

HPLC - High-performance liquid chromatography

IDA - Information Dependent Acquisition

LC – MS - Liquid chromatography–mass spectrometry

Lys - Lysine

m/z - Mass-to-charge ratio

MALDI - Matrix-assisted laser desorption/ionization

MAP2 - Microtubule-associated protein 2

MS – Mass Spectrometry

MS/MS - Tandem mass spectrometry

MSN - Medium spiny neurons

mTOR - Mechanistic target of rapamycin

nAChRs - Nicotinic acetylcholine receptors

NF-kB - Factor nuclear kappa B

NMDA - N-methyl-D-aspartate

NSC – Neural Stem cells

OXPHOS - Oxidative phosphorylation

Q - Quadrupole

RP – Reverse-phase

SCZ – Schizophrenia

SDS – PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP – Single Nucleotide Polymorphism

SWATH - Sequential window acquisition of all theoretical mass spectra

TOF – Time of flight

WHO – World Health Organization

INTRODUCTION

1. Introduction

1.1. Schizophrenia

1.1.1. Definition and description of Schizophrenia

It all started in the middle of the 19th century, European psychiatrists began to describe disorders of unknown cause, typically affecting the young and often progressing to chronic deterioration. Several names were given to describe the phenomenon but Paul Eugen Bleuler in 1908 was the one who masterfully introduced the term and concept schizophrenia (Scz), replacing Kraepelin's term "dementia praecox" ("dementia of early life").[1,2] Bleuler derived his concept from the Greek verb *schizein*, meaning splitting, merging with *phren* indicating "soul, spirit, mind".[3]

According to the World Health Organization (WHO), SCZ refers to "a severe mental disorder, characterized by profound disruptions in thinking, affecting language, perception, and the sense of self(…)"[4]. It typically begins in early adulthood and is characterized by poorly understood variable phenotypic expression, complex and multifactorial etiology with extensive genetic heterogeneity, leading to a significant diverse symptomatology and course of the disease.[5,6]

Available evidence suggests that numerous individuals are affected worldwide, and although it is treatable, it is among the most disabling and economically expensive medical disorders.

1.1.2. Epidemiology and economic costs Schizophrenia

Scz affects more than 20 million people around the world.[7] The lifetime prevalence has been estimated to be approximately 1% worldwide.[7] Gender differences in the incidence of the disorder have been documented, and even though there is no consensus related to this matter, it seems that more new cases of SCZ have been detected in men.[8,9]

Scz shows significant economic consequences. The economic burden can be reported as direct medical and nonmedical costs, indirect costs and intangible costs.[10] Direct medical costs cover expenditures for hospital and nursing home care (short- and long-term), emergency department visits, laboratory tests, outpatient follow-up,

pharmaceutical interventions and medical supplies.[10] Direct nonmedical costs include all the non-health care resources, including patient transportation, food and cost-associated social services.[10] Indirect costs are wholly defined as productivity losses due to morbidity and premature mortality[10] , where intangible costs are related to the decline in patients quality of life. Aspects such as side-effects of pharmaceutical interventions and stress and anxiety, both caused by the disease itself and also the treatment process are considered. However, this type of Scz-related cost is tremendously difficult to quantify, and therefore is often absent from economic studies.[10]

In England, SCZ costs approximately 16 billion euros per year, with around a third of this figure accounted for by direct expenditure on health and social care.[11,12] The substantial burden of disease is a reflection of two key features of Scz: early onset and tendency to chronicity [7] and, despite optimal treatment and assertive diagnosis, individuals have persisting or intermittent symptoms.[7]

1.1.3. Etiology

In the past decades, SCZ research has been focused in understanding the neurobiology of the disorder. Since antipsychotic drugs were found to have high affinity for dopamine receptors, the hypothesis that an over activity of dopamine system in the brain was behind SCZ pathology has arisen. [13-15] However, as SCZ is a multifactorial disease, it is thought to be caused by a combination of biopsychosocial influences including genetic, neuroanatomical and neurochemical anomalies. [7]

1.1.3.1. Genetics & environmental factors

It is now understood that genes and environment work along to influence the development of certain diseases, where genomic analysis of psychiatric disorders remains a work in progress. A large body of data collected from families, twins and adoptees consistently supported the involvement of a major, complex genetic component in SCZ development.[16] The search for candidate genes has proven difficult and has been hampered by clinical and genetic heterogeneity.

Scz has a significant genetic component with high heritable trait.[16] The genetic risk arises from different forms of DNA sequence variation: single nucleotide polymorphisms (SNPs) and copy number variants (CNVs).[17] The use of genome-wide association studies (GWAS), allow researchers to pinpoint and study genes or genetic locations that may contribute to an individual's risk of developing a certain disease. From previous studies, researchers have identified hundreds of SNPs as risk factors and contributors to Scz, however, the functional implication remains unclear.[18,19] Identifying SNPs only points to

risk loci, which does not always mean finding the disease-causing gene. Several studies have suggest a genetic involvement from the major histocompatibility complex (MHC) to Scz.[20,21] MHC is located on chromosome 6, known to contain several genes involved in immune function and known to be the strongest genetic predictors of individual's risk of Scz.[22] The results of the study propose that a variation in the gene complement component 4 (C4) could be involved in SCZ development, as it seems to promote improper synaptic pruning of neuronal circuits.[22] Meaning that, the higher the levels of C4 activity, the greater a person's risk of developing Scz. [22]

Relatively to CNVs, tend to occur in regions rich in genes as a type of duplication or deletion and only a few have been associated with SCZ and tend to impact genes involved in neuronal function.[23] A large deletion at chromosome 22q11.21 has been documented as significant susceptibility factor for Scz, where individuals with approximately 25% of 22q11.2 deletion normally manifest symptoms of psychosis.[23] The results from a study published in the British Journal of Psychiatry suggest that approximately 2.5% of individuals with SCZ carry at least one known pathogenic CNV.[24]

Since the initial observation that SCZ has a polygenic inheritance, associations between SCZ and genetic variants that predispose the brain to developing the disease have been reported, but only a small number can be considered as SCZ susceptibility genes. Disrupted-in-Scz (DISC1), Neuregulin 1 (NRG1), regulator of G protein signaling 4 (RGS4), dysbindin (DTNBP1) have been proposed as candidates.[25] For instance, DISC1 a gene locus originally identified in a Scottish family that plays key role in behavioral control and vulnerability for psychiatric disorders.[26] DISC1 is involved in many cellular processes including neurodevelopment, neuro-signaling, synaptic functioning and it interacts with several neuronal proteins reflecting the diversity roles in brain function.[26] NRG1 is a protein encoded by a large multiexon gene on chromosome 8p that plays multiple effects in nervous system development that are relevant to schizophrenia: neuronal migration, neuronal plasticity and modulation of neurotransmission.[25,26] Recently, NRG1 and DISC1 were shown to interact with each other, NRG1 influences DISC1 expression and they are thought to participate in a common pathway that regulates neurodevelopment.[26]

Last but not least, stress-inducing factors both in early life and in later life can influence and predispose schizophrenia. Early life factors such as obstetric complications can be divided into three groups: (i) complications of pregnancy (bleeding, preeclampsia, diabetes); (ii) abnormal fetal growth and development (low birth weight, congenital malformations, small head circumferences); and (iii) complications of delivery (asphyxia, emergency caesarean section).[27,28] Obstetric complications unarguably play a role in the etiology of Scz, but the nature and strength of this association are unclear.[28] Also, is it

evident that obstetric complications are neither necessary nor sufficient to cause Scz, however, it is surely a risk factor.[28]

Later life factors comprise migration, drug abuse and urbanicity.[27,29] These factors may not only be seen potential causative factors, but they also influence and confine vulnerability to schizophrenic patients during the course of the disorder.[27] It is not knew that individuals suffering from SCZ consistently show higher rates of substance abuse compared to the general population.[29] Studies have addressed the effect of cannabis on the course of Scz. The results suggest several clinical implications related to cannabis use: (i) increased relapse or re-hospitalization; (ii) poor adherence to treatment; (iii) adverse course of psychotic symptoms.[30] Still, the mechanism behind cannabis use and increased relapses and poorer clinical outcome in SCZ are not very clear. A cannabis component Delta-9-tetrahydrocannabinol (THC) was found to be the main psychoactive constituent, causing positive symptoms and neurocognitive changes.[31,32] The endocannabinoid system is able to modulate neurotransmission either at inhibitory and excitatory synapses in brain regions that regulate emotion, motivation and cognition. [31] Moreover, THC enhances striatal and mesocorticolimbic dopamine levels, therefore affecting dopaminergic system.[31] Hence this, cannabis does not cause any structural changes per se, but it affects brain areas relevant for emotion and cognition. [31]

The environment in which an individual's genes find expression is extremely important to the development and progression of the disease.[29,33]

1.1.3.2. Neurological factors

Different results have shown neurotransmitter activity is closely related to Scz. Consequently, aberrant signaling between dopaminergic, GABAergic and glutamatergic circuits may be involved in the aetiology.[34-38] This field of study is particularly significant since most of the pharmaceutical treatment options involve regulating these chemical messengers, as detailed below.

Dopamine Hypothesis of Schizophrenia

The classic dopamine hypothesis of SCZ postulates that the unusual and/or altered behavior associated with SCZ can be largely explained by an over activity of dopamine in the brain.[38,39] Dopamine is produced in the *substantia nigra* and ventral tegmental regions of the brain.[15] Dopaminergic pathways comprise the nigrostriatal, mesolimbic and mesocortical projections, which are implied (by the dopamine hypothesis) to be associated with the different types of SCZ symptoms.[15]

Two major lines of evidence support dopamine hypothesis: firstly, amphetamine, cocaine and similar drugs were shown to increase extracellular concentrations of dopamine in the brain and induce psychotic symptoms like the ones observed in schizophrenic patients [40,41]; and secondly, discovery that Reserpine and Chlorpromazine inhibits dopamine uptake and calm agitated mental patients. [40,42]

In the 70's, several studies associate the effectiveness of antipsychotic drugs to their affinity for dopamine receptors.[43,44] Seeman *et al.* discovered a particular receptor in the brain where antipsychotics would bind, when searching for a pathophysiological mechanism of psychosis [45]. During their search, the team discovered Haloperidol specific binding sites in brain striatum and consequently observed that dopamine binds to these sites with higher effectiveness compared with other neurotransmitters.[43,45] The antipsychotic target turned out to be a dopamine receptor - dopamine D_2 Receptor (D_2R), which subsequently triggered the screening and study of new antipsychotics.

Altogether, these findings were the origin for the dopamine hypothesis of SCZ and a cornerstone in psychiatry. [46,47] At this stage, the focus was on the over activity of dopamine and blockade of dopamine receptors to treat psychosis. [48,49] Dopamine

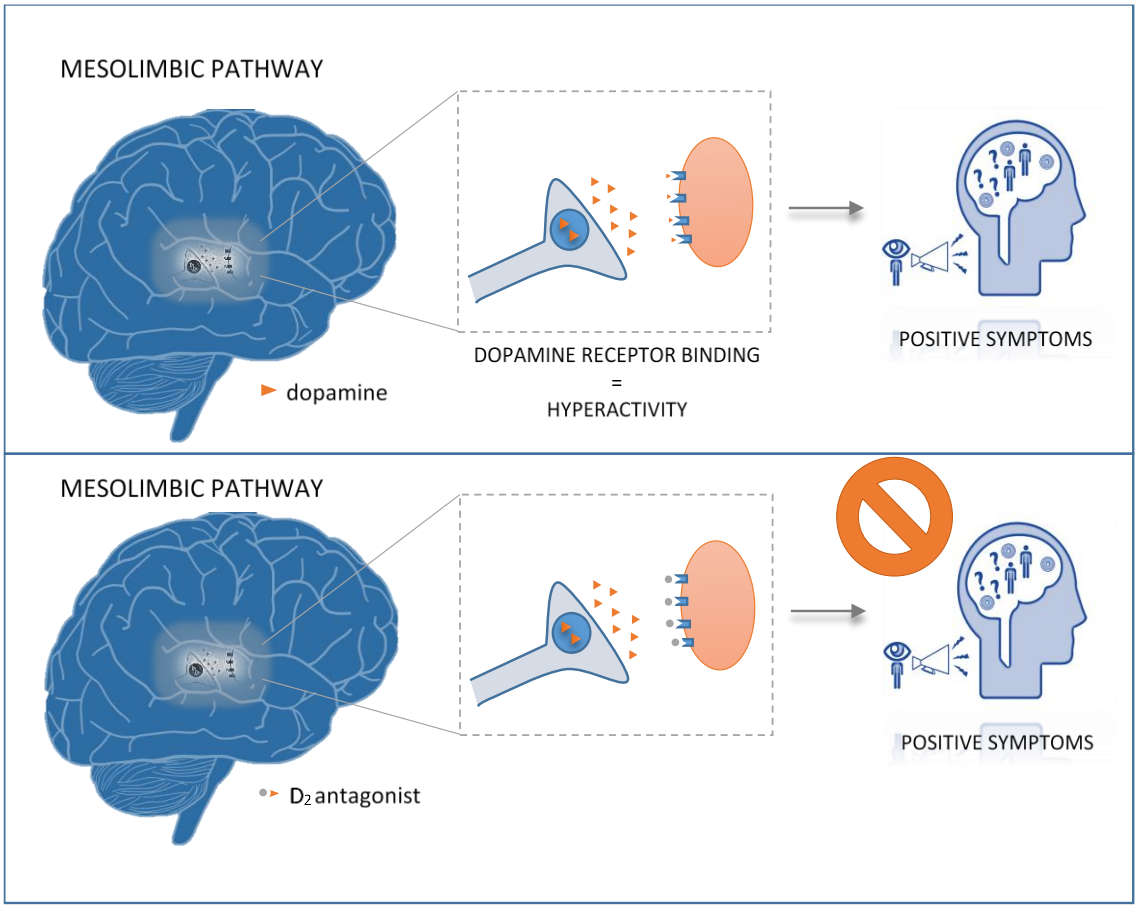


Figure 1.1- Dopamine hypothesis of Schizophrenia. A) Excessive DA activity in the mesolimbic pathway is associated with positive symptoms of schizophrenia such as hallucinations and delusions. **B)** Administration of antipsychotics reduce positive symptoms of schizophrenia by blocking the D2 receptors in the brain.

dysfunction leads to impairments in dopaminergic system in several brain areas including *substantia nigra*, ventral tegmental region, striatum, prefrontal cortex and hippocampus, which leads to schizophrenic symptoms as indicated in Figure 1.1.[50,51]

Glutamate Hypothesis of Schizophrenia

The bedrock of the glutamate hypothesis of Scz, proposed over two decades ago, involved clinical observations demonstrating that the administration of drugs blocking NMDA glutamate receptors could induce Scz-like symptoms in healthy individuals and exacerbate symptoms in schizophrenic patients.[52] Other evidence suggests a decreased glutamate activity in limbic brain structures likely involving the postsynaptic NMDA and/or AMPA subtypes of glutamate receptors is involved.[52]

Kornhuber's team purpose a role for glutamate in Scz, where their findings suggest that glutamate hypofunction may be involved in the etiology of Scz, when observing low cerebrospinal fluid (CSF) levels of glutamate in SCZ patients compared to controls.[53]

N-Methyl-d-aspartate (NMDA) receptors are involved in releasing dopamine into the striatum and frontal cortex in SCZ patients.[15] These receptors are known for playing several physiological roles, where appropriate signaling is essential for cellular homeostasis. Any perturbation in NMDA pathway, with enhanced or decreased activity is thought to result in neuropsychiatric pathologies.[54] Numerous theories provide evidence that NMDA-receptors hypofunction results in dopaminergic abnormalities and underlie many aspects of molecular, cellular and behavioral abnormalities seen in Scz.[55-57]

There is critical evidence suggesting a link between NMDAR and GABAergic systems in Scz, where abnormal dopamine signaling can be a consequence of modulatory abnormalities, for instance by NMDAR deregulation.[58,59]

GABAergic hypothesis of SCZ states that alterations in the GABA neurotransmitter system are also found in SCZ studies.[60,61] Post mortem, neuroimaging and electrophysiological studies provided evidence for disruption of GABAergic circuits related to NMDAR hypo-activity states in Scz.[58,59] The most described result is that GABA concentration is reduced in schizophrenic patients with respect to healthy controls.[62-64]

An orchestrated balance between excitation and inhibition brain neuronal transmission is essential for behavior and function, where an imbalance seems to be partly responsible for development of psychiatric disorders.[62]

1.1.4. Brain areas associated with Schizophrenia

Scz is not restricted to a specific brain region. One of the most consistently implicated regions in the pathophysiology is the dorsolateral prefrontal cortex (PFC).[51] This part of the brain is mainly responsible for executive functions including decision-making, strategizing and adjusting behaviors. It has been proposed that PFC dysfunction enhances DAergic activity, boosts subcortical DA neurotransmission and induces psychosis.[51] However, the mechanisms underlying enhanced DA function by PFC dysfunction remain unclear.

The hippocampus and the thalamus are also affected.[65] The existence of neuroanatomical and electrophysiological alterations may be behind brain connectivity dysfunction.[65] Volume deficits of the hippocampus in SCZ and first-episode psychosis have been consistently reported.[66,67] The clinical significance of hippocampus abnormality is not very clear, it is suggested that it may be related with specific brain regions influencing cognitive function.[66,67]

Basal ganglia is particularly important in Scz, as antipsychotics drugs currently in the market and routinely administered to patients tend to act in this part of the brain. [68]

Basal ganglia consist of four nuclei: the striatum, the globus pallidus, the sub thalamic nucleus and the *substantia nigra* that are involved in a range of functions including motor control, executive functions, behaviors and emotions. [69,70] All of these functions are disturbed in Scz, so it is not surprising the abundance of recent studies acknowledging an altered basal ganglia network present in the disorder. [68,70,71]

Specifically, the striatum has been in the spotlight lately. Abnormal striatal dopamine transmission is suggested to be observed in schizophrenic patients.[72,73] The striatum is the largest subcortical brain structure in the mammalian brain that receives glutamatergic and dopaminergic inputs from different sources, and serves as primary input to the basal ganglia system.[69] This brain area contains two different neuron populations known as projection neurons or medium spiny neurons (MSNs) and interneurons.[69,74] MSNs are GABA-ergic cells represent approximately 90-95% of neurons within the human striatum.[69,75] These express both D_1 and D_2-like receptors which are the major players in SCZ [74,75], and have a crucial role in motor control, habit formation and motivated behavior [76,77].

D_1 and D_2 receptors constitute two different efferent pathways: direct and indirect, where the direct pathway is formed by Striatonigral MSNs expressing D_1 receptors and the indirect pathway formed by Striatopallidal MSNs that express D_2 receptors.[74,78] Functional connectivity of MSNs is extremely essential not only due their

important roles in the brain but also because an altered function is observed in major psychiatric disorders.[76]

As mentioned before, there is a hyperdopaminergic function in the striatum.[73] One of dopamine's functions is to control thoughts and decision-making [79-81], acting as a "gatekeeper". However, although in theory, what is happening in SCZ is that due to hyperactivation of the dopaminergic system, the "gatekeeper" is uncontrolled and thoughts and impulses just flow over the brain, leading to hallucinations, delusions and abnormal and inappropriate behaviors and responses to a given task or situation.[80,82] Multiple components of the dopaminergic system may be involved in dopaminergic over activity and these include: increased DA synthesis and/or release; receptor number and/or affinity DA-mediated postsynaptic effector mechanisms, and decreased inactivation.

1.1.5. Symptomatology and Diagnosis

Schizophrenic patients usually experience a combination of positive, negative, cognitive, mood and motor symptoms whose severity differs from patient to patient through the course of the disorder.[6] Positive symptoms consists of delusions, hallucinations, confused thoughts and speech, they reflect reality distortions and are not experienced by all patients. These are considered "add-ons" to normal behavior. [6,12] On the other side, negative symptoms are thoughts or feelings normally present that are diminished in an individual who suffers from Scz. They represent emotionless, depression, lack of motivation and social withdrawal. [6,12] Besides the well-known positive and negative symptoms, cognitive symptoms constitute an important part of SCZ symptomatology.[83] Cognitive symptoms include poor "executive functioning", patients lose their ability to absorb and interpreter information and make decisions based on that information; inability to sustain attention and also problems with "working memory", patients find it difficult to keep recently learned information.[83] This type of symptomatology often interfere with the patient's ability to lead a normal life and earn a living, and causes a great emotional distress.

Although the symptomology is quite characteristic, SCZ diagnosis is not that clear. Research efforts have progressively shifted towards the identification of SCZ biomarker [84-86], that can be used in diagnostic, efficacy response (clinical outcome after intervention), pharmatoxicity (predictor of adverse drug-response), pharmacodynamics (predictor of drug-response), prognostic (future clinical course) and predictive (prior to intervention) [87]. However, no validated biological marker has been discovered and successfully used in clinical practice. Our group work have also assessed 25 publications where a total of 25 publications on peripheral SCZ biomarkers were presented, 14 from proteomics studies

performed in body fluids of patients searching for protein 15 markers, and using mass spectrometry.[88] Also empathizing the need for new panels of biomarkers in SCZ and the importance of this research field.

Diagnostic remains based on the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) [89], where evaluation of signs and symptoms in clinical interviews are assessed. [90] If for instance patients do not exhibit symptoms of psychosis such as hallucinations and/or delusions, the disorder can remain undiagnosed.[91] Moreover, misdiagnosis is also a common occurrence, once some SCZ symptoms are common in individuals with personality disorders.[9,91] Indeed psychiatric diagnosis is simultaneously too broad and too narrow [92], and there is no single symptom which is unique to Scz, as well as no definitive blood test or scan.[7]

1.1.6. Schizophrenia treatment

Until the advent of modern antipsychotics in the mid-1950s, SCZ treatments were limited.[93,94] Electroconvulsive therapy (ECT) was first introduced as a treatment for SCZ in 1938, by Ugo Cerletti and Lucio Bini.[95] Cardiazol, insulin or electric current were used to re-set the brain and this type of therapy was mostly indicated in pharmacotherapy augmentation, urgency of therapeutic response and in treatment resistance cases.[93,95] ECT controversial bust in since treatment was performed without anesthetic and often without consent, and far more treatments were given than is common now. Nowadays, the treatment is improved and successfully executed mainly in treatment resistant cases, in a controlled manner and given under general anesthesia and using muscle relaxants.[93,96]

Lobotomy procedures were also used and involved severing connections in the brain's prefrontal lobe.[93] The Portuguese neurologist Egas Moniz and the neurosurgeon Almeida Lima were responsible for spreading lobotomy procedure during the 1930s and 1940s, however, this surgery was gradually set-aside after antipsychotics discovery.[93,94]

Presently, a greater diversity of treatments are available, including combination of non-pharmacological and pharmacological approaches.

1.1.6.1. Non-pharmacological approaches

Psychotherapy is performed in patients with emotional problems and mental health conditions that aims to improve patient's well-being. The goal is to target symptoms, prevent relapses and re-integrate patients back into society by helping to foster healthy relationships and maintain employment for example.[96]

Several types of psychotherapy approaches exist and can be divided into three categories: individual, group or cognitive behavioural.[96] Individual psychotherapy includes counselling and supportive talks, whereas group therapy is more associated to interactive/social interventions.[96] Cognitive Behavioral Therapy (CBT), is a popular therapeutic approach that examines how beliefs and thoughts are linked to behavior and feelings.[97] It is focused on the construction and re-construction of people's cognitions, emotions and behaviors. [97,98]

Hence this, non-pharmacological therapies are an added value to pharmacological treatments, but not a substitute of medication.

With non-adherence rates ranging from 37% to 74% [96], other strategies to intervene are necessary to overcome this issue. Non-adherence is a problem throughout medicine but there are key factor such as the lack of illness awareness, direct impact of symptoms and social isolation that make it personally challenging in Scz.[96,99] Additionally, non-adherence is linked to relapse, rehospitalization, higher risk of suicide and poor quality of life.[100-102] Psychotherapies is useful in this matter as it can help educate patients about the importance of taking their medications, illness risks and effectiveness of treatment.[96]

1.1.6.2. Pharmacological approaches

The course and outcome of SCZ is variable and unpredictable.[99] SCZ is a chronic disorder, wherein the majority of patients experience multiple relapses characterized by acute psychotic exacerbation.[103] The illness influences all aspects of life, leading to treatment plans that consist of three major goals: 1) reduce, alleviate and eliminate symptoms, 2) maximize quality of life and adaptive functioning, and 3) promote and maintain recovery from the debilitating effects of illness.[104]

The story of antipsychotics

The "psychopharmacological era" was remarkable for three fundamental developments: 1) Chlorpromazine, although it was originally intended to potentiate surgical anesthesia due its anxiolytic properties, it was surprisingly beneficial for patients experiencing psychotic symptoms in 1950 [105]; 2) Reserpine, a natural substance obtained from the dried roots of *Rauwolfia serpentina* was also introduced in 1953 [106], and 3) Haloperidol in 1958, marked a new historical landmark in antipsychotic pharmacology treatment.[107]

First-generation antipsychotics (FGA), the so-called typical antipsychotics, are a class of drugs typically used for the treatment of acute mania, agitation and other

conditions. They are frequently prescribed in several mental conditions including: SCZ and schizoaffective disorder, bipolar disorders and delusional disorder.[108] Strong D_2 antagonists, act on dopaminergic system by blocking dopamine type 2 receptors.[108] Besides theirs powerful effect in diminishing, for instance, positive effects of Scz, they are also associated with high risk of extrapyramidal side effects (EPS), some of which are quite severe (acute dystonic reactions, parkinsonism, tardive dyskinesia).[108] The search for a powerful and effective antipsychotic with lesser side-effects is a continuous task.

Second-generation antipsychotics (SGA), the atypical antipsychotics, differ from typical antipsychotics by producing significantly less EPS and by having a different mechanism of action and receptor binding profiles.[108] Generally speaking, SGAs show preferential occupation for serotonin (5-hydroxytryptamine, 5-HT) receptors than D_2 receptors.[93,109] In detail, they have additional affinities for other receptors such as other serotonin subtypes (5-HT_{1A}, 5-HT_{2C}, 5-H_{T6} and 5-H_{T7}), dopamine receptors (D_1, D_3 and D_4) as well as histamine receptors H_1, adrenergic receptors (α_1 and α_2) and muscarinic receptors (M_1, M_2, M_3, M_4 and M_5).[109]

In 1970s, the development of clozapine opened the door to SGAs.[110] First prepared in the early 1960s as a potential antidepressant, but subsequently was seen as a potential antipsychotic.[110] It showed unique therapeutic benefits, with no disabling neurological side-effects, efficacy in treatment-resistant SCZ and also the ability to decrease the incidence of suicide.[110,111] For almost two decades (1970-1980) only one or two novel antipsychotics were approved, but the 90s and onwards reflected meaningful steps in attempting to improve psychopharmacological treatment for SCZ patients. Risperidone was approved by FDA in 1993 as well as olanzapine in 1996, followed by Ziprasidone in 2001 and Iloperidone in 2009.[93]

Nevertheless, the scenario is not as good as it seems**,** SGAs also have side-effects. Weight gain (although, also with FGA), glucose dysfunction and dyslipidemia are metabolic side-effects commonly feature of SGAs treatment.[108,109] Receptor-binding profile of atypical antipsychotics is involved in this matter, as drug affinity for Histamine H_1, Muscarinic M_3 and 5-HT_{2C} receptor is closely linked with an increased risk for diabetes; whereas 5-HT_{2A}, 5-HT_{1A} and D_2 affinity is more associated with weight gain.[109]

Adding to the first two generations of antipsychotic drugs, the “third generation” came into the limelight due to Aripiprazole discovery. Previous developed drugs have been dopamine-serotonin antagonists, however, Aripiprazole can modulate dopamine levels without completely blocking D_2 and 5-HT. [112] Short- [113] and long-term [114] studies have reported the success of this medication in relieving of both positive and negative symptoms of Scz. The resume for the three generations of antipsychotics can be visualized in Figure 1.2.

More recently, there are few new antipsychotics in the pipeline. For instance, Cariprazine which had FDA approval in 2015, performed well in both positive and negative symptoms.[115] It is a partial D_2 and D_3 agonist that modulates dopamine dysfunction, rather than extreme dopaminergic blockade.[115] Some of these new drugs are merely slight tweaks of current-market drugs, others provide novel mechanisms of action. The α/7 subtype of nicotinic acetylcholine receptors (nAChRs) has been seen as a particularly attractive therapeutic target for Scz.[116,117]

GTS-21 (3-(2, 4-dimethoxybenzy-lidene)-anabaseine), MEM3487 (N-[(3S)-1-azabicyclo [2.2.2]oct-3-yl]-1H-indazole-3-carboxamide hydrochloride) and TC-5619 ((2S, 3R)-N-[2-(pyridin-3 ylmethyl)-1-azabicyclo [2.2.2]oct-3-yl] benzo[b]-furan-2 carboxamide) are examples of drugs currently undergoing clinical trials that are α7 nAChR agonists.[117,118]

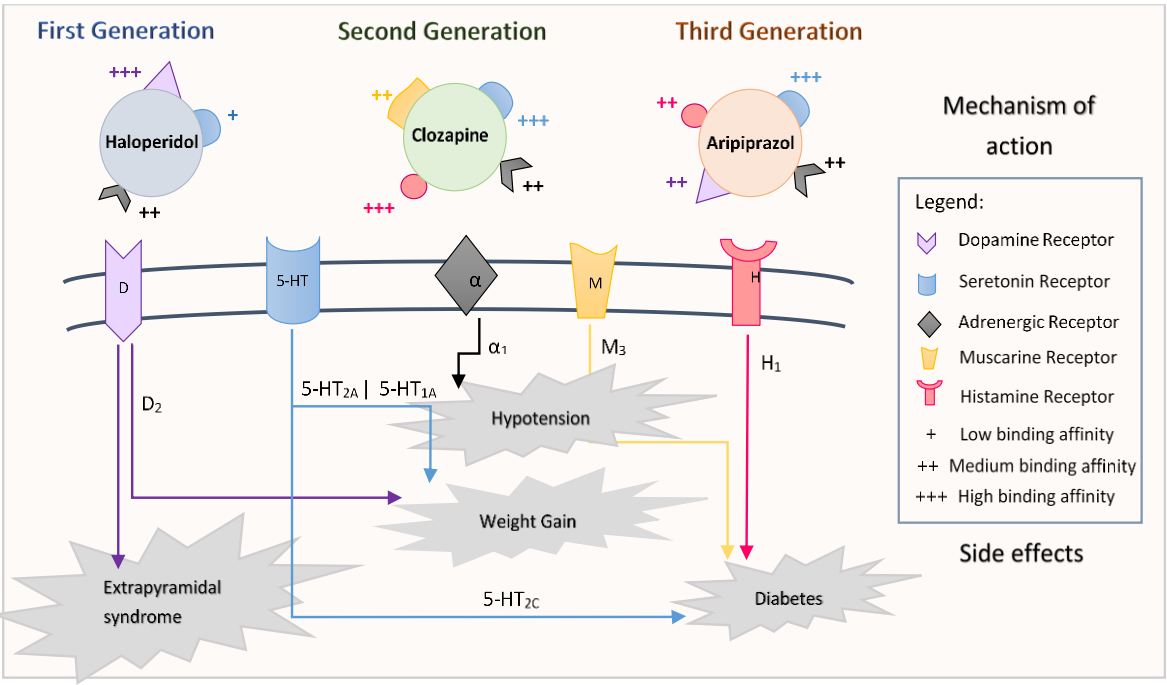


Figure 1.2 - Mechanism of Action and Side effects of antipsychotics. Haloperidol, Clozapine and Aripiprazol all belong to the same class of medication, antipsychotics, although from different generations. All show different binding affinities for different receptors, as well as different side-effects. Haloperidol, a first-generation antipsychotic is a major dopamine D2 antagonist, which has severe extrapyramidal side-effects and weight gain problems associated specific with D2 specific receptor blockade. Haloperidol further shows affinity for serotonin and adrenergic receptor, however, not comparable with affinity for D2. Clozapine, a second-generation antipsychotic demonstrate higher affinity for the serotonin and histamine receptor, while Aripiprazol mainly acts on serotonin receptors. Blockade of serotonin receptors is associated with weight gain as well as diabetes, therefore showing metabolic consequences. Adrenergic receptors are more involved in hypotension, where muscarinic and histamine receptors show increased risk for diabetes.

In summary, even though there is a wide spectrum of drugs, acting in different receptors and having different mechanism of action as it can be observed in Figure 1.2,

there is no medication neither therapy to prevent or cure Scz. The earlier the disorder is detected, the better chance there is to alleviate the symptoms, prevent relapse and improve patient well-being and social life aspects.[96]

1.2. Haloperidol

1.2.1. Definition and characteristics

Haloperidol is a butyrophenone, a first-generation antipsychotic agent commonly used in the treatment of SCZ and acute psychotic states.[119,120] Developed in Belgium in the late 1950s at the Janssen Laboratories, it remains on the WHO Model List of Essential medicines, the most significant drugs needed in a basic health system.[121,122] Also called by Haldol, commercial name, its structure is highlighted in Figure 1.3. The chemical designation is 4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one, molecular formula of $C_{21}H_{23}ClFNO_2$ and molecular weight of 375.864223 g/mol.[16,71] Despite the newer antipsychotics generation and extrapyramidal side effects, Haloperidol remains in the top 3 medication most commonly used in Scz.[123,124] It can be administered by multiple routes, including orally, UV infusion, intramuscular, subcutaneously, topically and rectally.[125] Bioavaibility, Tmax and half-life have different values, depending on the route of administration used.[125]

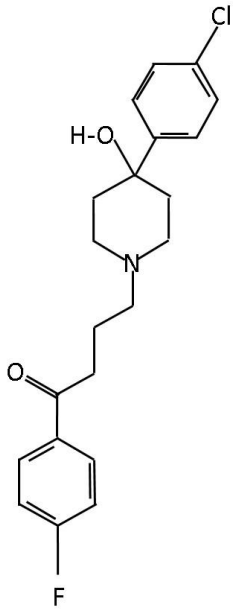


Figure 1.3- Haloperidol chemical structure.

Haloperidol is metabolized in the liver, the greatest proportion by CYP3A4. 125 It is noteworthy to mention that factors such as patients body weight, age, gender, ethnic pharmacogenetics differences and cytochrome P450 inhibitors and metabolic inducers influence Haloperidol metabolism.126 After a single oral dose and after reaching peak

plasma concentration, Haloperidol levels drop significantly with an apparent half-life ranging from 14.5-36.7hours.[125,126] It has been suggested that in order to reach a therapeutic response, plasma Haloperidol concentration should range from 4 µg/L to 20 to 25 µg/L.[127] A study from 2004 suggests that Haloperidol half-life is consistently longer after chronic administration comparing with acute dosing studies, being reported half-lives of up to 3 weeks.[126] This is particular important, once Haloperidol can remain in the body for longer than expected, thus either its beneficial and harmful effects may also be extended.[126]

Haloperidol is excreted by urine (40%) and feces (60%), with around 1% excreted unmetabolized.[125,127]

1.2.2. Mechanisms of action

A significant body of literature has shown that the principal brain target of antipsychotic drugs is the dopamine D_2 receptor.[46,47]

There are five metabotropic G-protein coupled receptors (D_1-D_5) that mediate dopamine signalling.[128] These receptors were classified as either D_1-like receptors (including D_1 and D_5 dopamine subtypes) or D_2-like receptors (D_2, D_3 and D_4)[128], based on their ligand affinity properties as well as their effects on intracellular cyclic adenosine monophosphate (cAMP) production.[129,130] D_1-like receptors activate adenylyl cyclase and thus stimulate cAMP production, while the activation of the D_2-like receptors inhibits adenylate cyclase activity and causes a decrease of the cAMP production.[129] Present in the brain, dopamine receptors are mostly found in high concentrations within the basal ganglia, limbic regions and frontal cortical areas.[128]

Specifically speaking about Dopamine receptor D_2, it was discovered by Philip Seeman who had named it “antipsychotic dopamine receptor”.[46] Back in the 70´s, the approach was to identify a site in the brain that was sensitive to clinically relevant concentrations of antipsychotics.[46] Early *post-mortem* studies showed increased D_2 receptor levels in the brain of patients with Scz.[131-133]

Haloperidol is known for its blocking capacity of post-synaptic mesolimbic D_1 but mainly D_2 receptors.[134,135] It is proposed to act on positive symptoms by reducing activity in the mesolimbic pathway [136], however, rapid beneficial effects are often followed by significant extrapyramidal side-effects such as parkinsonism, dystonia and akathisia.[137] Several authors suggest that there is an optimal therapeutic window sufficient to obtain antipsychotic effect (above 70%) but insufficient to induce EPS (less 80%).[128] Risperidone and Olanzapine belong to SGAs era, daily recommend doses of both drugs are within the suggested therapeutic window, whereas FGAs (like Haloperidol) normally occupy > 80% of D_2 receptors.[128] It is not only occupancy percentage that has impact and intensifies the

risk of antipsychotic side-effects. Binding of an antipsychotic to a receptor is a dynamic process with continuous association and dissociation. Quite a few studies have proposed that rapid displacement or dissociation of an antipsychotic is related to the lack of motor side effects, prolactin elevation and secondary negative symptoms.[138,139] Therefore, occupancy by time is also relevant.

How blockade of D_2 receptors by antipsychotics is transmitted to the signal transduction system remains unknown. Antipsychotics interfere with neurotransmitter function, usually blocking dopamine receptors and inducing behavioral, endocrine, motor-kinetic effects.

Haloperidol has also affinity for other types of receptors, 5-HT_{2A} and α_1 receptors (Figure 1.2).[140]

1.2.3. Haloperidol signaling

During the past few years several studies have provided evidence for the involvement of different signaling pathways in SCZ and antipsychotic drug action. D_2R are G-protein coupled receptors $G_{i/o}$ type receptors, consequently, the first characterized downstream signaling related to antipsychotics signaling are 3'-5'- cyclic adenosine monophosphate (cAMP)- associated pathways.[141] Most antipsychotics when exerting inhibiting action on D_2R, activate adenylyl cyclase activity, which in turn, catalyzes the conversion of ATP into cAMP. [141] Protein Kinase A (PKA) is then activated when cAMP levels are raised, where PKA takes part in synaptic modulation functions by phosphorylating receptor and ion channels at synapses.[142,143]

Haloperidol, and other antipsychotics acting on D_2R also prevent the assembly and activation of the β-arrestin 2 and phosphatase 2A complex.[141] This complex, when assemble, has the ability to dephosphorylate and deactivate protein kinase B (Akt), therefore enhancing Akt signaling.[141]

Akt/GSK-3 and Wnt pathways are altered in SCZ and are targets of antipsychotics.[144] Akt is a protein kinase that phosphorylates substrates on specific serine and threonine residues.[144] Besides its involvement in multiple cellular functions (metabolism, cell stress, cell-cycle regulation) it has particular significant functions in neuronal processes including neuronal cell size and survival, synaptic plasticity and also in intracellular trafficking of biogenic amine transporters such as DA.[144] Once active, Akt phosphorylates a number of molecules, including GSK-3, a particular important target. It is also a Serine/Threonine protein kinase and is a well-established downstream component of the phosphatidylinositol 3-kinase (PI3K) signaling pathway.[144] It is also key enzyme in negatively regulating the canonical Wnt/β-catenin signalling pathway, and it is involved in several signal transduction cascades including: glycogen metabolism; cell

development; gene transcription and it also participates in innate immune regulation and migratory processes.144 Wnt pathway has also been implicated in Scz.144,145 Wnt proteins are secreted glycoproteins that are involved in few neuronal processes such as synaptic modulation, regulation of synaptogenesis and synapse specificity.144

There is also evidence that Akt-mTORC1 (mechanistic target of rapamycin complex 1) pathway activation by Haloperidol leads to changes in specific protein synthesis and components of the translational machinery.[141,146] 4E-binding protein (4E-BP) and p70 ribosomal protein S6 kinase 1 (S6k1) are two main downstream effectors of mTOR and both upstream effectors of protein synthesis.[141,147] Ribosomal protein S6 is a target of S6K1 but it can also be phosphorylated downstream of PKA signalling.[141,147] So, there seems to be a convergence between the two pathways relatively to the ribosomal signaling following antipsychotic administration as indicated in Figure 1.4.

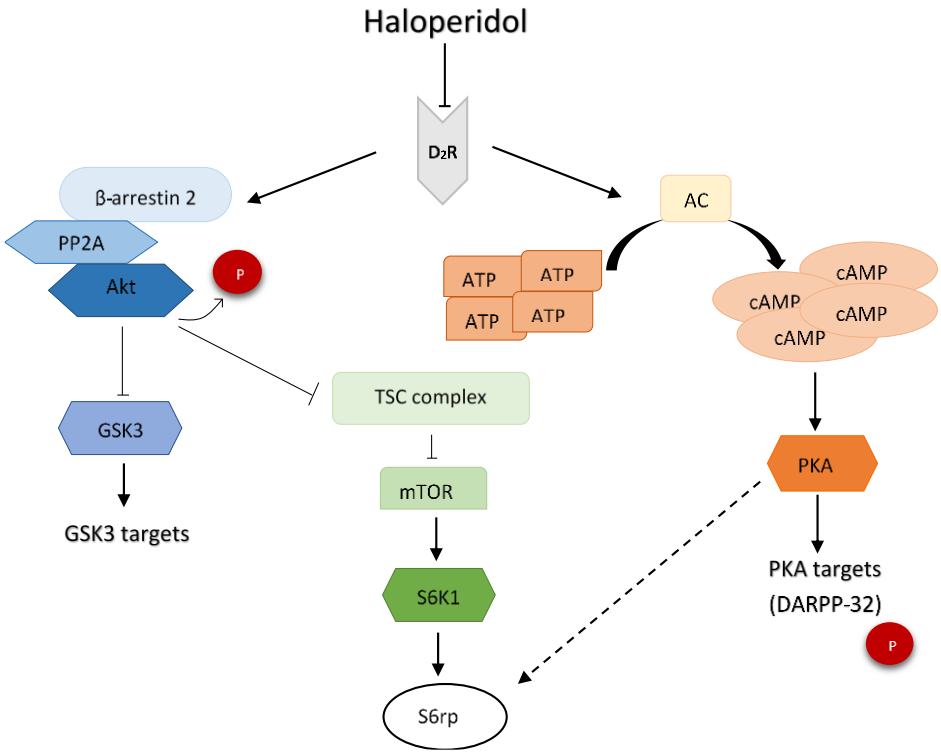


Figure 1.4 - Antipsychotic mechanisms of action. Blockade of D2 receptor is involved in a several signaling pathways. Haloperidol starts its action by inhibiting action on D2R, which leads to the activation of adenylyl cyclase and catalyzes the conversion of ATP into cAMP. cAMP levels raise and activation of PKA occurs. On the other side, Haloperidol prevents the assembly and activation of a complex formed by β-arrestin 2 and phosphatase 2, resulting in increased AKT activity. Phosphorylation and activation of Akt is followed by phosphorylation of GSK-3. Akt-mTOR pathway activation leads to changes in specific protein synthesis and components of the translational machinery. S6k1 is one of the main downstream effectors of mTOR and upstream effector of protein synthesis. Ribosomal protein S6 is a target of S6K1 but it can also be phosphorylated downstream of PKA signalling. Both AKT and PKA lead to activation of mTORC1 and S6 ribosomal proteins, which regulated protein synthesis.

Akt1 isoform has been shown to be reduced in different tissues from patients with Scz.[144,148] Individuals with SCZ express 68% less Akt1 in their lymphocytes relatively to

control subjects.[148] Akt1 was found to be reduced in the hippocampus and also frontal cortex of SCZ *post-mortem* brain samples, when compared to controls.[144] However, this loss of Akt1 levels may be reverted or prevented by antipsychotic medication. Studies from both acute and chronic Haloperidol treatment showed increased levels Akt1, and inactivation of GSK-3β by phosphorylation.[144]

In Scz, analysis of *post-mortem* patients confirmed decreased phosphorylation levels and GSK-3β protein levels in the frontal cortex, as well as, GSK-3β mRNA levels in the dorsolateral prefrontal cortex.[145]

Another potentially relevant signal pathway underlying the mechanisms of action of antipsychotics is the extracellular signal-regulated kinase (ERK). It has been shown that Haloperidol treatment activates the ERK signaling pathway in D_2R neurons in the striatum though an unknown mechanism. [149,150]

These are all small steps in understanding the intracellular signaling of antipsychotic drugs, in particular Haloperidol. Consequently, further research is mandatory to improve knowledge in this field as it is one of the most challenging issues in antipsychotic research. The discovery of upstream and downstream signaling that produces certain effect or response in the body.

1.2.4. Cellular processes affected by Antipsychotics

Proteomic studies have highlighted few cellular processes that may be associated with antipsychotic action. Changes in metabolism-related proteins such as Disrupted in Schizophrenia 1 (DISC1) and Akt pathway suggest that energy metabolism may be modulated by antipsychotics exposure.[141,151]

Changes in cytoskeleton, synaptic, metabolic and mitochondrial cellular processes have also been observed.[152,153] For instance, a recent study found that proteins associated with translation (rpS6 - translation ribosomal protein S6) , cytoskeleton (MAP2 – microtubule-associated protein 2) and morphological complexity (ARMS - *ankyrin* repeat-rich membrane-spanning protein) are produced in response to Haloperidol, *in vitro* and *in vivo*.[146] These processes are all related, as antipsychotic effects in cytoskeleton protein changes may be related to neural plasticity events that take place after long-term treatments and that impact neurotransmitter circuitry.[154]

Synaptic plasticity is consistently observed after treatment with Haloperidol.[142] Synaptophysin, a molecular marker for synaptic competence is decreased in SCZ and increased after Haloperidol treatment.[142] It seems that Haloperidol may act on synaptic reorganization and achieve functional and anatomical reconnection, reversing synaptic

disruption observed in Scz.[142] Haloperidol also alters synapse morphology and number. Studies have presented that rats treated chronically with Haloperidol show increased axon terminals, followed by increased number of vesicles per synapse, increased postsynaptic density and additionally, increased number of synapses in rat striatum.[142]

Other Haloperidol associated-cellular processes include: cellular assembly and organization [146]; nervous system development and function [155]; cellular function and maintenance; synaptic vesicle trafficking, synaptogenesis[156] and neurotransmitter release [157]. Haloperidol as also been shown to cause phosphorylation of proteins and to induce gene expression as observed in Figure 1.4.

Despite that, treatments for psychotic episodes in SCZ exist, however, there is limited mechanistic understanding and effective therapies to prevent or treat the cognitive impairments and deficit symptoms of Scz, which are the earliest and most constant features of the disorder.

1.3. Proteomics

Long-term administration of antipsychotics has been proposed for inducing changes in protein expression in the brain.[157-159] Extensive work has been carried to understand the biological and pathological basis of Scz. However, understanding and disentangling the molecular effects of medication remains poorly unstudied. Rodent studies focused mainly in the identification of proteins and cellular pathways affected by antipsychotics are desirable. Animal models also give a clear picture of the medication induced-changes, as they are better suited to remove the confounding factors inevitably present in the human samples.[157]

So, what can proteomics tell about antipsychotic mechanisms of action?

For over a century, *post-mortem* studies have been extremely important in the search for the structural and biochemical pathology of Scz. Brain collection allows testing of hypothesis in a substrate that cannot be fully recapitulated by animal models.[160] Nevertheless, as any other experimental sample, *post-mortem* tissue has variables that cannot be controlled. Manner of death, diagnostic certainty, co-morbid substance use and prior medication treatment are just a few examples that contribute to inconsistent results and possible mistaken findings.[160] Mental disorders treatment typically include long-term administration of antipsychotics that certainly have great impact in brain structure and chemistry.[161] The availability of subjects “off” medication for a period of time prior to death is scarce as it is implicit that schizophrenic patients take medication to control their symptoms.[160] Thus, there are many shortcomings allied to this type of experimental sample.

Regarding animal models of Scz, this disease is a complex chronic mental disorder with unclear etiology and pathophysiology that has different symptoms predominating at different stages. Several drugs are used to induce effects that resemble some of the symptoms of Scz, and animal models that express known neurochemical and behavioral alterations. However, SCZ diagnosis is interview-based that requires verbal report, it is not clear if animals are experiencing delusions and hallucination or thought disorders. Therefore, it is very hard to reproduce the human clinical picture in animals.[162]

1.3.1. Antipsychotic Research

1.3.1.1. Animal Models

Animal models are mostly employed in preclinical research to understand the pathogenic mechanisms of the disorder or, for initial drug trials.[163] For instance, in SCZ and other psychotic diseases it is difficult to translate animal findings to human, firstly because they are complex disorder and it's complicated to reproduce all the aspects and symptoms; and secondly because there are no effective techniques that can determine the proper emotional state of the animal, as most psychotic diseases are diagnosed mostly in interview-based.[162]

Nevertheless, animal models are suitable for understanding the mechanisms of action of antipsychotic drugs, where proteomic studies of rodent brain tissue after antipsychotic exposure have already led to interesting findings. Examples of it, is one study where the proteome and pathway effects of chronic Haloperidol treatment in mouse hippocampus was assessed using label-free LC-MS/MS approach.[164] The authors consider the findings inspiring and stimulating for further research into the cellular mechanisms associated with Haloperidol treatment and also assist in treatment biomarker discovery.[164]

In a different study, the authors focused on 2D-gel electrophoresis analysis to understand the effect of antipsychotic medication in rat mitochondria from cerebral cortex and hippocampus samples. [152] The results reveal a distinctly different expression of mitochondrial proteins, in particularly in oxidative phosphorylation pathway (OXPHOS), following antipsychotic drugs exposure (chlorpromazine, clozapine and quetiapine). [152]

Another study on chronic antipsychotic treatment with Clozapine and Risperidone showed altered cytoskeleton structure, as well as in some synaptic and regulatory proteins in the cerebral cortex of healthy rats.[165]

These are some examples of different proteomic studies and approaches used to explore antipsychotic drugs mechanisms of action.

1.3.1.2. Cellular models

Cellular models are also used to explore the mechanisms of action of antipsychotics. Besides their utility, the findings achieved by this type of model always requires validation testing using more intact physiological systems.[163] This is due to the lack of interaction that happens in the culture, which is a controlled environment. In other words, most of the body cells do not act alone and require feed-forward and feedback control from other cell types in the body, clearly absent in most *in vitro* cell culture systems.[163]

The effects of antipsychotics drugs on cell cultures have been assessed in several proteomic studies. Recently, a shotgun mass spectrometry analysis was performed in oligodendrocytes treated with the NMDA receptor antagonist MK-801, showing changes in protein levels associated with energy metabolism.[166]

Another example is a study where the effect of two antipsychotics (Haloperidol and Risperidone) was investigated in neural stem cells (NSCs).[167] It has been suggested that antipsychotic drugs have significant effects on NSC activities, however, the molecular mechanisms behind them are largely unknown. Protein expression profiles were assessed through a proteomic approach and from the results obtained, the authors suggested that at early stages both drugs showed common mode of action, while administration prolonged showed opposite actions.[167]

1.3.1.3. Human studies

In order to conduct a biomedical research study involving the participation of human beings it is necessary to go through a variety of ethical and legal issues that perturb values such as dignity, bodily integrity and privacy.[168] The role of human participants is to serve as a source of needed data, usually by providing samples and/or clinical information.

There are no proteomic studies carried out to investigate the effects of antipsychotic drugs in brain tissue samples from healthy human subjects, what it is either used is blood serum or plasma samples.[163]

Several examples can be mentioned, for instance, a study where multidimensional LC-MS was used to assess potential differences in the serum proteome of SCZ patients caused by olanzapine treatment.[169] The authors used blood samples from patients at baseline and after six weeks of Olanzapine therapy wherein they found changes in total protein levels, as well as, changes in phosphopeptides in response to antipsychotic therapy.[169] The results of this study may route to new insights in molecular etiology of SCZ and Olanzapine mechanism of action.[169]

A different study using the same antipsychotic was focused on the effects of Olanzapine on the glycosylation of serum proteins in SCZ patients.[170] In order to assess this question, the authors chose to use a proteomic approach in blood samples from acute paranoid SCZ patients (pre-treatment) and after six weeks of Olanzapine treatment (post-treatment). Olanzapine triggered changes in the glycosylation machinery associated with the biosynthesis of abundant serum proteins and this could induce a variety of changes downstream as glycosylation affects many cellular processes such as protein function, protein stability and immunogenicity.[170]

Altogether, proteomic studies are very helpful in unveiling medium long-term antipsychotics mechanisms of action. However, when using *Post-mortem* brain tissue samples of for example, Scz, or blood serum samples from schizophrenic patients, there are several confounding factor that need to be consider. For example, if assessing protein levels of a certain protein, lower levels seen in the brains of subject with SCZ could be due to illness or a consequence of chronic treatment with antipsychotic drugs. There is also the possibility that alterations observed are not only induced by antipsychotics but combined with the disorder at the same time.

1.3.2. Proteomic techniques

Proteomics is the large-scale study of the structure and function of proteins in a complex biological sample.[171] The current available proteomic tools allow large-scale, high-throughput analyses for the detection and identification of the proteome.[171] In general, proteomic approaches can be used to answer many different scientific questions and it can be used for different proposes including protein profiling; comparative expression between two or more proteins; protein-protein interactions; localization and identification of post-translational modifications.[171] In this work, quantitative and identity aspects of expression proteomics and its methodology will be discussed.

Quantitative proteomics provides quantitative information, either relative or absolute on existing proteins within different experimental conditions. Identification is an important step in proteomic studies, still, quantitative proteomics is indispensable for understanding global protein expression and modifications underlying the molecular mechanisms of biological processes and disorder states.[172]

1.3.2.1. Two dimensional gel electrophoresis

One of the most useful features of proteomics is the countless techniques employed to study protein level changes. Traditional proteomic two dimension gel

electrophoresis (2DE) approach have preceded, and accompanied, the birth of proteomics and are widely used to study proteomes.[165,173] It entails separation of proteins based on their charge (isoelectric point) in the first dimension and by mass (molecular weight) on the second dimension.[171] Unlike SDS-PAGE, 2DE gels produce complex maps of proteomes that are visualized as discrete protein "spots", and it can also resolve many more proteins. Protein profiling by 2DE is an accessible and economical method with high resolving power that provides a bird's-eye view of the entire sample, however, gel-to-gel variability can limit the quantitative accuracy and inhibit the detection of minor differences in expression. [171,174]

Nevertheless, analysis of complex cellular samples can be tricky and therefore this technique shows restricted reproducibility and small molecular dynamic range.[174] For example, the separation of too basic or too acidic, too large or too small proteins is usually very limited. However, it is no longer the exclusive separation tool used in modern proteomics. Mass spectrometry-based approaches have been successfully use, and can be associated with 2D electrophoresis, allowing accurate and sensitive quantitation and the ability to multiplex extremely complex samples through the application of robust bioinformatics tools.[175]

1.3.2.2. Mass Spectrometry

Shotgun mass spectrometry has been the elected method for studying the mechanism of action and molecular effects of antipsychotics in Scz.[163,176] Bottom-up proteomics methodology starts with sample-preparation that consists in protein extraction and digestion, by a sequence-specific enzyme such as trypsin, followed by peptides separation using high-performance liquid chromatography (HPLC) couple to tandem mass spectrometry (MS/MS) to identify proteins in a complex sample. [177]

Brief History

The discovery of mass spectrometry began with the British physicist Sir Joseph John Thomson, whose studies on electrical discharges in gases led to the discovery of the electron in 1897, and what later would be recognized with a Physics Nobel Prize in 1906. [178] In the first decade of the 20th century, Sir J.J Thomson went on to construct the first mass spectrometer for the determination of mass-to-charge-ratio of ions. The ions generated by discharge tubes were passed into electric and magnetic fields and ions were moving through parabolic trajectories and then detected on a photographic plate. [178] By the 1940s, mass spectrometers were commercially available and the technique was firmly established. [178]

Anatomy and principles of a Mass Spectrometer

Mass spectrometry (MS) is the study of matter through the determination of the abundance and the mass-to-charge ratio (m/z) of ions in the gas phase.[179] The mass spectrometer consists of 1) Ionization Source, which converts peptides or other molecules into gas phase ions to acquire positive or negative charges; 2) Mass Analyser, ions travel though this part and are separated based on m/z ratios and 3) Detector, ions are detected and useable signals are generated and recorded by a computer system (Figure 1.5). The data acquired is interpreted in a mass spectrum, which is a plot of relative intensities (ion abundance) versus the m/z values of the ions.[172]

Two ionization sources have revolutionized the use of mass spectrometers because of their "softness": Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).[172,179] These methods have the ability to ionize intact ions before mass measurements, which tend to be fragile and fragment when ionized by conventional ionization methods.[172]

ESI employs energetic electrons beams during ionization process and operates under vacuum, while analytes are already in the gas phase. ESI can generate multiple charged ions, directly from sample solution.[179] On the other hand, MALDI uses a matrix compound that absorbs and transfers energy from the laser, it produces singly charged peptide ions which make mass spectra interpretation very straightforward.[172] MALDI and ESI are complementary to each other, each having its own strength and weakness.

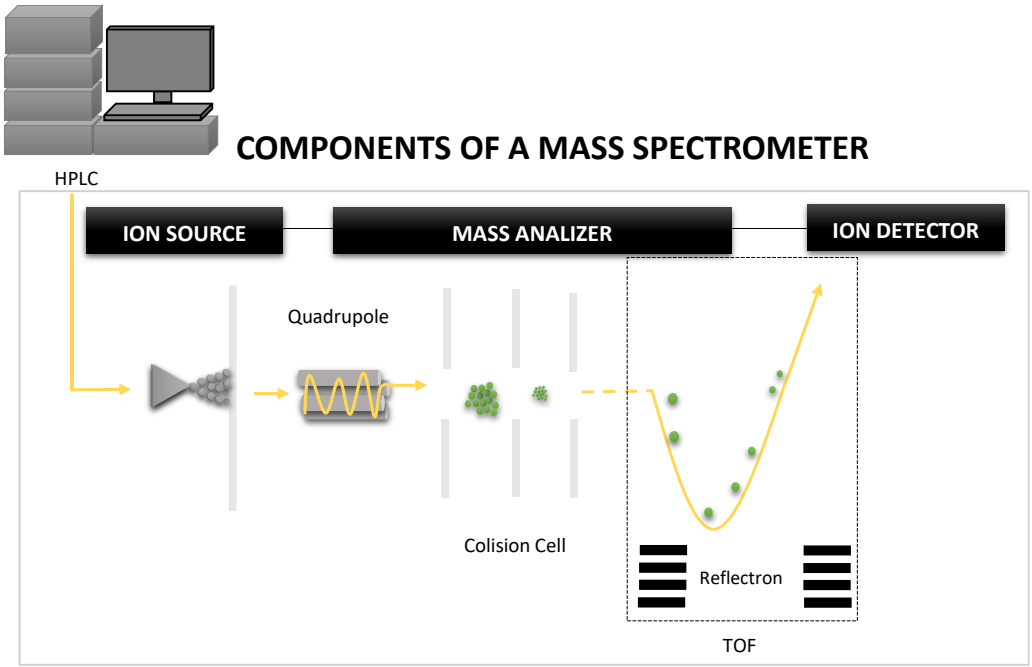


Figure 1.5 – Components of a Mass Spectrometer. The instrument consists in three major components that include an ion source, which is responsible for ionization of the sample; an analyzer responsible for separating the gas-phase ions that have been produced; and the detector system, in charge for detecting the ions and recording their relative abundance.

Relatively to the mass analyzer, is the component of the mass spectrometer responsible for separating the gas-phase ions that have been produced, based on their charge-to-mass ratio. Consequently, for multiply charged ions the apparent m/z values are fractional parts of their actual masses.[179] Several parameters are used to measure the performance of a mass analyzer: 1) mass range limit, determines the limit of m/z over which the mass analyzer can measure ions; 2) analysis speed and transmission, ratio of the number of ions reaching the detector and the number of ions entering the mass analyzer ; 3) acquisition and precision, the ability of the instrument to reproduce a mass measurement of a given compound; 4) mass accuracy, confidence in the m/z values; and 5) resolution, the ability of a mass analyzer to yield distinct signals for two ions with a small m/z difference.[179,180]

Quadrupole Analyzers, consist of four parallel metal rods where a direct voltage is applied to two of these rods, while the other two are linked to an alternating radio-frequency potential. That will cause all the charged molecules to accelerate and move away from the center line, the rate being proportional to their charge to mass ratio.[180] Quadrupole analyzers, either in the single or triple quadrupole configuration, are widely used in clinical biochemistry LC-MS applications.[181] Time-of-flight (TOF) analyzers, separate ions by time, instead of measuring the m/z of selected ions, all ions are pulsed down a field-free flight tube, and although they are pushed at the same time, the smaller ions will travel faster than the larger ions.[181] Consequently, TOF measures the velocity and time taken by the ions to travel down a flight tube and reach the detector, depending on their m/z ratio. All ions will reach the detector although at different times. [181] Unlike single quadrupole analyzers, TOF instruments have the ability to collect spectra very quickly and with high sensitivity.[181]

Last but not least, the final element of the mass spectrometer is the detector. It is responsible for recording the values reported by the mass analyzer. The detector must possess several key properties including high amplification, fast time response, low noise, high collection efficiency, same response for all masses, large dynamic range and narrow distribution of responses.[182] The output of the mass detector is the mass spectra.

Single and Tandem Mass Spectrometry

Mass spectrometry experiments are often referred to as being of the type single or tandem. The difference between the two is the number of measurements performed. Single mass spectrometry involves a single measurement, a tandem involves more than one-step of mass selection or analysis, and fragmentation is usually induced between the steps.[183]

Hybrid Analyzer, is a device for tandem mass spectrometry that consists of a combination of different mass analyzers. For instance, a combination of a selection quadrupole with a fragmentation cell and a TOF analyzer results in a hybrid quadrupole time-of-flight (QTOF) mass spectrometer.[181] It is also possible to design other type of instruments, where a quadrupole of a triple quadrupole MS can be switched between an ion trap mode and conventional quadrupole mode, this is known as QTrap instrument.

The methodology used in this study is LC-MS/MS, which consists of a chromatographic system couple to an ionization source, concretely an ESI source, coupled to the first mass-filtering device (Q1), which leads to a collision cell (q2) that can be filled with low-pressure gas for collision-induced dissociation (CID), followed by the TOF analyzer and finally the ion detector.[183]

1.3.2.3. Mass Spectrometry IDA and SWATH modes

In proteomics approaches, fragmentation mass spectra can be acquired using one of the following methods: “Data-Dependent Acquisition” (DDA), or “Data-Independent Acquisition”, (DIA).[184]

DDA also known as Information-dependent acquisition (IDA) is a powerful acquisition method for protein identification that maximizes the information obtained in a single LC-MS/MS analysis. In a classic DDA experiment, an MS survey scan is used to generate a peak list of all ions present in a sample in a given time point. The peak list is then subjected to a set of user-defined criteria filter, where the remaining ions are then submitted to fragmentation, product ion scan - MS/MS. This procedure is repeated throughout the duration of the acquisition, to generate large amounts of informative data. One of the major characteristic of this method is that unique and multiple survey scans can be specified as well as multiple dependent scans, as different collision energies can be established from the candidate masses subjected to product ion scans.[184] The most common use of IDA is for identification of compounds through “shotgun” techniques.

Sequential window acquisition of all theoretical mass spectra (SWATH) is a data independent acquisition method (DIA) which aims to complement traditional mass spectrometry-based proteomics techniques. It allows both relative and absolute quantification of samples an unlike DDA, does not select ions to be fragmented based on information in the precursor ion scan.[184,185] It allows a complete and permanent recording of all fragment ions of the detectable peptide precursors present in a sample.[185] Usually, a TOF instrument is used due to its fast and high resolution power and the data is acquired by repeatedly cycling through variable windows or swaths.[185,186] The result is, in a single

sample injection, time-resolved fragment ion spectra for all the analytes detectable within precursor range where the majority of the precursors fall in the 400–1200 m/z.[186]

Using SWATH method is an added value once it is powerful in evaluating whole proteome changes, besides the analysis of specific PTMs and establishing dynamic profiles of protein-protein interactions.[185]

1.3.2.4. Technical overview of sample preparation and separation in MS-Based proteomics

Sample preparation for bottom-up proteomics requires several treatment steps. After protein separation, one of the most important is protein digestion into peptides, usually performed enzymatically though proteolytic enzymes, where Trypsin is favoured.[187] It is robust, cheap, specific *qb* and it generates ideal mass range peptides for chromatographic separations (mass range of 500 to 3,000 Da). [187] It cleaves after lysine (Lys) and Arginine (Arg), with the exception when they are followed by a proline (Pro). [187]

Following enzymatic digestion and purification of proteins (to remove e.g. buffers and salts added during the sample preparation), these peptides are typically resolved according to their hydrophobicity by reversed-phase (RP). RP is one of the most used liquid chromatography format for separation that uses water-methanol or water-acetonitrile mixture as the mobile phase and C18 resins as the stationary phase.[172] After separation, peptides are eluted from the column and directly ionized by electrospray ionization before entering into the mass spectrometer.[187] LC-MS is the major platform used to analyses proteomic samples due to its high sensitivity, selectivity, accuracy, fast analysis times and small sample volumes.[187]

1.3.3. Applications

Mass spectrometry has been used in research and clinic applications as a very powerful technology to identify and quantify molecules. The most recent applications are focused towards biological problems by characterizing the whole proteome, metabolome, and performing drug discovery and toxicology research. This technology is extremely versatile and other analytical applications are routinely applied in pollution control, food control, doping, archaeology and forensic science, among others.[179]

With the ability to provide very specific identification, high sensitivity and simultaneous analysis of multiple analytes, mass spectrometry is now used in clinical laboratories. Different assays have been developed for identification and quantification of immunosuppressant, antiretroviral and anticancer drugs.[181] Toxicology screenings with

a wide range of drugs, toxins and their metabolites being easily detected.[181] It is also attractive to several areas of steroid biochemistry, quite useful for measuring low testosterone and dihydrotestosterone levels due to inherent problems with androgen immunoassays[188]; new-born screenings for inborn errors of metabolism are also widely performed.[189]

From a different perspective, mass spectrometry is also widely used in food sector, in the examples of pesticide residues analysis in food [190] or food allergen detection [191].

In proteomics, this methodology is widely applied in biomarker discovery, where protein identification and quantification are the major applications of mass spectrometry in proteomic field.

AIMS AND OBJECTIVES

2. Aims and Objectives

2.1. Aims

Haloperidol has been widely used since it was introduced and marketed in Belgium, October 1959. Despite its beneficial antipsychotic effect in SCZ and other mental disorders treatment, the literature reveals that relatively little is known about its long-term effects in the brain. Researchers around the world have been focused in understanding SCZ biology and pathology, while understanding the molecular effects of medication remains poorly unstudied. Proteomic alterations of *post-mortem* brain tissues provide valuable understandings into the pathophysiology of SCZ and try to explain medication effects, however, confounding factors of the disorder intercalate with medication effects. Nevertheless, rodent studies focusing mainly in the identification of proteins and cellular pathways affected by antipsychotics are necessary.

Consequently, this research project aims to narrow this knowledge gap and conduct an investigation to assess long-term molecular effects of Haloperidol in mice brain striatum. Identification of Haloperidol-associated changes is the main focus of the work as well as comprehend new insights in molecular networks and pathways affected by psychotropic medications.

2.2. Research objectives

The above aim will be accomplished by completing the following tasks:

Literature review regarding previous Haloperidol proteomic studies and mechanism of action

Subproteome fractionation of the striatum tissue from mice exposed to Haloperidol chronic therapy, in order to deepen the proteome coverage of the study

Proteomic identification and quantification of control samples versus samples from mice exposed to Haloperidol therapy using a label free quantitative mass spectrometry approach

Bioinformatics analysis of proteins identified and quantified by mass spectrometry

Knowledge progression of medication-associated changes related to antipsychotics mechanism of action

EXPERIMENTAL PROCEDURES

3. Experimental Procedures

3.1. Animal model and drug administration

Animal sample preparation was courtesy of Dra. Graça Baltazar's lab, with collaboration of Diogo Neto who have done Haloperidol injections (University of Beira Interior, Covilhã), Joana Pinto and Cátia Santa (Centro de Neurociências e Biologia Celular da Universidade de Coimbra) who assisted in striatum collection.

Young black male C57BL/6J mice were divided into three groups: control, vehicle treated; experimental group one, daily treated during 15 days (HA15); and experimental group two, daily treated during 30 days (HA30). Animals were maintained under 12 hours light/dark cycles at 22±1 ˚C with 55% humidity. Rodent's chow and water were accessible and animals were chronically treated during different time points.

To reduce animal stress, they were handled for one week previous to drug administration. Animals were then subjected to intraperitoneal injection of 1 mg/kg Haloperidol (BIOTREND Chemicals AG, ref. BG02211) for 15 or 30 days or injected with vehicle for 30 days. Haloperidol solution was prepared in absolute ethanol, heated up to 60˚C to complete dilution and stored at room temperature. All the solutions administrated were daily prepared in a sterile pH-adjusted solution of 0.9% NaCl with 0.13% HCl 5M. Haloperidol was administrated at 0.665 mM from 10 mM stock, while same volume of absolute ethanol was used in control solutions.

No differences were observed in body weight between littermate controls (26.7 ± 0.7 g) and treated 15 days with Haloperidol (27.4 ± 0.7 g) or 30 days with Haloperidol (26.6 ± 0.3 g).

All procedures were conducted in accordance with the national ethical requirements for animal research, and with the European Conventional for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

3.2. Sample collection

Before tissue collection, animals were anesthetized with 100mg/kg of solution containing Ketamine (87.5%) and Xylazine (12.5%) and sacrificed after. The brain was removed and dissected to obtain striatum. TEAB 0.5 M (triethylammonium bicarbonate

buffer) with phosphatases and proteases inhibitors (EDTA-free Protease Inhibitor Cocktail) was added to each tube. All samples were stored at -80ºC until use.

3.3. Sample preparation

3.3.1. Tissue Homogenization by Ultrasonication

To each microcentrifuge tube containing the striatum, 850 µL of Tris 0.05M (tris(hydroxymethyl)aminomethane) with phosphatase (PhosSTOP cocktail pills from Roche) and protease inhibitor (cOmplete, EDTA-free Protease Inhibitor Cocktail from Roche) was added. Striatum samples were homogenized by ultrasonication (Vibra Cell 130 watts, Sonics) with 2mm probe for 30 seconds at 40% amplitude with 1 second cycles and for 30 second at 50% with 1 second cycles. After that, samples were centrifuged (Centrifuge 5417R, Eppendorf) at 5,000*xg* for 5 minutes at 4˚C. The supernatants were saved for new microcentrifuge tubes and 500 µL of Tris 0.05M with phosphatase and protease inhibitor was added to the pellets. These pellets were also homogenized with 2mm probe for 30 seconds at 40% amplitude with 1 cycles and for 30 seconds at 50% amplitude with 1 second cycles and were centrifuged as performed previously. Supernatants were saved for the same microcentrifuge tubes as the ones from before and pellets were discarded.

3.3.2. Subproteome Fractionation

Samples were pooled for identification. A volume of 150 µL was taken from each sample and 250 µL of Tris was added to each pool. An ultracentrifugation (OptimalTM L-100XP, Beckman Coulter) using a 90Ti rotor at 144,000*xg* for 1 hour at 4˚C was performed. The supernatant corresponding to the soluble fraction was transferred to a 15mL plastic centrifuge tube. To the remaining pellets, 500 µL of TEAB 0.5M with protease and phosphatase inhibitors was added and the pellet was dissolved by ultrasonication with 2mm probe for 30 seconds at 40% amplitude with 1 second cycle and 30 seconds at 50% amplitude with 1 second cycles, this step was repeated until total dissolution of the pellet. Samples corresponding to the membrane-enrichment fraction were transferred to a 15mL plastic centrifuge tubes.

3.3.3. Protein Precipitation

From the homogenized, 250µl of the individual samples were used for protein precipitation and added to a microcentrifuge tubes. Cold acetone was added in order to make a volume ratio of 1mL sample: 6mL acetone. The sample tubes were inverted several times to mix, incubated for at least 20min in deep freezer (-80˚C) and centrifuged in the Eppendorf Centrifuge 5417R at 20,000xg and 4˚C. Acetone was discarded and the pellet was ressuspended in 250µL 2x Laemmli Sample Buffer (5% glycerol, 1.7% SDS, 100 mM DTT and bromophenol blue in 50 mM Tris Buffer at pH 6.8). The samples were spinned and transferred to new microcentrifuge tubes.

Both soluble and membrane-enriched fraction each pool were also precipitated following the procedure mentioned above. However, given the higher volume of sample, pools were centrifuged in the Thermo Scientific Heraeus Megafuge 40R Centrifuge for 20min at 4,100x*g* at 4˚C.

3.3.4. Protein Quantification

Protein quantification of the samples was assessed by 2-D Quant Kit (GE Healthcare). Bovine serum albumin (BSA) was used as standard and the assay was performed as indicated in the manufacturer protocol. The 2-D Quant Kit works by precipitating proteins, while leaving interfering substances behind, and then evaluating the concentration by colorimetric assay. The first step was to precipitate both the standards and the samples, followed by resuspension in copper solution. The assay is based on the specific binding of copper ions to protein, where unbound copper is measured with a colorimetric agent. The absorbance at 480 nm is inversely related to the protein concentration and it was measured on a Microplate Spectrophotometer (PowerWave XS, BioTek). A calibration curve was obtained from the standards and protein concentration of the samples was calculated (Figure 7.2 and Figure 7.1).

3.3.5. SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis)

From the three experimental conditions (control, 15 days of Haloperidol treatment and 30 day of Haloperidol treatment) pools were made using 150 µL of each sample. Soluble fraction and membrane fraction were separated by ultracentrifugation as mentioned before and quantified by 2-D Quant Kit. For the protein library creation, 50 µg of protein of each fraction of each pool were used and 2 µg of a recombinant protein (Green fluorescence protein and Maltose-binding periplasmic protein (malE-GFP) were

added and used as internal standard. Pooled samples fractions were denatured in the Thermomixer for 5 minutes at 95˚C. In order to promote the cysteine alkylation, acrylamide [40% acrylamide/bis solution (Bio-Rad)] was added to the samples. Pooled samples were electrophoretically separated in a pre-cast stain-free 4-20% SDS-polyacrylamide gel (Bio-Rad) using a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad). The samples ran for 66 minutes at 110V (Figure 3.1).

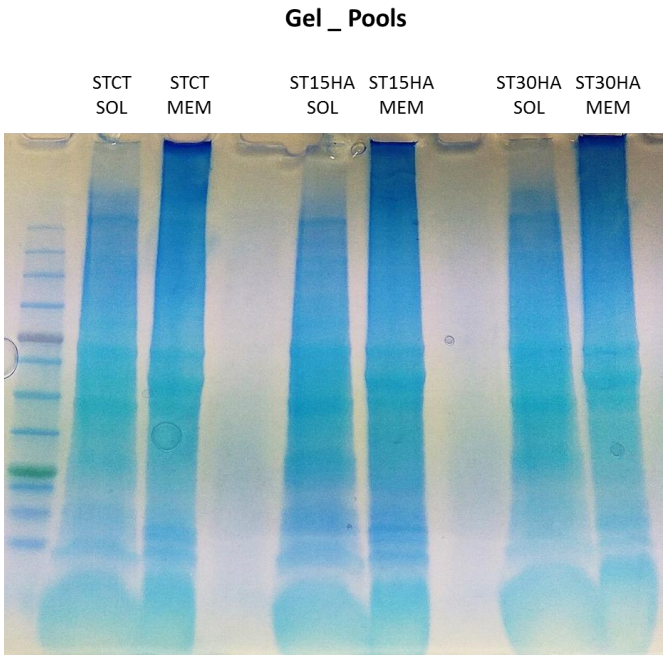


Figure 3.1 - SDS-PAGE of pooled samples. Pools were made using 150 µL of each sample and soluble (SOL) and membrane-enriched (MEM) fractions were obtained by ultracentrifugation. Each fraction was electrophoretically separated using Mini-PROTEAN Tetra Electrophoresis System and stained with colloidal coomassie.

3.3.6. Short-GeLC

For SWATH analysis, all the samples were electrophoretically separated as performed with the pools. Fifty micrograms of protein of each sample with 2µg of MalE-GFP were boiled in the Thermomixer for 5 minutes at 95˚C. GFP was used as internal standar. In order to promote the cysteine alkylation, acrylamide [40% acrylamide/bis solution (Bio-Rad)] was added to the samples. Samples were electrophoretically separated in a pre-cast stain-free 4-20% SDS-polyacrylamide gel (Bio-Rad) using a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad) and ran for 15 minutes at 110V.

3.3.7. Gel Staining

After electrophoresis, the gels were washed with deionized water and immersed in 50 mL of fixation solution (5,85mL of ortho-phosphoric acid (80%), 5g of ammonium sulfate, and 10mL methanol). For staining, Coomassie blue powder was added carefully with a strainer to prevent accumulation and left staining for 1hour. After staining, the staining solution was discarded and gels were transferred to new boxes filled with ddH_2O. Successive washes with ddH_2O were performed till the background of the gel was clear (Figure 3.2).

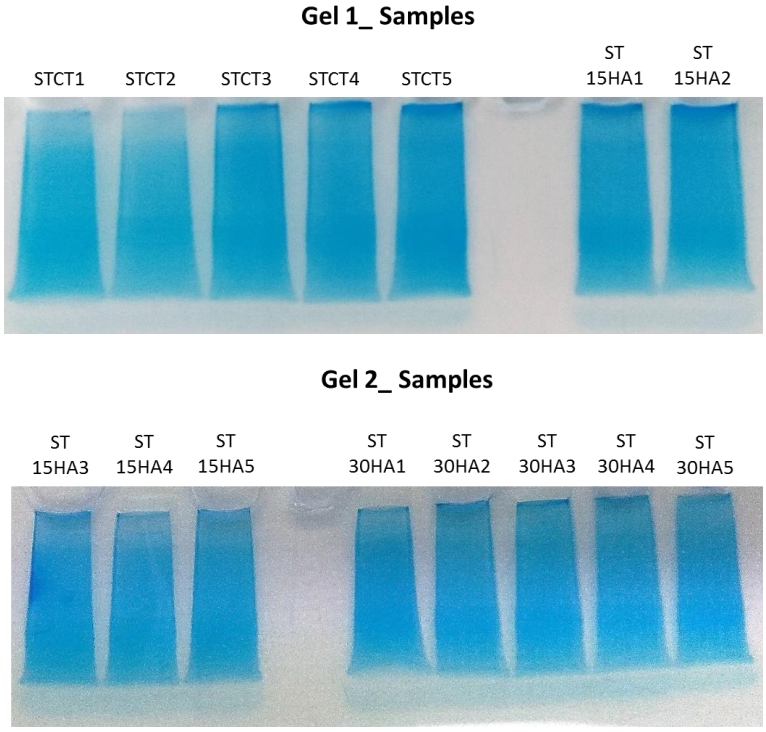


Figure 3.2 – Short-GeLC of Samples. For SWATH analysis, all samples were electrophoretically separated using a Mini-PROTEAN Tetra Electrophoresis System for 15 minutes at 110V.

3.3.8. Gel band processing

In the laminar flow chamber, a proper cleaned acetate sheet (with detergent, ddH2O and ethanol) was used to place the gels. Gel lanes were sliced into 6 (pool samples gel) and 3 (samples gel) bands of similar size using a scalpel blade. Gel pieces were transferred respectively to the wells of a clean 96-MW plate filled with 600µL LC-MS water. To distain the gel pieces the water was removed and 600µL of distain solution (50mM ammonium bicarbonate and 30% acetonitrile) was added. The plate was taken to the thermomixer for 15 minutes at 1050 rpm's and 25˚C. The distain solution was removed and the procedure was repeated. After another 600µL of distain solution and 15

minute in the thermomixer, the solution was removed and 600µL of LC-Water was added. The plate was shaken in the thermomixer for 10 minutes at 1050 rpm's and 25˚C. After that, the water was removed and gel pieces were dehydrated on the Concentrator Plus (Eppendorf) for 1 hour at 60°C. When the gel pieces were completely dehydrated, 75µL of trypsin at 0.01 µg/µl (Roche) was added until all the gel pieces were covered with the solution and incubated for 15 minutes at 4˚C. After this period, 75µL of 10 mM ammonium bicarbonate buffer was added and the plate was left overnight at room temperature in the dark, leaving trypsin reacting and digesting the proteins. After this digestion step, peptides extraction was performed and the tryptic solution (containing trypsin and some peptides) in the plate was collected to LoBind® microcentrifuge tubes (Eppendorf). The remaining peptides were extracted by adding solutions with an increasing concentration of acetonitrile (ACN). 100µL of 30% ACN in 1% formic acid (FA) was added and gel pieces were taken to the thermomixer for 15 minutes at 1200 rpm's and 25˚C. The solution with peptides was collected to the same tube containing the initial tryptic solution. The same process was repeated when adding 100µL of 50% and 98% ACN in 1% FA. The peptide mixtures were concentrated on the Concentrator Plus (Eppendorf) at 60ºC until dry.

3.4. Sample preparation for LC-MS

3.4.1. C18 peptide clean up

After peptide extraction, it is necessary to clean up peptides for compatibility with LC-MS run. So, 100µL of 2% ACN 1% FA were added to the evaporated peptides and sonicated on a Sonics 750 W using a cup-horn (2 min with 1 sec ON and 1 sec OFF cycles at 20% amplitude). In this step C18 OMIX tip was used in each sample. OMIX tip contains a small bed of functionalized monolithic sorbent (C18) inserted inside a pipette tip, working as a miniaturized solid phase extraction bed for peptide clean up prior to mass spectrometry. Briefly, 200 µL of 50% ACN were used to hydrate the tip columns and 300 µL of 2% ACN with 1% FA solution to equilibrate. The peptides were loaded into the column and this step was repeated five times followed by a washing step with 100 µL of 2% ACN with 1% FA solution. Peptides were eluted to LoBind tubes with 400 µL of 70% ACN and 0.1% FA and eluates were concentrated using the Concentrator Plus at 60 °C. When completely dried, 30 µL of 2% ACN 0.1% FA was added to each sample and the samples were sonication in cup-horn (2 min with 1 sec ON and 1 sec OFF cycles at 40% amplitude). After that, samples were centrifuged at 14,000*xg* for 5 minutes (miniSpin, Eppendorf) and the supernatants were transferred to the appropriate LC vials.

3.4.2. LC-MS data acquisition

After digestion and peptide extraction and clean up, samples were analyzed on Triple TOF™ 5600 System (ABSciex®) using information-dependent acquisition (IDA) for library generation and SWATH-MS acquisition of each individual sample for protein quantification. Firstly, peptides were separated by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column ChromXP™ C18CL (300 µm ID × 15 cm length, 3 µm particles, 120 Å pore size, Eksigent®) at 5 µL/min with a multistep gradient: 0-2 min linear gradient from 2 to 5 %, 2-45 min linear gradient from 5 % to 30 % and, 45-46 min to 35 % of acetonitrile in 0.1 % FA and 5% DMSO. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, ABSciex®) with a 50 µm internal diameter (ID) stainless steel emitter (NewObjective).

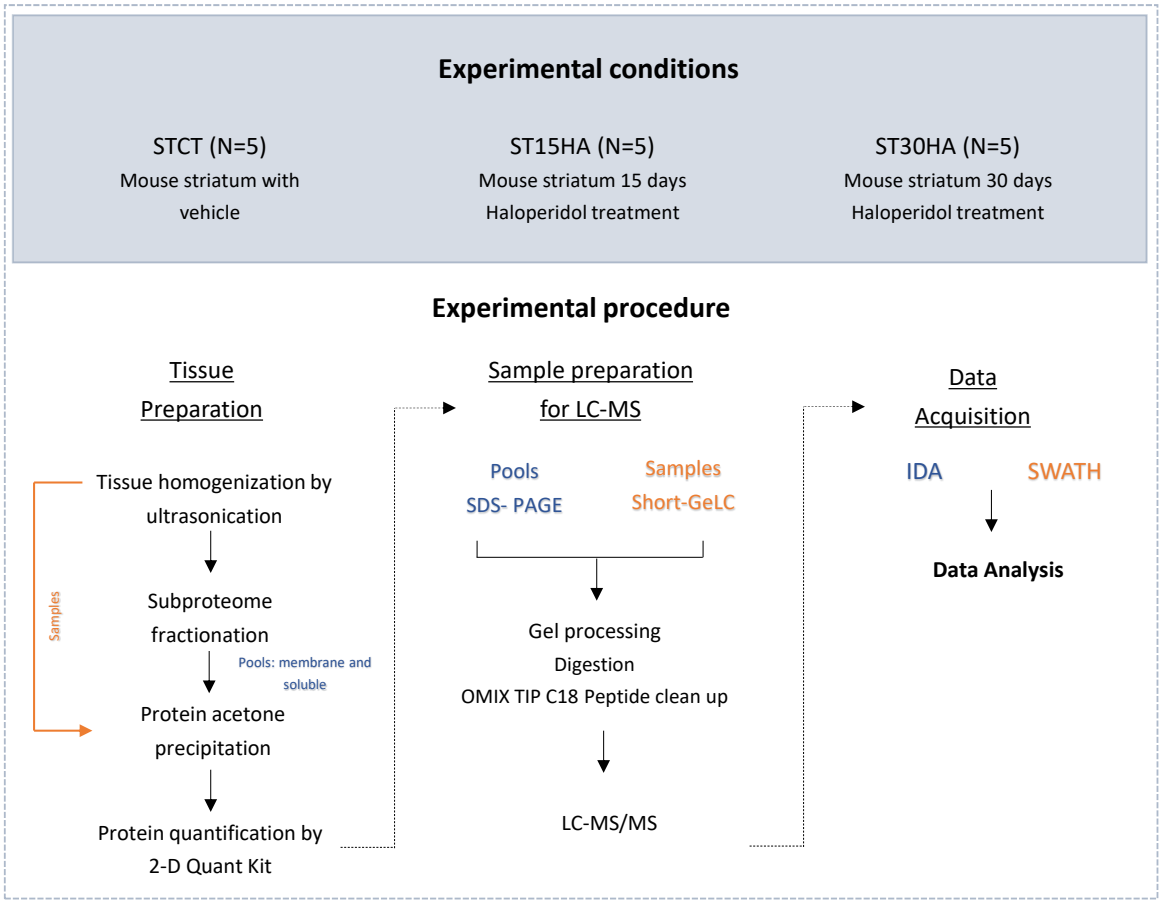


Figure 3.3 - Experimental design and procedures of the work. The first step is tissue homogenization of all the samples by ultrasonication, followed by Subproteome fraction of pooled samples. Protein acetone precipitation and protein quantification by 2-D Quant kit was also performed. Sample preparation for LC-MS was performed in two different running conditions. For pooled samples SDS-PAGE was executed and for all samples, Short-GeLC. For gel processing, trypsin was used for overnight digestion, followed by C18 peptide clean up. After sample preparation, samples were analyzed in the mass spectrometer and data acquisition was performed using IDA and SWATH methods.

For IDA experiments, the mass spectrometer was set to scanning full spectra (350-1250 m/z) for 250 ms, followed by up to 100 MS/MS scans (100–1500 m/z) using a dynamic accumulation time – minimum of 30 ms for precursor above the intensity threshold of 1000 – in order to maintain a cycle time of 3.3 s. Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 25 seconds (mass spectrometer operated by Analyst® TF 1.7, ABSciex®). Rolling collision was used with a collision energy spread (CES) of 5.

For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode and the same chromatographic conditions were used as described above for the IDA run. A set of 60 windows of variable width containing 1 Da for the window overlap was constructed, covering the precursor mass range of 350-1250 *m/z*. For instrument calibration, a 250 ms survey scan (350-1500 *m/z*) was acquired at the beginning of each cycle. SWATH MS/MS spectra were collected from 100-1500 *m/z* for 50 ms resulting in a cycle time of 3.25 s. The collision energy (CE) applied to each *m/z* window was determined considering the appropriate CE for a +2 ion centered upon this window as well as the CES was also adapted to each *m/z* window.

3.5. Data Analysis

3.5.1. IDA and SWATH data file analysis

IDA analysis fundamentally generates an identification library. Pooled sample files from the three experimental conditions (control, 15 day Haloperidol and 30 day Haloperidol) were combined and peptide identification was performed using ProteinPilot™ software (V5.0.1, Sciex®). Several parameters were set in order to do the correct search method. Species used, digestion type and alkylating agent are several important parameters. Uniprot_SwissProt database for mouse (April 2017 – last update) was the database used (malE- GFP sequence was also added to this database), digestion type was in gel-digestion, trypsin was selected as used enzyme and acrylamide as the alkylating agent. An independent False Discovery Rate analysis (FDR) was performed. This FDR analysis using the target-decoy approach, was used for quality assessment of the identified proteins. In other words, positive identifications were considered when proteins present 95% confidence (5% local FDR) with more than one peptide hit with individual confidence above 95% local FDR.

A library of precursor masses and fragment ions was then generated from IDA experiments and it was used for subsequent SWATH processing. The protein quantification was performed using Peak View Software v2.1 SWATH™ (Sciex). IDA library

was imported to the software along with the SWATH files. In order to align sample's retention time, malE-GFP peptides as internal standard and present in all samples, were selected. Subsequently, sample peptides were selected automatically from the library and processed using the Table 1:

Table 1 – SWATH filtering criteria

Peptide Filter	
Number of Peptides per Protein	15
Number of Transitions per Peptide	5
Peptide Confidence Threshold % (0-99)	98
False Discovery Rate Threshold % (0-100)	1.0
Exclude Modified Peptides	✓
XIC options	
XIC Extraction Window (min)	3.0
XIC width (ppm)	100

Protein levels were estimated by adding all the transitions from all the peptides for a given protein.

3.5.2. Software tools for protein analysis

Scatter Plot diagrams was performed using Excel. This type of diagram is used to show scientific XY data, to look for a relationship between the variables X and Y, in this case (ex: condition 15DHA vs Control). p-values and median were used.

Venn diagram tool (http://www.biovenn.nl/) from a web application BioVenn was used. This is a useful tool to visualize data in a logical way, allowing the users to see groupings and sets clearly [192]. In this type of analysis users can then quickly observe similarities and differences between the data sets. A list containing the accession name and number of proteins belonging to each condition to be compared were uploaded and the tool automatically generates a Venn diagram of the analyzed proteins and the distribution within the different conditions compared.

InfernoRDN was used to test if the samples correlate between themselves, as correlation plot.

MarkerView™ Software (V1.2.1.1) was used for principal component analysis analysis (PCA). The generated profile plots are used to confirm the behavior of selected variables across all samples. The following parameters were used in this analysis: weighting; pareto scalling.

The Graphical Proteomics Data Explorer (GProX) is a platform for comprehensive and integrated analysis and visualization of large proteomics datasets [193]. The program provides a range of functions for data analysis, including: Plots, Clustering, Enrichment tests and also Pathway Analysis. This type of analysis is mainly used for biological characterization. A Heatmap analysis was carried out, and a visual identification of the number of clusters potentially formed by the set of proteins being studies was observed. Two important aspects were considered: i) data standardization and ii) the hierarchical clustering of the proteins in the Heatmap. Standardization of the data is a fundamental aspect in this type of analysis because protein levels with different orders of magnitude are frequently observed in proteomics data. And, because different proteins cannot be directly compared, in this type of analysis what is comparable is the levels of the same protein among the different experimental conditions. Therefore, data standardization results in standardized protein levels that fit into the same scale of values allowing the simultaneous analysis of the entire dataset.[194] Other relevant point is the hierarchical clustering during the heatmap creation, which groups proteins with the same interaction profile. The clustering analysis was also performed using the unsupervised clustering fuzzy c-means algorithm implemented in the Mfuzz package, which is a soft clustering algorithm, noise-robust, and well-fitted to the protein profile data. Number of clusters, regulation threshold and membership values are also some parameters to be considered. Biological interpretation of the results was performed by Enrichment analysis. This starts with the attribution of GO (gene ontology) to the entire set of proteins analyzed, which can be imported directly from the UniProt database using the GProX software. Once we are looking for a better characterization of the mechanisms and pathways being altered, an analysis of the biological processes was performed. In this test several aspects were considered including: I) background cluster; II) statistical test; III) p-value adjustment; IV) minimum p-value and V) minimal occurrences.

Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/) is a database which was used for advanced pathway mapping[195]. These maps allow a better biological interpretation of data, representing molecular interactions and reaction networks.

GO enrichment analysis was performed using the web-based application Gene Ontology enRIchment anaLysis and visuaLizAtion tool – GOrilla (http://cbl-gorilla.cs.technion.ac.il/)

GOrilla helps to identify and visualize enriched GO terms in genes or proteins lists. The output of this analysis is visualized as a hierarchical structure, using a representation with color-coding that reflects the enrichment degree based on the p-value. All the analyses were done for the *mus musculus*, in the target ranked list of genes running mode

(unaltered proteins were used as background) and based on biological process. Diagrams were exported and presented as results.

3.5.3. Statistical analysis

Statistical analysis of results was tested using IBM SPSS Statistics version 19 assessed by Mann-Whitney U-Test. The test was used to identify the proteins that were altered by Haloperidol between the two time-points and control. The results were reported as statistically significant when p-value <0.05, and proteins which are outside a 1.2 fold factor were also considered as being changed between the control and the two Haloperidol groups.

RESULTS AND DISCUSSION

4. Results and Discussion

Scz is a complex disease fraught with obstacles to accurate diagnosis and effective treatment that are able to cover all symptomatology associated with the disease. Antipsychotics are the first line of treatment of Scz, where Haloperidol is one of the most frequently used drugs worldwide.[119] Much of the published results regarding molecular alterations in SCZ patients have been obtained using samples from medicated patients, or in some cases, patients that underwent treatment at some stages of their lives. However, the mechanism of action and molecular effects of antipsychotic medication are not completely understood, so, is not possible to ensure that changes observed in these studies are disease related and not drug related or even a consequence of chronic impairment. The importance of using samples from drug-naïve patients or animal models lacking the disease cannot be overestimated.

Proteomic analyses of the pharmacological profile of antipsychotic drugs on specific brain areas in rodent have been used, with the potential to uncover molecular networks and pathways affected by antipsychotic medication, and it may also contribute do understand the etiology of mental disorders.[164] The striatum is a brain area where subtle changes by Haloperidol should be most prominently expressed due to the high density of D_2 receptors [69]. Therefore, the study of brain striatum seems a natural path to follow for the understanding of the molecular effects of Haloperidol and for this reason it was the tissue studied in this project.

In this research project, three animal groups were studied: control (mouse striatum with vehicle); 15HA (mouse subjected to 15 days of Haloperidol) and 30HA (mouse subjected to 30 days of Haloperidol). Each of the condition contained five mice that were the biological replicates, where samples STCT1, STCT2, STCT3, STCT4 and STCT5 are from the control group; samples 15HA1, 15HA2, 15HA3, 15HA4 and 15HA5 are from the 15 day Haloperidol; and 30HA1, 30HA2, 30HA3, 30HA4 and 30HA5 are from the 30 day Haloperidol.

4.1. Protein Identification

In order to attest the number of proteins identified in each condition, the five sample replicates of each condition were combined (pools), ultracentrifuged and protein identification was performed in both fractions (soluble and membrane-enriched) of each pool by LC-MS/MS, as previously described. Identification of high numbers of proteins is essential to obtain a favorable IDA library to be used subsequently in SWATH analysis. The highest the number of proteins, the greatest the library content. Identification is obtained by fragmentation spectra, which is a characteristic of a given peptide. This fragmentation spectra is achieved as a result of the MS, that is set to scan precursor ions eluting at a certain time point from the chromatographic separation, and then followed by a selection of a limited set that is fragmented (usually the most intense ones).[185] IDA data was then subjected to database search by ProteinPilot™. Mouse (mus musculus) reviewed protein database was downloaded from UniProtKB (April 2017 Version) and used for database searches.

Four different protein libraries were generated: i) all samples; ii) control; iii) 15 day Haloperidol and iv) 30 day Haloperidol. From, Figure 4.1 it is possible to visualize that the total number of proteins identified is very similar between conditions.

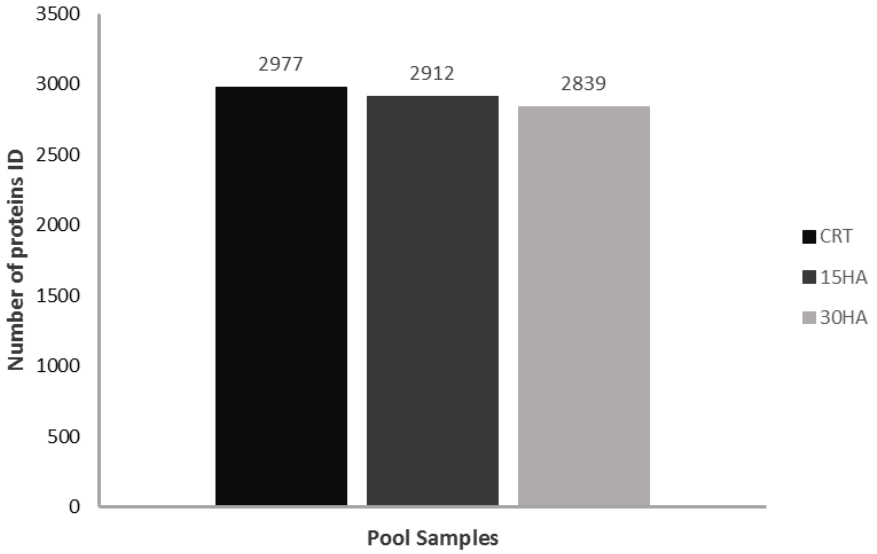


Figure 4.1 – Total number of proteins identified by Mass Spectrometry. To understand the number of proteins identified in each condition, the five sample replicates of each condition were combined and protein identification was performed by LC-MS/MS in both fractions of each sample pool.

Using the information from the identification library, it is also possible to understand the number of shared or unique proteins between the different experimental conditions. Venn analysis diagram provides a clear visual representation of data, allowing

the visualization of groupings and sets distinctly. The total number of proteins identified in the library was 3311. From Figure 4.2, comparison between the control, 15HA and 30HA revealed that about 72% (2443 proteins) were shared by all the conditions, where 215 were only identified in the control group; 167 in 15HA and 119 in 30HA.

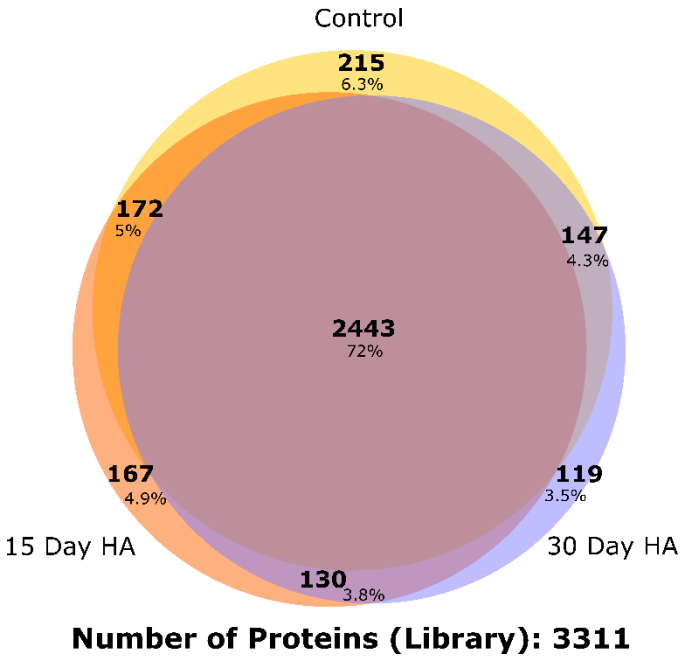


Figure 4.2- Venn Diagram illustrating the number of shared and unique proteins identified by Mass Spectrometry in the different experimental conditions. A high number of shared proteins (2443) were identified between all experimental conditions. The number of individually identified proteins is slightly different between conditions: 215 in control; 167 in 15 day of Haloperidol and 119 in 30 day of Haloperidol treatment.

4.2. Protein quantification

A SWATH-MS analysis was performed to find out which proteins were altered between the three experimental conditions. With SWATH-MS a complete record of the fragment ion spectra of all the analytes in the sample is generated with a certain m/z window. The acquired data from the chromatographic peak of each peptide is further used to determine the relative quantity of a certain protein. A total of 1366 proteins were quantified and passed the criteria mentioned in the experimental procedures chapter. For statistical analysis, Mann-Whitney U-test was performed. The results were reported either if i) they were statistically significant when $p < 0.05$ and/or ii) proteins presented a ratio lower than 0.83 or higher than 1.2. The Scatter Plot in Figure 4.3 represents the 1366 proteins quantified. Medians were used to make the Scatter Plot, also the indication of proteins with 20% regulation and p-value of 0.05 is represented. Comparisons between control and 15 days of Haloperidol (A); between control and 30 days Haloperidol (B); and between 15 days and 30 days of Haloperidol are (C) represented. The number of proteins

in each parameter is indicated in each plot. There are several conclusions that can be taken from these Scatter Plots observed in Figure 4.3:

Control condition vs 30HA is the comparison where major changes are observed; it has the major number of down-regulated, and also the major number of up-regulated proteins;

15HA vs 30HA is the comparison where less alterations between the conditions are observed; This may indicate a stabilization by several proteins that increase or decrease relative to the control condition, but stabilize at least after the 15 days of medication.

About 39 proteins (p-value <0.05) are down-regulated in control condition when compared to 15HA; this number increases when comparing 30HA to control, 65 down-regulated proteins (p<0.05)

About 13 proteins (p-value 0.05) are up-regulated in control condition when compared to 15HA; this number increases when comparing to 30HA to control, 24 up-regulated proteins (p-value<0.05).

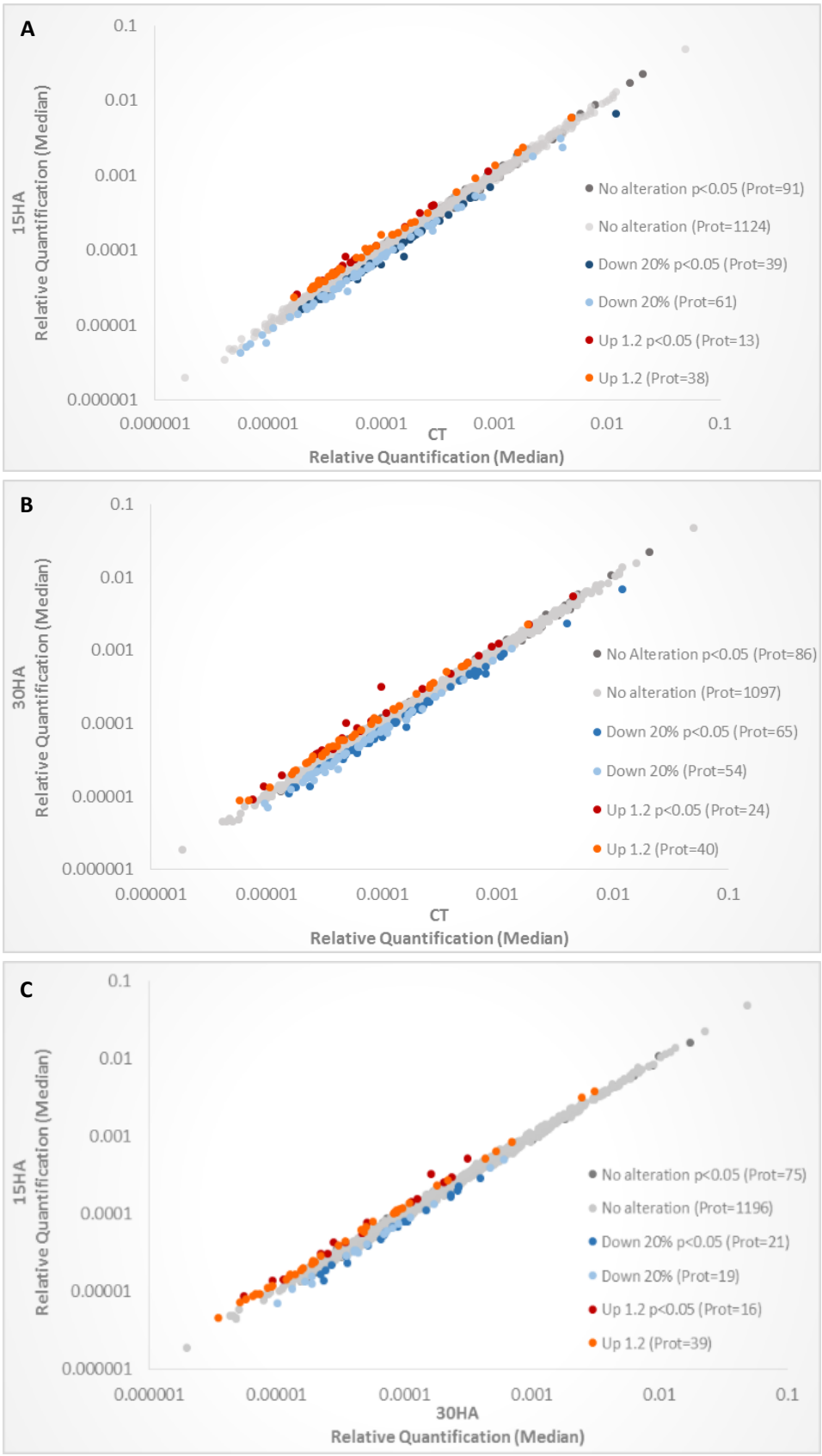


Figure 4.3 - Scatterplot representation of the 1366 quantified proteins (group median). Indication of proteins with 20% regulation, and p-value <0.05 (Mann-Whitney U-test). **A)** Comparison between Control and 15 day of Haloperidol; **B)** Comparison between Control and 30 day of Haloperidol; **C)** Comparison between 15 day Haloperidol and 30 day Haloperidol.

A correlation analysis between replicates from the three experimental conditions was performed in order to assure that no technical or biological problem (including sample handling, processing or innate animal biological alteration) occurred with any of the samples. From this analysis a correlation table was obtained (supplementary data Figure 7.3). The data points out to correlation values above 0.9, which allow to proceed the analysis with a certain confidence.

A HeatMap analysis was also performed for all the replicates of each experimental condition (supplementary data Figure 7.4). No distinct expression pattern could be observed between the three experimental conditions. When evaluating the HeatMap something dissimilar was noticed in respect to replicate two from control condition (STCT2). It shows a specific pattern which makes it differentiate from the other control samples.

A PCA analysis to confirm the behavior of all samples was performed. From the profile plots of all proteins that quantification filter quality (1366 proteins) the distinct sample profiles was not clear (supplementary data Figure 7.5), so the list was restricted to the proteins that pass the statistical test with p-value <0.05 or/and have a 20% variation (459 proteins). As observed in Figure 4.4. Replicate 2 from control condition is uncorrelated with the rest of control sample replicates, and it may represent a different profile of the sample.

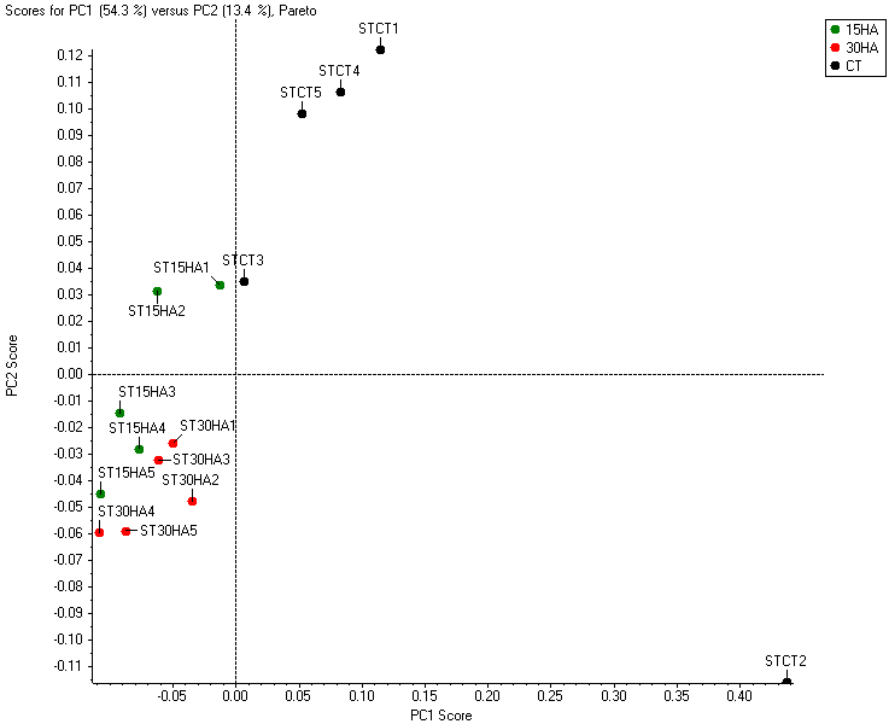


Figure 4.4 – PCA analysis of the 459 altered proteins. Each dot represents a replicate from the three experimental conditions (CT: control; 15HA: 15 day of Haloperidol treatment; 30HA: 30 day of Haloperidol treatment). PCA analysis was performed with altered proteins that pass the statistical test with p-value <0.05 or/and 20% variation. A clear separation of the three experimental conditions profile was obtained. Control samples are separated from samples that received 15 or 30 days of Haloperidol along the first principal component.

Most importantly, control samples are separated from samples that received medication.

After this preliminary analysis 459 proteins were chosen to be used in the rest of the analysis. The 1366 proteins that initially passed the quantification quality filters were restricted in order to simplify the rest of the analysis, and most significantly, try to analyze proteins that apparently would have more impact. As it can be visualized in the PCA analysis performed, when the list of proteins was restricted (459), a well clear separation is obtained. PCA of all proteins (supplementary data Figure 7.5) a separation is also evident, however, not so clear. In this way, the 459 proteins are contributing to this separation profile, while using the 1366 may be masking some differences.

All the replicates from each experimental condition have shown the same expression pattern, therefore instead of individual replicate values, further analysis were performed with medians.

Replicate two from control condition (STCT2), although it demonstrates a different behavior comparatively to the other control replicate in the heatmap and PCA mentioned above, and after critical consideration, it was included in the rest of the analysis. Including this sample did not alter sample medians and the correlation values above 0.9 between this replicate and all the other, plus in the PCA analysis it fitted closer to the right group, control condition.

4.3. Protein expression profile

From the 459 were altered, a heat map and unsupervised clustering analysis of the protein expression values was performed, from which six different clusters with well-defined expression profiles, Figure 4.5.

In the Heat Map, the data is displayed in a grid where each row represents a protein and each column represents a sample. The color and intensity of the boxes is used to represent changes (not absolute values) of protein expression. The red represents down-regulated proteins and green represents up-regulated proteins. Black represents unchanged expression. Hence this, Heat Map is a common and of easy interpretation method for visualizing protein expression. The results from Figure 4.5-A show an opposite pattern between control condition and Haloperidol conditions (either in the 15 days or 30 days of treatment). There are about 48% down-regulated proteins and 40% up-regulated between 15HA and control condition. Relatively to the 30HA, the results show a 48.5% of down-regulated proteins and 34% up-regulated when comparing to the control condition. Also, there are 8%, about 44 proteins that are diminished at 15 day of Haloperidol and

increased at 30 day of Haloperidol and about 10% (52) proteins that are increased at 15 day of Haloperidol and diminished at 30 day of Haloperidol relatively to control.

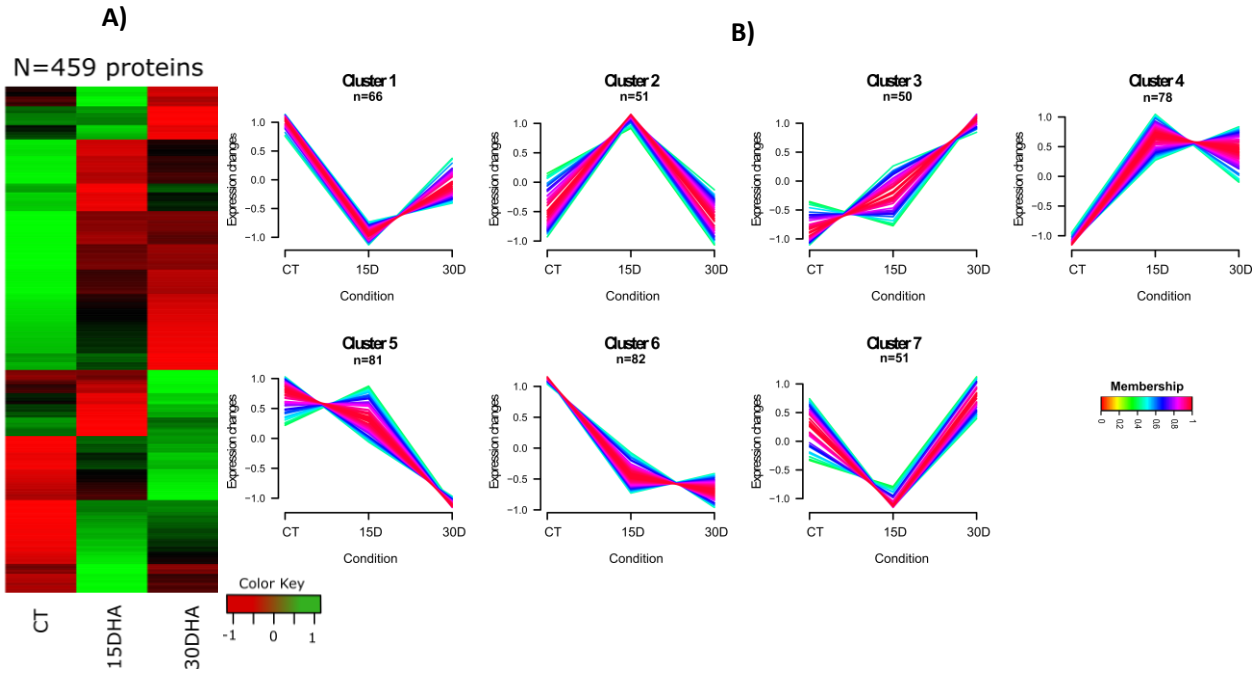


Figure 4.5 - Characterization of altered protein expression profile under Haloperidol treatment. (A) Row-clustered heat map showing the median standardized expression levels of the 459 proteins under Haloperidol treatment. **(B)** Dynamic profiles of altered protein expression under Haloperidol treatment. Profiles were obtained by an unsupervised clustering analysis performed for the standardize expression levels, with an upper regulation threshold of 2 and lower regulation threshold of 0.5, established for the higher and lower expression levels of each protein. The "n" indicates the number of proteins within each cluster. Membership color values represent how well the protein profile fit the average cluster profile.

From the clustering analysis, there are two distinct patterns that can be observed (Figure 4.5). For instance, in cluster 1, 2, 4 and 7 it seems that there is a peak in protein expression at 15 days of Haloperidol and there is a tendency to return to normal at 30 of day Haloperidol. Contrary to what is observed in cluster 3, 5 and 6, where protein expression changes drastically after 30 days with Haloperidol when comparing to control.

Cluster 5 and 6 are the ones that have more proteins associated. Although both clusters have similar decreasing pattern, in cluster 5 it can be observed a slight tendency to maintain protein expression, although with treatment, protein expression is indeed reduced. On the other hand, proteins in cluster 6 show an opposite behavior. Protein expression decreases drastically at 15 day of Haloperidol, however at 30 day of Haloperidol it stops decreasing and protein expression is almost maintained.

4.4. Enrichment Analysis

In order to understand and interpret this type of protein expression changes, a common approach used is to perform a gene ontology (GO) enrichment analysis based on the functional annotation of the differentially expressed proteins. This is a useful approach to evaluate if differentially expressed proteins were significantly enriched in any particular biological process.

This type of analysis results in a similar representation as a heat map, where each cluster from Figure 4.5 was tested for overrepresented GO using unregulated proteins as background.

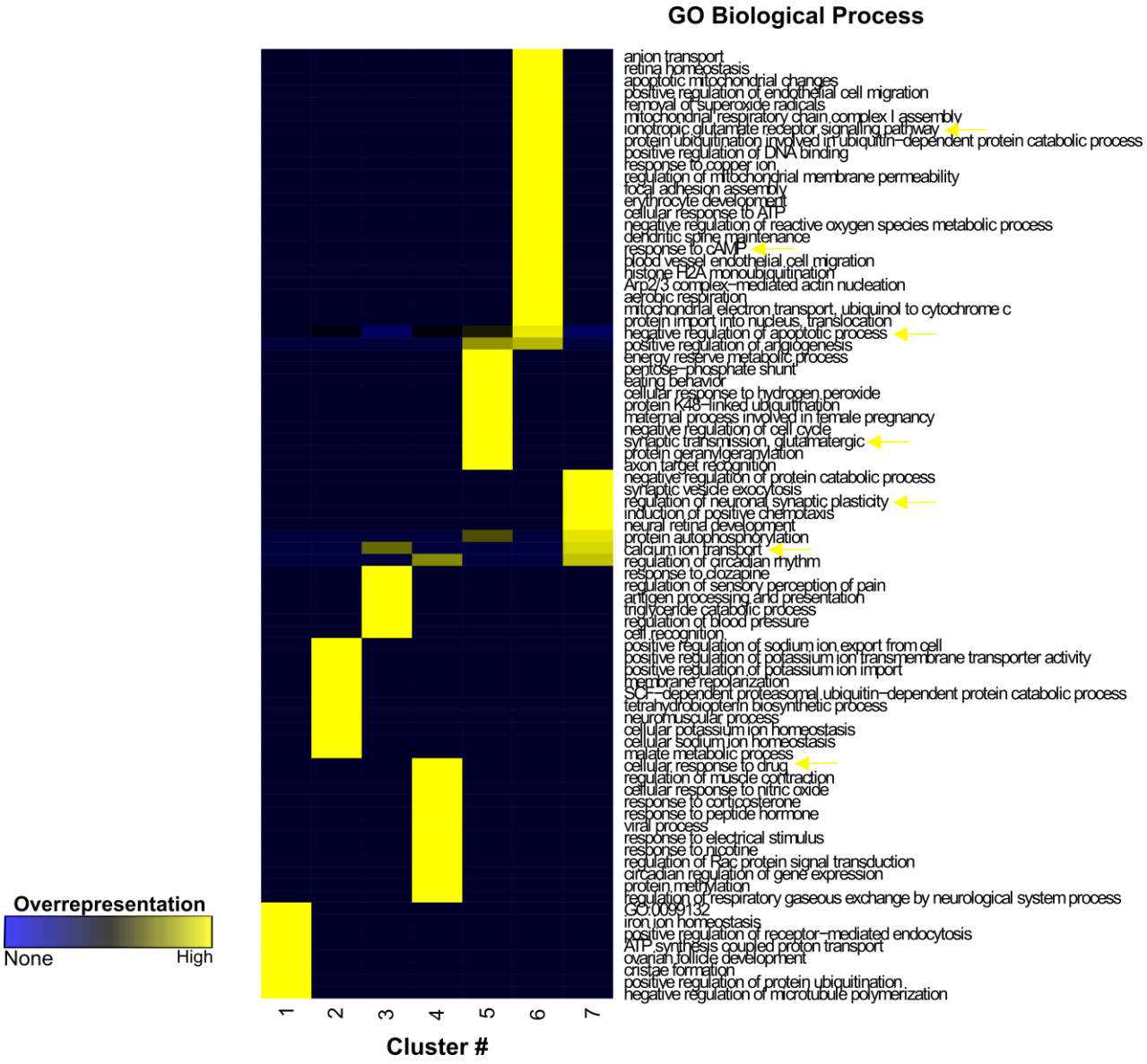


Figure 4.6 - Overrepresented gene ontologies for biological processes for each cluster. Each cluster from Figure 4.5 was tested for overrepresented GO compared with the unregulated proteins using a Binominal statistical test with Benjamini-Hochberg adjustment, minimum p-value of 0.05 and minimum occurrences of 2. The arrows represent several biological processes that are going to be discussed.

Through the analysis of the Biological Process GO annotations from Figure 4.6, there are processes enriched and some examples include: metabolic processes, negative regulation of apoptotic process, calcium ion transport, cellular response to drugs, receptor regulation and synaptic transmission. However, to observe specific or deeper changes within these processes and molecular interaction or reaction networks, a KEGG pathway analysis was performed.

4.5. KEGG Pathway Analysis

From several pathways that were altered, including apoptosis, calcium signaling and oxidative phosphorylation, the metabolic pathway was the most highlighted and the one with more associated changes between conditions. Figure 4.7 shows possible parts or entire paths of the metabolic pathway that may be affected by Haloperidol medication.

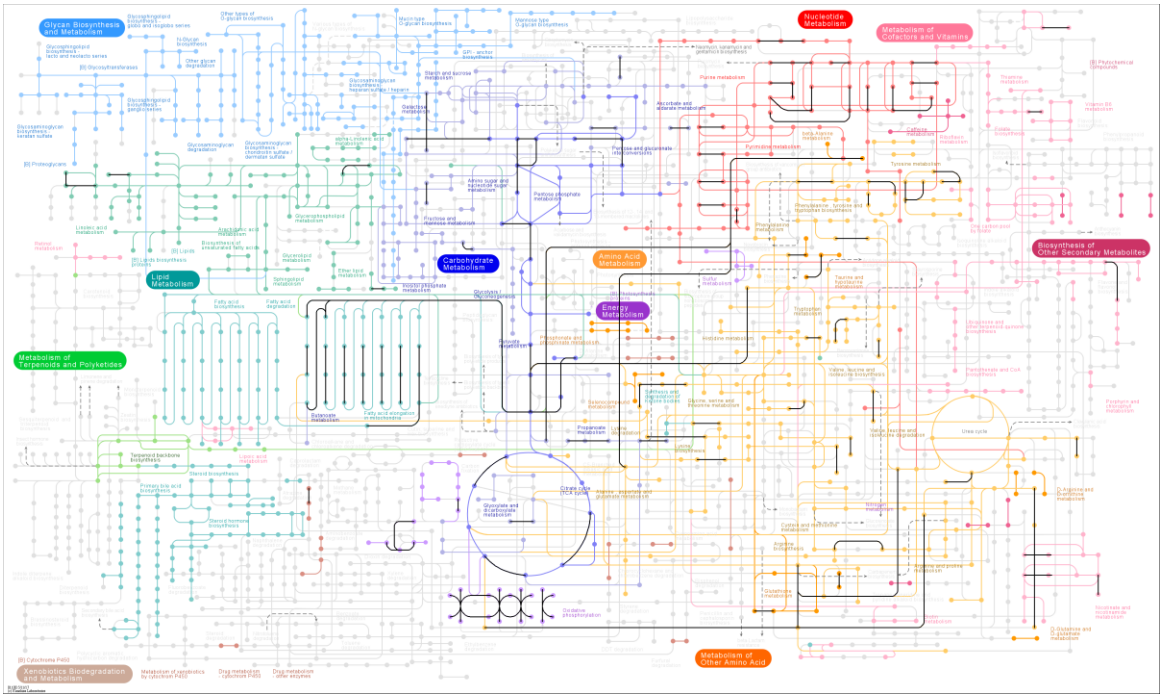


Figure 4.7 – KEGG Pathway Analysis of the 459 altered proteins. KEGG Mapper Search&Color Pathway was used. The search was against *mmu (mus musculus)* where all the objects (proteins) were inserted one per line. The search included aliases. In this figure 89 proteins are represented and just shown deprived of any representation related to their expression. Color coding: black represents altered processes within the metabolic pathway.

Impaired glucose metabolism was described in psychiatric patients even before antipsychotics were introduced in the market.[196] Schizophrenic patients show repeatedly impairments in glucose metabolism and mitochondrial dysfunctions independent of treatment, so it is not new and there are well documented abnormalities of the metabolic

pathway in SCZ patients.[157,196] Treatment with antipsychotics have also been strongly associated with alterations in proteins belonging to metabolic pathways involved in energy metabolism such as oxidative phosphorylation, suggesting antipsychotics induce impairments in metabolism. [157] In Figure 4.8 are represented metabolic changes induced by Haloperidol observed in the present study.

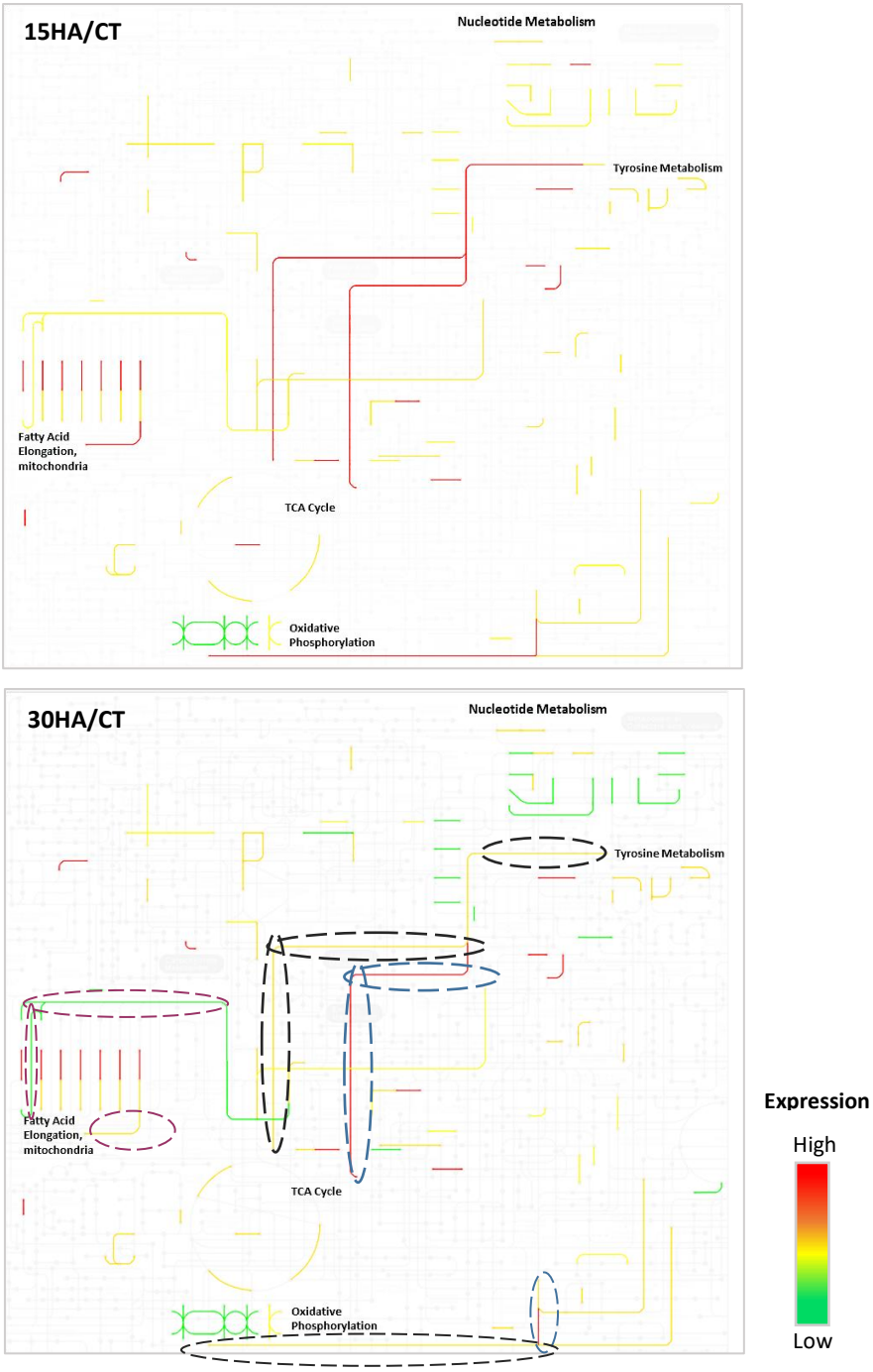


Figure 4.8 – KEGG Metabolic Pathway analysis of differentially expressed proteins. Comparison between 15HA/CT; 30HA/CT. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2) Black circles: GABA-T; Blue circles: GAD; Purple circles: fatty acid related proteins

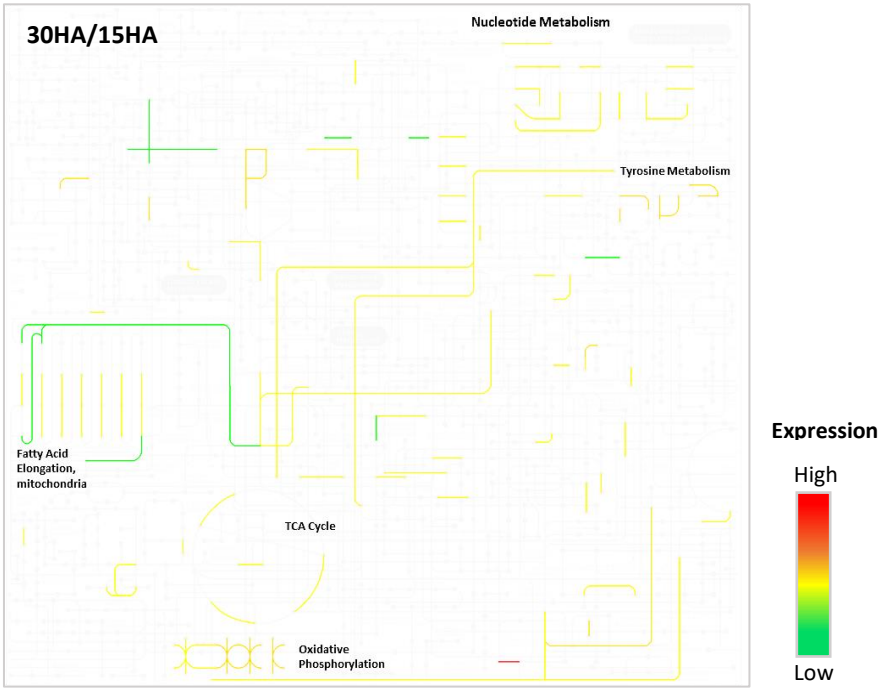


Figure 4.9 - KEGG Metabolic Pathway analysis of differentially expressed proteins. Comparison between 30HA/15HA. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2)

KEGG pathway analysis has shown that from the 459 altered proteins, 89 are related with metabolic changes. Nucleotide metabolism, amino acid metabolism, TCA cycle, oxidative phosphorylation, fatty acid elongation and GABA biosynthesis are the most evidently altered ontologies by Haloperidol medication (Figure 4.8).

Relatively to GABA biosynthesis, the results show two altered key proteins: 4-Aminobutyrate aminotransferase (GABA-T), which is responsible for GABA catabolism; and glutamate decarboxylase (GAD), which participates in GABA biosynthesis by the decarboxylation of glutamate. Several reports in the literature conducted by multiple research groups have consistent findings of reduced GAD expression in post-mortem studies of Scz.[197] Due to long-term antipsychotic treatment before death, it is relevant to understand if these findings are only consequence of the disease or if medication effects also contribute to it. By analyzing KEGG metabolic pathway, there is a relevant increase of GAD protein, both with 15 day and 30 day of Haloperidol comparing to control condition. Conversely, this enzyme expression is decreased when comparing 30 day with 15 day treatment. This pattern considerably changes when comparing GABA-T expression. It is increased at 15 day of Haloperidol comparing to control, however, its expression is slightly decreased at 30 day of Haloperidol. This reduction is more visible when comparing

30 day to 15 day treatment. GABA-T is a GABAergic gene that has been associated with the development of neuroleptic-induced extrapyramidal symptoms.[198]

Regarding to GAD increased expression with Haloperidol treatment, this findings are consistent with a study that evaluated long-term effects of typical (haloperidol) and atypical (clozapine) antipsychotic drugs on the GABAergic system, where the authors also found increased expression of GAD.[199] What can be suggested from this analysis is that reduced expression of GAD observed in post-mortem brains does not appear to be influenced by antipsychotic medication, as the results of the present study demonstrated the contrary. Additionally, it is tempting to propose that reduced GAD can be a consequence of schizophrenic pathophysiology and that maybe Haloperidol is trying to compensate this effect.

Regarding neurotransmission system, the molecular pathophysiology of SCZ encompasses synaptic disturbances. [200,201] Synaptic-related molecular changes do not appear to be result of chronic antipsychotic mediation, firstly because there is evidence that monkeys receiving chronic antipsychotic medication do not show reduced expression of synaptic markers; secondly, schizophrenic subjects not receiving antipsychotic medications at the time of death show synaptic alterations; and thirdly antipsychotic medication treated subjects with diagnoses other than SCZ show no apparent synapse-related pathology.[200] It is suggested that the disease process of SCZ is likely to affect both presynaptic and postsynaptic elements, where postsynaptic receptor expression changes have been reported in the monoamine, glutamatergic and GABAergic systems.[200]

However, synaptic plasticity, a process of rewiring and strengthening neural circuits, is regularly observed after Haloperidol treatment.[142] It is though that Haloperidol could be involved in synaptic reorganization and may reverse a pathological disruption in SCZ[142]; it also documented changes in synapse morphology in the striatum.[142]

It is hypothesized that activation of PKA by Haloperidol is able to activate transcription factor which can regulate gene expression, for instance CREB activation plays a very important role in neuroplasticity.[142] On the other hand, the involvement of glutamate receptors is also associated and have a critical role in synapse formation and stabilization, as synaptic stabilization seems to require the post-synaptic activity of NMDA and AMPA receptors. [142]

Haloperidol-induced alterations on GABAergic and Glutamatergic can be visualized in Figure 4.10.

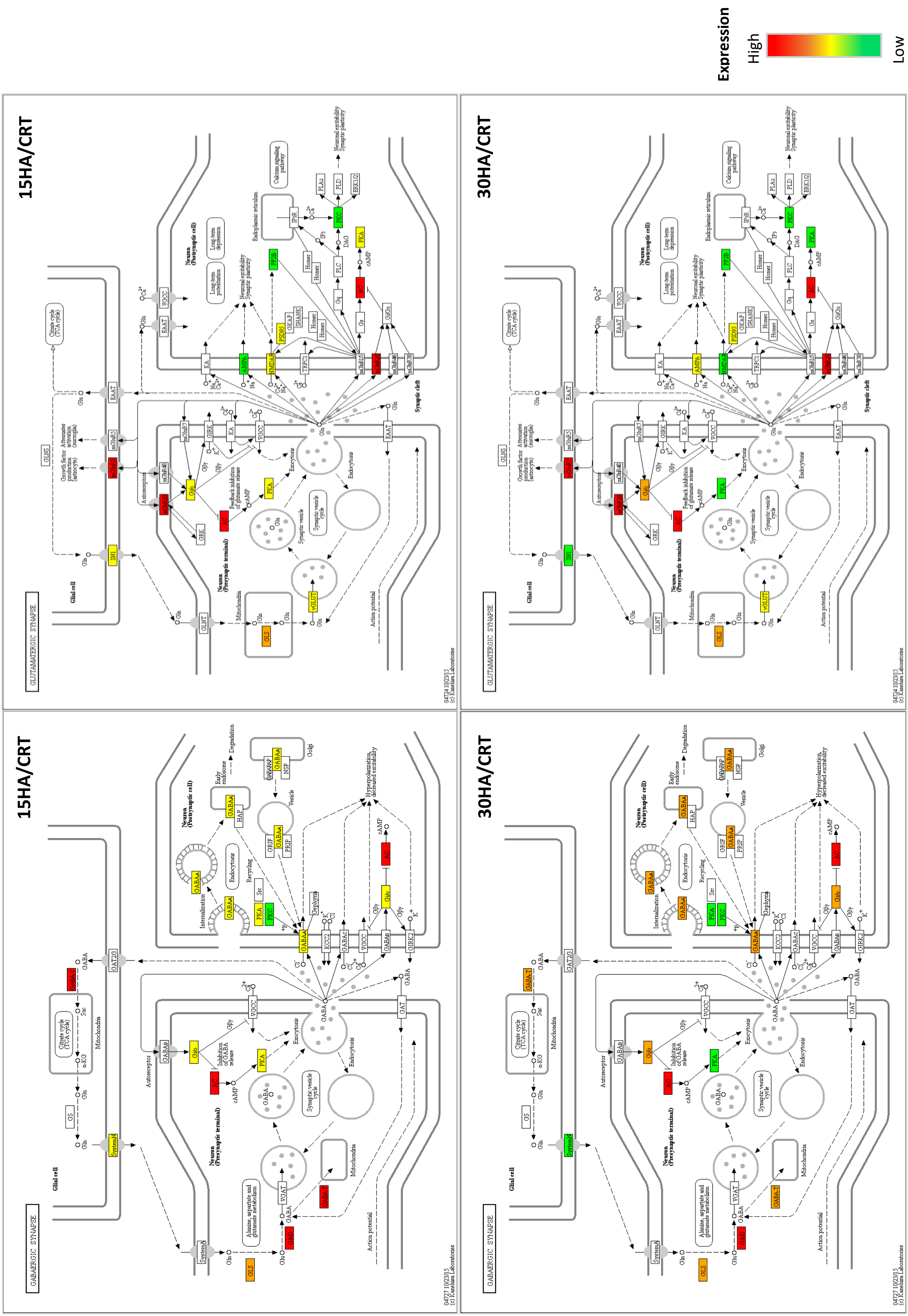


Figure 4.10 GABAergic and Glutamatergic synapses. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2).

At 15 day of Haloperidol treatment, up-regulation of GAD and GABA-T can be observed, supporting previously mentioned results and suggesting an increase in GABA synthesis in the presynaptic terminal. GABA release is negatively regulated by GABAB autoreceptors, where PKA activation potentiates the process. In the postsynaptic terminal, a generalized decrease in GABAA receptor is observed. GABAA receptors are responsible for fast inhibitory neurotransmission, however, it may suggest that GABA transmission is decreased. At 30 day of Haloperidol a slight decrease is observed in GABA-T, on the other hand, GAD maintains its increased expression. In the postsynaptic terminal, the decrease in GABAA receptors at 15 day of treatment is contradicted at 30 day treatment. This data suggests that, GABAergic synapse is being modulated by Haloperidol. It is possible that at 15 day GABA biosynthesis and degradation processes are increased due to GAD and GABA-T increased expression, which can mean a reduced amount of GABA going to the synaptic cleft and therefore it can explain the reduction in GABAA receptors. On the other hand, at 30 day with Haloperidol the scenario is quite different and it seems that GABA transmission is recovered. GAD increased expression is maintained, where GABA-T is slightly decreased comparing to 15 day of treatment. The increased in expression of GABAA also suggests that a decline in degradation is occurring, which allows more GABA to go onto the synapic cleft and therefore reaching GABAA receptors in the postsynaptic terminal.

Concerning glutamatergic synapse Figure 4.10, at 15 day Haloperidol it stands out an expressive increase of the metabotropic glutamate receptors, mGluRs 2 and 3 both in presynaptic and postsynaptic terminal, and in glial cell. Oppositely, vesicular glutamate transporter (VGLUT) in presynaptic terminal is decreased, as well as, NMDA and AMPA receptors in postsynaptic terminal. Postsynaptic density protein 95 (PSD-95) and Serine/threonine-protein phosphatase 2 (PP2B) are also affected showing a decreased expression relatively to control. When considering alterations induced with 30 day treatment the scenario is quiet similar, although AMPA and NMDA receptors look as if their expression as changed from 15 day of treatment. The values presented AMPA receptors varies from 15HA to 30HA although maintained the same tendency, specifically

AMPA receptor expression has a fold-change increase of 1.24 from 15 day to 30 day of treatment, but keeping a slight decreased expression in relation to the control condition; and NMDA receptor maintains the expression (fold-change of 0.95) from 15 day to 30 day of treatment.

mGluRs 2/3 traditionally by coupling to $G_{i/o}$ subunits of G-proteins, is able to inhibit adenylyl cyclase activity and other signaling pathways.[202] It also inhibits neurotransmitter release from glutamatergic, GABAergic and neuromodulatory (dopaminergic) presynaptic terminals.[202] At the presynaptic terminal, mGluR2 mainly acts as autoreceptor, it

suppresses the excessive glutamate release by a negative feedback mechanism in order to keep synaptic homeostasis[203], where in glia cells these receptors are able to increase glutamate uptake by increasing the expression of glial glutamate transporters[204]. Regarding Scz, alterations of group II mGluRs are not very consistent.[203] Most of research studies do not find significant alterations, reporting unchanged mGluR 2/3 protein expression in schizophrenic patients. [203] From this data, modulation of glutamatergic synapses by Haloperidol is observed, where it seems to interfere with glutamate levels in the synapse, and consequently neuronal firing. Hypofunction of glutamate has been implicated in SCZ pathophysiology, where antipsychotic drugs are thought to perform their beneficial effects against schizophrenic symptoms by strengthening excitatory transmission in critical brain areas. A considerable amount of evidence also supports a therapeutic potential of group II receptor agonist for SCZ treatment, suggesting mGluR2/3 modulation a very attractive strategy.[205]

Relatively to dopaminergic synapses (Figure 4.11), there are also changes induced by Haloperidol treatment. This changes were expected because this drugs binds preferably in the D_2 receptors as described in the introduction.

Tyrosine hydroxylase (TH), the enzyme responsible for catalyzing the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) is increased at 15 day of treatment, as well as , the enzyme monoamine oxidase B (MAOB) which is involved in dopamine metabolism. The scenario is identical between the two experimental time points either at 15 day or 30 day of Haloperidol. Increased activity of TH attest to intensification of DA synthesis at middle-term and long-term effects of medication. This effect is observed in other study, where TH activity was assessed after short-term exposure to Haloperidol.[206] The author suggests that suggest that DA synthesis (TH activity) considerably increased in both nigrostriatal structures, which leads to the activation of the whole system of transmitter turnover.[206] Our results also show an increase MAO-B activity at 15 and 30 day of Haloperidol, which also happens after short-term administration of the drug.[206] It seems that these short-term changes in DA synthesis and metabolism continue even after chronic-Haloperidol administration.

Summarizing, Haloperidol treatment seems able to modulate both GABAergic, Glutamatergic and Dopaminergic synapses, what is not fully understood are the exact consequences of this modulation, but it is suggested that possibly, it interferes with synaptic plasticity.

Long-term potentiation (LTP) and long-term depression (LTD) are enduring changes in synaptic strength, induced by specific patterns of synaptic activity.[207] In schizophrenic patients, cognitive abilities such as memory and learning are impaired. LTP

is the enhancement of signal transmission at a synapse, whereas in LTD synaptic transmission is reduced.[208]

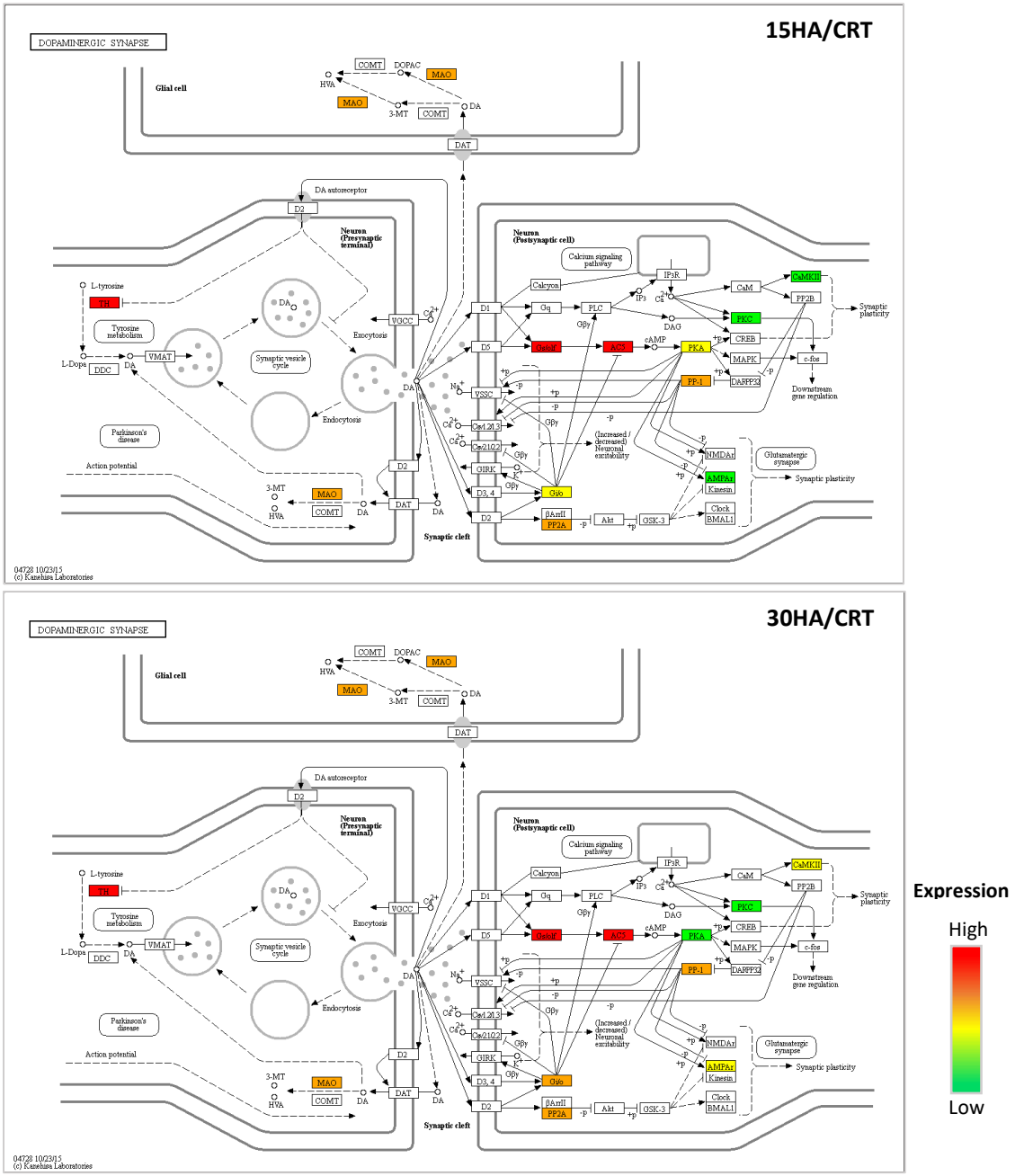


Figure 4.11 – Dopaminergic Synapse affected by Haloperidol medication. The differential colours observed in the maps represent the different protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2).

These mechanisms are believed to be the synaptic processes underlying learning and memory, so implicated in Scz.[208] Regarding LTP, the most common form is dependent of NMDA- type glutamate receptors, where activation of certain enzymes such as cAMP, PKA, PKC and CAMKII are thought to be essential for the induction of this specific form of LTP.[207] Protein phosphatase 1 and calcium/calmodulin-dependent protein phosphatase Calcineurin are also believed to be involved in LTD.

Relatively to the long-term potentiation KEGG representation, it can be observed in Figure 4.12 with 15 day of treatment NMDAR and AMPAR are decreased, as well as, CaN, CaMKII, PKC and PKA, suggesting interference in LTP activation. On the other hand, PP1 and Rap1 show increased expression. At 30 day of treatment, glutamate receptors continued showing decreased profile, along with PKC and CaN, whereas CaMKII expression is even more reduced. Comparatively to 15 day, PP1 expression it's maintained at 30 day, while Rap1 protein expression raises.

The vast majority of studies suggest that antipsychotics are associated with LTP impairment, however, several studies also suggest no effect or even improvement.[207] The inconclusive findings could be explained by differences in drug concentration, physiology of the animals or even receptor binding profile of the drugs.[207] The data presented in this study suggests that Haloperidol indeed affects LTP, where it seems that proteins necessary for LTP induction such as cAMP, PKA, PKC and CaMKII show decreased expression after treatment relatively to control.

Fatty acid biosynthesis and degradations is also altered by Haloperidol medication. Several enzymes, particularly acyl-CoA synthetase long-chain, acetyl-CoA acyltransferase, acetyl-CoA C-acetyltransferase are associated with these processes and show diminished expression relatively to control condition.

Other altered expression of proteins after treatment can be visualized in Figure 4.9, sharpen changes are observed at 30 day of Haloperidol comparing to 15 day Haloperidol. Thus, it seems that significant metabolic changes occur in the first weeks of treatment, where in the following weeks these variations start to stabilize. Additionally, this improvement in brain energy/metabolism at the beginning of treatment can be related to the clinical efficacy and/or side effect profile of antipsychotic medication, in this case Haloperidol. Changes between 30 day and 15 day treatment confirm that metabolic alterations stabilize, once protein expression of metabolic proteins decrease. These findings showing alterations in proteins belonging to metabolic pathways are supported by other studies [157,158,164]

Major changes are detected in mitochondrial respiratory chain, particularly in oxidative phosphorylation (OXPHOS) system. In Figure 4.13 it can be observed a generalized decrease of mitochondrial respiratory chain proteins between 15 day Haloperidol and 30 days of Haloperidol comparing to control condition. A clear reduction is noticed when analyzing complex I, III, IV and V either 15 day or 30 day of Haloperidol versus control condition.

NADH dehydrogenase enzymes are also decreased, as well as several proteins from cytochrome c reductase and cytochrome c oxidase complexes. However, a generalized increase in all these proteins is observed when comparing 30 day Haloperidol

with 15 day treatment. The importance of mitochondria in neuronal function is enormous, as neurons critically depend on mitochondrial function[209]. For instance, neurons need mitochondria energy to establish membrane excitability, to execute complex processes such as neurotransmission and neuron plasticity, for neuronal development and connectivity and many other processes.[209,210]

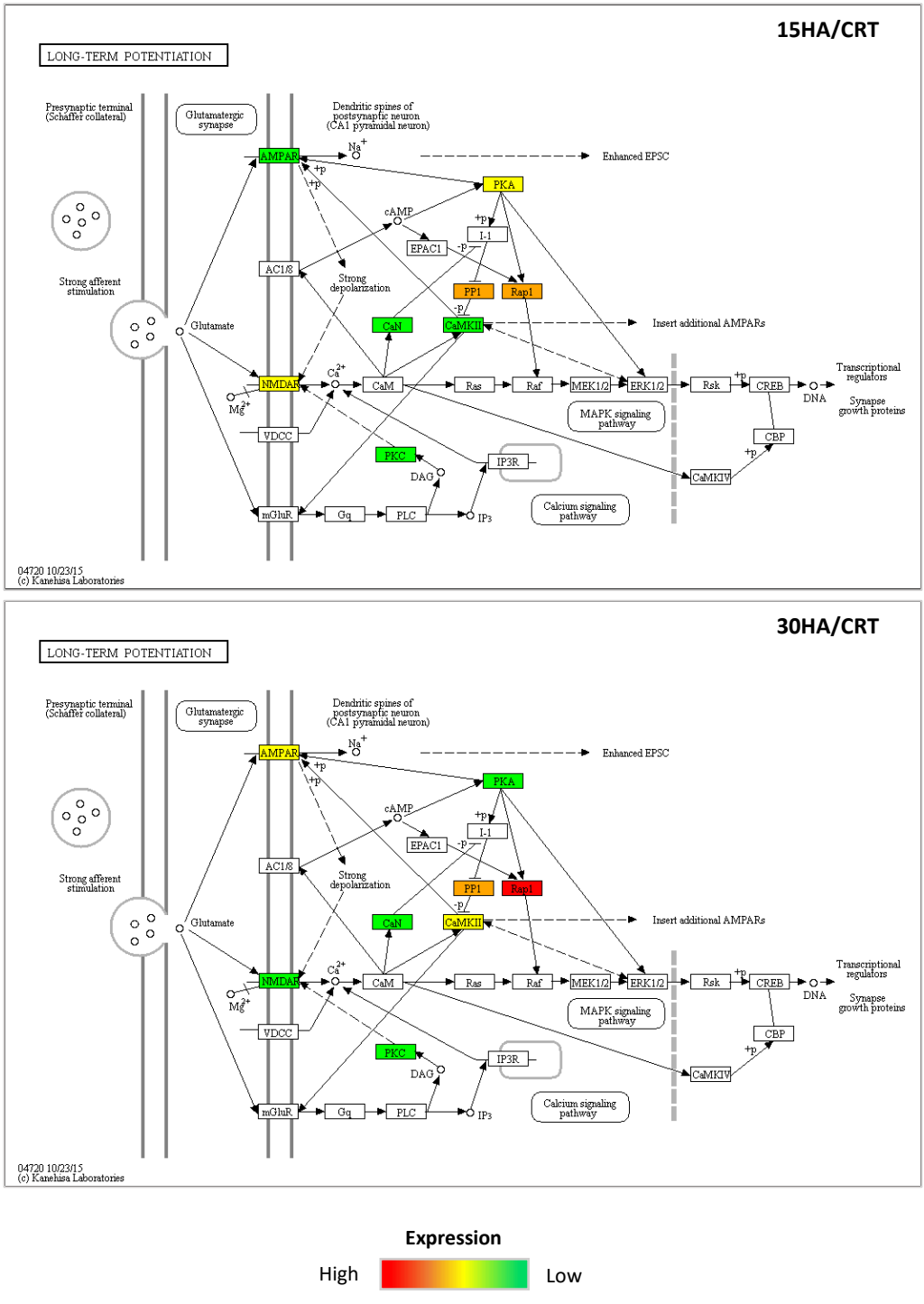
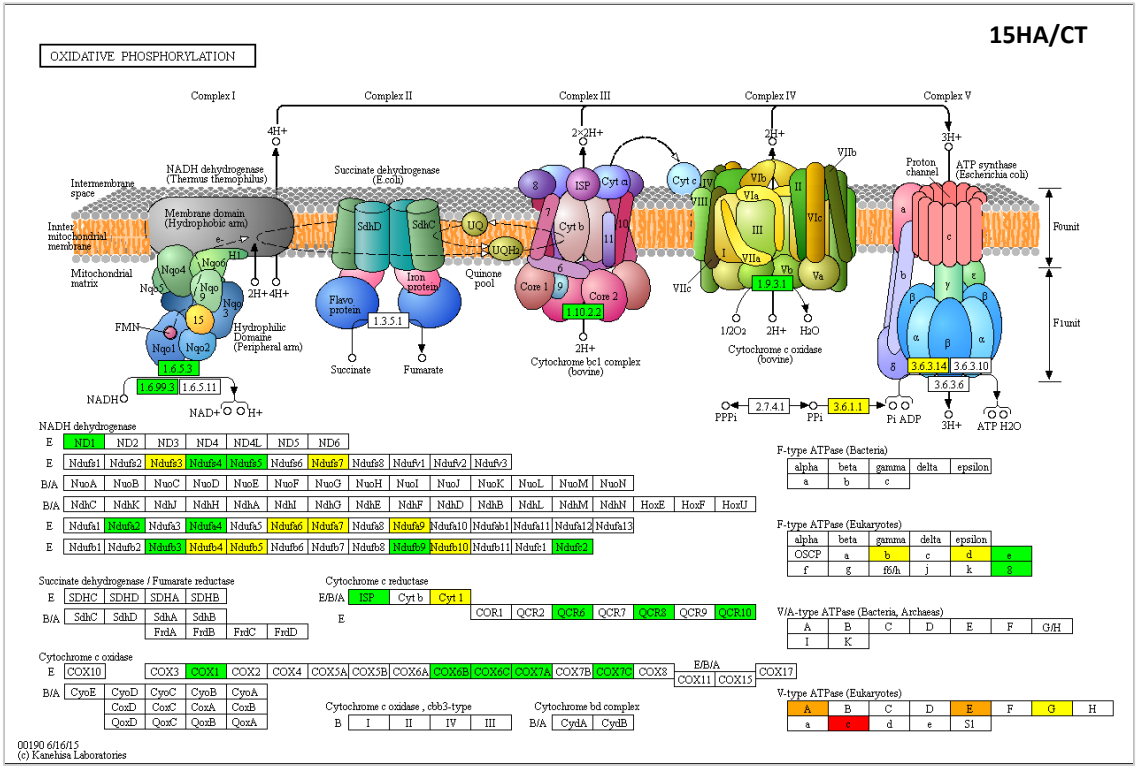
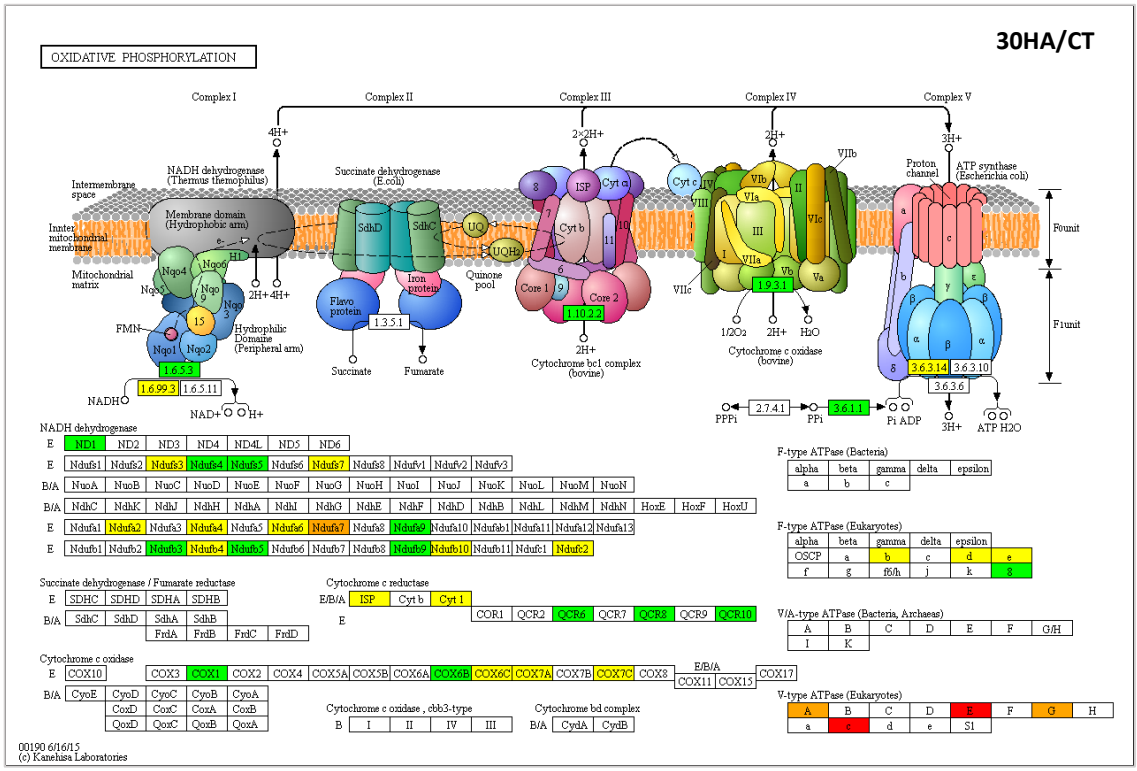


Figure 4.12 – Long-term potentiation affected by Haloperidol. Comparison between 15HA/CT; 30HA/CT. The different colors observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2).





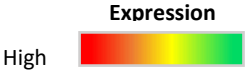
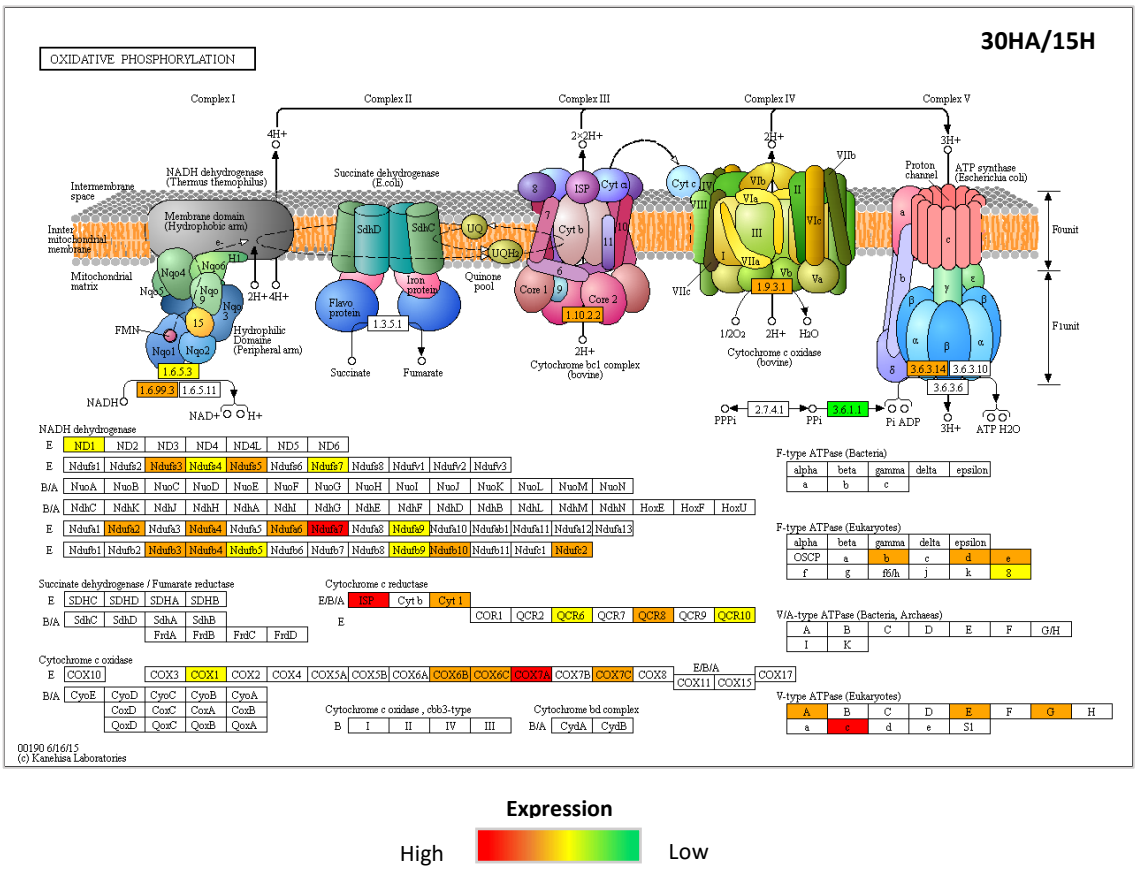


Figure 4.13 – KEGG Oxidative Phosphorylation Pathway analysis of differentially expressed proteins. Comparison between 15HA/CT and 30HA/CT. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2)



Expression

High — Low

Continuation of Figure 4.13 - KEGG Oxidative Phosphorylation Pathway analysis of differentially expressed proteins. Comparison between 30HA/15HA. The different colours observed in the maps represent the different protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2)

Additionally, this energy is provided by converting metabolites into ATP through OXPHOS, [209] making this system extremely important. Several independent lines of evidence have associated impairments in mitochondria functions to SCZ patients.[210] These studies have suggested that a decreased energy metabolism is observed in several brain areas of schizophrenic patients, and also that an OXPHOS deficit seems to be particularly involved.[210]

Studies in the brain of SCZ patients have shown a reduction in complex I activity and also in cytochrome c-oxidase.[211,212] However, other studies also reported the capacity of antipsychotics to interact with the mitochondrial OXPHOS system.[209] Reduction or inhibition of complex I activity as observed in this experimental study (Figure 4.13) is confirmed by other studies.[213,214] Consequently, the reduction observed in complex I activity in brain samples of medicated schizophrenic patients may be correlated with the ability of antipsychotic medication, specifically Haloperidol, to influence/inhibit complex I activity. Once more, the significance of this experimental study is highlighted, as one particular characteristic attributed to SCZ may not be exclusively part of the physiopathology but also by the medication. Our study corroborates with a study

published in 1997, although they have used normal human brain cortex and a different experimental design.[215] The authors of this study suggested the specific inhibition of mitochondrial complex I may be the cause of extrapyramidal side-effects.[215] However, the mechanism of action behind is stills unknown.

Mitochondrial dysfunction, specifically inhibition of complex I, oxidative stress and free radical induction have been suggested to be involved in Haloperidol neurotoxicity and consequently for extrapyramidal side-effects.[216] A theoretical hypothesis is described in Figure 4.14, when antipsychotics block dopamine receptors, an increased turnover and metabolism of dopamine occurs [217,218], and can lead to an increased hydrogen peroxide production[216], resulting in oxidative stress. On the other hand, dopamine is metabolized either by oxidation by monoamine oxidase to 3,4-dihydroxyphenyl acetic acid where this reaction produces hydrogen peroxide, or by autoxidation, yielding superoxide radicals.[217] Haloperidol metabolization implies an oxidation into pyridinium metabolite, thought to be a mitochondrial toxin.[219] Haloperidol was suggested to potentiate increases in oxidative stress or free radical-mediated levels of toxic metabolites in rat.[217] Therefore, it is tempting to speculate that a decrease in brain energy metabolism by Haloperidol may play a role in the mechanisms of action of antipsychotics and that it can modulate brain energy metabolism.

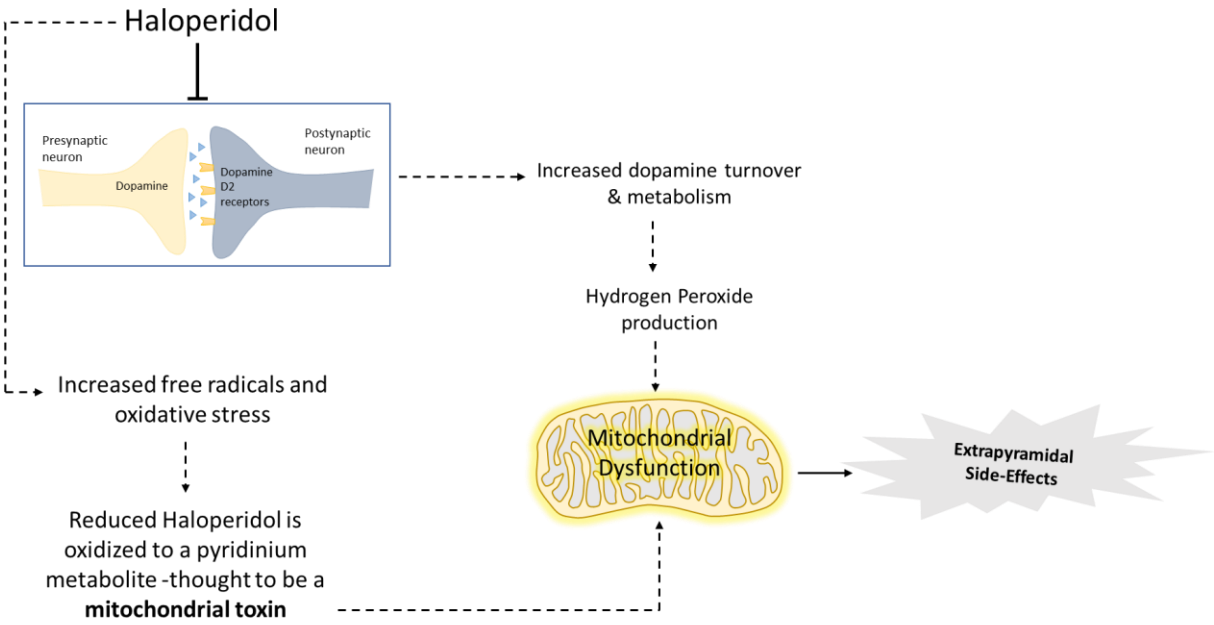


Figure 4.14- Theoretical hypothesis how Haloperidol can contribute to extrapyramidal side-effects. (See text for details)

On the other side, extrapyramidal side-effects such as Tardive Dyskinesia and Parkinsonism could be explained by alterations in energy metabolism, especially by mitochondrial dysfunction induced by free radicals and oxidative stress.

Besides their significance for supplying cellular energy to the cells, mitochondria and OXPHOS are also involved in a range of other cellular processes such as calcium signaling, cAMP/protein kinase A (PKA) signaling, and apoptosis.[209,210] As previously mentioned, the primary characterized downstream pathways related to antipsychotics signaling are cAMP-associated pathways due to the fact that D_2R are G-protein coupled receptors Gi/o type receptors.[141] As stated on the literature, the majority of antipsychotics when blocking dopamine receptors enhance the activity of adenylyl cyclase, increase cAMP levels and activate PKA.[141] This is also supported by our results as observed in Figure 4.15. cAMP pathway is clearly affected by Haloperidol, where medication effects induces its activation.

Returning to mitochondrial dysfunction, it leads to impaired energy metabolism, perturbed calcium homeostasis, increased ROS, oxidative stress and apoptosis.[220] For instance, calcium signaling pathway and apoptosis are two processes that were observed altered in KEGG analysis.

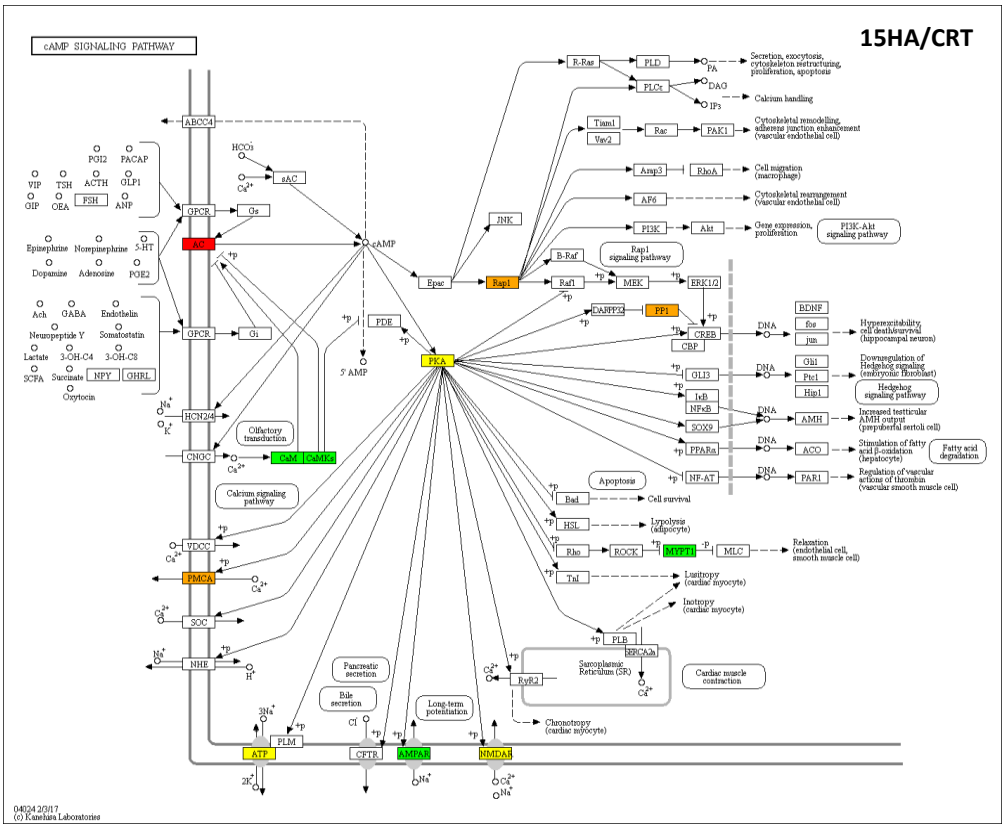
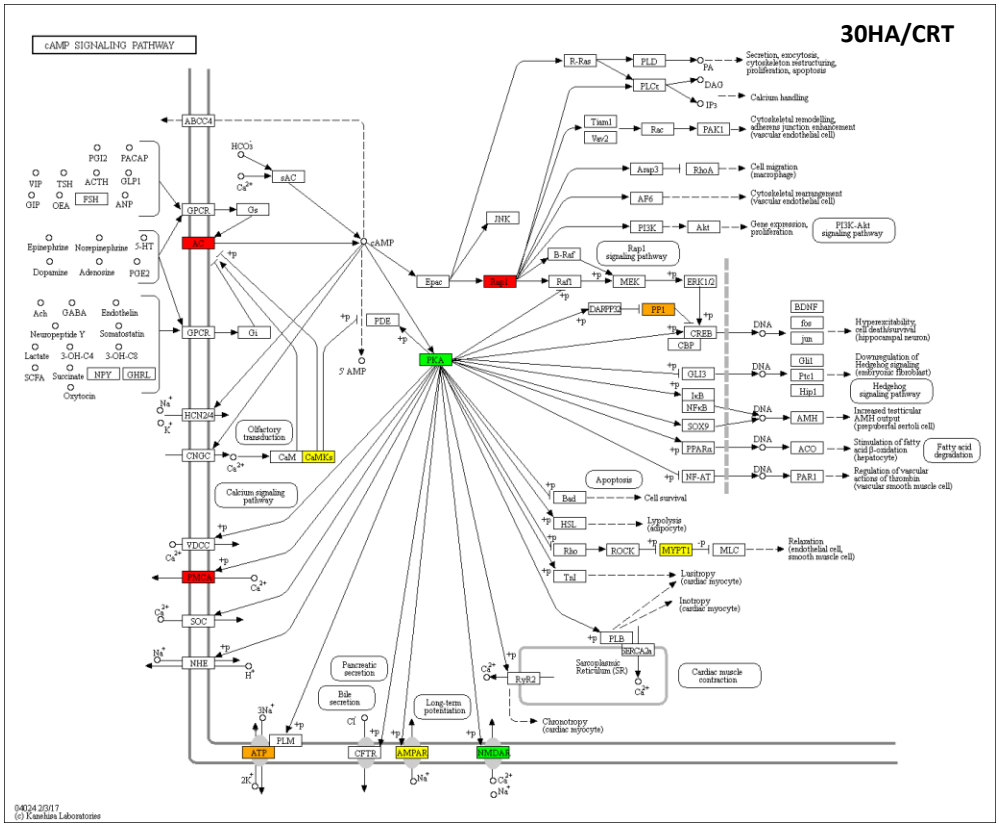
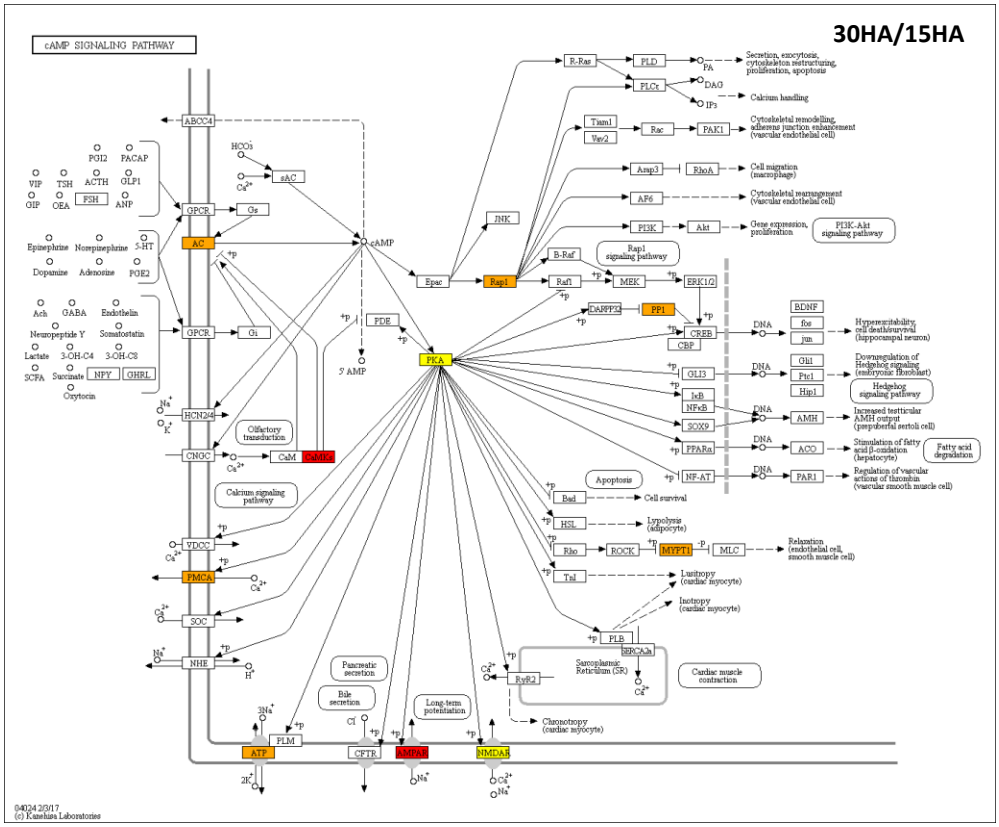


Figure 4.15 – KEGG cAMP Signalling Pathway analysis of differentially expressed proteins. Comparison between 15HA/CT and 30HA/CT. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2).





Continuation of Figure 4.15 - KEGG cAMP Signalling Pathway analysis of differentially expressed proteins. 30HA/15HA. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2).

Calcium is one of the cell's most common second messenger that is involved in a variety of vital cellular functions including signaling pathways, regulation of neuronal functions, synaptic plasticity, just to mention a few.[210] Several factors can influence Ca^{2+} homeostasis. Insufficient function of Na^{+}/K^{+} ATPases can lead to instabilities of ion transmembrane gradients efflux of K^{+} and efflux of Na^{+}, Cl^{-} and Ca^{2+}.[220] NMDA receptors also share responsibilities in the influx of Ca^{2+} into the cells.[210] From our experimental data analysis relatively to this matter (Figure 4.15), it can be observed that Na^{+}/K^{+} - ATPase, AMPAR and NMDAR are decreased at 15 days of Haloperidol treatment relatively to control. At 30 day of Haloperidol an increase in expression of Na^{+}/K^{+} - ATPase is observed, whereas glutamate receptors continued with a reduced expression. Plasma membrane Ca^{2+} ATPase (PMCA) is shown to be increased in both experimental conditions, although at 30 day of treatment the tendency is to increase. PMCA is a transport protein present in the plasma membrane of cells and its function is to remove Ca^{2+} from the cell. When looking closely to calcium signaling pathway (Figure 4.16), the two proteins responsible for Ca^{2+} extrusion are altered relatively to control condition. PMCA and the plasma membrane Na^{+}/Ca^{2+} exchanger (NCX) are increased in Haloperidol condition, however, PMCA increase is enhanced at 30 day of treatment.

Nevertheless, this is not the only Ca^{2+} related proteins affected by Haloperidol. Antipsychotics also affect multiple calcium calmodulin dependent proteins.[221] Calcineurin (CaN), a calmodulin dependent protein phosphatase was recently found to be altered in schizophrenic patients and also by repeated antipsychotics administration in rats.[221] In the results, both CaN and calcium/calmodulin dependent protein kinase II (CAMKII) are diminished when comparing 15 day of Haloperidol to control condition, as well as with 30 day of treatment. CAMKII expression increases from 15 to 30 day, with a variation of about 20%.

So it seems, that these changes in calcium dependent proteins and transport increases in time-dependent manner, in other words, the increase in expression is consistent with increase time of treatment. These results are corroborative of a study published in 2009. Where treatment with Haloperidol show decreased levels of CaMKIIα, CaMKIV, CaMKKα, CaMKKβ and CaN in the striatum of Sprague-Dawley rats.[221] The consequences of altering CaM-related proteins are not completely understood.

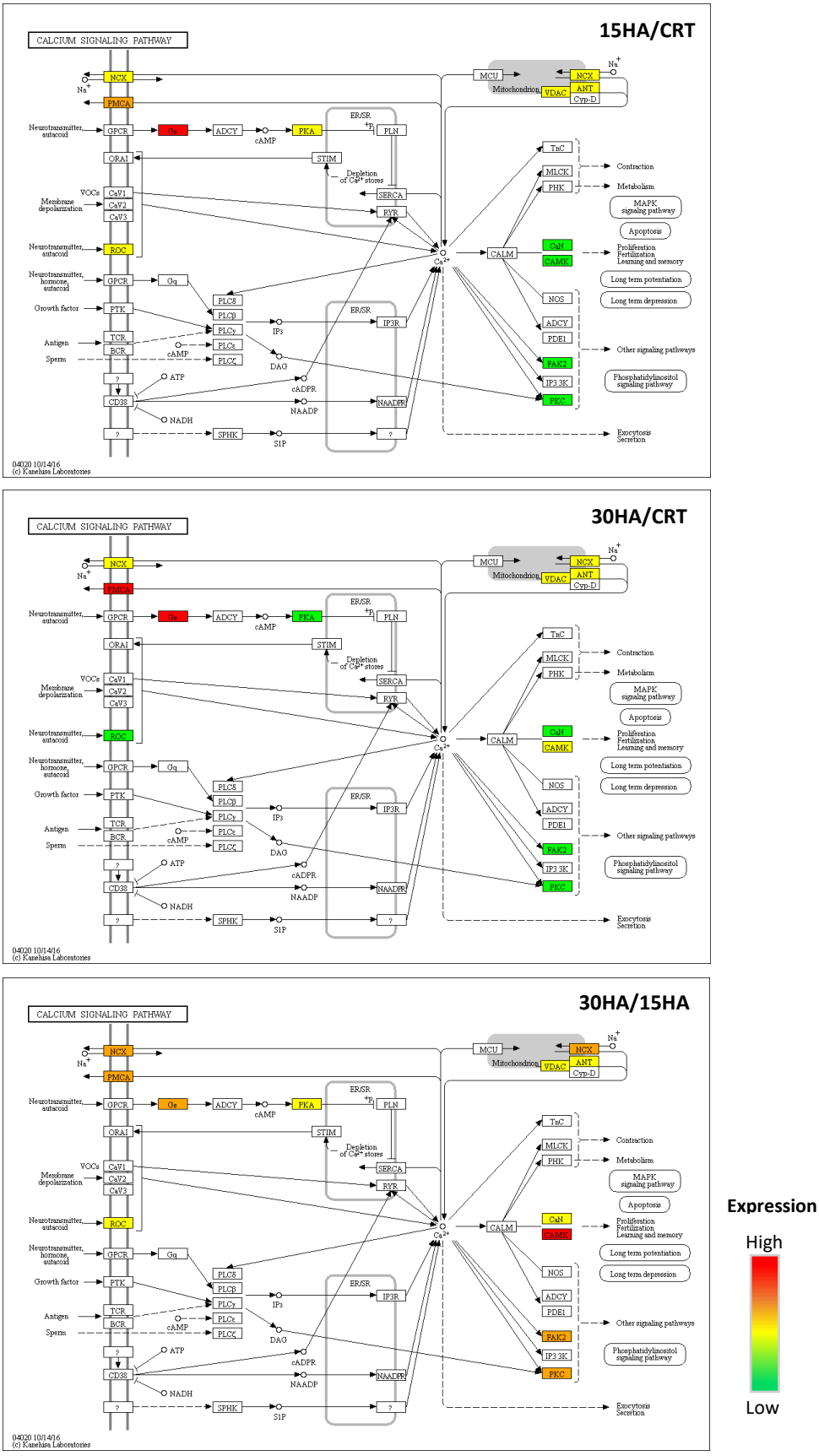


Figure 4.16 – KEGG Calcium Signalling Pathway analysis of the differently expressed proteins. Comparison between 15HA/CT; 30HA/CT and 30HA/15HA. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2)

Rushlow and colleagues suggest that altered activity of CaMKIV could induce changes in gene transcription through regulatory factors such as CREB.[221] Also, CaMKIV regulates PKA, Akt and MAPK signaling pathways, and it is well known that all these pathways are affected by antipsychotic medication.[221] CaMKII and CaN have also important roles in glutamate and dopamine receptors regulation, and both glutamate and dopamine are strongly implicated in antipsychotic drug actions.[221]

Concluding, CaM and CaM-related proteins participate in the regulation of several signaling pathways that are involved in the mechanisms of action of antipsychotic drugs. These alterations in these calcium proteins may elucidate the interrelationship between the diverse signaling pathways. [221]

Programmed cell death, apoptosis, has been linked to mitochondrial and calcium signaling distresses. There are a variety of insults able to trigger apoptosis and these include over-activation of glutamate receptors, mitochondria dysfunction, Ca^{2+} impaired signaling and oxidative stress.[220] A light increase of CaN expression is observed at 30 day of Haloperidol, and this is where apoptotic events may initiate. Calcineurin causes translocation of pro-apoptotics factor into the mitochondria, hence triggering apoptosis.[220] From Figure 4.17 increased calpain, a calcim-dependent proteolytic enzyme, is observed in the striatum of mice with 15 day of Haloperidol medication. Calpains are activated by elevated Ca^{2+} and they surely have a critical role in apoptosis, although their functions are not completely understood.[222] Apoptosis inducing factor (AIF) is also showing as altered in KEGG apoptosis analysis. At 15 and 30 day of Haloperidol AIF show decreased expression relatively to control, however, a tendency to increase is observed when comparing 30 day to 15 day treatment. This protein also participates in apoptosis and it's involved in initiating a caspase-independent pathway of apoptosis.[220] β-Actin and B-type Lamin are also perturbed by Haloperidol medication, where their expression is expressively increased both at 15 and 30 day of treatment. β-Actin is one of six different actin isoforms belonging to the non-muscle cytoskeletal actins. [223] This type of actin is involved in several functions in a cell that support cell survival and adaptation to a changing environment.[223] On the other hand, Lamins are required for proper cell cycle regulation, chromatin organization and apoptosis.[224] Several studies have indicated that the expression and stability of lamin proteins is altered in response to oxidative stress, which in turn is tightly coupled to cell proliferation, cellular senescence, apoptosis.[224] This alteration observed in B-type Lamin expression induced by Haloperidol medication may have been encouraged by mitochondria dysfunction that was previously described.

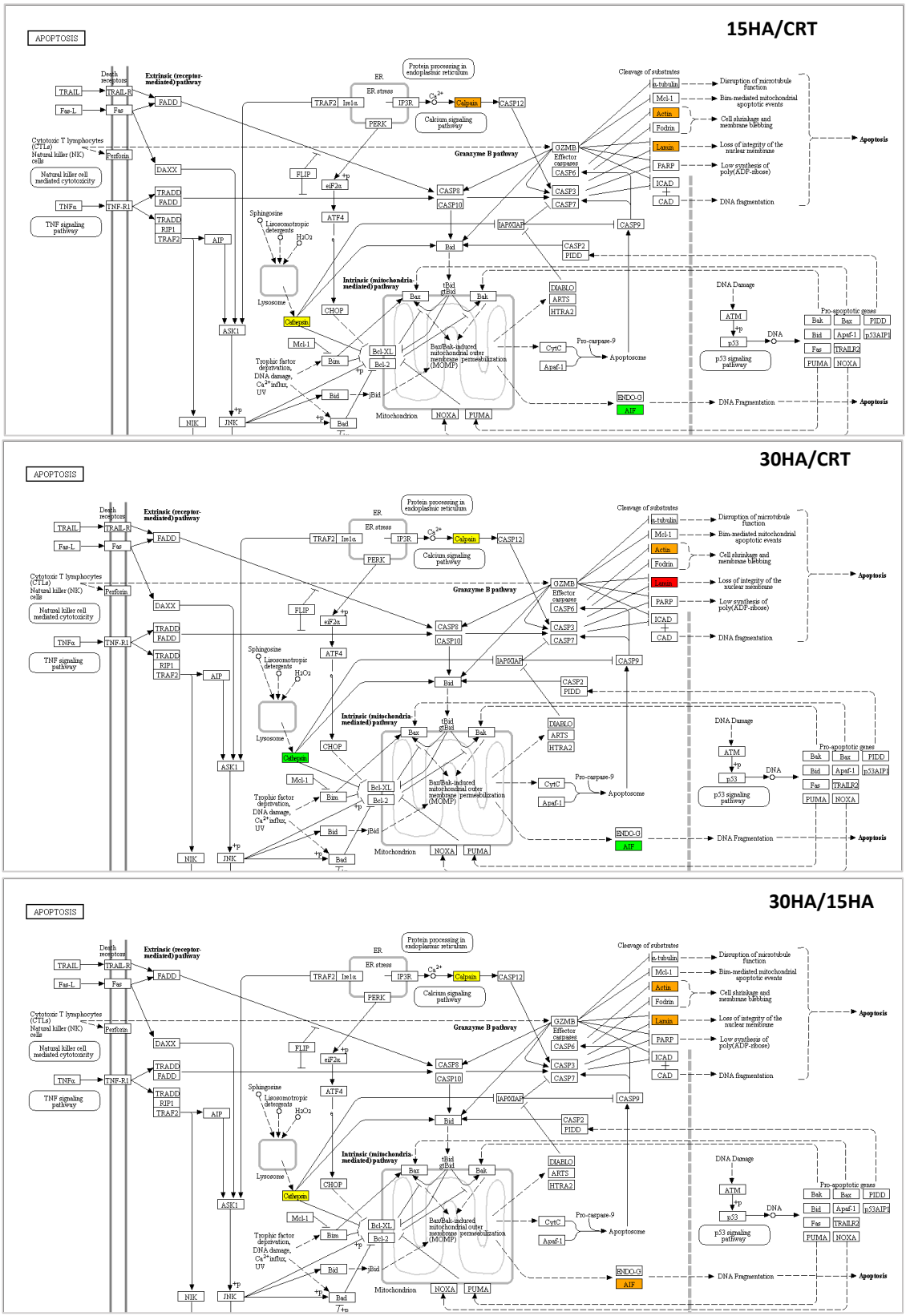


Figure 4.17 - KEGG Apoptosis Pathway analysis of the differently expressed proteins. Comparison between 15HA/CT; 30HA/CT and 30HA/15HA. The different colours observed in the maps represent the differential protein expression. Color coding (Green: < 0.83; Yellow; < 0.99; Orange: > 1 and Red: > 1.2)

From a GOrilla analysis that was performed to the proteins attributed to some of the clusters from Figure 4.5, positive regulation of cell death was enriched (Figure 4.18). By observing cluster 5 dynamic profiles, a tendency of positive regulation of cell death to decrease is notorious after 30 day or chronic Haloperidol treatment. This suggests that processes that modulate cell death are maintained at 15 day of treatment, however, a drastic decrease is observed at 30HA, and suggesting that at 30 day the body is trying to overcome changes induced by Haloperidol.

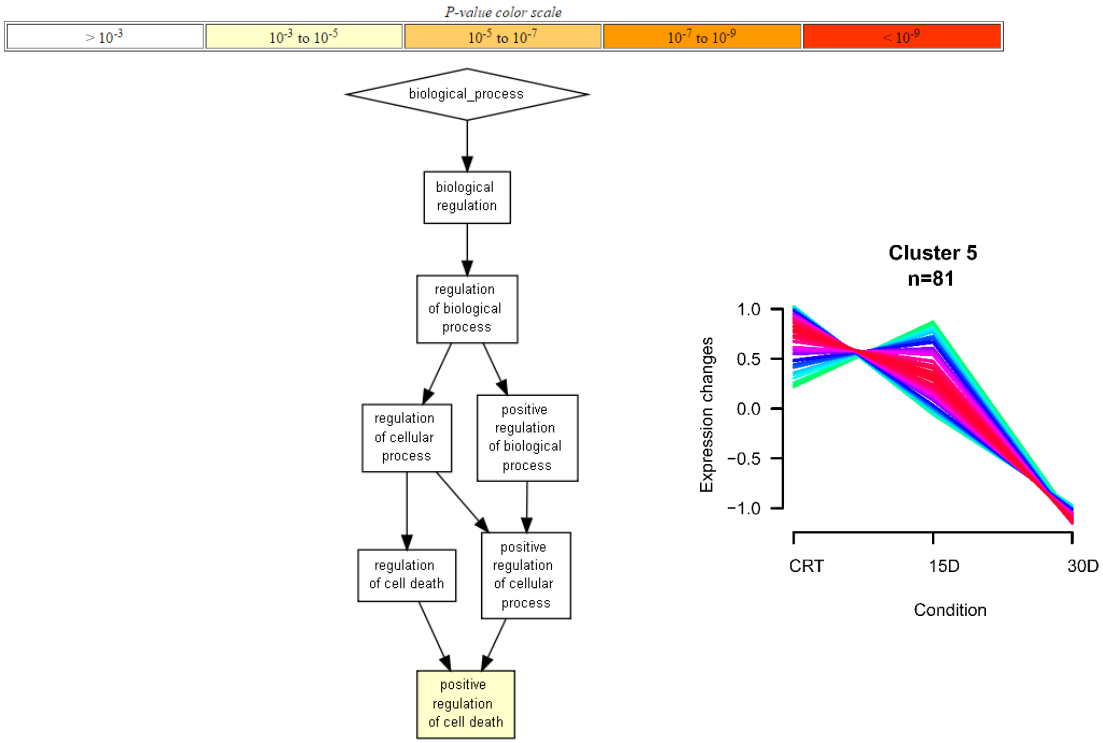


Figure 4.18 – GOrilla analysis of cluster 5 from Figure 4.5. Positive regulation of cell death was enriched by Gorilla analysis. The p-value for this GO term was $7.01E^{-4}$. For this analysis a background and a target list were use. Unaltered proteins were used as background, while the target list were all proteins belonging to cluster 5 (n=81).

Negative regulation of cell death as well as negative regulation of apoptotic process and negative regulation of programmed cell death were enriched processes in Gorilla analysis (Figure 4.19). In this case, negative regulation of these processes means that there are processes or events preventing and reducing cell death. It is possible that at control condition events preventing cell death are increased, yet, with Haloperidol medication there is a severe reduction in these processes, suggesting that prevention of cell death has been affected.

One curious observation is that chronic treatment of Haloperidol decreases both positive and negative regulation of cell death. What appears to be suggested by these

results is that, Haloperidol may interfere with cell death as well as in cellular surviving signaling.

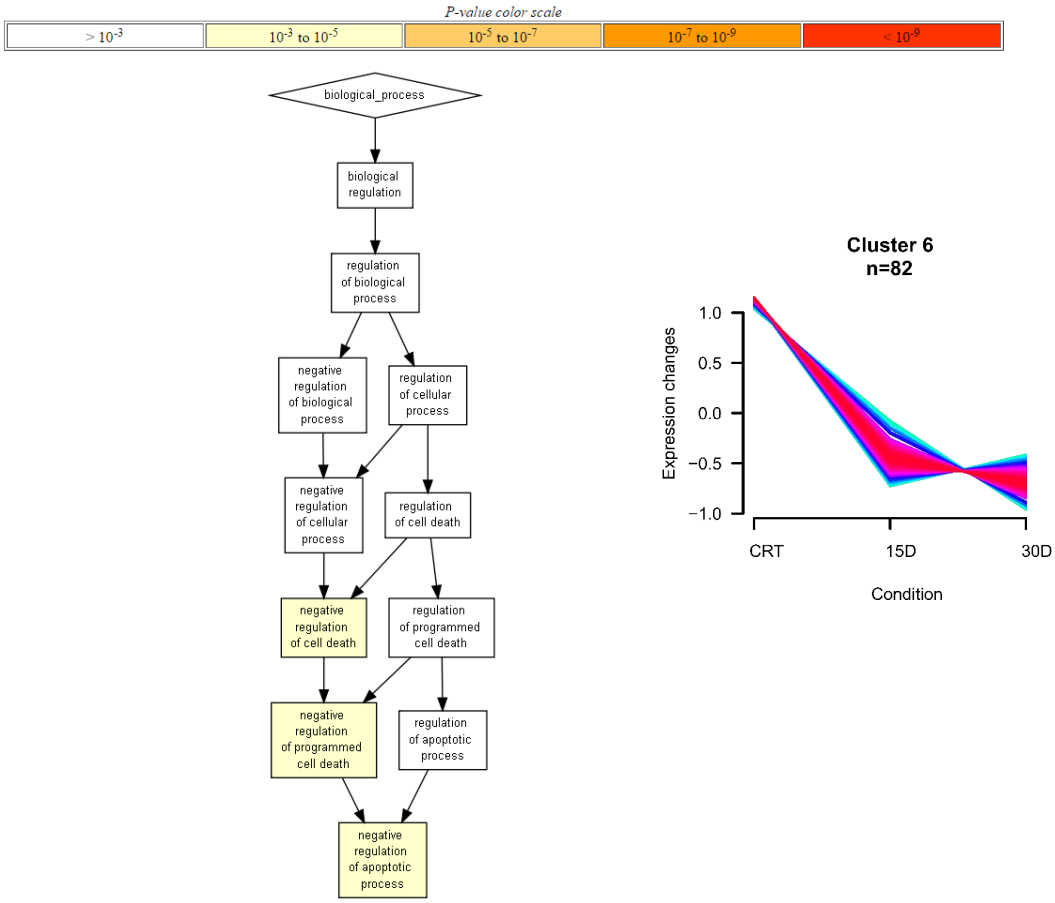


Figure 4.19- GOrilla analysis of cluster 6 from Figure 4.5. Negative regulation of cell death, negative regulation of apoptotic process and negative regulation of programmed cell death revealed to be enriched by Gorilla analysis. The p-value were $3.77E^{-5}$, $1.91E^{-4}$ and $1.41E^{-4}$ respectively. For this analysis a background and a target list were use. Unaltered proteins were used as background, while the target list were all proteins belonging to cluster 6 (n=82).

The results obtained from this study suggest that Haloperidol is able to induce expression of proteins linked to oxidative stress, calcium signaling and apoptosis. This goes in accordance with a study published Ahmed EU et *al* [225] that also verified the implication of Haloperidol in these cellular processes.

Although, protein expression altered levels are not very high, the number of modulated proteins and respective pathways is substantial. This observations are expected once the study was performed with long-term exposure to the antipsychotic, where is expected to have some degree of stabilization. This leads to a vast number of

altered proteins obtained with smaller values of fold-change than what would probably be observed if short-term exposure was studied.

In summary, several processes are being affected/modulated by Haloperidol chronic medication, such processes include metabolic pathways, calcium signaling as well as neurotransmission and apoptosis.

CONCLUSION AND FUTURE PRESPECTIVES

5. Conclusions and Future Perspectives

The main goal of this study was to identify and understand long-term proteomic medication-associated changes of striatum from mice treated with Haloperidol, as the molecular mechanism responsible for differential therapeutic efficacy and expression of side effects remains unclear.

In this study young black male C57BL/6J mice were divided into three groups: control condition (vehicle), 15 days of daily Haloperidol and 30 days of daily Haloperidol (intraperitoneal injection of 1 mg/kg of Haloperidol). The duration of treatment was intended to meet chronic treatment as well as the dosage attributed to be clinical comparable to the ones used in human clinical environment.

Firstly, identification of proteins was performed by LC-MS/MS in the three experimental conditions (control, 15 days of Haloperidol and 30 days of Haloperidol). The total number of proteins identified was similar between conditions (around 2900) and in total the protein library comprised 3311 proteins confidently identified.

SWATH methodology was used to allow the quantification of high number of proteins with high confidence. After the application of stringent quality filters, 1366 were quantified with confidence. However, the list was restricted to 459 proteins, as proteins with p-value<0.05 or 20% variation were the ones with capacity do distinguish control from drug conditions in PCA and were chosen for further analysis.

KEGG analysis revealed three major pathways altered with Haloperidol treatment: metabolic, calcium signaling and apoptosis. For instance, disturbances in the mitochondrial respiratory chain, particularly OXPHOS and complex I inhibition. As it was expected, synaptic alterations after Haloperidol treatment were observed. The results suggest that GABAergic, Glutamatergic and Dopaminergic synaptic transmission were being modulate by Haloperidol. This is not surprising once Haloperidol interferes with dopamine D_2 receptors and consequently affects other neurotransmitters and signaling pathways. The exact consequences of this modulation is not well clarified, but possibly, it interferes with synaptic plasticity.

Several CaM-related proteins were affected by Haloperidol treatment as well as Ca^{2+} extrusion proteins, suggesting disturbances in calcium signaling pathway. Relatively to apoptotic processes, they are also being modulated by this antipsychotic medication. It is possible that Haloperidol may interfere with cell death along with cellular survivival signaling. Some proteins related to apoptosis are altered after treatment, this

interference is possibly caused by mitochondrial impairment and perhaps by oxidative stress.

The pathways suggested in this study as being altered by Haloperidol medication are in some way or another connected. The alterations observed could be directly modulated by Haloperidol or could be influence by each other. Differences between the two time points of drug administration are also observed, suggesting that different changes and adaptations may occur and continue to occur at chronic treatment.

Altogether, these findings highlight several pathways affected by Haloperidol chronic treatment that are involved in the molecular effects induced by this medication. This study also elucidates new directions for recognizing and differentiate disease related or medication related changes.

In the future would be interesting to select key proteins from the pathways suggested to be affected by Haloperidol medication and validate them, for instance, in a new cohort of samples and by using other techniques as immunoassays; It would be also interesting to understand what the consequences of these modulations are and if they are related to the diverse effects and side effects observed in schizophrenic patients.

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Milovancevic;Danielle Posthuma;John Powell;Alexander L Richards;Brien P Riley;Douglas Ruderfer;Dan Rujescu;Engilbert Sigurdsson;Teimuraz Silagadze;August B Smit;Hreinn Stefansson;Stacy Steinberg;Jaana Suvisaari;Sarah Tosato;Matthijs Verhage;James T Walters;Multicenter Genetic Studies of Schizophrenia Consortium;Psychosis Endophenotypes International Consortium;Wellcome Trust Case Control Consortium 2;Elvira Bramon;Aiden P Corvin;Michael C O'Donovan;Kari Stefansson;Edward Scolnick;Shaun Purcell;Steven A McCarroll;Pamela Sklar;Christina M Hultman;Patrick F Sullivan, S. R. C. O. D. K. C. J. L. M. A. K. K. S. A. S. E. B. A. L. C. J. J. C. M. F. Y. K. S. H. Genome-wide association analysis identifies 13 new risk loci for schizophrenia. *Nature Genetics* **45**, 1150-1159, doi:doi:10.1038/ng.2742 (2013).
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SUPPLEMENTARY DATA

7. Supplementary Data

From quantification of individual and pool samples (membrane and soluble fractions) was performed using 2D- Quant Kit, a calibration curve was obtained.

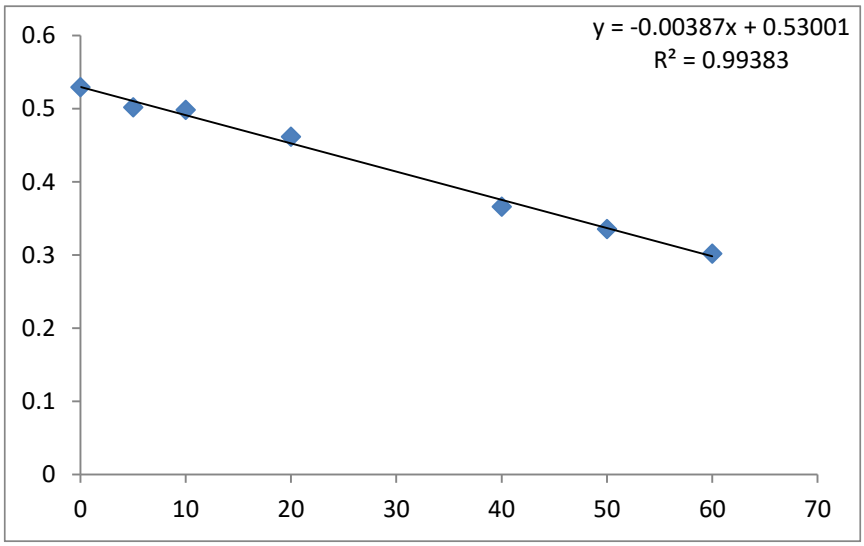

Figure 7.2 – Calibration curve obtained by 2D-Quant Kit using BSA as standard protein.

sample	µg	Vol applied	[] mg/mL
STCT1	21.32041344	10	2.132041344
STCT2	25.06718346	10	2.506718346
STCT3	37.2118863	10	3.72118863
STCT4	50.90697674	10	5.090697674
STCT5	35.40310078	10	3.540310078
STCT sol	16.66925065	10	1.666925065
STCT mem	43.67183463	10	4.367183463
15HA1	31.39793282	10	3.139793282
15HA2	22.6124031	10	2.26124031
15HA3	31.78552972	10	3.178552972
15HA4	32.43152455	10	3.243152455
15HA5	28.68475452	10	2.868475452
15HA sol	16.92764858	10	1.692764858
15HA mem	41.0878553	10	4.10878553
30HA1	21.4496124	10	2.14496124
30HA2	30.10594315	10	3.010594315
30HA3	40.3126615	10	4.03126615
30HA4	34.24031008	10	3.424031008
30HA5	37.5994832	10	3.75994832
30HA sol	12.27648579	10	1.227648579
30HA mem	35.66149871	10	3.566149871

Figure 7.1 – Protein concentration of individual and pool samples (soluble and membrane-enrichment) before SDS-PAGE.

A correlation analysis using MarkerView™ was performed to all replicates of the three experimental conditions to assess if no technical problem (including sample handling and processing) occurred with any of the samples. From this analysis a correlation table was obtained (Figure 7.3).

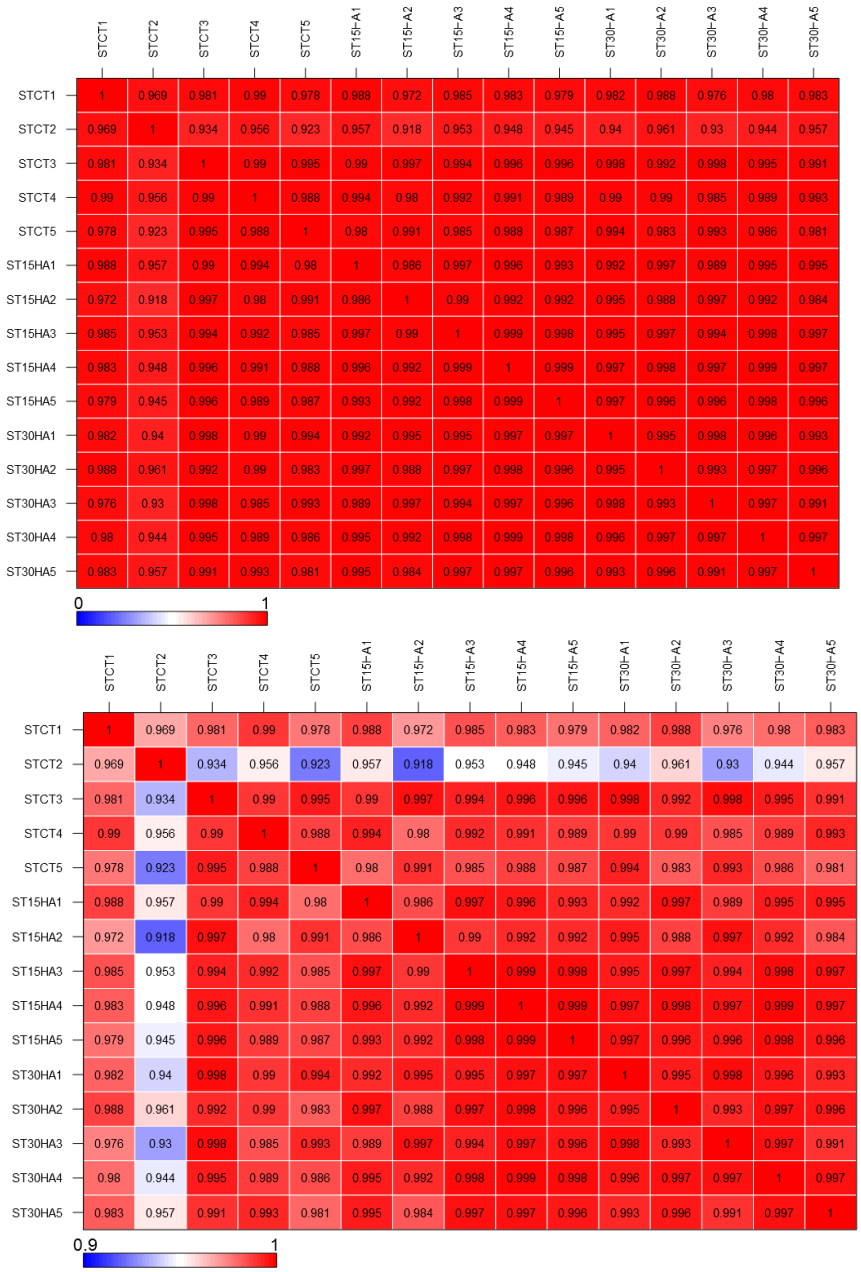

Figure 7.3 – Correlation profile of all samples used in this experiment. No changes are observed and all samples are within similar correlation values and above 0.9.

No significant changes are observed, the correlation values are above 0.9, which allow to continue the analysis with a certain confidence.

A HeatMap analysis was also performed for all the replicates of each experimental condition (Figure 7.4).

It is clear that protein profile expression could not be properly viewed in this HeatMap, where all proteins that passed the statistical test were included.

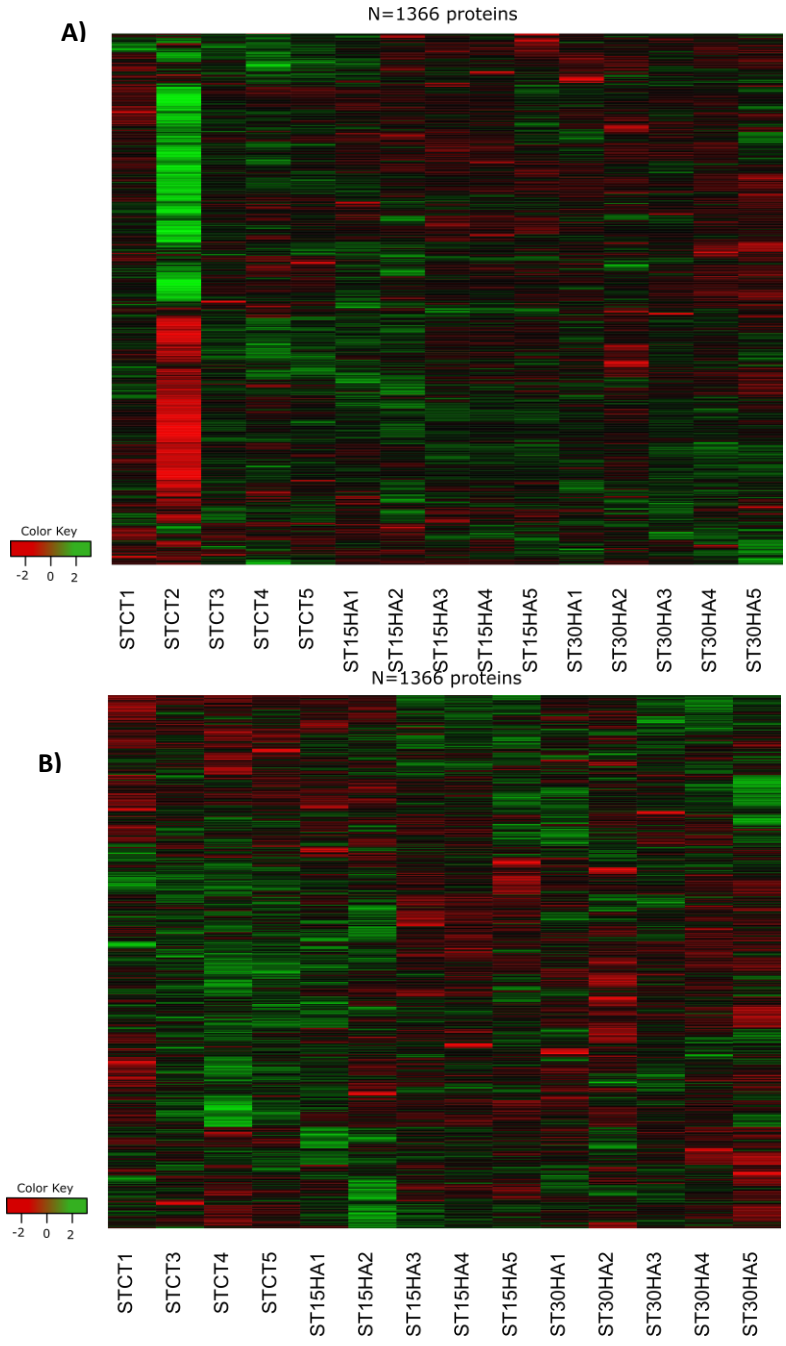

Figure 7.4 – Expression profile of all proteins. Row-clustered heat map showing the quantification expression levels of all replicates for the three experimental conditions. A) Heatmap analysis of all sample replicates of each condition. B) HeatMap analysis without replicate two from control condition.

Control replicate two stood out for its unique profile, especially when comparing to other control condition replicates. From Figure 7.4 it is possible to visualize the difference in expression profile of STCT2. Two heat maps were generated, Figure 7.4 – A reveals protein expression profile of all replicates, where Figure 7.4 – B is the same analysis although STCT2 was not included. It could be conclude that STCT2 has a different expression profile comparatively to the other control replicates.

PCA analysis was performed in order to access how the different replicates of the three experimental conditions would behave. In this analysis all the proteins (1366) from the statistical test were included. There is a tendency for control samples to separate from Haloperidol samples. However, due to the large number of proteins included, this separation is not very clear. Fifteen day of Haloperidol did not separate from samples that receive 30 days of treatment.

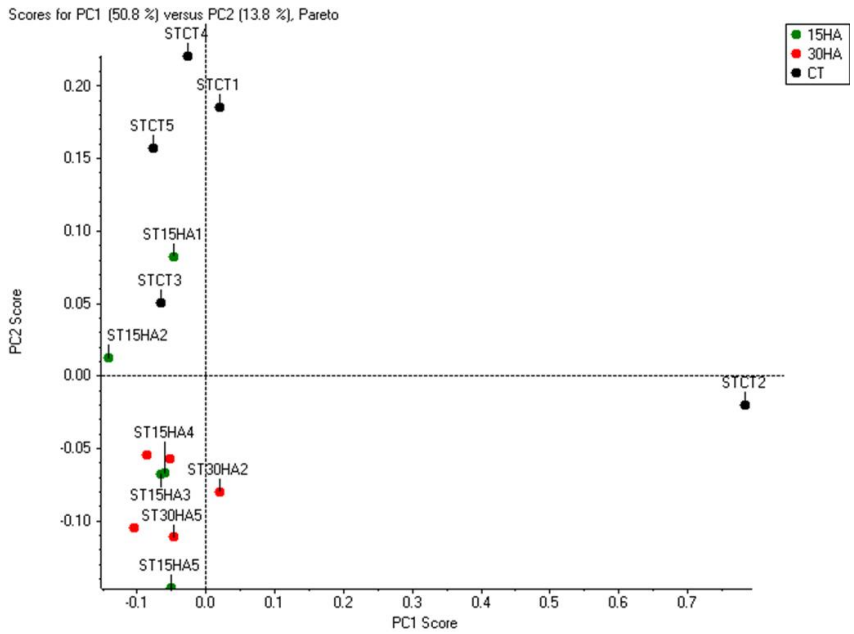

Figure 7.5 – PCA analysis of the 1366 proteins. Each dot represents a replicate from the three experimental conditions (CT: control; 15HA: 15 day of Haloperidol treatment; 30HA: 30 day of Haloperidol treatment).

Attachments

Title: Circulating biomarkers in schizophrenia – a proteomics perspective

Running title: Schizophrenia's circulating biomarkers by mass spectrometry

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Title: Circulating biomarkers in schizophrenia – a proteomics perspective

Abstract

Schizophrenia (SCZ) is a very serious, heterogeneous and debilitating major mental disorder. This is one of the most debilitating medical conditions and is economically devastating, being listed by the World Health Organization (WHO) among the top 20 leading causes of disability worldwide. The diagnosis of SCZ is performed predominantly by recurring to interviews of the patients and by analyzing the patient's history, as there is to date no biochemical test to aid in this diagnosis. On the other hand, SCZ treatment is mostly based in psychotropic medication with high rates of ineffectiveness, lack of coping and severe side effects. For all these reasons, it is of the utmost importance the current research on the pathophysiology of the disease but also the search for new panels of biomarkers, able to aid in the correct diagnosis and stratification of the patients, prognosis, and prediction of treatment effectiveness.

In this review, a total of 25 publications on peripheral SCZ biomarkers are presented from proteomics studies performed in body fluids of patients searching for protein markers, and using mass spectrometry. To date such proteomics studies have already been achieved in CSF, serum, plasma, peripheral blood cells (namely, mononuclear cells, T-cells and red blood cells), saliva, and sweat, being of the utmost importance for the schizophrenia research field but still lacking validation and clinical translation.

In summary, a general overview of the results from these 25 studies, as well as the challenges and future perspectives of the field are here presented and discussed.

Introduction

Schizophrenia (SCZ) is categorized as a psychotic disorder, and is a very serious, heterogeneous and debilitating major mental disorders.[1] Schizophrenia is one of the most disabling medical conditions and is economically devastating, being listed by the World Health Organization (WHO) among the top 20 leading causes of disability worldwide [2].

This disease is characterized by its chronic psychotic symptoms, with a prevalence of about 0.7% worldwide [3], having an usual onset in late adolescence or adulthood, although rare infant and late-onset cases have been reported, and is frequently preceded by a prodromal phase [1, 4].

The disease is characterized by a diverse set of symptoms: (i) positive, such as delusions, hallucinations or thought disorder; (ii) negative, like reduced interest, motivation and social interaction; and (iii) cognitive dysfunction symptoms, as impaired attention, learning and memory; where the positive symptoms tend to relapse and remit and the negative and cognitive symptoms tend to be chronic and the main responsible for the patients' poor social functioning [4].

In general, the diagnosis is achieved only after the first psychotic episode and is based in descriptive criteria and may be considered clinician dependent, as there is to date no biochemical test to aid in this diagnosis. On the other hand, SCZ treatment is mostly based in psychotropic medication and it is predicted as being ineffective for about 40% of the patients at a first approach, consequently, around 60% of the patients end up abandoning the treatment due to lack of coping and also severe side effects.[5, 6] This way, it is of the utmost importance the current research on the pathophysiology of the disease but also the search for new biomarkers, especially in body fluids, able to aid in the correct diagnosis and stratification of the patients.

In this review, we aim to examine the publications on peripheral SCZ biomarkers published to the present, in particular the studies on proteomics, searching for protein markers, using the leading non-target technique for discovery of the field: mass spectrometry. Also, a critical view on the future perspectives of the field in what concerns expected results and new technical developments are presented.

The need for biomarkers in psychosis

The diagnosis of SCZ is performed predominantly by recurring to interviews of the patients and by analyzing the patient's history. The diagnosis is then achieved by verifying the fulfilment of operational criteria specified in international guidelines such as the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) [7] or the International Classification of Diseases, 10th revision (ICD-10) [8]. In fact, psychiatry remains the only branch of medicine that does not use routine laboratory tests for diagnostic purposes, mainly due to the fact that the biology of the diseases are poorly known, the preclinical models present many challenges and drawbacks and specially due to the fact that the brain is not easily accessible.[9] This way, schizophrenia diagnosis remains descriptive and a syndromic concept, and it has been argued by many that this classification of the disease may have been an impediment for research in this area.[10]

In the psychiatry field, a biomarker (or panel of biomarkers) may be useful: (i) to correctly diagnose and stratify a psychiatric patient, especially important in this field where several diseases may have overlapping clinical symptoms, or even (ii) to better classify at-risk individuals, (iii) it may be used for prognosis or (iv) as therapeutic monitoring, and (v) predictive of therapy compliance [9, 11, 12]. In summary, the definition of a biomarker

states that it should be a quantifiable or identifiable physical characteristic that is closely and exclusively related to a given physiological state. This way, biomarkers can be used to assess physiological conditions, such as good health or disease, toxicity or drug treatment responses. [13]

Throughout the years, many research groups have been studying SCZ with numerous and varied strategies. In the genetics field the studies have been performed using genome wide association studies (GWAS), next-generation sequencing, copy-number variations and microarrays, with different study designs using the general population, twins and family studies [14, 15] linking SCZ with rare genetic variations and stating that SCZ has a strong genetic component. The genetic liability is estimated to be close to 80%, although the heterogeneous manifestations of the disease indicate a non-Mendelian complex mode of inheritance.[15, 16]

Many years and numerous studies later, more than 100 distinct loci containing fairly common alleles of small effect are now known to exist [17], also several copy number variants that may individually confer an elevated risk for schizophrenia have been identified [18] (for a review on the subject refer to [19]). This way it is understood that there is a substantial contribution of genetic factors for the pathophysiology of schizophrenia, but it is also understood that this contribution is not exclusive and does not explain the cause of the disease entirely. While the complexity of the subject is starting to be well accepted, it is believed that much of the genetic risk for schizophrenia is still to be uncovered and the majority of the genetic discoveries still do not have a clinical application. [1] Nonetheless some authors have already suggested that chromosomal microarray analysis of clinically relevant CNVs, with a prevalence of around 5%, should start being used as a clinical diagnostic test. [20]

Given all this, a genetic trait associated with schizophrenia can give clues about increased risks and susceptibility of disease manifestation, but the molecular alterations or deficits are ultimately reflected in the phenotype, as in the proteome and metabolome [11]. Other well established characteristic of schizophrenia physiology is the importance of environmental factors and insults [21, 22], whose impact is difficult to anticipate with a genomic analysis, but which are reflected in the molecular phenotype. Moreover, the genome is static while the proteome and metabolome are dynamic [12, 23], making it possible to quantitatively track a molecule over time, being this way possible to have clues about the progression of the disease, correlating the levels with the severity of the symptoms or anticipating therapeutic outcomes.

Overall, it is possible to state that the need for biomarkers for psychiatric diseases at different levels (diagnosis, prognosis, treatment resistance,…) are urgent and that to achieve this not only gene expression and DNA variations must be studied and determined, but also the abundance and modifications of the proteins and their distribution at the subcellular level.[14] In particular, efforts to find protein altered patterns in circulating fluids have increased interest once this fluids are relatively easier to access than the brain in order to perform clinical test but firstly to work in discovery research.

Proteomics contribution for schizophrenia's biomarker research

The term "proteomics" was firstly introduced in 1995 and it was used to define the large-scale analysis of the entire composition of proteins of a cell line, tissue, or organism.[24] Proteomics aims to describe and understand biological processes based on the qualitative or quantitative comparison of proteomes [14], especially in diseased versus control cases. The great development of this area may be attributed to the technological advances in mass spectrometry (MS), optimization in sample preparation and computer

sciences that are now able to deal with the large amount of information that is nowadays generated by the MS-based technologies [25, 26]. With proteomics approaches, several levels of information may be obtained: (i) identification of the proteins in a sample at a given moment; (ii) expression levels of the proteins or quantitative proteomics; (iii) identification or quantification of post-translational modifications (PTM) of those proteins (phosphorylation, glycosylation, acetylation, …); (iv) determination of protein-protein interactions; (v) proteomic functional studies to unravel to role of a protein, constituents of functional complexes its localization, translocation, among others [24, 26].

In the beginning of MS-based large screening proteomics approaches it was possible to characterize proteins in complex mixtures, but these approaches were mainly qualitative, while successfully identifying a high amount of proteins from one sample they fail in quantifying the expression levels of these [27]. Over the last few years, the proteomics field has turned to quantitative approaches, especially due to technological advances, and this has become the analysis of choice when comparing proteomes, once most of the interesting biological alterations are slight differences in the amount of a protein present in a given situation and not an abrupt alteration stating its presence or absence [28].

These improvements in MS-based proteomics techniques have paved the way to reach the long wanted goal of having an exhaustive characterization of all proteomes at specific moments, especially in diseased states, from where biomarkers for diagnosis, prognosis or therapy could be identified, validated and introduced into clinical practice. This is where proteomics field stands at the moment, where it is being directed to the use of proteomics in the clinical setting although it mainly remains in the discovery phase of biomarkers; and with psychiatric research it is not different. [23]

In general, the history of proteomics in schizophrenia research is very similar to the history of proteomics itself [24, 29]. The first reports of untargeted proteomic large screenings in schizophrenia begin by using two-dimensional electrophoresis (2-DE) [30] where the first dimension is an isoelectric focusing step (IEF) followed by the second dimension which is a standard SDS-PAGE. In this approach the quantitative information is retrieved from the analysis of the intensity of the staining of a given spot in the gel, which is then picked and the proteins are identified by MS. Later on, an improvement of this technology was introduced, two-dimensional difference gel electrophoresis (2D-DIGE) [31], where the separation steps remain the same but the proteins are directly labelled with fluorescent dyes (CyDies), enabling the analysis of multiple samples in the same gel, this way improving the reproducibility of the quantitative results.[32]

Although 2-DE techniques have been extremely important in transforming protein analysis into an “omics” approach, these methods have some drawbacks as the lengthy sample preparation and the difficulty of detection of some subtypes of proteins, like membrane proteins or due to dynamic range limitations.[32] Throughout the years the community has turned to other methodologies that can measure the quantitative levels of the entire set of proteins, and that can be divided into two main groups: (i) labelling techniques, which require the chemical, metabolic or enzymatic stable isotopic labelling of the samples prior to MS analysis; (ii) label-free techniques, which are gaining increasing interest due to improvements in accuracy and sensitivity of MS instruments and data processing algorithms.[33]

All the above-mentioned methods are, in general, untargeted techniques aiming to identify and quantify all the proteins in a complex sample in relation to another, usually diseased proteome versus control. Nonetheless, some of these methods may be used

for targeted approaches or even for absolute quantification, being the gold standard of MS-based absolute quantification the multiple reaction monitoring (MRM) method.[34]
There are numerous studies employing these techniques in psychiatric research. Initially, the great majority of the studies were performed in *post-mortem* brain tissue and it is easy to find large screenings in several brain areas, such as frontal [35] and prefrontal cortex [36]; the corpus callosum [37]; the temporal lobe [38] or the hippocampus [39] (citations as examples, for an extensive review refer to [14]). Later on, other tissues [40, 41] or cell populations [42] and body fluids (Tables 1 and 2) or even animal models (e.g. [43, 44]) started to raise interest in the search of the physiological characteristics of the disease, but especially in the quest for biomarkers.
As schizophrenia is considered a brain disease the most natural way of studying the disease is through the brain, and when talking about proteomics this means *post-mortem* brain tissue [45], as just exemplified above. Nonetheless, and although very informative, *post-mortem* brain tissue has its drawbacks, as the susceptibility of confounding factor, namely medication or age and chronicity of the disease, and the fact that the tissue is static, with no possibilities of being manipulated, perturbed or having longitudinal samplings. [46, 47] This way, the current proteomic studies have been focusing in peripheral tissues or fluids, and with the integration of all these analysis it is expected to emerge a more complete picture of the disease in what concerns onset, progression and responsiveness.[47]

Table 1 – Proteomic studies of schizophrenia and schizophrenia biomarker discovery using mass spectrometry in CSF.

Year	*Number of Samples*	*Method*	*Diagnosis*	*Treatment*	*No. proteins*	*Altered Proteins*	*Altered Pathways*	*Reference*	*Observations*
2003	10 SCZ; 10 CRT	2-DE/ MALDI-MS	Schizophrenia	-	Total ID: 54	APOA4 ↓	Lipoprotein metabolism	[48]	Haptoglobin, fibrinogen, complement component 3 and Gc-globulin were also altered, but not statistical significant
2006	58 first-episode SCZ; 16 DEP; 5 OCD; 10 AD; 90 CRT	SELDI-TOF-MS	Paranoid schizophrenia; Depression; Obsessive-compulsive disorder; Alzheimer's disease	Drug naive	-	40-amino acid VGF32-62 peptide ↑ and TTHY↓	-	[49]	Different distribution between SCZ and CRT; 80% sensitivity and 95% specificity in the validation study
2006	35 SCZ; 36 CRT	2-DE/ MALDI-MS	Schizophrenia	Chlorpromazine	Total ID: 80	APOE↓; TTHY tetramer↓; TRFE↓; RET4↓; Ig Kapa↓; Ig Gama↓;HPT↓; APOA1↓; A1AG2↓; TTHY monomer↑; TETN↑; APOJ↑; A1AT↑;ALBU↑;	Retinoid transport	[50]	-
2007	54 first-episode SCZ; 24 IPS; 70 CRT	H-NMR and SELDI-TOF-MS	Paranoid schizophrenia; Prodromal State of Psychosis	Drug naive	-	VGF (VGF23-62) peptide ↑; TTHY↓;	Metabolism	[51]	Significant differences between CRT and SCZ; no significant differences between IPS and CRT, and IPS to SCZ
2007	10 first-episode SCZ; 10 CRT	label-free nano-LC MS^E	Paranoid schizophrenia	Drug naive	Total ID: 77	-	-	[52]	Clear difference between CRT and SCZ observed in PLS-DA scores
2008	41 SCZ; 40 CRT	SELDI-TOF-MS	First episode Paranoid Schizophrenia or brief psychotic disorder	Drug naive	Total ID: 1	APOA1↓	Neuronal and glia metabolism	[40]	Also decreased in the liver, RBC, serum and post-mortem brain samples

2010	17 first-episode SCZ; 10 CRT	2-DE/ MALDI-TOF/TOF	Paranoid Schizophrenia	Atypical Antipsychotics	Total ID: 6	APOE ↑; PTGDS↑; APOA1↑; TTHY↓; TGFR1↓; CCDC3↓;	Lipid metabolism	[53]	540 protein spots in SCZ 2-DE; 542 in CRT; 468 matched spots
2012	11 SCZ; 20 AD; 20 CRT	SELDI-TOF-MS	Sporadic Alzheimer's Disease; Schizophrenia	Neuroleptics and/or antipsychotics	Total ID: 15 Aβ-peptides	sAPPα↑; Aβ1-42↓	Neuronal plasticity and survival	[54]	-

SCZ: Schizophrenia; CRT: Control; LC: Liquid chromatography; MS: Mass Spectrometry; TOF: Time-of-flight; 2-DE: Two-dimensional gel electrophoresis; MALDI: Matrix-assisted laser desorption/ionization; DEP: Depression; OCD: Obsessive compulsive disorder; AD: Alzheimer's disease; SELDI: Surface-enhanced laser desorption/ionization; IPS: Prodromal state of psychosis; H-NMR: Proton nuclear magnetic resonance; PLS-DA: Partial Least Squares Discriminant Analysis;

Biomarker discovery in CSF

It is natural that the quest for biomarkers is generally performed as closely as possible to the source of the question being studied, and in what concerns neuropsychiatry (and neurology in general) and the diagnostic purpose of biomarker discovery, the closest to the living brain that it is possible to get is to analyze the cerebrospinal fluid (CSF).[11]

CSF, or liquor, is a body fluid that occupies the ventricular system surrounding the brain and the spinal cord, and is rich in molecules, like proteins, peptides and small metabolites, that are either products of or have physiological properties in the central nervous system (CNS).[55, 56] It is estimated that at a given time each person has 150 mL of CSF, and the production turn-over is of 500 mL per day, meaning that CSF is extremely dynamic and characteristic of the physiology at the moment of collection.[11]

The protein content of CSF may be very variable and is estimated to have values between 0.18 to 0.58 g/L [57], which corresponds to about 0.5% of the concentration of proteins in serum [58], and presents a high dynamic range with proteins like albumin accounting for much of the protein content.[59] Nonetheless, even the total amount of protein present in the CSF has diagnostic potential, for instance an increase in protein content in the liquor is observed in infection or multiple sclerosis.[57]

CSF is collected upon the request of a lumbar puncture, where a small volume of the liquor is collected for clinical analysis (for instance for protein and glucose levels assessment, cell counts and microscopic examination, or culture) [57] and it can be collected using controlled and standardized procedures which help reducing variability at the collection step.[59] CSF collection is considered a fairly invasive procedure, implicating that new collections, either to have more sample volume or to perform longitudinal studies, are not easy to perform. Thus, proteomic analysis of CSF is feasible but implicates robust and sensitive techniques.[11]

After a careful search in public repositories (namely PubMed and Web of Knowledge) and with bibliographic mining, we were able to locate 8 schizophrenia studies using MS-based proteomics approaches to study the CSF as documented in Table 1. From the analysis of this table it is possible to understand that the first study of the kind was performed in 2003, and that several studies followed this first one until 2012. Since then we were not able to find other studies using mass spectrometry in CSF of schizophrenic patients.

The majority of these proteomic studies were performed using 2-DE followed by MALDI-TOF-MS [48, 50, 53] or by using SELDI-TOF-MS [40, 49, 51, 54]. Only one study [52] uses label-free MS^E, an approach where the fragmentation spectra of virtually all the peptides resulting from protein digestion is acquired.[60]

In general, a low number of proteins are identified in these studies, but some of the studies claim to be able to distinguish between schizophrenia and healthy groups using either the relative quantification of the detected features (exact mass and retention time) in MS^E analysis [52]; or by using the up-regulation of a VGF-derived peptide and the down-regulation of transthyretin, in this case achieving a specificity of 95% and a sensitivity above 80% in distinguishing SCZ from controls but not being able to distinguish from patients in the prodromal state [49]. Several other studies also propose some molecules as a biomarker of SCZ, such as Wan et.al. [50] which proposes transthyretin and apolipoprotein E as possible markers for diagnosis and optimal treatments; or Martins-de-Souza and colleagues, which propose Apolipoproteins E and A1 and prostaglandin-H2 D-isomerase as a putative panel of biomarkers in CSF for SCZ diagnosis [53]. In another very complete study of CSF, Apolipoprotein A1 was found

down-regulated in CSF, liver, red blood cells (RBC), serum, and in *post-mortem* brain tissue regardless of the treatment and chronicity of the disease, evidencing that some alterations in CNS can have correlated results in systemic organs and fluids.[40]

Given all this, CSF is a truly valuable source of information from where many potential biomarker may arise, and also many clues to understand the pathophysiology of SCZ, as it is documented by these (and other) studies described. Nonetheless, we believe there is still space for the non-targeted large screening approach in the CSF especially due to the constant improvement of the mass spectrometry field. Even so, this fluid does not meet entirely the definition of a good fluid for routine clinical evaluations because its collection is invasive and the amount of material is relatively small, [61] this way the study and validation of possible biomarker candidates will probably be translated to more readily available biological fluids.

Table 2 – Proteomic studies of schizophrenia and schizophrenia biomarker discovery using mass spectrometry in blood serum and plasma.

Year	*Number of Samples*	*Method*	*Diagnosis*	*Treatment*	*No. proteins*	*Altered Proteins*	*Altered Pathways*	*Reference*	*Observations*
									SERUM
2007	22 first episode SCZ; 23 CRT	Label-free LC-MS^{E}	Paranoid Schizophrenia	-	Total ID: 1709 (50%); 232 (95%)	zinc finger , BTB domain-containing protein 38 **less abundant**; APOA1 **most abundant**	-	[62]	Technical paper
2010	22 first-episode drug-naive SCZ; 33 CRT	Label-free nano UPLC-MS^{E}	Paranoid Schizophrenia	Drug naive	Total ID: 1411; significantly different: 10	CD5L↓; IGHM↓; F13B↓; TRFE↓; APOD↓; APOA1↓; FETUA↓; APOA4↓; APOA2↓; APOC1↓	lipid metabolism; molecular transport; immune response	[63]	-
2012	20 first-episode drug-naive SCZ ; 20 CRT	Depleted MARS-14 IMAC + LC-MS^{E}	Paranoid Schizophrenia	Drug naive	Detected: 694; Total ID: 312; Significantly different: 35; Phospho altered: 72	K2C6B↑; FCN3↑; SRBS1↑; NUCB1↑; K1C9↑; NUDT6↑; **ALS2**↑; IBP3↑; **MAST1**↑; **CFAB**↑; **C4BPA**↑; **FHR3**↑; **ITIH3**↑; **CO6**↑; AGRE1↑; CAH1↓; RET4↓; LRRC7↓; FR1L6↓; KI21B↓; TETN↓; KIF27↓; **APOA1**↓; **APOA2**↓; **MYOF**↓; FIBA↓; **CCD57**↓; **SMC1A**↓; K1C14↓; **PHLD**↓; LIFR↓; XIRP1↓; WDR19↓; SMC4↓; SAGE1↓ (proteins in bold also have altered phosphorylation pattern)	Acute phase; Complement and coagulation system; Immune Response	[64]	Of the 72 proteins showing phosphorylation changes, 59 showed no significant changes in expression level; FCN3 and RET4 validated by ELISA

2012	Proteomics sample set: 10 SCZ; 10 CRT; Validation sample set: 47 SZ; 53 CRT	Depleted Label-free Nano-LC-MS/MS	Schizophrenia	Proteomic sample set: drug-naïve; Validation sample set: 29 drug-naive; 18 drug-naive during 8 weeks;	Total ID: 1344; 192 used for PLS-DA (27 SCZ-related)	CO8B↑; CD5L↑; DOPO↑; IGHG4↑; IGHM↑; KNG1↑; PI16↑; PGRP2↑ ITIH4↑; PLTP↑; IPSP↑; IGK@ protein↑; IGL@ protein ↑; AMPN↓; APOC2↓; APOF↓; C4BPB↓; APOL1↓; FA7↓; GGH↓; ICAM2↓; ALS↓; isoforms 2 of ITIH4↓; LBP↓; PROS↓; ZNF57↓ (SCZ related proteins)	Complement cascade pathway	[65]	Validation set: were divided in three groups and received four weeks treatment with clozapine (26 patients); chlorpromazine (15 patients) or other drugs such as risperidone (6 patients)
2014	Model: 166 SCZ; 201 CRT; Validation: 76 SCZ; 103 CRT Test group: 44 SCZ; 41 CD	Magnetic beads for peptides MALDI-TOF/TOF-MS	Schizophrenia, Chronic diseases: 29 hypertension; 12 diabetes	Drug naive	Total ID: 10 altered peptides	Fragment of Fibrinopeptide A m/z: 1206.58 AUC of Roc above 0.98	-	[66]	-
2015	26 SCZ; 26 CRT	C8 magnetic beads or C18/TiOtips MALDI-TOF-MS	Schizophrenia	Olanzapine; Clozapine; Aripiprazole; Flupentixol; Haloperidol; Zuclopenthixol; Promethazine; Quetiapine; Risperidone;Paliperidone ; Flupenthixol; Hydroxyzin; Levomepromazine; Melperone; Amisulprid	Total Detected: 94; Significantl y different: 11 protein ions from TiO; and 5 from C8	m/z 3177 suggested to be fragment of Apolipoprotein A1	-	[67]	-
2015	44 SCZ; 26 DP; 40 CRT	IMAC30 SELDI-TOF-MS and MALDI-TOF/TOF	Schizophrenia, Depression	Drug naive	Significantl y different: 91 protein peaks	N-terminal fragment of fibrinogen ↓	-	[68]	

									PLASMA
2006	19 SCZ	2-DE/ MALDI-MS	Schizophrenia	Chlorpromazine	Total ID: 56	TTHY tetramer ↑	-	[50]	Responders vs non-responders in pre and post-treatment
2006	22 SCZ; 20 CRT	2-DE/ MALDI-TOF-MS	Schizophrenia	Clozapine	Total ID: 66; Significantly different: 7	HPT-α2 chain↑; HPT-β chain↑; A1AT↑; CFAB↑; APOA4↑; APOA1↓; TTHY↓	Inflammatory, Immune response	[69]	To exclude drug treatment effect, plasma from 11 SCZ patients was collected before medication and after 2 months of medical treatment (clozapine)
2007	25 SCZ; 9 CRT	2-DE/ MALDI-MS	Schizophrenia	8 patients w/ Chlorpromazine; 8 patients w/ Clozapine	Total ID: 7	APOA1↓	Lipoprotein	[70]	Adult male Sprague-Dawley rats: 9 rats w/ Clozapine; 9 rats w/ Chlorpromazine; 8 rats received ddH_2O; Total proteins ID: 29; Significantly different: 18; APOA1, APOA4, APOAE ↑ w/ Chlorpromazine (negative control)
2007	42 SCZ; 46 CRT	2-DE/ MALDI-MS	Schizophrenia	Chlorpromazine	Total ID: 20; significantly different: 6	HPT↑; A1AT↑; SAMP↑; AMBP↑; ANT3↑; VTDB↑	Inflammatory, Immune response	[71]	-
2014	80 first-episode SCZ	2-DE/ MALDI-TOF/TOF	Schizophrenia	Risperidone	Total ID and altered: 18	APOA1↑; C4B↑; CFB↑; NEB↑; C8B↑; ZN185↑; PLMN↑; HEMO↑; HNMT↑; GBP1↑; FGG↑; TRFE↑;	Metabolism	[72]	Investigate changes in protein expression before and after treatment

						ALEX↓; RET4↓; K1C9↓; K2C1↓; VINC↓; GELS↓			
2016	29 SCZ; 93 CRT; 25 BD	nano-LC–MRM-MS	Schizophrenia, Bipolar Disorder	Quetiapine, Risperidone, Amisulpride, Aripiprazole, Haloperidol, Antidepressant (SCZ); Lithium, Bupropion, Valdoxan, Lamotrigene, Valproic acid, Quetiapine, Antidepressant (BD)	Total ID: 42; significantly different: 19	ANT3; APOA1; APOA2; APOA4; APOC1; APOC2; APOC3; APOC4; APOL1; C1QC; CFB; C3; F13B; FCN3; HSP70 escort protein 2; HRG; KLKB1; PEDF; RET4 (Patients – SCZ and BD vs Control)	-	[73]	Significant dissimilarities between BD and SCZ patients: A2AP, ANT3, APOB, APOD and APOF

Biomarker discovery in Peripheral fluids

For over a decade, peripheral fluids' analyses for disease investigation, and biomarker discovery in particular, has increased in the psychiatric field.[12] As it can be observed in Table 2, blood-based analysis accounts for most of such studies, especially with the analysis of serum and plasma, although blood cells' analysis is growing in interest (Table 3).[74] The rationale behind this is that, even if psychiatric disorders are brain diseases, its consequences can be sensed throughout the entire body; and most importantly, the access to fluids like blood is considerably easier than the access to the living brain or even to CSF.

It is a long standing fact to know that there is strong integration of brain and a variety (if not all) of physiological conditions of the body altering the contents of body fluids, where the classic example is the "fight-or-flight reflex" [75] in which there is a strong interplay between the hypothalamic-pituitary-adrenal axis. It has been demonstrated that there are dynamic changes correlating alterations in brain and blood, and vice-versa [47, 74], and in the case of schizophrenia some alterations of hormones, cytokines or growth factors can be replicated both in brain and blood stream [74, 76].

This whole-body concept of psychiatry is starting to give some answers, and in recent investigations it is stated that molecular signatures may be found in the periphery, especially in circulating blood, as are the examples of studies for schizophrenia [77, 78], major depressive disorder [79], and bipolar disorder [80].

In summary, peripheral fluids present themselves as extremely valuable for psychiatric research and biomarker discovery and validation, once molecular changes due to the disease causes, chronicity or drug responses, for instance in proteins' levels or PTM patterns, may be measured directly in the blood (Table 2 and 3), or even in less conventional fluids as saliva or sweat (Table 3). The collection of these fluids presents the valuable advantage of being non-invasive (urine, saliva, sweat, tears…) or minimally invasive (blood), allowing for multiple collections and the collection of meaningful amounts for the analyses to be performed. On the other hand, these fluids also present challenges, such as the extremely high dynamic range of plasma or serum, where a dozen of proteins represent more than 90% of the protein content; the more laborious procedure to obtain pure blood cell specimens [81]; or the still not standardized collection of fluids like saliva, sweat or tears [82-84].

Given all this, an extensive search for schizophrenia mass spectrometry-based proteomic studies in peripheral circulating fluids was performed. On Table 2 are presented the studies published on blood serum and plasma, spanning from 2006.

After this search 7 studies on serum of schizophrenic patients and 6 on plasma were uncovered. The preferred quantitative method of analysis of the serum samples was label-free MS, using LC-MS^E [62-64] or MS1 signal intensity [65-67]. On the other hand, from the 6 studies in plasma, 5 used 2-DE-MS analysis [50, 69-72] and one study using targeted mass spectrometry MRM technique to quantify 42 plasma proteins [73].

As expected some of the studies used depletion of the most intense proteins prior to the analysis [64, 65, 68]; and some of the studies also focused on PTM analysis, namely phosphoproteins and differential phosphorylation patterns [64, 67]. Zhou et al focused on the peptidome of serum by using magnetic beads to capture the peptides, identifying 10 altered peptides, one of which being identified as a fragment of fibrinopeptide A with a receiver operating characteristic analysis (ROC) showing an area under the curve of 0.981 between schizophrenia and controls, and 0.999 for schizophrenia and other chronic diseases.[66] And Al Awan and colleagues prepared an integrative study,

analyzing proteomics and metabolomics data suggesting a profile signature of the pathology found in serum [67].

Mass spectrometry proteomics studies of schizophrenia using less conventional fluids as specimens for analysis are presented on Table 3. The use of blood cells, for instance, has the advantage of circumventing the dynamic range issue of serum and plasma. And this way, total population of peripheral blood mononuclear cells (PBMC), specific lymphocytes subpopulations as T-cells (both stimulated or unstimulated), or red blood cells (RBC) obtained from the blood stream of SCZ patients have already been studied [41, 85, 86]. Also one study using saliva [87] and another using sweat [88] have already been studied in the context of proteomics of schizophrenia.

Red blood cells are an easily accessible cell population of the blood stream, and taking advantage of it Prabakaran et al analyzed RBC proteins using 2D-DIGE methodology, being able to find 8 altered proteins, 4 of which related to oxidative stress, corroborating earlier findings in post-mortem brain tissue and validating blood cells as good specimens for the search for SCZ biomarkers [41].

In the study of T-cells from minimally medicated SCZ patients, α-defensins were found altered and able to discriminate the 2 groups (SCZ and control) in a PCA analyses; this differential expression was further validated by an ELISA assay finding the up-regulation of α-defensins both in affected and unaffected monozygotic twins, indicating these proteins as possible early indicator of SCZ risk [86]. In order to study stimulated and unstimulated PBMCs from drug-naïve SCZ and control individuals, Herberth and colleagues chose a LC-MS^E and were able to quantified hundreds of proteins [85]. From these, 18 proteins were found to be statistically altered between first onset SCZ patients and controls, with more pronounced alterations when the cells are stimulated; this phenotype was found to be rescued when a cohort of chronically ill medicated patients was analyzed, suggesting that these proteins are modulated by medication and may have diagnostic potential in first episode patients.

The study using saliva as specimen for the analysis of samples from SCZ and bipolar disorder (BD) found several proteins altered between the disease states and controls, and was not able to find statistically meaningful alteration between SCZ and BD patients [87].

Although the studies relating SCZ and sweat odors span back to 1969 [89], and sweat has already been used to analyze the distribution of antipsychotics, like clozapine [90], only one proteomics study was found that searched for SCZ biomarkers in sweat [88]. In this study, spectral counting label-free approach was used to compare several pooled samples from SCZ and controls individuals and select 30 proteins to be validated by targeted MRM-MS, where 17 proteins showed a differential expression of at least 2-fold between the SCZ and control pooled samples.

Table 3 – Proteomic studies of schizophrenia and schizophrenia biomarker discovery using mass spectrometry in less studied biological fluids, like peripheral blood cells, sweat and saliva.

Year	*Number of Samples*	*Method*	*Diagnosis*	*Treatment*	*No. proteins*	*Altered Proteins*	*Altered Pathways*	*Reference*	*Observations*
2014 (Saliva)	32 SCZ; 17 BD; 31 CRT (smokers and non-smokers)	Label-free peak intensity HPLC-ESI-MS	Schizophrenia, Bipolar Disorder	-	Total ID: 8	DEF1↑; α-defensin-2↑; DEF3↑; DEF4↑; S10AC↑ ; CSTA↑; CSTB glutathionyl↑; CSTB cysteinyl↑; (Patients – SCZ and BD vs Control)	Innate Immunity	[87]	No statistically significant observed between SCZ and BD groups
2012 (Sweat)	23 SCZ; 55 CRT	Label-free spectral counting LC-MS/MS; MRM-MS	Schizophrenia	Risperidone; Olanzapine; Quetiapine; Clozapine; Paroxetine; Escitalopram; Lorazepam; Benztropine	1st set Total ID: 150; 2nd set Total ID: 185; MRM: 30	ZA2G↑; ANXA5↑; ARG2↑; BLMH↑; CALL5↑; CASPE↑; CDSN↑ ; CSTA↑; DCD; Desmoglein↑; DJ-1↑; G3PDH↑; KLK11↑; KRT10; PRDX1↑; PEBP1↑; S100A7↑; THIO↑; PIP↓;	Metabolic process; Biological regulation; Development process; Multicellular organismal process; Response to stimulus	[88]	Altered protein: MRM results for sweat peptides/proteins chosen from Scaffold list after LC−MS/MS of 2nd set of pooled samples
2008 (T-Cells)	15 SCZ; 15 CRT (SELDI-TOF)/ 6 SCZ; 18 CRT (ELISA)	SELDI-TOF MS and ELISA	Schizophrenia	-	Total ID: 108 peaks	α-defensins ↑	Immune response	[86]	-
2011 (PBMC)	19 SCZ (12 first-onset; 7 chronically ill); 19 CRT	LC-MS^{E}	Schizophrenia	12 Drug naïve; 7 Antipsychotic treated	Total ID unstimulated: 185; Stimulated: 441;	Unstimulated PBMCs: CNDP2 ↑; Uncharacterized protein KIAA0423↑; LDHB↑; COTL1↓; GPI↓; HSP72↓; Stimulated PBMCs: ALDOC↑; GAPDH↑;	Glycolytic pathway, Immune response	[85]	Validation cohort: 8AN, 7AT, 13 HC

					Significantly different: 19 between drug naïve SCZ and CRT	HNRPK↑; LDHB↑; MYH14↑; MYH15↑; NAMPT↑; PGK1↑; PPIA↑; TPIS↑; PKLR↑; PGAMA4↑; CH60↓;			
2007 (RBC)	20 SCZ (13 treated / 7 naïve); 20 CRT	2D-DIGE / LC-MS/MS	Schizophrenia	8 Drug naïve; 13 antipsychotic-treated (atypical antipsychotic medication);	Total ID: 418; Significantly different: 8	SBP1↑; GSTA3↑; PRDX5↓; HS71L↓; ALBU ↓; APOA1↓; SPTA1↓; ACTB↓	Oxidative Stress	[41]	-

ELISA: Enzyme-Linked Immunosorbent Assay; IMAC: Immobilized metal affinity chromatography; CD: Chronic disease; MRM: Multiple reaction monitoring; BP: Bipolar disorder; HPLC: High-performance liquid chromatography; ESI: Electrospray Ionization; 2D-DIGE: Difference gel electrophoresis

General Overview and challenges

Proteins found to be changed on SCZ patients in peripheral fluids were gather on Figure 3. In this figure, the proposed altered proteins in the published studies were organized by biological fluid; once the same protein was found altered in more than one biological fluid it was listed in a “common proteins” column, meaning that there are more evidences for the regulation of those particular proteins in the peripheral fluids of SCZ patients.

In total, there are dozens of differently expressed proteins which were found altered between the 25 projects analyzed in this review (Tables 1, 2 and 3). From all these proteins only 18 of them were found modulated in at least 2 different fluids; and although in many cases this fact may act as a preliminary validation of the potential of that protein as a SCZ biomarker, in some of the cases the results in the different studies are not corroborating, having opposite differential expressions, as is the example of protein tetranectin which was found upregulated in the CSF study [50] and downregulated in the serum study [64]. On the other hand, some other proteins have been consistently and repeatedly identified as altered in several fluids, as transthyretin which has been found downregulated in CSF [50, 51, 53] and plasma [69], but upregulated in plasma after medication [50].

From the analysis of this integrative Figure 3, it is possible to note that from the 18 “common proteins” 7 belong to the family of apoliproteins, and these represent some of the most replicated modulated proteins in peripheral fluids in SCZ. Nonetheless, all the other proteins presented in this figure have potential to be further studied, validated and possibly integrated in a panel for SCZ diagnosis, prognosis or even treatment response prediction; even though these large screening approaches still present themselves with many challenges.

Overall, this preliminary data overview on peripheral biomarkers of SCZ is very encouraging in the pursuit for a proper biomarker panel for the disease. Nonetheless, the fact that SCZ is a very a complex and heterogeneous disorder should always be taken into account.[91] Moreover, it is even suggested that further knowledge of the disease physiology and higher stratification of the patients may be needed, where biological and clinical markers may help distinguish different sub-sets of SCZ patients and may not have the classical diagnostic potential (SCZ versus non-SCZ).[92, 93]

Additionally, some important factors are many times overlooked in these screening studies, such as comparing if the candidate proteins show similar patterns in other psychiatric diseases, meaning that those alterations may arise from common phenotypes of the pathologies and not be an indication of the unique trait of each one [93], which is the final goal of a biomarker.

Another challenge in the research of psychosis biomarkers is the sample set and patient recruitment that is necessary for all studies, but in particular for these large screening studies. First-onset drug-naive schizophrenia individuals are the preferred subjects of study, and their recruitment has a low rate leading to longer durations of the projects and consequently longer storage times and higher variability.[94, 95] On the other hand, the recruitment of chronically ill medicated patients is easier but comes with the disadvantage of making it necessary to clearly distinguish drug from disease-related alterations, which is not as straightforward as it may seem and it is a factor that is many times disregarded in these studies, explaining in some extent the low reproducibility of the findings.[96]

This way, the use of well established, well-matched sub-pools of disease and control subjects, with enough replicates to generate powerful analysis should be used. Another

important step is the validation of the interesting findings with different methodologies and most importantly with different and bigger cohorts of individuals. [14]

A primary challenge in the search for biomarkers is the easy access to sufficient amounts of high-quality body fluid [97], for instance the use of CSF. Although it is still the circulating fluid that more closely reflects pathophysiological alterations caused by neurological disorders, and although it is in communication with cerebral extracellular fluid and is less hampered by confounding factors, problems could be associated with CSF sampling as it is still an invasive procedure [97] although it tends to become more routine and safe.

Moreover, blood collection standardization is a necessary step in any blood study aiming to report findings on potential biomarker. In order to decrease study variation, blood collection protocols should have all identical time of collection, must use the same coagulants and blood processing methods, where these methods should be extensively and accurately described. [93] Furthermore, standardized collection also allows investigators to replicate studies with samples that match the initial pilot data.

In summary, the idea that the establishment of standard operating procedures (SOP) for each step of the process, comprising collection, handling, preparation and storage is appearing as mandatory in order for the results of the different research centers to be integrated and the identification of powerful biomarkers may be reached. [98] Moreover, the publication of entire proteomics datasets from these exhaustive proteomic studies, rather than just positive findings, is crucial for the mandatory integration of all the information being acquired by the scientific community at the present.[14]

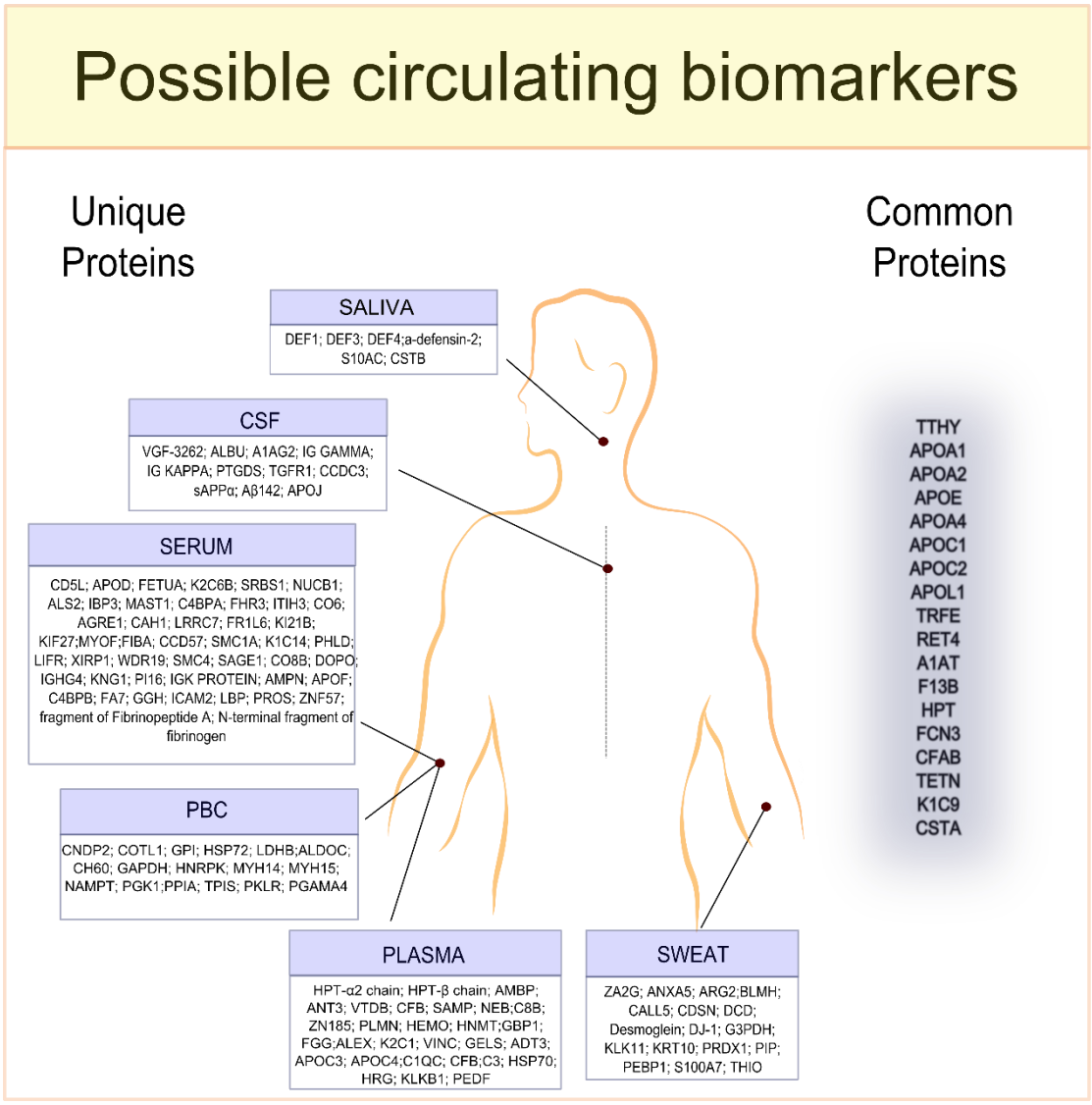

Figure 1 – Summary illustration depicting the proteins found altered in the several studies of each fluid. The uniquely altered proteins for each fluid are represented, as well as the proteins found altered in at least 2 different biological fluids.

Future Perspectives and conclusion

This review gathers a compilation of proteomics studies of schizophrenia using mass spectrometry, focused on studies using body fluids. Comparative proteomics analysis is extremely important and of highest clinical interest as it may reveal potential biomarkers for diagnosis, choice of therapy, refine prognosis and treatment prediction. In certain pathologies, it is not the presence of certain proteins per se that make them markers but rather their expression, the alterations in expression as well as their state. Moreover, in many cases it is not just one single protein but a set of proteins that is indicative of a certain disease. In this way, emerging proteomic platforms have facilitated the identification of several biomarker candidates by the simultaneous measurement of thousands of proteins. Before proteomic tools can be routinely used in the clinical laboratory, further work is necessary to enhance the performance and reproducibility of established proteomics approaches.

Sufficient and high-quality sample, sample collection standardization and type of approach are some of the methodological limits of the existing studies that should be overcome or improved.

The use of multidisciplinary approaches in SCZ studies is appearing as mandatory strategy. Due to the multifactorial picture of schizophrenia, possibly one type of approach is not sufficient to discover one specific biomarker. A combination of imagining techniques, neuropsychology, electroencephalograms (EEGs) and proteomic approaches should be interesting and challenging to identify a multifactorial signature of the disease. [95] Additionally, this type of combined approach may help to overcome present difficulties in biomarker discovery such as the issue of antibody availability and lack of comprehensive proteome coverage. [95]

Also, the exploration of less classical peripheral fluids, like sweat, saliva or urine, are starting to arise and will probably be intensely studied in the future once they present themselves as easily accessible fluids, although still lacking standardization of collection and processing also. Urine is an easily accessible biological fluid, extensively studied in many fields but still not explored in the proteomics of psychiatry, being this a gap in the investigation of the field.

Since we are in the era of Bioinformatics, current studies have been focused on protein expression and protein-protein interactions around certain genes in order to fill the gap between genomics and proteomics. [97] Although most of the published studies are promising, they are based on small sample size, have low power or have not been replicated, which can interfere with further research. Multi-site studies, data sharing, multivariate biomarker studies, and combined use of “omic” data are some alternatives to overcome these methodological limitations. [99] Moreover, there is a critical need for complementing and integrating studies with different profiling methods (proteomics, transcriptomics, metabolomics).

Altogether, mass spectrometry offers the most holistic, integrated system for clinical analysis of patient samples, seeking both known and unknown biomolecules (Tables 1 2 and 3). Mass spectrometers can analyze proteins, peptide fragments, small molecules, antibodies, metabolites, and lipids. This collection, obtainable from a single platform, can generate the total sum of a patient’s physiological state needed for quick and proper diagnosis, exact treatment selection, and therapeutic monitoring. [23] The categorization of patients based on proteomic biomarker profiles for optimized treatment is the form of personalized medicine that is aimed to be reached in the near future.

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