Faculdade de Engenharia da Universidade do Porto

FEUP

UNDERSTANDING THE PROTECTIVE EFFECTS OF NEUROSTEROIDS AND ENDOCANNABINOIDS IN SCHIZOPHRENIA USING hiPSC-DERIVED NEURONS

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to my mother,
for all your love and devotion

Abstract

Psychiatric and neurodevelopmental disorders are characterized by alterations in neural circuits that emerge into cognitive deficits. The cellular mechanisms that underpin the plasticity and dynamics of these conditions, namely Schizophrenia, remain unclear. Thus, proper model systems are required for further investigation into the underlying aetiology. Moreover, while animal models and post-mortem studies have provided important insight, both have limitations and it is necessary to explore the effects in human neurons.

Estrogens have been widely documented by their effects not only in the regulation of the reproductive behavior but for its effects in different cognitive functions in both male and female. Recent studies have classified 17-β estradiol (most representative active form) as a neurosteroid and neuromodulator, demonstrating its ability to modulate neuronal activity and being able to manifest rapid effects in specific areas of the brain. Moreover, testosterone as an estrogen precursor participates on estradiol "*de novo*" synthesis in the brain being also reported to influence neurobiological processes as stimulation of neurite outgrowth [1].

During adolescence, the development of Schizophrenia has been associated to the use of cannabis [2]. Investigations over the effects of the plant-derived cannabinoid in the human brain enabled the discovery of the endogenous cannabinoids (endocannabinoids), synthesized within mammal tissues. Endocannabinoids modulate the human brain since early stages of development, influencing neurobiological processes as cell proliferation and differentiation [3].

Therefore, this thesis investigates the effects of neurosteroids and endocannabinoids in Schizophrenia, with the aim to identify potential novel therapeutic avenues. In order to achieve this objective I differentiated cortical neurons from induced pluripotent stem cells derived from healthy control males and studied the effects of the different treatments on neuronal morphology.

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To you my beloved Tiago, for supporting this decision that kept us away for so long. Thank you my sweet-heart for being with me in this stressing and so important time of my life. For putting a smile on my lips as only you know how to do and to refill my heart with love and joy at every blink of your eyes.

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Abbreviations and symbols

AA2P	L-Ascorbic acid 2-phosphate
AEA	anandamide
AMPARs	α- amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANOVA	Analysis Of Variance
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
CBR1	Cannabinoid receptor 1
CBR2	Cannabinoid receptor 2
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DAPT	N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester
DLPFC	Dorsolateral Prefrontal Cortex
DMEM:F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
DPN	Diarylpropionitrile (selective agonist for estrogen receptor ß)
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
eCBs	Endocannabinoids
ER	Estrogen Receptor
ESCs	Embryonic Stem Cells
EXP	Experiment
E2	17ß- estradiol
E8	Essential 8 media
GPCRs	G-protein coupled receptors
GPR55	G protein-coupled receptor 55
GPR1/ GPER30	G protein-coupled estrogen receptor 1
HBSS	Hank's Balanced Salt Solution
hiPSC	human induced Pluripotent Stem Cell
ICC	Immunocytochemistry
IGF-I	Insulin-Like Growth Factor-I
iPSCs	induced Pluripotent Stem Cell

mGluRs	Metabotropic Glutamate Receptors
MRI	Magnetic Resonance imaging
NDDs	Neurodevelopmental Disorders
NGF	Nerve Growth Factor
Ngn3	Neurogenin 3
NMDAR	N-methyl-d-aspartic acid
NP	Neural passage
NPCs	Neural progenitor cells
OVX	Ovariectomized
PDL	Poly-d-lysine
PFC	Prefrontal Cortex
PPT	Propyl pyrazole (selective agonist for estrogen receptor α)
q-PCR	quantitative polymerase chain reaction
Rocki	Rock (Rho-associated protein kinase) inhibitor
SCZD	Schizophrenia Disorder
SERM	Selective Estrogen Receptor Modulator
SDAMs	Serotonin-dopamine activity modulators
SMADi	SMAD (small mothers against decapentaplegic) inhibitor
SOP	Standard Operating Procedure
SZ	Schizophrenia
WAY200070/WAY	Selective agonist for estrogen receptor β (ERβ)
WHI	Women's Health Initiative
2AG	2-Arachidonylglycerol

α	Alpha
β	Beta

Chapter 1.

Introduction

1.1. Motivation

Schizophrenia is a severe neurodevelopmental disorder, nowadays defined as a chronic, disabling mental disorder. Several approaches have been used to investigate the disorder. Pharmacological, magnetic resonance imaging (MRI) and post-mortem studies have revealed a decrease in brain volume, aberrant neurotransmitter signalling, reduced dendritic arborization and impaired myelination in schizophrenic brains. However, despite the investigations that surround this disorder, the clinical treatment has low efficacy since the aetiology and neurobiological systems that underlie the disease are poorly understood. The complex genetic and varying environmental risk factors that contribute to this disorder make the need of proper model systems of supreme relevance to further its study and to develop therapeutics.

The use of cannabinoids during adolescence has been considered as a potential risk factor of Schizophrenia for individuals with genetic predisposition for the disorder [4]. Further studies revealed that cannabinoid receptors can be also activated by endogenous cannabinoids/endocannabinoids. The endocannabinoid system has been reported to influence neuronal development since early embryonic stages [3], being involved in cell proliferation, differentiation and cell survival. Further investigations have been revealing the effects of endocannabinoids in cognition and the potentialities of its use in the treatment of different disorders including Schizophrenia [2]. Therefore it is important to understand the effects and mechanisms of this system for future medical application.

Moreover neurosteroids, such as estrogens and testosterone, have a great influence in cognitive and memory processing, which has been demonstrated by their ability to modulate synaptic plasticity in several areas of the brain [5]. Clinical studies have demonstrated that an adjunct treatment by means of administration of estrogens, namely 17β-estradiol, resulted

in the enhancement of the symptomatology of the patient [6]. Some potential mechanisms have been proposed. However the precise mechanisms that explain estrogen's effects in Schizophrenia are still unclear.

1.2. Objectives

This thesis will investigate the effects of neurosteroids, estrogens and testosterone, and endocannabinoids in Schizophrenia, with the aim to identify potential novel therapeutic avenues. In order to achieve this objective, neurons were differentiated from induced pluripotent stem cells derived from healthy control males, providing a proper model system required for the study of this neurodevelopmental disorder. Using human iPSCs derived neurons, the protective effects of neurosteroids and endocannabinoids in Schizophrenia were investigated.

1.3. Work structure

The following document aims to cover the theoretical concepts explored during the thesis as well as to provide a full report of the project developed with the referred aims. Therefore the report will be divided into six chapters, including "Chapter 1 – *Introduction*". The second chapter *"State of the art"* will provide an overview about *Neuroscience (*brain structure, neuronal development and neuritogenesis), *Schizophrenia* as a neurodevelopmental disorder and the importance of dendrites and synapses; the role of *Estrogens* in the brain and influence on neuronal connectivity; the *Endocannabinoid system*; and *Schizophrenia "in a dish"*, where it is explored the use of hiPSCs as an *in vitro* model system to differentiate glutamatergic cortical neurons. Then in chapter three, "*Materials and Methods",* the different methodologies used are described: *Cell Culture, Immunocytochemistry, Image analysis* and *Statistical analysis*. Moreover Chapter 4 exposes the results observed in the experiments, firstly reports the *optimization experiments* and then the *Neurosteroids and endocannabinoids effects on neurite outgrowth*. "Chapter 5 – *Discussion*" will discuss the limitations of hiPSCs and drawbacks found during the development of the project and will debate on how the different treatments affected neural development. Finally, "Chapter 6 – *Conclusions"* summarizes the information previously exposed.

Chapter 2.

State of the art

2.1. Brain structure

The human brain is the most complex and at the same time extraordinary organ in the human body, not only for the excitement of its unknown but for its fundamental role on the interplay of the different functions that contribute to the homeostasis of our body.

This organ is protected by the skull, being suspended in cerebrospinal fluid and isolated from the bloodstream by the blood-brain-barrier. It is included in the central nervous system that comprises the brain stem (midbrain, pons and medulla oblongata), the spinal cord and finally the brain, which includes three major regions, the diencephalon (thalamus and hypothalamus), the cerebellum and the cerebrum/cerebral hemispheres, represented on figure 2.1.

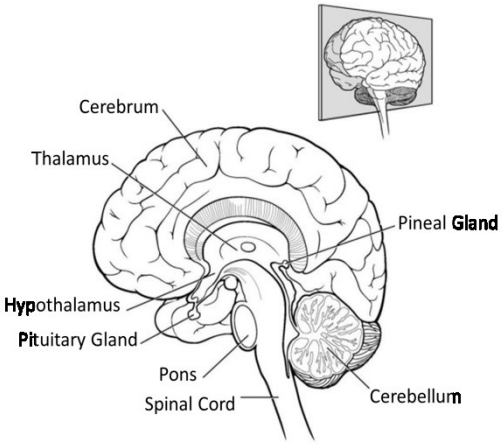


Figure 2.1. Central Nervous System, structural regions. *Adapted from* [7]

The cerebrum regulates the most complex functions and sees its structure stratified in a thick outer layer, the cerebral cortex and three deeper layers, the basal ganglia, the

hippocampus and the amygdaloid nuclei. The basal ganglia associated to motor functions, the hippocampus to memory storage and the amygdaloid nuclei to emotions, involving autonomic and endocrine responses.

The cerebral cortex is structurally divided in four lobes: frontal, temporal, parietal and occipital lobes, responsible for different functions. This structure can also be categorized based on the process complexity of the tasks, controlled by each region, as *primary* or *association* cortex, to perform basic motor or sensory functions or to associate different types of information to do more complex processing. In order to map the cerebral cortex with human behaviour, several approaches have been explored, from magnetic resonance imaging (MRI) and positron emission tomography (PET) in healthy volunteers, to mapping during brain surgery and insights from patients with lesions in specific areas of the cortex. The brain mapping has been evolving over the time and nowadays the four lobes that constitute the two cerebral hemispheres have specialized functions. The occipital lobe processes visual information; the parietal lobe is associated to somatosensory functions; the temporal lobe with hearing; and the frontal lobe is responsible to process motor functions, control movement and plan future actions.

It is important to refer that sensory and motor functions present a *contralateral* action, motor information from one side of the brain lead to an action on the opposite side of the body, the same happens to sensory information (arriving from one side of the spinal cord will be processed on the opposite hemisphere of the cerebral cortex). Moreover despite the similarities of the two hemispheres, they are not completely symmetrical in structure nor in function. The anatomical structures of both left and right hemispheres can be observed on figure 2.2.

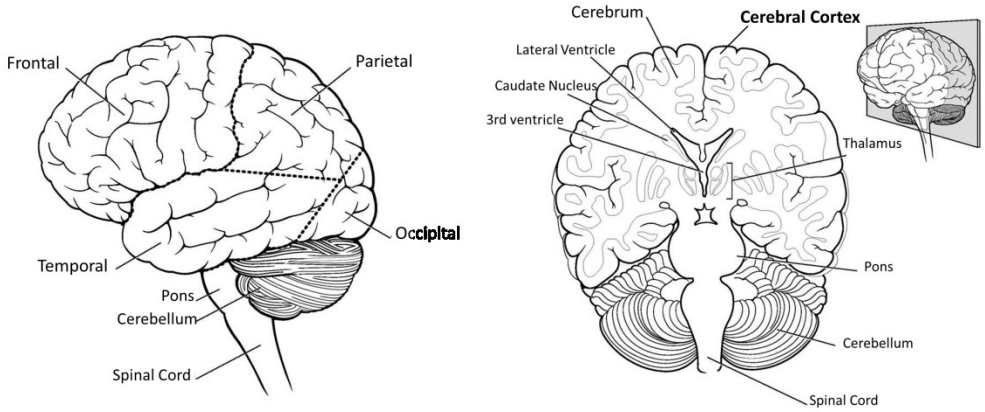


Figure 2.2. Lateral and coronal view of the brain. Anatomic representation of the left hemisphere and respective lobes (left); coronal section of the brain (right) indicating the outer layer of the cerebrum, the cerebral cortex. *Adapted from* [7].

2.2. Cerebral cortex and neuronal development

The following topic will primarily focus on the formation of the different layers of the cerebral cortex to then explore the development of the neuronal structure, the different stages involved, namely the formation of the immature neurite into the single axon which is the first manifestation of neuron polarization.

2.2.1. Cerebral cortex - layers

The cerebral cortex presents an organized structure of 6 layers, characterized by the type of neuron and additional elements (figure 2.4), being numbered from the pial surface to the white matter (outer to deeper layers).

Depending on the type of staining, the structures that define these layers can be identified. The Golgi stain allows the visualization of both dendrites and cell soma, whereas Nissl and Weigert stain only identify the cell soma and proximal dendrites, and axons (myelinated fibres) respectively.

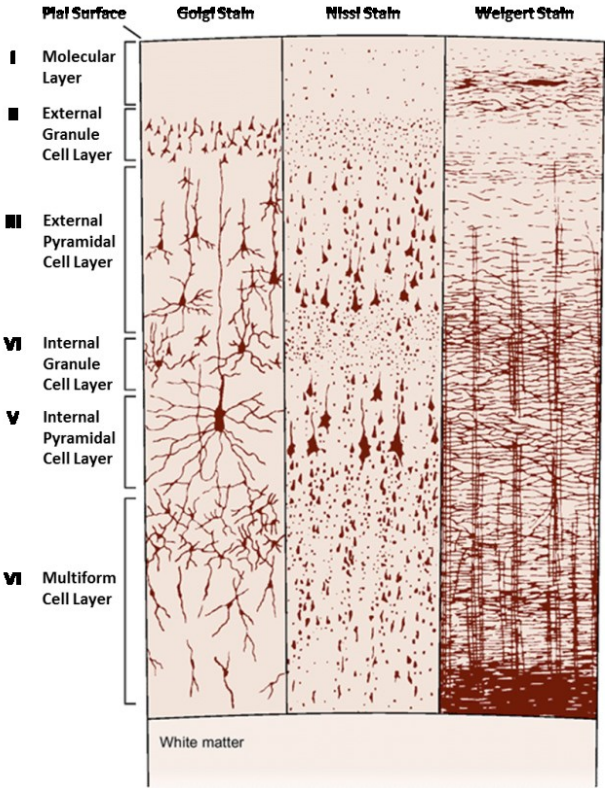


Figure 2.3. Six Layer structure of the cerebral cortex.

Adapted from **[8]**.

The neurons distributed along the structure have been identified as *projection neurons* and *local interneurons*. Layers III, V and VI present projection neurons, characterized by a pyramidal shape cell body and the use of glutamate as a primary transmitter (excitatory aminoacid). Local interneurons are GABA-ergic neurons that make use of the inhibitory neurotransmitter amino-butyric acid (GABA), being present in all layers and representing 20-25% of the total number of neurons.

The different layers represented on figure 2.3 have been termed not only by the type of neurons present but also by its extra constituent elements. The outer layer of the cerebral cortex, *Layer I* has been defined as *molecular layer*, consisting in dendrites of deeper cells and axons. *Layer II*, *external granule cell layer* contains granule cells (small spherical cells). As the previous, *Layer III* is also named according to its composition and position, as *external pyramidal cell layer*. Pyramidally shaped neurons are the type of neurons featured in this layer. *Layer IV* is defined as *internal granule cell layer* named by the presence of granule cells, as layer II. *Layer V,* as layer III contains pyramidally shaped neurons, glutamatergic neurons, as referred before. Therefore it is defined as *internal pyramidal cell layer*. At least the inner layer, *Layer VI* termed *polymorphic or multiform layer* is constituted by different types of neurons being the border of axons between to and from the cortex.

Each layer of the cerebral cortex receives or sends information for different regions of the brain or the human body. The organization of the cerebral cortex in a stratified structure allows a higher performance and processing of information, increasing the efficiency of the inputs-outputs, which would not be expected on an unorganized frame.

2.2.2. Neuritogenesis

During neuronal development, a neuron forms two different types of processes, multiple dendrites and a single axon. Besides the morphological differences, these two different structures differ on the composition and structural components.

The different stages that comprise this process have been documented using different neuronal cell types. The following study used rat embryonic hippocampal neurons, represented below on figure 2.4.

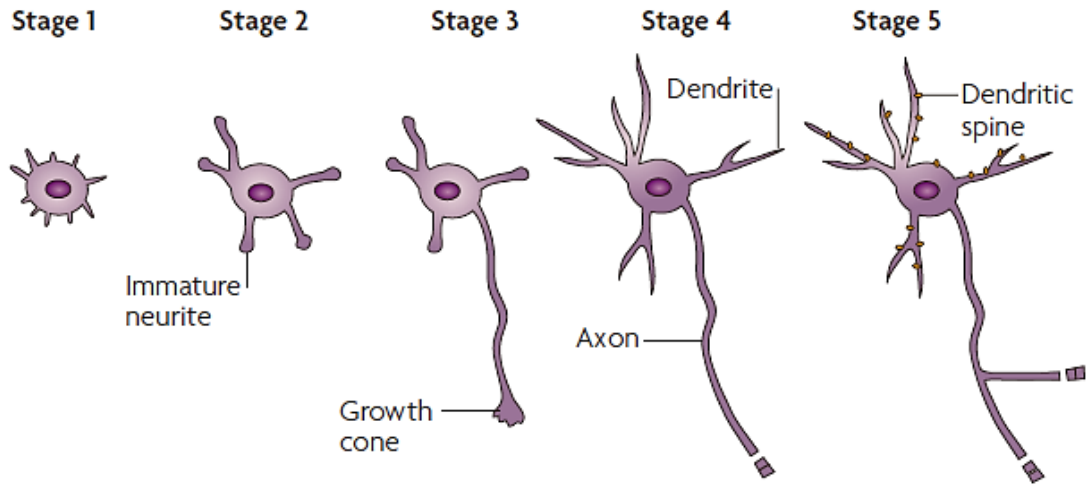


Figure 2.4. Neuronal development. Morphological changes of rat embryonic hippocampal neurons in culture. At least 7 days are needed to reach stage 5. *Adapted from* [9].

Immediately after being plated, little protrusions named lamellipodia attach the cell to the substratum, surrounding the cell and completing **stage 1**. **Stage 2** is defined by the elongation of these little projections that are now defined as immature neurites, extending to a maximum length of approximately 20μm. At this point the neurite growing process is represented by neurite elongation and retraction, with no significant difference between neurites. After 24 hours, one of the neurites starts to elongate faster than the others that remain steady. This leads to the formation of the axon and the polarization of the cell, **stage 3.** The following stage, **stage 4**, is marked by neurite maturation, which develop into dendrites along with axon elongation. Finally neuronal connectivity is possible with the formation of dendritic spines at **stage 5** [10].

2.3. Schizophrenia

The society has been carrying the burden of mental disorders for centuries. Since the ancient ages, symptoms associated with psychiatric diseases have been terrifying human beings. Mental disorders such as Schizophrenia were previously associated to popular beliefs, or as a result of possession or gods' punishment for immoral behaviour. Schizophrenia symptomatology has been documented over the time, from the Stone Age until the present [11].

Besides the complexity of the disorder, the history of Schizophrenia is not linear. The 19th Century observed an emergence in mental health development, and in 1878 during studies with young adults suffering from dementia, Emil Kraepelin named the disorder as "*dementia praecox*" (early dementia), characterized by a decline of cognitive process.

However it was only in the 20th Century that Eugen Bleuler first defined the term "Schizophrenia". In 1911 Bleuler defined Schizophrenia as the four "***A's***", flattened **A**ffect, **A**utism, impaired **A**ssociation of ideas and **A**mbivalence [12]. Over the years researchers have attempted to classify the disorder but the complexity and unknown etiology of Schizophrenia causes a constant revision of the concept.

Nowadays it is defined as a chronic, neurodevelopmental disorder affecting 1% of the world's population [13]. Schizophrenia is a syndrome, diagnosed by the clinical observation of the signs and symptoms of the patient, because of its unknown cause.

2.3.1. Etiology and pathophysiology

Schizophrenia is a heritable developmental disorder with an estimated heritability as high as 80% and it manifests in late adolescence or early adulthood with a higher lifetime risk of developing the disease for males than for females [14]. It is not only devastating for the patient that experience a lifetime of disability and emotional distress that in worst scenarios can lead to suicide (10%), but also for the family since these individuals are overrepresented

among homeless, unemployed, unmarried, socially isolated and chronically hospitalized. It is estimated that 40% of schizophrenics suffer from substance abuse, 20% are homeless and 10% commit suicide as previously referred [15].

Several approaches have been used to investigate Schizophrenia, although its aetiology is unknown. Pharmacological, magnetic resonance brain imaging and post-mortem studies have revealed a decrease in brain volume, aberrant neurotransmitter signaling, reduced dendritic arborization and impaired myelination in the brains of schizophrenic patients.

Observations from brain imaging of first-episode patients registered decrease on the whole brain volume mainly in the gray matter of the hippocampus, basal ganglia and thalamus, and increased ventricular volume [16]. On chronically ill individuals the frontal and temporal gray matter areas of the cortex registered the most significant volume deficits, but whether the deficits are due to the disease or the long-term antipsychotic treatment remains unclear.

Moreover functional MRI tests have detected cortical hyperactivity and hyperconnectivity between the cortex and hippocampus in SCZ patients at rest, in contrast to a reduced activation of the cortex during working memory tasks [15].

Despite the investigations that surround this disorder, its treatment is lacking since the aetiology and neurobiological systems that explain the disease remains poorly understood.

The discovery of the psychosis-inducing effects of dopamine-releasing drugs, such as amphetamine, triggered the emergence of the “dopamine (DA) hypothesis” of SCZD. This hypothesis proposes that an excessive activation of D2 receptors caused the positive symptoms of Schizophrenia. Anti-psychotics and later atypical anti-psychotics were introduced as a form of treatment since they allowed the blockage of the D2 dopamine receptor. This form of treatment focused on the dopamine hypothesis reduced the positive effects (delusions and hallucinations) but failed on the improvement of the cognitive deficits. Therefore more recently, the treatment of Schizophrenia has focused on the cognitive symptoms leading to the hypothesis of Schizophrenia as a ”glutamate disorder” which explains that the cognitive deficits may result from the low activity of the NDMA receptor on GABA interneurons in the prefrontal cortex [13].

Therefore, in order to develop more effective treatments for Schizophrenia, it is important to understand the implications of the large number of genetic liabilities associated with Schizophrenia and the developmental pathways that are disrupted.

2.3.2. Diagnosis

Schizophrenia is a devastating and disabling mental disorder affecting 1% of the world's population[17]. It affects the way people think, interact with others and connect to the world. This brain disorder is different from person to person and persists in cycles (relapse and remission phases), with psychosis as the hallmark of Schizophrenia.

Self-portraits reflect people's inside, the way they think and connect to the world, which have been used by clinicians as a way to understand their patients, namely psychotic individuals.

Figure 2.5. Schizophrenic self- portrait of a female 19 year old patient [18].

The features detected in clinics which allow the diagnosis of Schizophrenia are divided into three categories, **positive symptoms**, **negative symptoms** and **cognitive symptoms**. However, positive symptoms comprise the most representative clinical feature used for the performance of the diagnosis.

Positive symptoms, also referred as "*psychotic symptoms*", include delusions (false beliefs), hallucinations, paranoia and psychosis, normally expressed as hearing voices; whereas negative symptoms comprises symptoms reflecting a disruption if normal emotions and behaviors, as flattened affect, impaired attention, social withdrawal, alogia (poverty in the amount of speech) and anhedonia (reduced capacity to experience pleasure) [14]. Cognitive impairment is expressed by deficits in attention, memory, planning and social-emotional processing [19]. As well as negative symptoms, this last category is harder to recognize by clinicians. However they are considered to be a leading cause of the disorder.

The diagnosis of Schizophrenia is performed trough a diagnostic criteria, since until now no pathogenic symptoms were found for an accurate diagnosis of the disorder. The criteria are fulfilled if the patient presents two of the following symptoms, according to DSM-5 (*Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*)[20]:

- Delusions
- Hallucinations
- Disorganized speech
    - Disorganized or catatonic behaviour
    - Negative symptoms

Moreover, delusions, hallucinations or disorganized speech must be one of the experienced symptoms; and continuous disturbances must be present for or least 6 months, having one month with active symptoms.

2.3.3. Current Therapeutics

Nowadays the etiology of Schizophrenia is still unclear and to be revealed. Therefore the treatments applied are based on the symptomatology of the patient. These include antipsychotic medication and psychosocial treatments that focus on eliminating the symptoms of the disease.

The *first generation* antipsychotics also called *typical antipsychotics* are dopamine D2 antagonists and include different drugs that exert also an effect on other receptors, such as serotonin type 2 (5-HT2), alpha1, histaminic, and muscarinic. This first class of antipsychotics present a high rate of extrapyramidal side effects (rigidity, bradykinesia, dystonias, tremor, and akathisia). [21] Chlorpromazine (Thorazine) was the first antipsychotic introduced in the treatment of SZ. [22] Moreover Haloperidol (Haldol), Perphenazine (Etrafon, Trilafon) and Fluphenazine (Prolixin) are some other *typical antipsychotics* commonly used to overtake the positive and negative symptoms.

The *second generation* or a*typical antipsychotics* introduced on the 1990s helped to surpass the side effects caused by the first drugs designed. Despite being dopamine D2 antagonists, present lower rates of extrapyramidal adverse effects. This class comprises several drugs, including Clozapine (Clozaril), Asenapine (Saphris), Iloperidone (Fanapt), Lurasidone (Latuda), Olanzapine (Zyprexa), Paliperidone (Invega), Quetiapine (Seroquel), Risperidone (Risperdal), Ziprasidone (Geodon) and Cariprazine (Vraylar). However they show higher rates of metabolic adverse effects and weight gain.

Recently another class has been emerging, *Serotonin-dopamine activity modulators (SDAMs),* which embraces Aripiprazole (Abilify, Abilify Maintena, Aristada) and Brexpiprazole (Rexulti). [21] These new forms of treatment approved by FDA in 2014 and 2015, respectively, are characterized to act as antagonist at 5-HT2A and noradrenaline alpha1B/2C receptors, and as partial agonist at 5-HT1A and dopamine D2 receptors. [21]

Schizophrenia treatment with antipsychotics attenuates patients' positive and negative symptoms. It includes two main phases: an acute and a maintenance phase (life-long). The first characterized by high doses, followed by a reduction of the dosage on the maintenance phase. A relapse requires the increase of the drug concentration, which is then reduced to the minimum required to prevent the experience of further episodes/relapses. Moreover different medications need to be tested in order to find the best treatment and dosage, since not all patients respond in the same way.

Despite the medication given to schizophrenic patients, different approaches are also used aiming the integration of these individuals in society and to live in community. Psychosocial treatments help schizophrenics with their everyday challenges that are mainly caused by the disease, such as difficulty with communication, self-care, work, and forming and keeping relationships.[22] Furthermore the understanding of family members about the disorder in order to help their loved one and to learn strategies and problem-solving skills is of extreme importance (family education), namely to help with the treatment and to avoid relapses.[22] As previously mentioned, nowadays, SZ treatment is focused on both positive and negative symptoms. Despite not being as perceptive as other symptoms (hallucinations and delusions), cognitive impairment interferes with the capacity to work, to socialize and to live independently. The cognitive remediation includes several forms of therapy such as cognitive rehabilitation, cognitive enhancement, or metacognitive therapy that work on the idea that the brain is plastic and that neurocircuitry can be improved.[23] Vocational rehabilitation and assertive community treatment represent another two models of intervention that help schizophrenic patients on fighting the disease.[23]

2.3.4. Neurodevelopmental disorders

Neurodevelopmental disorders, such as Schizophrenia, are caused by a wide range of factors, including biological (cellular and molecular), as well as environmental factors. These disorders share the fact that the disease onset occurs during the periods of ongoing maturation and development that results in alterations of the developing Central Nervous System (CNS) leading to an abnormal neural development and brain function. The complex genetic etiologies and varying environmental effects that contribute to these disorders demand proper and relevant model systems to further the study of these diseases and for the development of therapeutics. The study of these mechanisms in patients or animal models is difficult because of its variety, but the use of cell-based models namely human cell-based can be ideal experimental paradigms to investigate the disease [1].

2.3.5. Candidate genes

Twin and adoption studies have demonstrated a substantial familial aggregation in Schizophrenia. For instance the risk of illness is higher in monozygotic twins than in dizygotic. However some studies have shown that the risk of developing the disorder is an interaction between environmental and genetic factors, for e.g. about 11% of twin studies indicate a strong environmental influence on the development of SCZD [24].

The current leading candidate genes of Schizophrenia encode proteins with a multifaceted role in the nervous system. Its structure is known for its complexity and the encoded proteins have extensive and complicated interactions with other molecules. There have been

innumerous investigations in order to identify the genetic risk factors for Schizophrenia, such as family based linkage studies, candidate gene association studies which consist of biological hypotheses that led to the discovery of rare variants such as 22q11.2 deletion [25] and a chromosomal translocation in chromosome 11 disrupting the DISC1 gene in a Scottish family [26].

On other hand genome wide association studies (GWAS) have made interesting progress on identifying alleles and genomic regions not previously associated with the illness. This powerful technique is a DNA-based, hypothesis-free method which analyses genetic variation between individuals in a large population, being capable of uncover single-nucleotide polymorphisms (SNPs), micro-deletions and copy number variations (CNVs) related to a particular condition [27].

Despite the inconsistent findings there are some candidate genes for Schizophrenia as neuregulin 1 (NRG1), disrupted-in-Schizophrenia 1 (DISC1), and dystrobrevin binding protein 1 (DTNBP1). It is hoped that genome wide association studies will clarify the existing studies regarding the genetics of Schizophrenia.

2.3.6. Importance of dendrites and synapses

The complexity of neuronal network allows the performance of brain functions including cognitive behaviour, learning, memory and social behaviour. The communication of neurons in the neural circuitry is possible by dendrite morphogenesis which involves the development of dendrite branching, dendrite arbors and dendrite spines during neurodevelopment. Central nervous system diseases namely neurodevelopmental disorders (NDDs) such as Schizophrenia are characterized by the presence of neural circuitry defects that has been seen as a launch pad for novel therapeutics [28].

Dendrites play a key role in brain function, helping in the process of receiving information and to generate appropriate responses. During neurodevelopment and in adulthood the brain suffers different network alterations that highlight its plasticity and dynamics, such as changes in dendrite branching, spine number and morphology [29].

The number of dendrites and the pattern formed by their extension is intrinsically associated with the management and itinerary of the information in the nervous system. This information is processed by neuron-to-neuron communication through junctions called synapses. Since dendrite branching and synapse formation are essential in the structural and functional plasticity of the brain, defects in the neuronal circuits significantly contribute to structural and functional deficits observed in NDDs as Schizophrenia [30].

Neuroimaging and post-mortem studies have demonstrated a decrease in volume of cerebral cortex in schizophrenic patients [31]. Alterations in connectivity and in dorsolateral prefrontal cortex (DLPFC) involved with working memory tasks is evident in schizophrenic patients, which may explain the functional deficits in working memory suffered by these individuals, and supports the hypothesis of Schizophrenia as a disorder of altered

connectivity. However these alterations in neuronal circuitry and the reduction in brain volume are not attributed to a decrease in the total number of neurons but to abnormalities of pyramidal neurons including smaller somal volumes, decreased dendritic arbor size and branching and reduced dendritic spine density, represented below on figure 2.6 [32].

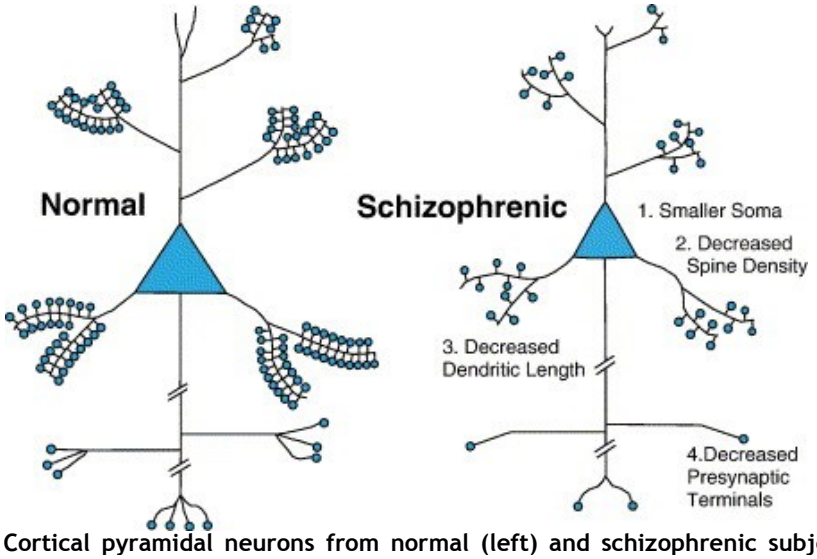


Figure 2.6. Cortical pyramidal neurons from normal (left) and schizophrenic subjects (right). Differences in soma volume (1), spine density (2), dendritic length (3) and presynaptic markers (4) [33].

The pathologies of DLPFC circuitry implicate different components. Pyramidal neurons that represent 75% of cortical neurons, interneurons, which comprise 25% and axons from neurons that project to the dorsolateral prefrontal cortex, neurons in the thalamus and from DA-containing neurons in the mesencephalon innervate targets in the DLPFC [34].

In DLPFC the spine loss occurs particularly in layer 3 pyramidal neurons (figure 2.7). Since the total number is not altered, the density reduction is explained by an increase of space between neurons, caused by lower axon terminals and spines per neuron.

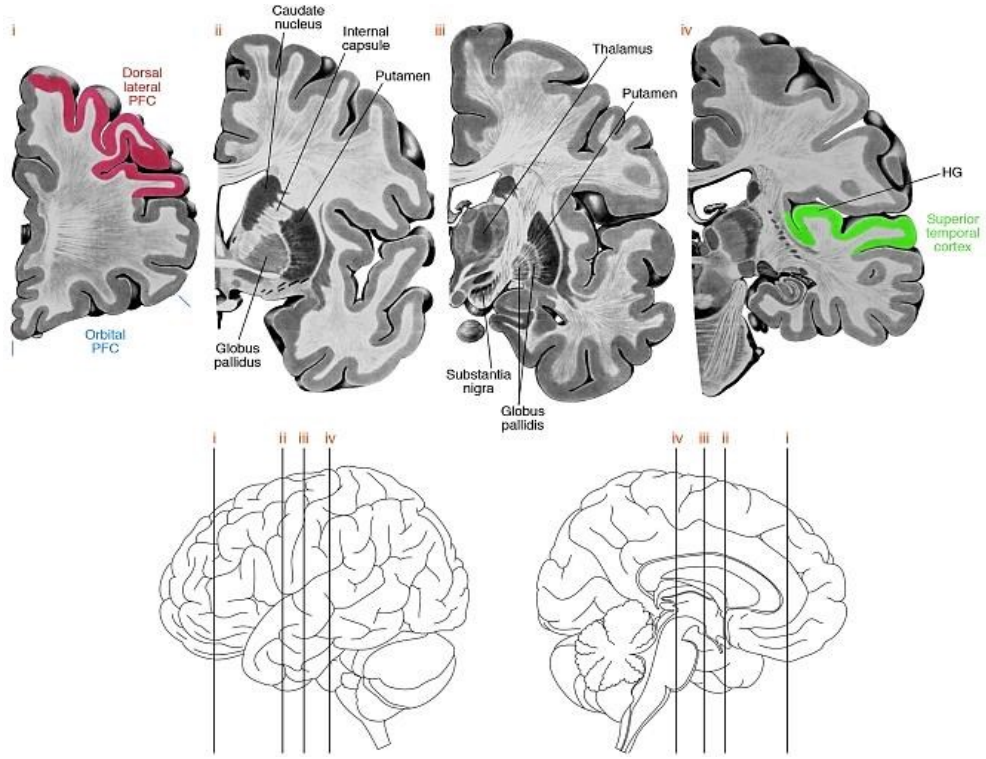


Figure 2.7. Regions involved in neural circuitry disturbances in Schizophrenia. [34]

The hypothesis of Schizophrenia as a neurodevelopmental disorder also supports these alterations in connectivity. Therefore the molecular mechanisms behind these abnormalities are under investigation, and have implicated the expression of proteins that regulate spine size and maintenance. The next page presents a table (table 2.1) that summarizes important factors involved in the regulation of dendritic morphogenesis, the function and its specific role.

Table 2.1. Regulation of dendritic morphogenesis by different factors/molecules.

Factor	*Function*	*Role in dendrite morphogenesis*
Secreted Factors, Cell Surface Receptors and Cell adhesion molecules		
BDNF	Neurotrophic factor	Increases dendrite branching [35]
Reelin	Extracellular matrix glycoprotein	Increases dendritogenesis [36]
Wnt family	Secreted glycoproteins	Promotes dendrite complexity [37]
Ephrins and Eph receptors	Receptor tyrosine kinases and their ligands	Promotes dendrite arborization [38]
Semaphorins	Secreted and transmembrane proteins	Initial step of neuronal polarization and promotes the origin of dendrites, controls dendrite bifurcation and complexity of basal dendrites [39]
Notch	Cell surface receptor	Promotes dendritic complexity[40]
Cadherin-catenin cell adhesion complex	Cell adhesion complex, participates in signalling and regulation of actin cytoskeleton	N-cadherin, δ-catenin,p120ctn and β-catenin promote dendritic branching[41]
Synaptic Scaffolding Proteins And Regulators		
PSD95	Postsynaptic density scaffolding protein	Stop signal for proximal dendrite branching [42]
Cypin	Protein that binds to the PDZ domains of PSD-95 and decreases localization of PSD-95 at the postsynaptic density	Promotes dendritic branching[43]
LAP family of proteins	Densin-180 and Erbin— postsynaptic density proteins	Promotes dendritic branching (Shank, δ-catenin)[44]
Regulators of the cytoskeleton		
Rac/Rho/Cdc42	Small GTP binding proteins	Activation of Rac and Cdc42 promote the extension of neurites, while activation of Rho mediates the retraction of neurites[45-47]

2.3.7. Dendritic spines

As referred previously, dendritic spines are key interveners in the neuronal circuitry and communication neuron-to-neuron. Dysfunctions in these structures have been documented and linked to neurodevelopmental disorders. Therefore, the following topic introduces important notions about this structure, necessary for forward concepts and for a better understanding of its role in modulating connectivity.

Dendritic spines are described as little protrusions presented along neuron's dendrites. These structures present a "mushroom" shape, formed by a neck and followed by a spine head. The complexity of the post-synaptic density structure and its constituent elements are still being investigated. However, as represented on figure 2.8, is known that dendritic spines are actin-rich, presenting at the post-synaptic density, neurotransmitter receptors as glutamate receptors, scaffold proteins (e.g. PSD-95) and signalling proteins as small GTPases.

The cytoskeleton of dendritic spines is primarily composed of actin, allowing the dynamics of spines shape and size [48, 49]. Therefore changes in the actin cytoskeleton directly determine spine structure and morphology. Different stimuli and signalling pathways can be involved and induce the formation, elimination and alterations of these protrusions. A type of G-proteins involved in this regulation, are small-GTPases, which have the ability to regulate gene transcription and the dynamics of the cytoskeleton. Small-GTPases can be divided into five different families: Ras, Rho, Rab, Sar1/Arf and Ran; and binding to GDP and GTP regulate different cellular processes, according to the type of cell and small-GTPases family. Rho- and Ras- family are important candidates to control neuronal functions, by their ability to regulate cytoskeleton morphology and gene transcription [50].

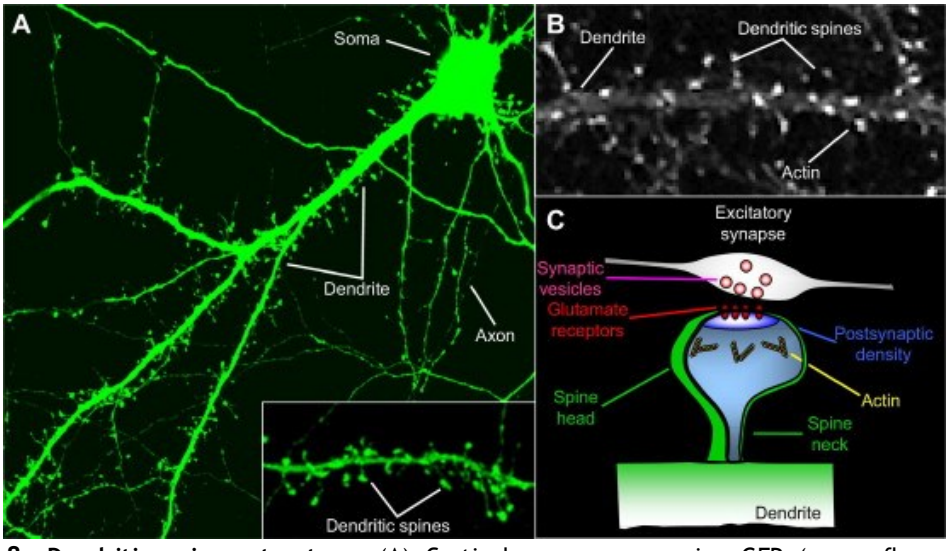


Figure 2.8. Dendritic spines structure. (A) Cortical neuron expressing GFP (green fluorescence protein). Below a higher magnification of the dendritic spines is shown. (B) Immunofluorescence image of a dendrite cortical neuron stained for phalloidin (marker for β-actin) (C) Scheme representing a mature dendritic spine in contact with an axon. [49]

2.4. Estrogens

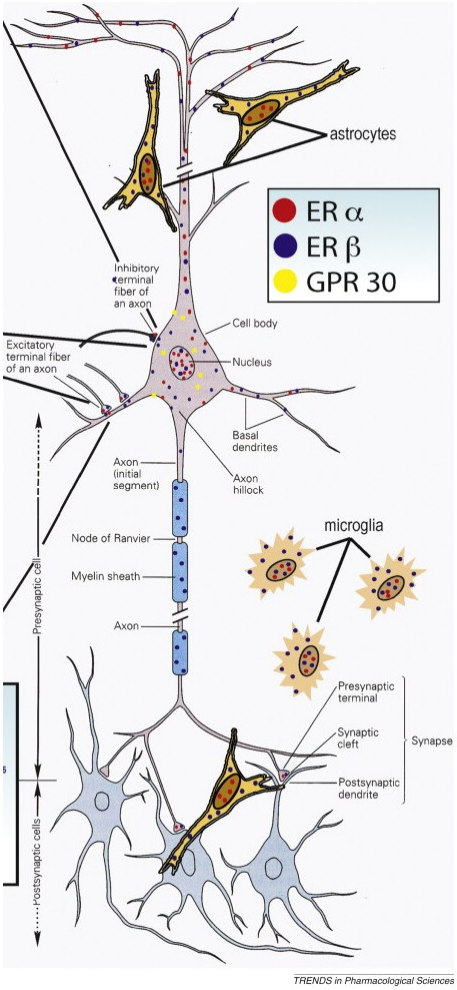
The influence of steroid hormones, including estrogens have been object of innumerous studies, for having influence in a wide range of physiological functions, from reproduction to development and cardiovascular functions, to its activity in the nervous system. These compounds are a class of steroid hormones, of which 17β-estradiol is the most representative and biologically active form[50]. Estrogens have been reported to exert effects in different areas of the brain such as the hippocampus and cerebral cortex, modulating cognitive and memory functions in both animal models and humans.

The following chapter will focus on estrogen role in the nervous system, the molecular mechanisms involved in its activity, the evidence of estrogens as a neurosteroid and neuromodulator and the resulting potential in treatment of psychiatric disorders as Schizophrenia.

2.4.1. Role of estrogens in the cortex

Despite our lack of understanding the precise molecular mechanisms that explain estrogen's effects on the synaptic plasticity, several findings have demonstrated estrogen's influence in different areas of the brain, modulating cognitive function within minutes to hours.

Estrogen receptors ERα, ERβ and GPER1 (a G protein-coupled ER 1 or GPR30) are expressed in different areas of the brain, and are present in different cell-types, including neurons, astrocytes, oligodendrocytes and microglia (figure 2.9) [51]. ERs can be included in the nucleus, cytoplasm and membrane subtypes and may be associated with organelles (mitochondria, synaptic vesicles and dendritic spines) [51]. However it is important to note that GPER1 is an estrogen sensitive receptor and can be activated by multiple agonists, consequently having a more complex pharmacology.



gure 2.9. Expression of ERs in different cell types. *Adapted from* [51].

The ER subtypes are placed in distinct regions of the brain and lead to different responses in the neuronal circuitry. The expression of ERα is higher in the hypothalamus compared to ERß, and is present in the mammalian forebrain. On the other hand ERß is highly expressed in the cortex and hippocampus [50].

Classic estrogen receptors can be divided into two different subtypes, ERα and ERß. The structure includes an N-terminal domain (defined as "A/B domain"), a conserved DNA-binding domain (with two Cys4 zinc fingers and defined as "C domain"), a C-terminal ligand-binding (the "E domain") and finally a C-terminal ("F domain"), figure 2.10. The subtypes present 97% and 59% homology, at the DNA-binding domain and ligand-binding domain, respectively.

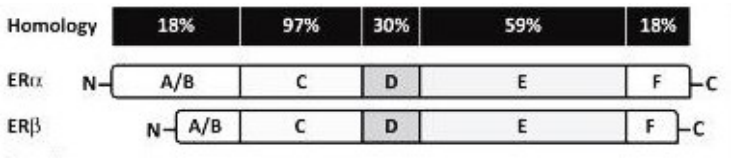


Figure 2.10. Domain structure of the human ERα and ERß estrogen receptors, and homology (%) between the two subtypes. *Adapted from* [52].

As previously mentioned, estrogens have been reported to exert actions in the brain. Several studies have documented the rapid influence on cognitive functions, which relies on cortical and PFC (prefrontal cortex) processes. For instance, the production of estrogens in the cortex of zebra finch song birds allowed social behaviours, and in rodent models, estrogen administration enhanced object recognition [53, 54]. These studies highlight the potential application of estrogens in modulating cognitive symptoms in Schizophrenia.

Moreover aromatase, an enzyme that converts androgens into estrogens, shows significant expression in response to stimuli in the hippocampus and cortex, in rodents, non-human primates and humans. The presence of this enzyme at pre-synaptic terminals places the synthesis of estrogens at the ideal location to act at the post-synaptic structures. Indeed, previous studies indicate that this *"de novo"* synthesis of estrogens in the brain can enhance the modulation of neuronal connectivity, and that aromatase inhibition leads to a loss of dendritic spines in cultured cortical neurons [55].

2.4.2. Signalling cascades

As explained in Chapter 2 (*2.3.5. - Importance of dendrites and synapses*), the communication and processing of information in the brain is performed through neuron-to-neuron communication at synapses. These structures are manly located at dendritic spines, which may change their morphology in response to different stimuli (neuromodulators) and thereby lead to alterations of the neuronal network. Therefore, it is important to study the

mechanism underlying estrogen's effects on neuromodulation and synaptic plasticity and its potential use to as a therapy for neurodevelopmental disorders. The following chapter will then focus on the signalling cascades involved in estrogens action in the nervous system, in order to uncover the processes that underlie estrogens' activity.

2.4.3. Rapid Modulation of neuronal connectivity

Estrogen exerts its effects either by long-term actions or by a rapid modulation of neuronal connectivity. The canonical concept considers estrogens as endocrine signals, mediated by the regulation of gene transcription, resulting in long-term effects that can take hours to days to manifest. The study of the rapid modulation of neuronal connectivity has been increasing in recent years. These studies examine the involvement of estrogen receptors in intracellular signalling cascades in different areas of the brain, and hypothesize that ERs are located in or associated with the plasma membrane. The rapid activity of estrogens will be discussed in more detail, regarding their potential use as treatment in neurodevelopmental disorders, and as the key of this thesis.

The rapid activation of membrane-signalling cascades can be triggered by estrogens either directly or by the transactivation of other receptors. It is still unknown which ERs activate this process and if its localization is integrated or associated with the plasma membrane. In order to address these questions, different studies have been performed.

Several studies have implicated the rapid initiation of membrane-signalling pathways by 17ß-estradiol treatment of neurons, resulting in memory processing enhancement. The signalling pathways include the extracellular signal-regulated kinase (ERK) pathway, the phospholipase C (PLC) pathway, Protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K)/Akt or Protein kinase B (PKB) and Protein kinase A (PKA) pathway. The administration of 17ß-estradiol in an ovariectomized young and middle age mice enhanced object recognition tasks, activating ERK1/2 and PI3K/Akt. The inhibition of these kinases resulted in no improvement of object recognition.

Besides the classic ERs, ERα and ERß and GPER1, some sensitive ERs have been documented, which may explain the different signalling profiles in varying brain regions activated by estrogens. This includes the cell surface signalling molecules ERX and STX-sensitive Gq-membrane estrogen receptor (Gq-mER) [56]. Moreover, different isoforms of ERs can be expressed in different organelles within a cell, thus distinct signalling mechanisms may be activated [56, 57].

Regarding the localization of ERs, the use of membrane impermeable estrogen have supported the hypothesis of 17ß-estradiol binding to the plasma membrane of neurons, and then initiating the signalling cascades. An example is the use of BSA-FITC-17ß-estradiol, that resulted in an increase of $Ca2+$ levels and phosphorylation of ERK1/2, demonstrating the bonding to extracellular sites. Additionally, in ovariectomized mice 17ß-estradiol activates ERK1/2 signalling resulting in the enhancement of object recognition. The binding of 17ß-

estradiol to the plasma membrane supports the presence of ER at the cell surface. However it is believed that the classic ERs, ERα and ERβ, do not contain hydrophobic residues/motifs that could enable its presence at the plasma membrane. As such, the capacity of these receptors to enable this localization is still unclear.

At the membrane, ERs can modulate rapid signalling cascades directly or through other receptors. As shown in figure 2.11, several mechanisms are thought to be implicated in this process. The classic ERs, as represented below, are considered to interact with caveolin proteins and or with scaffold proteins, in order to initiate the membrane signalling cascades.

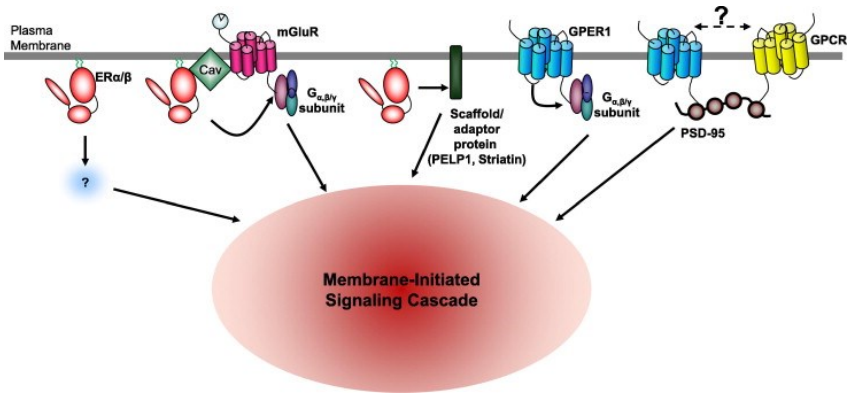


Figure 2.11. Mechanisms involved in rapid estrogen modulation of membrane-signalling cascades [49].

ERα and ERβ have been demonstrated to couple with mGluRs (metabotropic glutamate receptors) independent of glutamate.

A study using hippocampal neurons of female rats reported that after stimulation with 17β-estradiol, ERα triggered mGlu1a signalling, leading to Gq-mediated stimulation of PLC, PKC and inositol triphosphate (IP3) and to ERK1/2 dependent CREB (cAMP response element-binding protein) phosphorylation. ERα and mGlu1a interaction required the expression of caveolin-1. Moreover both ERα and ERβ activated mGlu2, decreasing Gi/o-coupled in cAMP, reducing L-type calcium channel-dependent CREB phosphorylation, which is essential for the expression of caveolin-3.The protein complexes formed by ERα and ERβ with caveolin-1 and mGlu1a, have been implicated in enhancing object recognition. Additionally GPER1 has been shown to be able to associate with other GPCRs, and to interact to TrKB receptors [58, 59].

The initiation of signalling cascades have also been documented to be triggered by ER association with scaffold proteins. The PELP1/MNAR, proline-, glutamic acid-, -leucine rich protein (PELP)1 or modulator of non-genomic actions of estrogen receptor (MNAR) have been shown to associate with ERα, ERβ as well as GPER1 to mediate membrane-signalling pathways [60]. These findings were shown in breast cancer cells and its role in neurons is still unclear. Another scaffold protein involved in membrane-signalling initiation by estrogens is striatin (also represented in figure 2.11). Striatin has been localized at the cortex and striatum (subcortical part of the forebrain), more specifically at the synapses [61]. It is expressed at

the dendritic spines, which binding to caveolin-1 may have influence in rapid ER induced signalling.

The localization of GPER1 is controverisal. As a 7-transmembrane protein, some studies reported the localization of this receptor not only at the plasma membrane, but also at the Golgi apparatus, endoplasmic reticulum, mitochondrial membranes and microtubules [62, 63]. In order for GPER1 to initiate signalling, this GPCR couples to Gα and β/γ subunits (seen in figure 2.11 above) when localized either at the membrane or at intracellular sites, by activation of 17β-estradiol that has the ability to pass through the membrane.

2.4.4. Estrogens modulation of dendritic spines

Dendritic spines, as introduced in chapter 2.3.7 are believed to be involved in memory and cognitive function, being a key intervener in brain circuitry. This structure, in response to stimuli, suffers alterations in shape/size, being formed or eliminated, and resulting in changes of communication between neurons.

The dynamics of dendritic spines, in response to estrogens, will be discussed below.

Depending on the brain region that is being treated, it has been suggested that 17-β estradiol modulates dendritic spines by the activation of distinct signaling cascades. As mentioned previously, the presence of ERs varies from tissue to tissue. Therefore the regulation of dendritic spines by estrogen stimulus is dependent on the estrogen receptor that is present at the specific area of the brain. The use of ERβ-selective agonist (WAY-200070) in rat cortical neurons demonstrated a NMDAR-independent effect in the formation of dendritic spines, contrary to what happens with hippocampal neurons, which depend on NMDAR activity when propyl pyrazole (PPT, ER-α selective agonist) is applied.

The increase in dendritic spine density by estrogen stimulation has been consistently documented. However it is still necessary to understand how the ERs are activated to lead to the modulation of the dendrites. Studies have revealed the involvement of 17-β estradiol in the spinogenesis modulation via distinct pathways, in both cortical and hippocampal neurons.

The stimulation of the dendritic spines of young cortical neurons has been linked to c-SRC/Rac1/Cdk5/WAVE1/Arp2/3 pathway and a RhoA/ROCK-2/moesin cascade. In more mature cortical neurons, 17-β estradiol induced filipodia has been related to the signaling via Rap/ERK/AF-6 pathway.

In hippocampal neurons spinogenesis by 17-β estradiol stimulation has been associated with the activation of p-LIMK/p-cofilin cascade that controls the polymerization of actin (thought to be performed via RhoA/ROCKdependent pathway).

Several studies have documented estrogens effects in hippocampus and cortex of rat and mice models. It has been reported that when stimulated by 17-β estradiol, cortical neurons transiently increase their number in dendritic spines, returning to baseline levels after (30 min in the cortex, and 2hours later in CA1 hippocampal neurons) [64, 65]. The newly formed

protrusions have been described to have a "thin" morphology (figure 2.12) and to be functional [50]. Its functionality could be explained since an overlap with pre-synaptic terminals was detected, which in non-functional are absent (no synaptic connections are formed).

Moreover, time-lapse images demonstrated that while the new formed spines resultant from the stimulus were preferentially eliminated at the end, pre-existent spines were not affected by 17-β estradiol, suggesting that the existent network is not affected [55].

Rapid activity dependent synaptic tuning has been implicated in the Long Term Potentiation (LTP) and Long Term Depression (LTD). [66] The rapid modulation of neuronal connectivity by 17-β estradiol has been shown to increase LTP, in particular. As described previously, the glutamate receptors AMPARs and NMDARs are essential in synaptic structure and plasticity.

Srivastava and coworkers have demonstrated that an acute treatment of rat cortical neurons with 17-β estradiol resulted in a transient removal of GluA1-containing AMPARs and insertion of GluN1 containing NMDARs from synapses, returning to the initial state after 60 min. [55] As represented on figure 2.12, this formation of "silent synapses" is developed by deletion of AMPARS, which is implicated in the modulation of neural circuits. In hippocampal neurons 17-β estradiol exert actions by mediation of distinct pathways, which is explained by the different signaling mechanisms existent in the different cells.

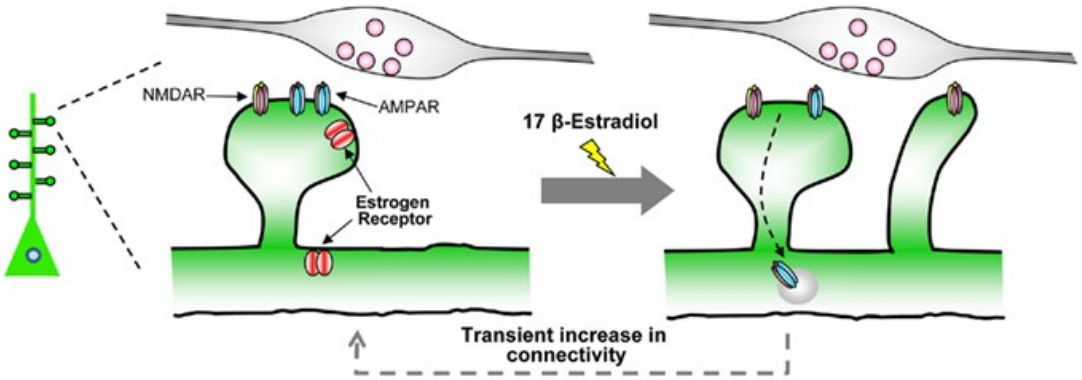


Figure 2.12. Transient increase in connectivity induced by 17-β estradiol stimulation [50].

Neuronal stimulation by estrogens seems to result in the formation, followed by elimination of transient spines, having almost no effects on pre-existing spines. It has been proposed that the rewiring of adult brain could be enhanced by strengthening existent synapses or by increasing the number of functional synaptic connections. Therefore the

question on how estrogens modulate and enhance neural function and cognition needs to be investigated further.

2.5. Endocannabinoid system

The endocannabinoid system is a neuromodulatory system that plays different roles in important physiological processes not only in the brain but also in other regions. This system has been associated to the pathophysiology of several diseases as neurodegenerative [67], psychiatric [68], stress-related conditions [69], pain and inflammation [70] and immunological disorders [71].

After being synthesised in response to different stimuli, endocannabinoids activate their specific targets and trigger biological responses depending on the cell type and tissue that are affecting [72]. The two types of cannabinoid receptors that have been identified so far are CB1R and CB2R [73, 74].

The endocannabinoid 2-Arachidonylglycerol, that will be referred as 2AG, is an agonist of the cannabinoid receptors CB1R and CB2R, and also activates the orphan receptor GPR55 [75]. The effects of these endocannabinoids in neurite outgrowth were studied, which can possibly suggest the presence of these receptors on hiPSC cortical neurons.

2.5.1. Therapeutic potential

Schizophrenia implicates genetic risk factors, involved in important developmental processes, from early stages to late stages of development. A prior knowledge of the risk genes, would mean that an effective treatment that targets the genetic predisposition disorder would be the best option. However this is not feasible at such an early stage of development. Therefore, a late stage of postnatal brain development (adolescence), where the brain is highly plastic and still in development is a more appealing phase to investigate novel therapeutics. The understanding of environmental stimuli allied with the genetic predisposition for the disorder would not only clarify its etiology and pathophysiology but would also help the development of new drugs.

During adolescence, cannabis abuse has consistently been linked to Schizophrenia [76], namely on individuals with the genetic predisposition for the disorder. Despite being involved in important cellular processes such as neural progenitor proliferation, neuronal migration, and axonal growth, the endocannabinoid system has also been implicated on the regulation of neurotransmitter systems, including glutamatergic, GABAergic, and dopaminergic synaptic functions. [77]

There is still some controversy regarding the acceptance of Schizophrenia has a dependent risk factor since the majority of individuals who consume cannabis to not develop

the disorder. Its use may contribute to the onset of SZ, however by itself does not lead to the development of Schizophrenia. Genetic risk factors, characteristic of the disorder, and/or other environmental risk factors need to be involved. Moreover a functional polymorphism of catechol-O-methyltransferase (COMT) that encodes a major dopamine degradation enzyme has been identified and associated with the influence of cannabis in psychosis.[78] Adding to the information given by genetic studies, post-mortem, neuroimaging and cerebrospinal fluid (CSF) have shown the involvement of the endocannabinoid system in SZ. Post-mortem studies have reported an increase of the cannabinoid receptor, CB1R, in different brain regions, as the cerebral cortex. [79] Magnetic resonance imaging studies performed on either cannabis users or cannabis-exposed schizophrenic patients, have reported a reduction in brain volume in regions rich in CB1R. Furthermore it has been verified that the use of cannabis during adolescence leads to a greater volume loss than in post-adolescence. [80]

Endocannabinoids regulate axon growth, coordinating a proper guidance and patterning for both GABAergic interneurons and pyramidal neurons during brain development. [81] The role of endocannabinoids in neuronal circuitry can be also observed on its influence in synaptic signalling. Endocannabinoids present a retrograde signalling function, by a suppression of neurotransmitters release at both excitatory and inhibitory synapses, via CB1R signalling. The release of endocannabinoids at post-synaptic regions is regulated by calcium influx with neuronal depolarization through different factors: NMDA receptor, voltage-dependent calcium channels (VDCC), and calcium release from intracellular stores. [82] As mentioned above, CB1R, is expressed in several regions that control cognition and emotion (the hippocampus, amygdala, and cerebral cortex). The influence of the eCBs on synaptic plasticity has been reported in short-term depression and long-term depression, for instances. [77] Moreover a co-treatment with antipsychotics in rodents, using a CB1R receptor antagonist (AVE1625), revealed an improvement of cognitive function and reduction of typical antipsychotics side effects.[83]

The eCBs plays important roles during brain development, namely at an embryonic and early stage. However it is at a postnatal phase that researchers have been focusing their attention in order to find new strategies to fight the disorder. This phase is targeted not only for being more practically feasible, but for being a critical period of plasticity and maturation of neurotransmitter systems and neuronal circuitry as a whole.

Following a few studies are presented (table 2.2) in order to show the potentialities of the endocannabinoid system as a potential therapeutic avenue of several diseases, namely Schizophrenia.

Diagnosis	*Outcomes*
Schizophrenia	CBD (Cannabidiol) enhanced anandamide signaling being as effective as amisulpride, a standard antipsychotic [84]
Schizophrenia	Cannabis does not cause psychosis by itself [85]
Bipolar disorders	Cannabis use was associated with better neurocognitive function, but the opposite the opposite occurred for schizophrenia subjects. [86]
Epilepsy	50 % reduction in seizures by the use of oral cannabis extracts, in 1/3 of children presenting different forms of eppilepsy. [87]
Cancer	Cannabis use "is perceived as highly effective" by some patients with advanced cancer. [88]

Table 2.2 - Clinical trials showing the potentialities of the eCB system has a novel therapeutic avenue

2.6. Schizophrenia "in a dish"

2.6.1. Stem cells

Early in development, the fertilized egg that gives rise to the entire organism has the ability to differentiate into all cell types, a characteristic known as totipotency (from Latin totus – "whole", "entire" and potentia – "ability", "power"). During development cells start to lose this ability, being labelled according to this capacity.

Stem cells are unspecialized cells capable of rewiring or producing new stem cells (through mitosis) that according to the environment where are inserted can differentiate into specialized cells with specific functions. These cells can be divided in two types, embryonic stem cells and adult stem cells. Embryonic stem cells are undifferentiated cells from a 3 to 5-day embryo that can divide for a long period of time, being capable of developing the tissues of the three germinative layers (ecto, endo and mesoderme). An adult stem cell can be found in a mature tissue among differentiated cells, acting as a repair system for example during injury and in disease. However, not only are they present in a very small number, their

ability to differentiate is limited since they can only specialize into the cell types of the tissue where they reside.

Pluripotent stem cells can differentiate into all cell types apart from cells of the amniotic sac and placenta, being present at the early stages of the embryo. Multipotent stem cells capacity to differentiate is limited, since they can only develop into a limited number of cell lines.

Not so long ago researchers believed that after a cell differentiation into a specific cell type their ability to originate another type of cell was lost. The researcher Conrad Hal Waddington compared the development of an organism and specifically of a single cell to the fall of a ball by the slope of a mountain (figure 2.13). The journey performed by the cell resulted in its differentiation into a specific cell, and a different route would lead to the formation of another cell type.

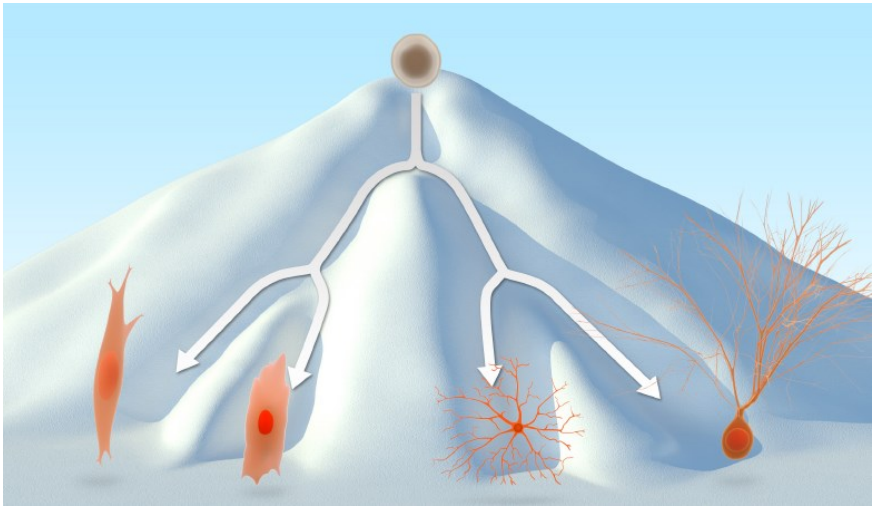


Figure 2.13. Conrad Hal Waddington comparison of the human body to a landscape [89].

2.6.2. Induced pluripotent stem cells (iPSCs)

In 1962, the embryologist Jonh B. Gurdon contradicted the previous theory of cell development in one straight direction, by its beliefs that all cells contain the genetic information necessary for an organism to form. Exploring his hypothesis, this researcher eliminated the nucleus of a frog's egg and replaced it by the nucleus of a somatic cell (specialized cell), from the intestine of a tadpole. Contrarily to the predictions of many, his experiment resulted not only in the formation of a tadpole but in the generation of a full-grown frog, demonstrating that the genetic material of mature cells have the same potential as embryonic cells. [90]

After 40 years Shinya Yamanaka, by studying the pluripotency of embryonic stem cells, found out that only 4 genes were required for a somatic cell (fibroblast in the case of his experiment) to return to its initial pluripotency that enables the formation of all cell types.

Identifying the genes that encoded the transcription factors required for the pluripotency of embryonic stem cells, Yamanaka and coworkers were able to demonstrate that specialized cells could be reprogrammed into pluripotent cells, named induced pluripotent stem cells (iPSCs). Discovering the genes required for fibroblasts to become pluripotent (Oct3/4, Sox2, Klf4 and c-Myc), he introduced these genes in the cell by retroviral transduction. The iPSCs formed were then injected into mouse embryos, attesting their similarity to embryonic stem cells (ESCs) [91].

In 2012 Sir Jonh B.Gurdon, together with Shinya Yamanaka were awarded the Nobel Prize in Physiology or Medicine, *"for the discovery that mature cells can be reprogrammed to become pluripotent"*[92].

The iPSCs technique introduces a wide range of possibilities, not only negating the need for embryos in the generation of embryonic stem cells but also for providing the opportunity for each individual to have their own pluripotent stem cells (figure 2.14).

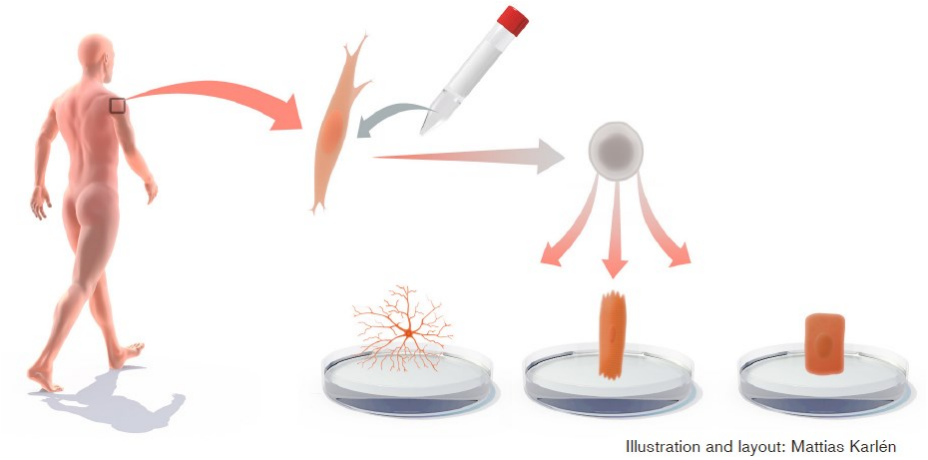


Figure 2.14. Illustration of the iPSCs ability to differentiate into different cell types [89].

2.6.3. Human induced Pluripotent Stem Cells (hiPSCs)

After Yamanaka discovery, the experiment was expanded to human cells, and in 2007, induced pluripotent stem cells were produced from a human fibroblast, leading to the generation of the first hiPSCs (human iPSCs) [93]. The hiPSCs were reprogramed from human somatic cells by transduction of the same transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) used on the mouse somatic cells, hiPSCs are able to differentiate into the cell types of the three germ layers.

Since then, numerous experiments have been performed to produce hiPSCs derived from mature adult cells of different cell types. Reprogramming somatic cells to iPSCs enabled innumerous applications, providing the opportunity to generate patient and disease-specific

stem cells to model human diseases, helping drug discovery, and enabling mechanistic studies. hiPSCs offer a new perspective of how things work without the drawbacks and ethical issues associated with the use of human embryonic stem cells, not to mention in the advantages of using the cells from the patient itself to study a certain condition.

In order to understand the pathophysiology of different disorders, animal models are used to simulate human conditions. Depending on the disease, after reprogramming the somatic cell, the resulting hiPSCs can be differentiated into the desired cell lineage.

Neurodevelopmental disorders have been studied for many years, but no significant advances in therapeutics have been achieved. Post-mortem studies have provided information regarding the abnormalities present in the brain circuitry, and animal models have helped to understand the mechanisms associated with the disease and the function of specific genes. However, the human brain features significant differences compared to rodents that result in cellular and molecular mechanisms different enough for the developed therapeutics not to be adequate for humans.

Despite providing important information for the understanding of psychiatric disorders, the possibility of studying the development and function of live human neurons is by far more attractive and has more potential than any model that could be possibly used. hiPSCs can be differentiated into neurons and glial cells, under appropriate growth factors and cell culture conditions for the study of neuronal development and function. Therefore with hiPSCs, it is possible to access patient's genetic information and elucidate the mutations associated with the disease and its severity. The differentiation of hiPSCs in neuronal progenitors can be achieved by the inhibition of the SMAD signalling pathway, which can be further differentiated into specific neuronal cells, such as pyramidal neurons, referred in the 2nd chapter as the most representative neurons in the cortex and the cells that will be used on the investigation of this thesis [94, 95].

2.6.3.1. Human neural development

Neuronal differentiation of hiPSC represents the *in vitro* phenomena that occur *in vivo* during the development of the embryo, throughout neural induction. The following segment will clarify the stages of human neural development and in vitro differentiation creating a bridge between both processes.

After being reprogrammed from somatic cell types, hiPSC demonstrates the pluripotency of the inner cell mass of the blastocyst. Therefore these cells have the ability to differentiate into the three germ layers, endo, meso and ectoderm.

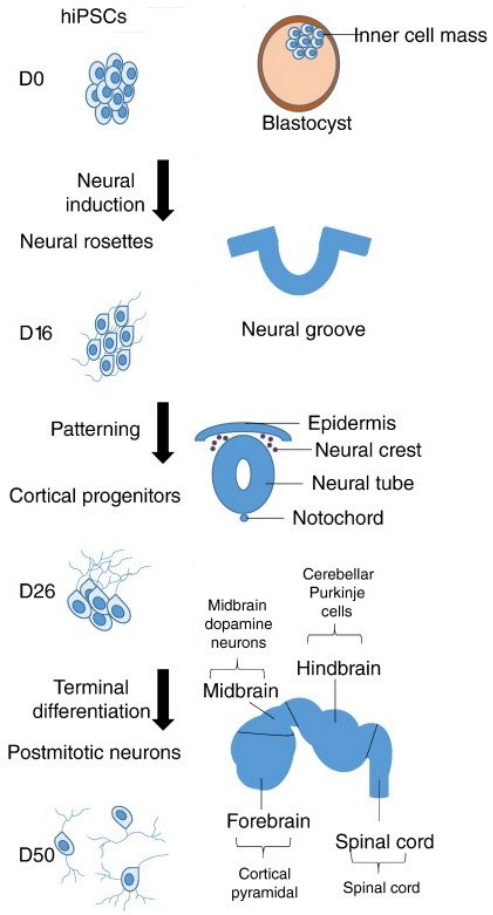


Figure 2.15. ***In vitro*** **differentiation of hiPSC and human neural development, analogy between processes.** *Adapted from* [96]

In human neural development, neural induction is triggered when the ectoderm is stimulated by the notochord, structure formed between the endo and the ectoderm. Signals diffuse from the notochord into the ectoderm, now called neuro-ectoderm, which leads to the development of the neural plate and then to the entire nervous system. The remaining ectoderm forms the epidermis. The bone morphogenetic proteins (BMPs), Wnt and Fibroblast growth factor (FGF) signalling pathways are believed to be associated to neural induction.

The neural plate progressively wraps itself forming the neural tube and the neural crest (neural plate borders) that will give origin to the peripheral nervous system. Morphogens as Wnts, FGFs, retinoic acid and Sonic Hedgehog (Shh) will allow the development and patterning of the primitive regions of the central nervous system: forebrain, midbrain, hindbrain and spinal cord.

Neural induction can be achieved by dual inhibition of the SMAD signalling pathway that includes the BMP inhibition and TGFß pathways. The differentiation of hiPSC into neuronal cells is therefore initiated by the induction of a neuroectoderm, and the formation of neural rosettes. Neural rosettes are morphological markers of neural differentiation, reminiscent of structures of the neural tube that present all properties of the neural plate [97]. Neural induction can be induced and differentiated into different populations of neural progenitors using various small molecule antagonists or endogenous inhibitors. The combination of several signals, specific to certain regions and cell types allows the differentiation into distinct neuronal cell types. However numerous limitations are associated to the direct differentiation of hiPSC, including cell culture of impure population of cells and differentiation efficiencies and divergences related to differentiation efficiencies, from protocol to protocol.

Chapter 3.

Materials and Methods

3.1. Cell Culture

3.1.1. hiPSC lines – reprogramming process

In this study four cell lines, derived from two healthy volunteers (two clones each) were considered (table 3.1). The hiPSC lines were generated and reprogrammed from keratinocytes of healthy volunteers, transduced by lentivirus expressing OCT4, SOX2, KLF4 AND C-MYC [98]. Quality control assays were performed to demonstrate the pluripotency of the iPSC cell lines ensuring the quality of the new reprogrammed lines [99].

The iPSCs cell lines were maintained in E8 medium in an humidified chamber at 37ºC (5% O_2, 5% CO_2), on geltrex coated plates. Medium was replaced every day and cell colonies were split with versene every 3-7 days when reaching optimal density. HBSS was used to rinse wells before passage. Further details about the different reagents used can be consulted on the annexes.

Table 3-1 - Cell lines included in the study. Control (CT) male (M) cell lines, M1 and M2. The last two digits identify the clone (e.g.: CT.M1 has two clones, CT.M1.11 and CT.M1.04 cell lines)

CT.M1	*CT.M2*
CT.M1.11	CT.M2.05
CT.M1.04	CT.M2.42

3.1.2. Neuralization Protocol

The four cell lines referred before on table 3.1 were differentiated for 30 days, involving three different stages as schematized on figure 3.1.

Neuralization of hiPSC was induced by dual inhibition of the SMAD signalling pathway. Cells were introduced to neuralization medium 50% N2 (Life Technologies, 17502-048): 50% B27 (Life Technologies, 17504-044) supplemented with SMADi (1µM dorsomorphin + 10µM SB431542) for 7 days and incubated at 37°C (5% CO_2; 20% O_2).

After formation of a uniform neuroepithelial sheet, reached at day 8, cells were carefully passaged (1:1) to 6 well plates in order to obtain large cell clusters. For this purpose cells were lifted using accutase, spun down (2x, 2min, 900rpm) and ressuspended in DMEM:F12, plated in N2:B27 **only** (no SMADi). Neuralization medium (N2:B27) was changed daily and cells were passaged at days 13 (np2) and 17 (np3), ascorbic acid (AA2P) was introduced from days 13 (np12) onwards. Reaching 100 % confluence, cryopreservation of neural progenitor cells (NPCs) was conducted, creating a stock of NP cell lines. Cells were gently thawed, plated on geltrex coated plates and cultured in neuralization medium N2:B27 containing bFGF. Medium was changed daily and cell passaging was performed at least once a week (confluence ≃ 100%). Terminal plating (day 20) was performed in poly-d-lysine (PDL) and laminin (2µg/cm^2) on 24 well coated plates, in N2:B27 + bFGF medium. NPCs were then cultured for the following 7 days in differentiation medium, B27 supplemented with DAPT and AA2P. The medium was finally changed to B27 and AA2P, with no differentiation stimuli to let cells grow until day 30/fixation.

Further details regarding the reagents used during the procedure and the neuralization medium during differentiation can be found on tables 6.1, 6.2 and 6.3 of the annexes.

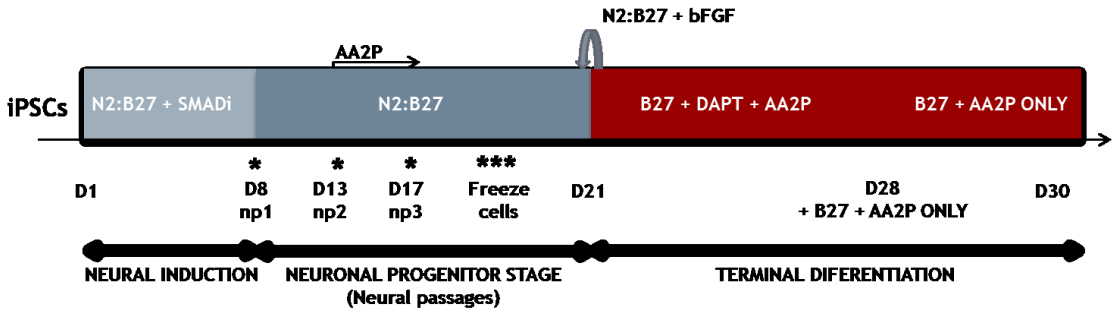


Figure 3.1. Representation of the direct differentiation of iPSC to cortical neurons. Three stages are involved. Neural induction is initiated at day 1 (D1), lasting for 7 more days. Neuronal progenitors are obtained on the second phase (D8 – D21). Terminal differentiation is initiated from D21 – D30. Np: neural passage.

3.1.3. Treatments

Cells were differentiated for 30 days. At day 29 of differentiation, neurons were treated for 24hours being fixed immediately after (day 30), figure 3.2. Cortical neurons were treated with 17β-estradiol (also defined as E2), three selective estrogen receptors agonists (PPT, WAY200070 and G1), testosterone and an endocannabinoid agonist (2AG), table 3.2. For each of the four cell lines, triplicates were performed (# EXP**M1**, # EXP**M2** and # EXP**M3** – '**M**' for **M**ultiple cell lines).

Table 3-2 - Final concentrations used in the treatments of the for cell lines at days 29.

Treatment	***Concentration***	***Company [Catalogue Nr.]***
17β – estradiol / E2	10nM	SIGMA [E8875]
WAY 200070	10nM	Tocrins Bioscience [3366]
G1	10nM	Tocrins Bioscience [3577]
PPT	100nM	Tocrins Bioscience [1426]
Testosterone	2nM	SIGMA [T1875]
2AG	1μM	Tocrins Bioscience [1298]

Figure 3.2. Time length of cortical differentiation. Cells are treated at day 29 (treatments summarized on table 3.2) and ICC (Immunocytochemistry) starts 24hours later (day30).

3.2. Immunocytochemistry (ICC)

At day 30 of differentiation, cortical neurons were fixed in 3.7% formaldehyde (w/v; in 4% sucrose in PBS) for 20 min. Cells were permeabilized and blocked for 2 hours using a blocking solution (2% goat serum, 0,1% Triton X-100 in PBS), and then incubated overnight with primary antibodies diluted in (2% goat serum in PBS). On the following day, cells were incubated with secondary antibody solution (2% goat serum in PBS) for 1hour and 5-10 minutes in DAPI (Sigma: D9542 w/v; made in PBS at 1:10'000 dilution). Cells were washed twice in PBS and DABCO (1,4 diazobizyclo[2,2,2]octane) antifade solution was added to retard

photobleaching (see table 6.4 on the annexes for further information about the recipe). Table 3.3 summarizes the antibodies used in the project.

Table 3-3 – Primary (shaded) and secondary antibodies (below) used on ICC.

Antibody target	***Type***	***Host Species***	***Dilution***	***Company [Catalogue Nr.]***
Anti-MAP2	Polyclonal	Chicken	1:1000	Abcam [ab92434]
Phospho-Akt (Ser473)	Polyclonal	Rabbit	1:200	Cell Signalling [Antibody #9271]
Chicken	Alexa Fluor®488	Goat	1:1000	Life technologies [A11039]
Rabbit	Alexa Fluor®568	Goat	1:750	Life technologies [A11036]

3.3. Image analysis

Images were captured using a 20x objective of a Zeiss Axiovert Fluorescence Microscope, 3 images were taken per condition. Neurite Outgrowth, an application of MetaMorph® Software (automated image analysis software) was utilized to study neurite outgrowth of cortical neurons under the effect of estrogens, testosterone and 2AG. Several features could be collected however only mean outgrowth per cell (μm/cell) and mean branches per cell (nr/cell) were considered. ImageJ 1.49 was used for additional image processing, whenever needed.

3.4. Statistical analysis

Raw data was initially studied and organized using Excel to then be statistically analysed with Graphpad Prism 6. Since more than two groups were being studied a one way analysis of variance (ANOVA) was performed using the Fisher's LSD method in order to individually compare the effects of each treatment to the control condition. This way each comparison stands alone and is not affected by the mean value of other conditions/groups.

Chapter 4.

Results

4.1. Optimization Experiments

In order to investigate the effects of estrogens, testosterone and 2AG in hiPSC derived forebrain/cortical neurons it was utilized the neuralization protocol briefly described before. However some aspects had to be improved to fulfil the aims defined and to overcome the challenges found on the way.

hiPSCs are extremely sensible cells, namely because of the extended periods in vitro. The first issue related to cell culture not only of hiPSC but all type of cells, is contamination. The control and quality of the reagents used not to mention on the cell handling according to the established standard operating procedure (SOP) of the laboratory is of extreme importance, specially given the long time periods needed to obtain mature neurons. In order to avoid major issues as cell death, contamination, cell lifting it is important to have a safety and caution cell culture handling.

A proper coating is essential and has influences on several aspects as: cell adhesion and cell clumping, and even cell death. Prior to terminal differentiation plates are coated with PDL and laminin as referred on *Chapter 3*. Preliminary experiments were resulting on the formation of cell clusters and agglomerates of cells, and leading to the detachment of the monolayer, and consequently cell death. This forced to an update of the protocol since no suitable results could be obtained in these conditions. Therefore two parameters were changed, the time of incubation of poly-d-lysine and the concentration used for the laminin coating. The alteration of the period of incubation to overnight for both reagents and a decrease on laminin concentration allowed the improvement of the results. The time of incubation of PDL used to promote the laminin adherence for the formation of a robust matrix for neuron attachment, was increased from 3 to 24 hours.

It is important to note that an half medium change was the ideal procedure since a complete change would expose cells and increase the force of suction that could potentially lead to cell lifting.

Cell clumping was also influenced by the type of plate used. Cells tend to be sparser on a 24 well plate than on a 96 well plate as figure 4.1 clearly exemplifies. Even after changing laminin concentration cells form clusters on the 96 well plates (figure 4.1 (A)), whereas on the 24 well plate (figure 4.1 (B)) cells are sparser and presented as single cells.

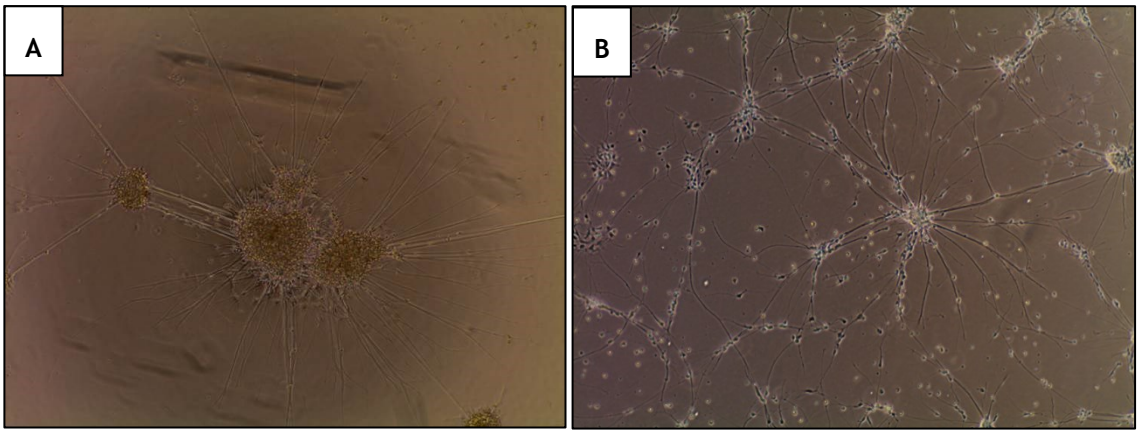


Figure 4.1. Difference of cell density in two different types of plates. (A) 96 well plate and (B) 24 well plate at day 29 of differentiation (CT.M1.11 cell line). Images taken with an EVOS® XL Core Microscope, 10x objective. Note: Scale bars were not available, which does not represent a problem since images were not taken to perform measurements but to have a record during *in vitro* differentiation.

The image analysis using Neurite Outgrowth Software requires cell plating at a low cell density since cells are traced individually. This parameter was improved gradually, having the best results (lower cell density) at the end of the project. An optimization of cell density also represented a challenge since the established protocol did not include cell counting. Cell density was controlled based on supporting documents [100] and on the volume of cell medium used to resuspend cells. Figure 4.2 illustrates how cell density was optimized along the different experiments. *#EXPM1, #EXP M2* and *#EXP M3* were performed in different time points, showing a decrease in cell density as the protocol was being upgraded.

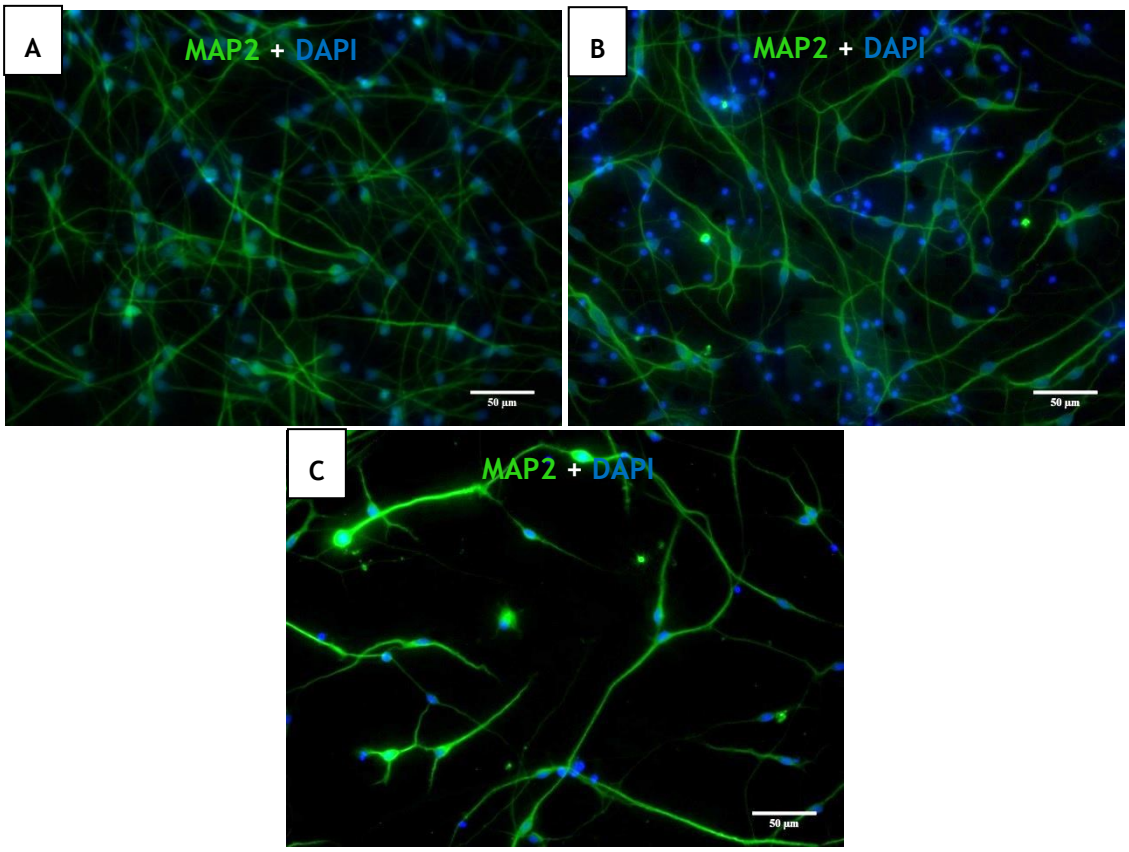


Figure 4.2. Cell density optimization. A decrease in cell density is observed along the experiments. Figures A, B and C are representative images of CT.M2.42 cell line of experiments #EXP M1, #EXP M2 and #EXP M3 respectively. Images taken with Zeiss Axiovert Fluorescence Microscope. Scale bars 50µm (A, B and C).

4.2. Image Analysis – MetaMorph

After taking a minimum number of 3 images per condition, the MetaMorph application Neurite Outgrowth provided segmented images and numerical results defining the morphology of the neurons concerning both cell bodies and neurites.

The application enabled the isolation of the cell bodies and identification of the neurites, based on the contents and wavelengths of the images. For this analysis two images were needed, the image containing the cell bodies and attached neurites (neurons) and images containing the stained nuclei. Images containing information regarding both cell bodies and neurites will be from now on referred as “MAP2 images” and nuclei images as “DAPI images”.

The isolation of the entire neurons is possible only with the information of the MAP2 image. However adding the use of the DAPI image provides a higher accuracy since it allows the correlation of the stained nuclei with the cell bodies of the MAP2 image. It enables a one to one correlation between cell bodies and nuclei.

In order to segment each image it is needed to set up setting values suitable to the image content (morphology of the features and intensity of the pixels). Working on the MAP2 image these settings enable to specify threshold values to separate the cell bodies from the background and establish dimensions of the cell bodies and neurites. Prior to segmentation, images are calibrated and background subtraction (statistical correction) is performed. For each experiment (#EXP M1, EXP M2 and #EXP M3), which includes the four cell lines referred previously, default settings were optimized and held constant. The resulting images represented on figure 4.4 not only help on the calibration of the settings, obtained through the analysis of multiple sample images, but also how the different cell lines behave on each experiment and between experiments.

The physical characteristics of the traced neurons (figure 4.4) were plotted on an Excel file. However only two parameters were studied, mean outgrowth and mean branches.

Mean outgrowth (μm/cell) is calculated by the average of the total neurite length, skeletonized outgrowth corrected for diagonal lengths, per cell. The mean branches per cell correspond to the total number of branch points/branching junctions by the number of cells present on the resulting traced image (positive cell body/nuclei). The different neuronal structures are represented on figure 4.3 for a better understanding of the defined parameters.

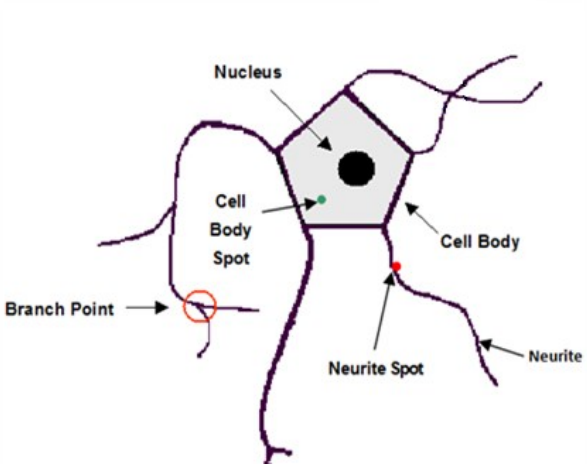


Figure 4.3. Neuronal primary structures. Nucleus, cell body, neurite and branch point physical characteristics can be found.

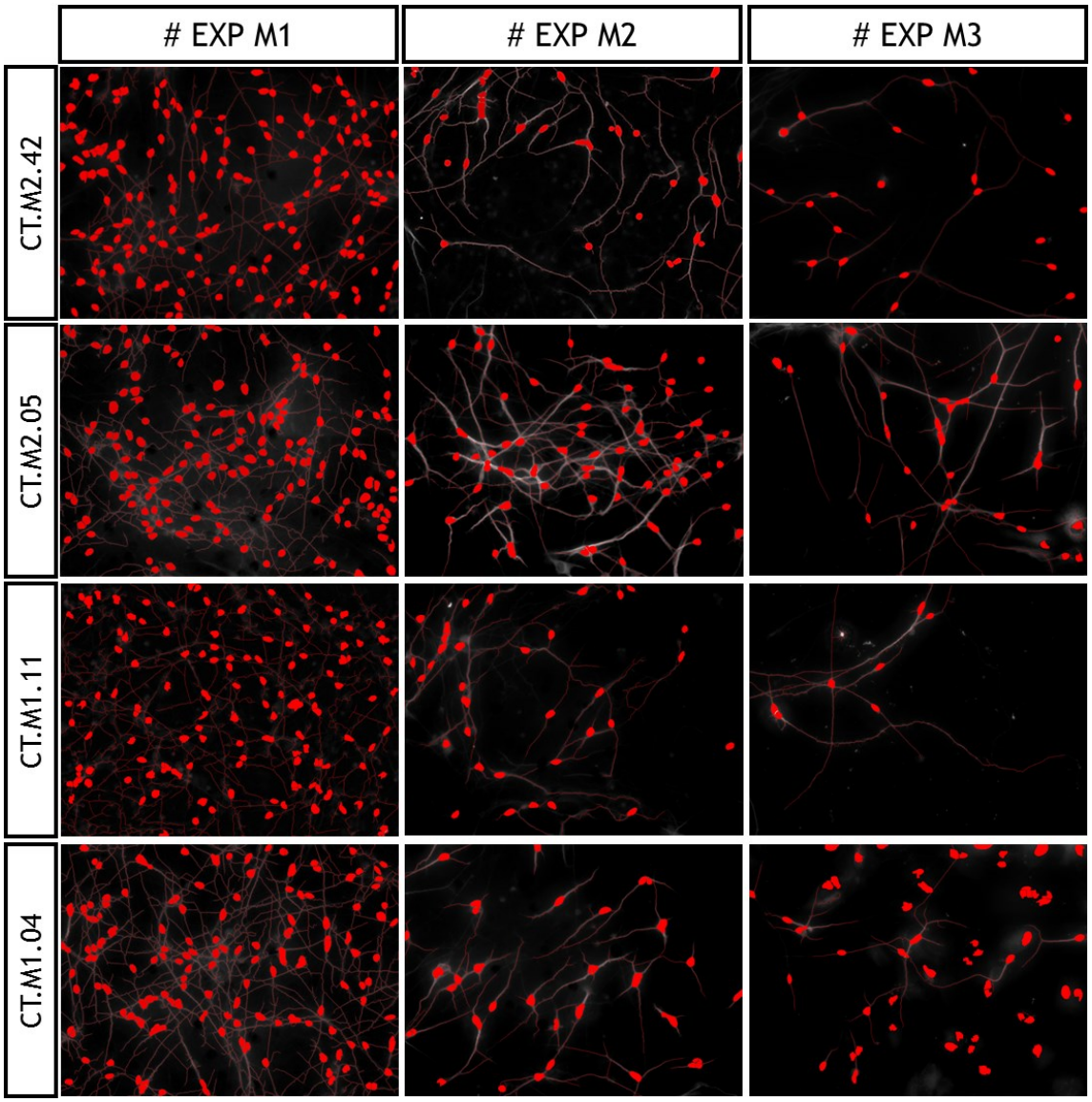


Figure 4.4. Segmented images after process and analysis with NeuriteOutgrowth. Four cell lines were used (CT.M2.42, CTM2.05, CT.M1.11 and CT.M1.04), having three replicates (#EXP M1, #EXP M2 and #EXP M3). Images taken with a 20x objective using a Zeiss Axiovert Fluorescence Microscope.

4.3. Neurosteroids and endocannabinoid effects on neurite outgrowth

The neuralization protocol used enables the differentiation of hiPSC into forebrain/cortical neurons, as previously reported [96, 99]. After formation of a monolayer of neuroepithelial cells induced by SMAD inhibition, structures named neural rosettes form, presenting the apical-basal polarity of hNPCs. These structures have been identified previously by the expression of ZO-1 in the apical lumen and nestin positive cells on the surroundings [99].

In this dissertation the expression of these markers was not tested. However the typical rosette structure of the neural progenitors of the cell lines was confirmed during cell culture

of the NPCs. Figure 4.5. illustrates the neural progenitor cells (neural passage 10) prior to the last stage of differentiation. As in vivo, neural stem cells polarize in a 2D structure in order to obtain the neural tube structure as referred before on Chapter 2 (*2.6.3.1 - Human Neural Development*).

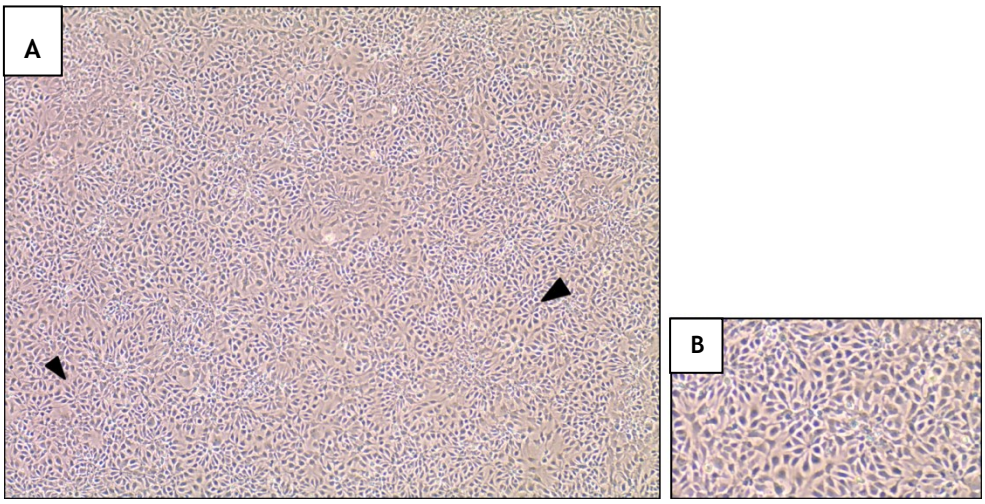


Figure 4.5. Rosette structure of neural progenitors. The cell line CT.M1.11 illustrates the morphologic characteristics of the cortical neural progenitors. Arrows point to the typical rosette structure of these cells. (A) This radial pattern is present in the all image. (B) Magnification of a section of image A, allows a closer analysis of the NPCs in culture before being terminally plated. Images were taken with an EVOS® XL Core Microscope (10x objective) during cell culture. Note: Scale bars were not available, which does not represent a problem since images were not taken to perform measurements but to have a record during *in vitro* differentiation.

After 30 days of differentiation forebrain/cortical neurons were generated. Forebrain consists of both glutamatergic and GABAergic interneurons that can be differentiated from hiPSCs using different inductive signals [101, 102]. However the established protocol in our lab is focused on the differentiation of hiPSCs in glutamatergic projection neurons for being the most representative neuron cell type in the cerebral cortex. On the following page, figure 4.6, shows the unipolar pyramidal morphology that characterizes this cell type and distinguishes from the multipolar morphology that interneurons present, as referred before on Chapter 2 (*2.2.1* - Cerebral Cortex Layers).

Estrogens, testosterone and endocannabinoids have been associated to affect neuritogenis of different neuronal cell types by activation of distinct signalling pathways [5, 103, 104]. However up to now the study of its effects on the morphology of hiPSCs derived cortical neurons that attest the response of these cells to the referred treatments has been poorly documented [96].

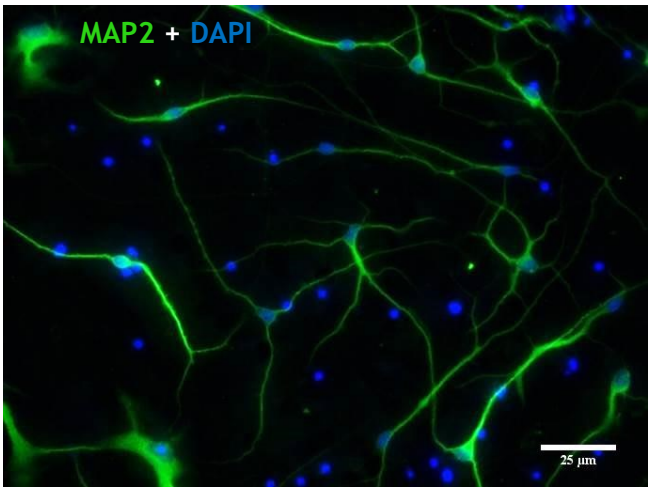


Figure 4.6. Unipolar pyramidal structure of glutamatergic cortical neurons. hiPSCs neurons differentiated for 30 days; MAP2 positive (green) and stained for DAPI (blue). Young immature neurons presenting an unipolar pyramidal morphology, poor in neurite ramifications. Images taken with Zeiss Axiovert Fluorescence Microscope. Scale bar 25µm.

Each hiPSCs cell line was differentiated for 30 days leading to the generation of cortical pyramidal neurons. This experiment was repeated three times (Experiments M1, M2 and M3) knowing that per experiment four cell lines were differentiated simultaneously.

In this dissertation, the influence of estrogens, testosterone and 2AG in neural development was studied after an acute treatment of 24 hours, at day 29 of differentiation. Neurite outgrowth was evaluated using MetaMorph and according to two parameters, mean neurite length (µm/cell) and mean branches per cell (nr/cell). The mean values of both parameters were normalized to the mean of the control condition of each experiment, i.e. the average of each condition was divided by the average of the control, per experiment. The following pages will explore the results of the three experiments performed for each cell line.

Figure 4.7 plots the results of CT.M2.42 cell line, showing the influence of the different treatments compared to the control. Despite the disparities between experiments possibly caused by a number of factors as cell density, referred before (Chapter 4 (4.1- Optimization Experiments)), it is possible to observe an increase in neurite length (figure 4.7 (A)) in the presence of E2 (EXP M1), PPT and 2AG (EXP M2), and 2AG (EXP M3). Positive effects of these conditions in the number of branches per cell (figure 4.7 (B)), enhances the results of the previous parameter and the effect of E2, PPT and 2AG on neurite development.

As it has been referred the three experiments were executed for each cell line. Regarding CT.M2.05, the results obtained for both parameters consistently show an influence of the ER-ß agonist (WAY 200070) on neuronal development, measured not only on neurite length (figure 4.8 (A)) but also on the number of branch junctions per cell (figure 4.8 (B)), observed on EXP M3.

CT.M1.11, a clone derived from another patient (M1), did not show any statistical significant results, neither on neurite outgrowth (figure 4.9 (A)) nor on the number of branches per cell (figure 4.9 (B)), relative to the control.

Finally CT.M1.04 responds to the presence of PPT, agonist of the estrogen receptor ER-α, by a positive effect on the number of neurite branch points per cell (figure 4.10 (B)), expressed on EXP M2. Moreover, an increase of the neurite length relatively to the control shows the response of this cell line to the endocannabinoid 2AG (EXP M3).

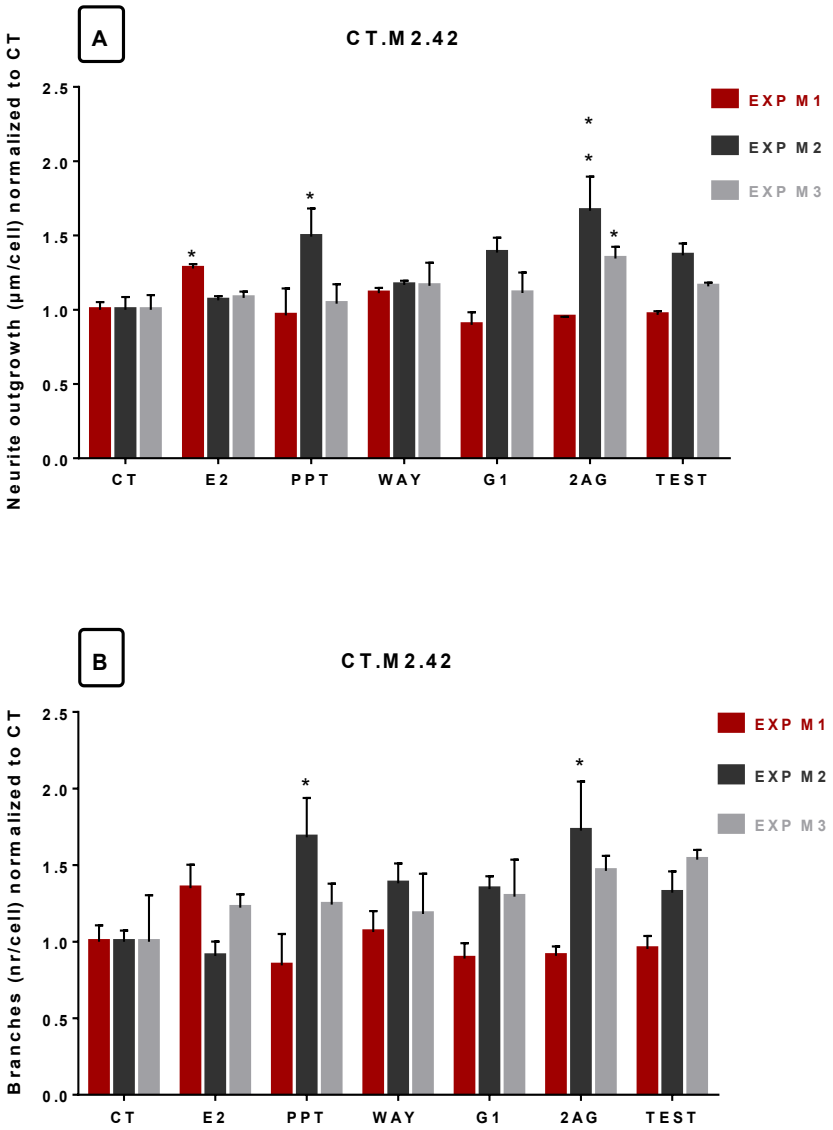


Figure 4.7. Effects of the different treatments on neurite outgrowth (A) and number of branches per cell (B) on CT.M2.42 cell line. Treatments with 17β - estradiol (E2) on experiment M1 and treatment with PPT and 2AG on Experiment 2, leads to an increase of neurite outgrowth relatively to the control (vehicle-treated cells) of CT.M2.42 cell line. 24h treatments with PPT AND 2AG resulted on a significant increase on the number of branches per cell. Quantitative analysis of the total neurite length by the total number of neurons; and analysis of the total number of branches by the total number of neurons; values of each experiments M1, M2 and M3 were normalized to the respectively control mean value (mean ± SEM). An one-way ANOVA, with Fisher LSD method was performed, * represents statistical significance of each group treatment relatively to the control.* $p < 0.05$; ** $p < 0.005$. Experiment M1, N = [110 ; 214] cells; Experiment M2, N = [16 ; 57] cells; Experiment M3, N = [22 ; 68] cells.

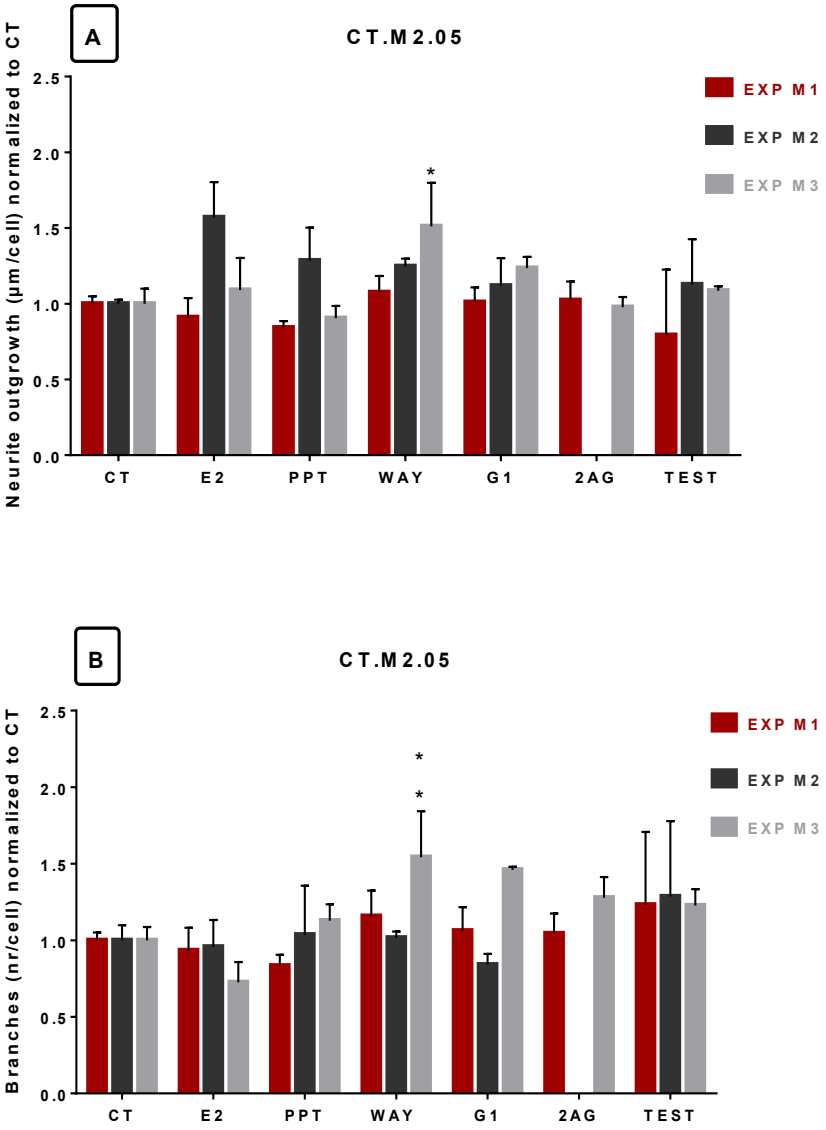


Figure 4.8. Effects of the different treatments on neurite outgrowth (A) and number of branches per cell (B) on CT.M2.05 cell line. 24h treatment with WAY200070 (ERß agonist) resulted on a significant increase on both neurite length (A) and number of branches per cell (B). Quantitative analysis of the total neurite length by the total number of neurons; and analysis of the total number of branches by the total number of neurons; values of each experiments M1, M2 and M3 were normalized to the respectively control mean value (mean ± SEM). An one-way ANOVA, with Fisher LSD method was performed, * represents statistical significance of each group treatment relatively to the control.* $p < 0.05$; ** $p < 0.005$. Experiment M1, N = [128; 242] cells; Experiment M2, N = [15; 76] cells; Experiment M3, N = [12 ; 59] cells.

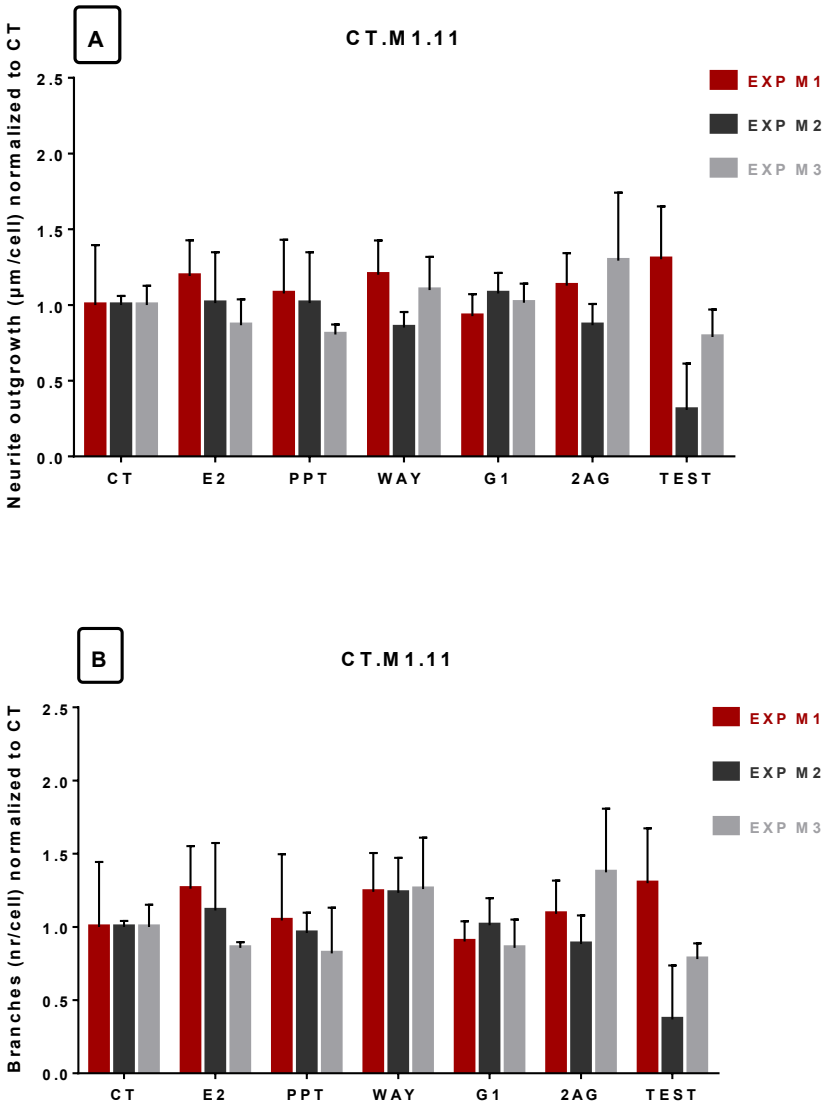


Figure 4.9. Effects of the different treatments on neurite outgrowth (A) and number of branches per cell (B) on CT.M1.11 cell line. The treatments performed at day 29 of differentiation did not show statistical significance between experiments. Normalized mean values are similar or even lower than the control condition. Quantitative analysis of the total neurite length by the total number of neurons; and analysis of the total number of branches by the total number of neurons; values of each experiments M1, M2 and M3 were normalized to the respectively control mean value (mean ± SEM). An one-way ANOVA, with Fisher LSD method was performed, $p < 0.05$; ** $p < 0.005$. Experiment M1, N = [68 ; 232] cells; Experiment M2, N = [22 ; 70] cells; Experiment M3, N = [1 ; 18] cells.

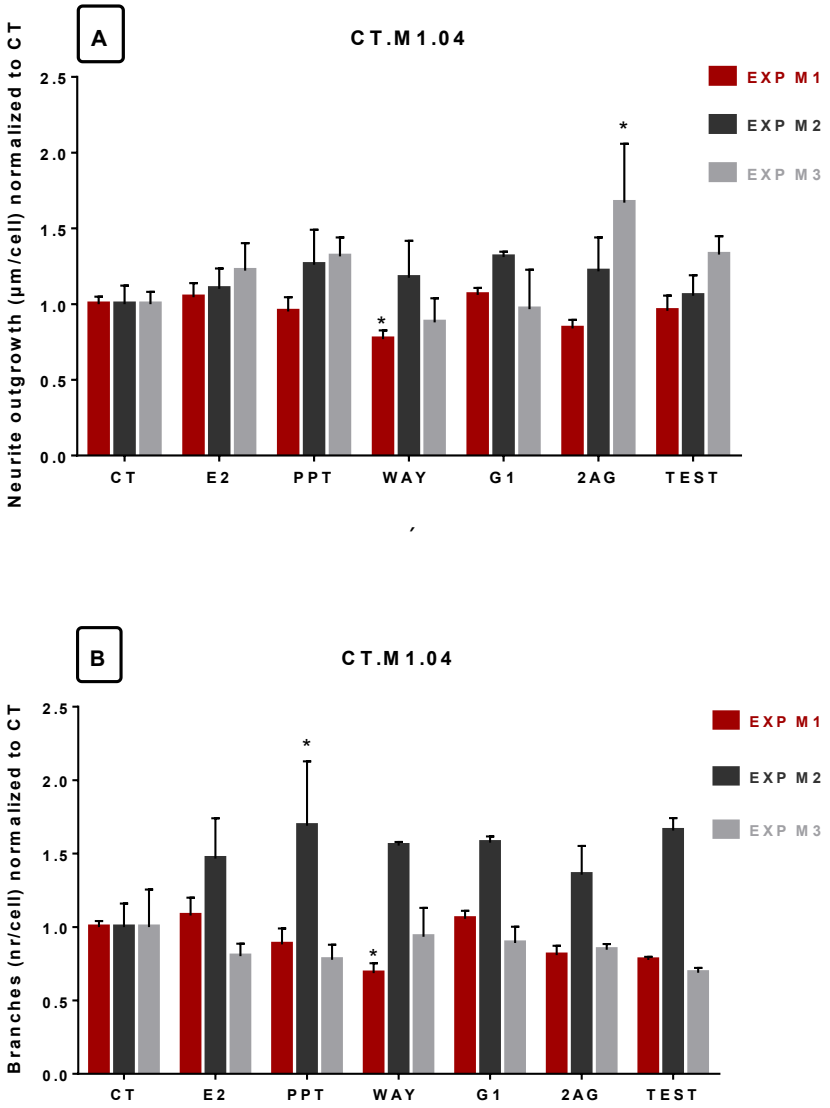


Figure 4.10. Effects of the different treatments on neurite outgrowth (A) and number of branches per cell (B) on CT.M1.04 cell line. The endocannabinoid 2AG influences neurite outgrowth (A) (EXP M3); neurite branching (EXP M2) (B) increases with the treatment with the ERα agonist PPT, and a negative effect is verified for the ERß agonist WAY. Quantitative analysis of the total neurite length by the total number of neurons; and analysis of the total number of branches by the total number of neurons; values of each experiments M1, M2 and M3 were normalized to the respectively control mean value (mean ± SEM). An one-way ANOVA, with Fisher LSD method was performed, * represents statistical significance of each group treatment relatively to the control.* $p < 0.05$; ** $p < 0.005$. Experiment M1, N = [54 ; 210] cells; Experiment M2, N = [23 ; 106] cells; Experiment M3, N = [22 ; 65] cells.

Under the conditions used in this study, cells respond mainly to the treatments summarized on table 4.1. The positive response to estradiol, which binds non-specifically to the classical estrogen receptors, ER α and ERß, and GPER1, and response to PPT and WAY, ER α and ERß specific estrogen receptors agonists, suggest that these cells express both estrogen receptors α and ß. Moreover, despite the limitations associated to inter- and intra-patient variability and differences in cell density between experiments, the endocannabinoid 2AG

(agonist of CB1, CB2 and GPR55 cannabinoid receptors) increased neurite outgrowth and neurite branching in two cell lines (CT.M2.42 and CT.M1.04, clones of distinct patients), and twice on the same cell line (EXP M2 and EXP M3, CT.M2.42). These results suggest the presence of CB1 or/and CB2 receptors on young differentiated cortical neurons.

Table 4.1 - Summary of the treatments considered statistically significant (one-way ANOVA, with Fisher LSD method). A negative effect (-) is also represented, meaning a decrease in neurite outgrowth and nr of branches per cell, relatively to the control condition.

Experiment / *Cell line*	*Experiment M1*	*Experiment M2*	*Experiment M3*
CT.M2.42	E2	PPT 2AG	2AG
CT.M2.05	-	-	WAY
CT.M1.11	-	-	-
CT.M1.04	(-) WAY	PPT	2AG

Chapter 5.

Discussion

5.1. Neurosteroids effect on neurite outgrowth

The results support the notion of estradiol as a neurotrophic factor, in the way that regulates neurite outgrowth during neuritogenis and differentiation of cortical glutamatergic hiPSC derived neurons.

Steroid hormones influence the nervous system having the ability to affect cognition as it has been studied over the time. Some controversy regarding positive and negative symptoms caused by estrogen therapy have been discussed in the area. A study in 2003 of Women's Health Initiative (WHI) of a long treatment with conjugated equine estradiol and medroxyprogesterone in post-menopausal women over 65 years, reported a decrease in cognitive function along with risk of dementia and stroke [105]. However, this controversy between studies, reporting opposite effects caused by estrogens has been attributed to the fact that human female brain is responsive to estrogens till a certain time, existing an "window of opportunity" [106] following menopause till when the hormone can exert positive effects, after this stage estrogen treatment leads to negative effects.

Despite the adverse effects pointed to this hormonal treatment, as cardiovascular problems, stroke and risk of developing cancer [105, 107, 108], several positive effects have been reported suggesting the potentialities of estrogens as a therapeutic avenue for the treatment of several neurodevelopmental disorders as Schizophrenia [109]. A recent study by Weickert et al., using raloxifene, a selective estrogen receptor modulator (SERM) synthetically synthesized, demonstrated the enhancing effects of estrogens over cognition in both human male and female. In this controlled-placebo study it was tested whether oral administration of raloxifene (120mg) of young middle-aged men and women with Schizophrenia add an impact on cognition improving the symptoms of the disorder, comparing

to the placebo. The adjunctive treatment of this SERM demonstrated improvements on attention/processing speed and memory in both genders. Moreover a transdermal treatment of 8 weeks with 17-ß estradiol (200μg and 100μg) showed the enhancement of positive and negative symptoms of female schizophrenic patients relatively to placebo, having a better performance at 200 μg than 100 μg relatively to the placebo, in a large-scale randomized-controlled trial [6].

Human post-mortem studies and genetically-modified organisms have been used to clarify the cellular and molecular mechanisms that address the effects of estrogens in the human brain. Post-mortem studies have the inherent limitation of being a non-living tissue, not providing the full picture of estrogens effect namely during development. In the other hand animal models do not fully recapitulate the pathophysiology and the genetic complexity of the human brain. However is the use of animal models that has been the main source of information of estrogens neuroprotective actions.

Studies involving animal models, either *in vitro* or *in vivo*, have reported the enhancing effects of estrogens, namely 17-ß estradiol, in learning and memory, as well as in behavioural tests. 17-ß estradiol influence over cognition has been associated to the ability of this hormone to modulate neural circuits, influencing the formation of neuronal processes, dendrites and axons, as well as dendritic spines and synapses [103, 110].

Multiple studies have demonstrated a time- and dose- dependent response to estrogen treatment, having a critical time and concentration on which the positive response to the therapeutics is optimal. Several studies using ovariectomized (OVX) female rodents have demonstrated impaired performance in working memory and spatial navigation, able to be reversed by acute treatment with 17ß-estradiol. Luitne et al., performing an acute treatment 30 minutes prior and post-training on OVX female rats, showed an enhancement on memory acquisition and consolidation [111]. In the same study, the administration of 17ß-estradiol 2 hours post-training did not reflected any effect, revealing that estrogens have a specific time frame of action to induce positive effects. The same was verified in an acute treatment of OVX rats performed immediately post-training with the selective agonist receptors ERα and ERß, PPT and DPN, that did not exert an effect when performed 60 minutes after. This time dependent response to estrogen treatment suggests that estrogens enhance cognition in a rapid and transient way as proposed before [112].

In regards to the dose/concentration of administration, it is important to highlight the behaviour assumed by estrogen treatment. It has been proposed that estrogens assume an inverted U-shaped dose response curve, suggesting that there is an optimal dose where estrogens are most effective [111, 112].

Previous studies, using neural stem cells (hNSCs) or neural progenitor cells hNPCs have studied the expression of the different estrogen receptors and consequent effect in cellular function, showing estrogenic influence not only on proliferation but also on differentiation in

the human brain. hNPCs derived from fetal cortex were reported to be affected by a time- and dose- dependent 17β-estradiol treatment, increasing proliferation [113]. It was also addressed the expression of the estrogen receptors, ERα and ERβ, revealing an enhancing response to ERβ over ERα, mediated via a MEK/ERK1/2-dependent pathway [113]. The expression of ERs on hNSCs derived from fetal mesencephalic tissue and a dose dependent response to 17β-estradiol treatment following *in vitro* differentiation was also demonstrated. Moreover the use of hESCs has also been showing the influence of ERs activation in neural development, demonstrating an higher expression for ERβ over ERα in neurons differentiated from hESCs lines [114]. Furthermore in order to understand the influence of 17β-estradiol and of each estrogen receptor, Ca^{2+} oscillations were analysed demonstrating the rapid action of 17β-estradiol and a higher activation of ERβ, capable of changing Ca^{2+} signalling [114].

The use of iPSC technology as a model to understand estrogens effects in human neural development it's still in its preliminary stages. Recently Shum et al. (2015), demonstrated that hiPSC derived cortical neurons are responsive to estrogenic treatment. hiPSC derived neurons were differentiated for 35 days, being treated with 17β-estradiol for 24 hours at day 34. A positive response on dendritic branching was observed, relatively to the control, showing the ability of estrogens to alter neuronal structure [96]. These results are consistent with what it is observed on the current dissertation, morphologic alterations in 17β-estradiol treated neurons (24 hours at day 29), represented by an increase in neurite length/cell which is also corroborated by the increasing number of neurite branches.

The data presented here also suggests the expression of the classic estrogen receptors ERα and ERβ, revealed by the increasing values of the two studied parameters relatively to the control. This was shown performing a 24 hour treatment at day 29 of differentiation, using 17β-estradiol and also the specific receptor agonists PPT, WAY and G1. As agonists of the classic estrogen receptors (ERα and ERβ), PPT and WAY activated estrogenic signalling, as demonstrated by previous studies already referred. However there is still some controversy regarding the expression of ERβ over ERα [114]. Moreover the expression of GPR1, activated by the agonist G1, was not significant when compared to the control following a 24 hour treatment of hiPSC derived cortical neurons. A couple of reasons can be pointed to the response to G1 treatment. As referred before, estrogen treatment is dose- and time-dependent, presenting an inverted U shape dose response. Therefore two different reasons can be suggested, the dose used was not adequate and different concentrations have to be tested to find the optimal concentration where GPR1 is expressed; or since estrogens actions are rapid and transient, the neurite outgrowth analysis of 24h treated neurons could have failed the time point that this receptor activation was stimulating neuritogenesis. In order to discard this hypothesis different time points should be tested and mRNA and protein analysis should be performed to further verify the presence of this receptor.

Estradiol synthesis occurs mainly in the ovary, but it is also produced in other tissues as bone, adipose and nervous system not only in females but also in males [5]. The synthesis within the brain, independent from the peripheral steroidogenesis, allows the modulation of several neurobiological processes. Estradiol/17β-estradiol synthesis involves mainly two phases [5], the first in the mitochondria that leads to the conversion from cholesterol to pregnenolone, and the second in the endoplasmatic reticulum, that ends with the catalysis of testosterone into estradiol by the enzyme aromatase. Three different sources of estradiol have been suggested: ***peripheral estrogens***, produced and circulating outside the nervous system; ***androgens*** (estrogen precursors) localized in the nervous system; and local synthesis from ***cholesterol*** [112].

It has been reported that nanomolar concentrations of estradiol are required to induce rapid cellular and molecular responses, which is not verified in the plasma that presents low picomolar levels of this steroid [115]. In order to produce rapid responses, androgens, as testosterone, or cholesterol are proposed as estradiol main sources [112].

As an estrogen precursor, testosterone could have possibly triggered estradiol synthesis and influence the morphology of the differentiated glutamatergic cortical neurons. Despite having higher levels of neurite outgrowth and neurite branching relatively to the control, observed in some cell lines, the 24 hour testosterone treatment did not reveal statistically significant results. This could have been possibly due to the variability between experiments and cell lines, related not only to the limitations intrinsic to the use of iPSC technology, but also to the differences of cell density observed between experiments.

The regulation of neuritogenesis (see Chapter 2 (2.2.2 - Neuritogenesis), for further details about this stage) by estradiol and the mechanisms involved in this process are still not clearly understood. Depending on the brain region and neuronal cell type, different mechanisms may be involved and influence the different stages of neuronal development that include axogenesis, dendritogenesis, spinogenesis and synaptogenesis.

Arevalo et al., provided important insights on the molecular basis of estrogen regulation of neuritogenesis, which has been suggested to involve different signalling pathways. The initiation of MAPK cascade trough the activation of the signalling kinase Ras has been reported to influence neuronal development, which is activated by estradiol phosphorylation of c-SRC [103, 116]. Moreover, activation of the protein kinase C by estradiol [117] and increased levels of Ca^{2+} have also been associated to the regulation of this signalling cascade and posterior activation of the transcriptor factor c-AMP response element binding (CREB). Adding to this estradiol interacts with other factors such as the insulin-like growth factor-I (IGF-I) and the brain derived neurothropic factor (BDNF), interveners in neuronal development. IGF-I an important regulator of neuritogenesis activates MAPK and phosphoinositide-3 kinase (PI3K) cascades [118], known to be also regulated by estradiol. Moreover estradiol increases BDNF levels and the expression of TrkB (BDNF receptor), which

interacts with MAPK signalling cascade [119]. Therefore estradiol modulation of neuritogenis by stimulation of BDNF is proposed to be performed via MAPK signalling [103]. It has been also reported that notch signalling can be modulated by estradiol [120], promoting neuritogenesis by notch inhibition and consequently increasing the proneural gene, neurogenin 3 (Ngn3) [121].

The estradiol influence on neuritogenesis have been attributed to the activation of c-Src/Ras/ERK/CREB signalling and interaction with Notch signalling, IGF-I and BDNF. Complex signalling cascades are involved adding to the interplay with external factors and expression, which depend on the brain region and neuron cell type. Therefore future studies are needed in order to clarify the molecular mechanisms that explain estradiol influence on neuritogenesis, namely in humans and under pathologic conditions.

5.2. Endocannabinoids effect on neurite outgrowth

The treatment with the endocannabinoid 2AG, agonist of the cannabinoid receptors CB1R, CB2R and GPR55 resulted in an alteration of the hiPSC derived glutamatergic cortical neurons morphology. The response at such an early stage of maturation provides evidence for the presence of cannabinoid receptors since embryonic stages till adulthood [3]. Despite the opposite behaviour of CB1 and CB2 receptors along neuronal differentiation, with an increase of CB1 levels and a decrease of CB2 concentration, at a neural progenitor stage the activation of both CB1 and CB2 receptors have been associated to influence cell fate, proliferation, differentiation and migration [122]. In adulthood the expression of the cannabinoid receptors is quite heterogeneous, CB1 receptors are mainly present at central and peripheral nervous system and CB2Rs are expressed in immune cells in and outside the nervous system [75] .Within the central nervous system the CB1 receptor is present at high concentrations in the basal ganglia, frontal cortex, hippocampus and cerebellum [2], being expressed namely at the GABAergic interneurons and glutamatergic neurons [123]. The differentiation of iPSC into glutamatergic cortical neurons and posterior alterations of the neuron structure that resulted from treatment with the endocannabinoid 2AG suggests the presence of the cannabinoid receptor CB1. However further studies would be necessary to confirm this observations.

The stimulation of neurite outgrowth and neuronal differentiation has been associated to the nerve growth factor (NGF) receptor, TrkA and G-protein coupled receptors (GPCRs) [104]. GPCRs can be activated trough different mechanisms, as Drd2 dopamine D2 receptors in cortical neurons [124], serotonin 1-B receptors in thalamic neurons [125] as well as cannabinoid receptors. The stimulation of the cannabinoid receptors on Neuro2A cells by the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2AG) have been implicated on neuron differentiation and neurite outgrowth [126]. The activation of these receptors,

coupled to GPCRs inhibits cAMP production by adenylyl cyclases and activates protein kinases as Src, MAPK 1/2 and Akt [104], resulting in the stimulation of neurite outgrowth and modulation of neuronal differentiation. CBR1 has been associated to the activation of these kinases and activation of the transcriptional factors CREB and Stat3 [104], known to be involved in neuritogenesis [9]. Additionally, PI3K-PAX6 a signalling pathway that regulates neuronal differentiation, has been found to be activated in response to cannabinoid signalling [127] delineated from an in silico gene regulatory network approach. Therefore, my data shows that the introduction of the endocannabinoid during neurite elongation changed the regular path of neuritogenesis by an increase of neurite length and complexity, explained by the number of branches. This alteration in neurite outgrowth has been reported by previous studies on Neuro2A cells [126] and has also been assessed to have effect in axon growth, the following stage of neuritogenesis. Harkany and colleagues using a CB1R knockout pyramidal neuron-specific and GABA-ergic interneuron specific mouse along with endocannabinoid signalling, found that eCBs act as axon guidance cues that lead to proper patterning of this neurons during brain development [128, 129].

The implications of the cannabinoid signalling in hiPSC derived neurons it's still in its preliminary steps and future experiments are needed to confirm the obtained results. Moreover it is mandatory to first standardize the protocol till further experiments are performed. However, despite the limitations engaged to the use of iPSC and the drawbacks found during the development of the project, under these conditions cells showed a positive response to endocannabinoid stimulation, which encourages further studies on this regards.

5.3. hiPSC differentiation : limitations

The 24 hour treatment of the differentiated hiPSC cortical neurons resulted in divergent effects between experiments and cell lines, not only between clones from the same patient but also among clones of the same individual. Therefore several questions can be addressed, firstly in respect to the different results obtained for each cell line, between experiments; and secondly in regards to the different responses between the four cell lines.

As referred before, the image analysis software MetaMorph (Neurite Outgrowth) requires a low density of differentiated cells, which was achieved at the end of the project, on the third experiment. As illustrated on figures 4.2 ("Cell density optimization") and 4.4 ("Segmented images after process and analysis with NeuriteOutgrowth") the differences on cell density between experiments are visible in all four cell lines, decreasing from EXP M1 to EXP M3. For the parameters that are being analysed it is required a low density of cells.

Cells plated at high cell density leads to the differentiation of cells tightly spaced that causes the overlap of neurites of distinct neurons, as observed on EXP M1 (figures 4.2 (A) and 4.4 (EXP M1)). With the use of a lower number of viable cells, cells are sparser and can be

analysed individually. Branch junctions can be distinguished from cross points (overlap of different neurites of the same neuron or different neurons) by changes in direction of neurite skeleton segments. However a high cell density increases cell overlapping, which could possibly influence the acquisition of the number of branch junctions per cell. As it is represented on figure 4.4, neurite tracing was not affected by the high values of cell density, since neurons are segmented. However it is important to note that neurites localized on the images boundaries can elongate beyond its limits. Therefore the full length of these neurites is not being quantified. Despite plating cells at a low cell density, achievable by cell counting before terminal plating, a possible solution and future approach to surpass this drawback and improve neurite outgrowth analysis is the use of a lower magnification, such as a 10x objective. This way neurons full length is detected and more accurate values are obtained for the total number of neurons figured in the image.

The hiPSC technology has a number of limitations, from technical to reproducibility and time consuming issues.

In order to obtain gene expression similar to the brain, hiPSC neural progenitors need 8-16 weeks of cell culture [130]. Therefore having the purpose of studying a neurodevelopmental disorder hiPSCs were differentiated for 30 days into immature glutamatergic neurons characterized by a pyramidal shape and the presence of neurites, demonstrating how hiPSCs cell culture is a time consuming *in vitro* experiment. This limitation was surrounded to some extent with the cryopreservation of the cell lines and posterior addition of bFGF that allowed having stocks of cell lines at neural passage 3.

Another important issue related to the use of hiPSCs is the variability between hiPSCs lines, among independent clones derived from a single patient and from hiPSC derived from different patients. Differences detected in different cell lines, phenotypic variations and even genetic mutations force the use of several cell lines in order to acquire consistent results. However a high cost of cell culture limits this solution that leads to the use of a low number of cell lines in most cases. In the present study four cell lines derived from two different patients were used.

Despite deriving from the same individual, inta-patient variability was verified. Different responses were observed from clones derived from the same individual. The cell lines CT.M2.42 and CT.M2.05 had a positive response to E2, PPT and 2AG, and WAY respectively, demonstrating how two cell lines from the same donor can exhibit different responses. Moreover the morphology of hiPSCs derived neurons from CT.M1.04 was affected after treatment with PPT and 2AG, whereas CT.M1.11 did not respond to any treatment. Genetic variability possibly derived from the reprogramming process is one of the cause that is normally given. Adding to this spontaneous mutations occur naturally being harder to avoid. Therefore high quality control protocols are needed such as morphological, gene expression and karyotype analyses, tests for contaminants, and with RNA and DNA methylation profiling.

Furthermore the use of neural progenitors at earlier passages will decrease the possibility of mutations to occur.

Table 4.1 provides a summary of the responses of the different cell lines, showing both intra-patient variability but also inter-patient patient variability. Divergences between hiPSC cell lines derived from different patients were observed, despite the consistent response to PPT and 2AG between clones of different patients.

Inter-patient variability has been commonly documented, such as changes on differentiation efficiencies and electrophysiological properties, which has been associated to the identity and sex of the donor [131, 132]. The creation of isogenic hiPSC cell lines has the ability to overtake this limitation, despite requiring genetic manipulation as has been proposed [96].

The different responses observed between the four cell lines, leads to the question of reproducibility. In order to attest the reproducibility of the technique and protocol used, and to confirm the effects obtained by the acute steroid and endocannabinoid treatments at day 29, it is needed to reproduce the experience and standardize the protocol.

Over the time, with the advances of stem cell technology different solutions have been implemented in order to surpass these limitations and reproduce results. The use of standard protocols, cell culture media and differentiation kits have been increasing in the market [96], for instances, in order to minimize these limitations and obtain consistent neuronal cultures.

Chapter 6.

Conclusions

Schizophrenia is a psychiatric disorder with unknown etiology and no treatment, being patients only medicated to attenuate the symptoms. Therefore investment has been made in demand of potential treatments not only of the positive and negative symptoms but also to take over the cognitive deficits. In order to achieve this goal the use of adequate model systems is necessary.

Over the past few years iPSC technology has been evolving and demonstrating to be a proper system for the study of neurodevelopmental disorders as Schizophrenia. Therefore hiPSCs were used in order to study the effects of two potential therapeutics of this disorder. Neurosteroids, estrogens and testosterone (estrogen precursor), have been demonstrated to enhance acquisition and memory consolidation, on both males and females [109]; and also endocannabinoids, reported to express differently from early embryonic stages until adulthood, but to affect cognition [2], which is highlighted by the consumption of plant-derived cannabis, an environmental risk factor to develop Schizophrenia [133].

Therefore the effect of neurosteroids and endocannabinoids was studied using hiPSCs derived cortical neurons, which was analysed using MetaMorph, an image analysis software. Despite the variability and reproducibility of the results between experiments and cell lines, intra- and inter- variability characteristic of iPSC use, the different treatments stimulated neuron elongation and ramification that mirrors an increase in neuron arborization.

Glutamatergic cortical neurons differentiated for 30 days were responsive to 17β-estradiol and to the classical ERs, ERα and ERβ, by the activation of the correspondent receptors performed by PPT and WAY200070 agonists, which was observed by an increase in neurite length as well as in number of branches per cell. Treatment with the endocannabinoid, 2AG, also reflected an increase in neurite outgrowth providing evidence of its role in neuritogenesis.

Further studies are needed, firstly to standardize the protocol to then reproduce the experiments and verify the obtained results. Moreover as a future experiment, different concentrations of the drugs should be tested namely for G1 and testosterone, which did not change neuron structure. Western Blot tests and quantitative q-PCR, for instances, can be also used as a way to study the signalling network that triggers neurite outgrowth by the different treatments used.

Despite the variability of iPSC and the drawbacks of this preliminary approach, the data presented here demonstrates the ability of hiPSCs to model Schizophrenia and to study possible treatments. The use of this model allowed the investigation of the different treatments in neuritogenesis revealing promising results, alluring for future research.

Annexes

Section A - Neuralization protocol – reagents

Table A.1 - Catalogue number of the reagents used in cell culture.

Reagent	Company : Catalogue Number
Geltrex	Life Technologies : A1413302
HBSS	Invitrogen : 14170146
Versene	Lonza : BE17-711E
E8 Medium	Life Technologies : A1517001
ROCKi	Sigma : Y0503
Accutase	Invitrogen : a1110501
N2 Supplement	Life Technologies : 17502-048
DMEM/F12	Sigma : D6421
B27 Supplement	Life Technologies : 17504-044
Neurobasal Medium	Life Technologies : 21103-049
L- Glutamax	Life Technologies : 35050-038
SB431542	Cambridge Bioscience : ZRD-SB-50
Dorsomorphin	Sigma : P5499
AA2P	Sigma : A4403
bFGF	Life Technologies : 13256-029
B-mercaptoethanol	Life technologies : 31350010
Insulin, Human recombinant (10mg/mL)	Sigma : I9278-5Ml
NEAA	Life technologies : 11140-050
DAPT	Sigma : D5942

Table A. 2 - Preparation of N2 and B27 medium for a total volume of 50mL.

N2 medium (50 mL)		B27 medium (50 mL)	
DMEM/F12	48,5 mL	**Neurobasal medium**	48,5 mL
N2 serum	500µL		
L-glutamax	500µL	**B27 serum**	1,0mL
NEAA	500µL		
B-mercaptoethanol	350µL	**L-glutamax**	500µL
Insulin	25µL		

Table A.3 - Neuralization medium used during neuronal differentiation. Concentration of the reagents.

Days	Neuralization medium (reagent)
Day 1 - Day 8	N2 medium (table 9.2 (a)) (50%) B27 medium (table 9.2 (b)) (50%) SMADi (1µM dorsomorphin + 10µM SB431542)
Day 8 - Day 12	N2:B27
Day 13 - Day 17	N2:B27 + AA2P (200µM)
Day 17 - Day 20	N2:B27 + AA2P + BFGF (10ng/mL))
Day 21 – Day 27	B27 + AA2P + DAPT (10µM)
Day 28 – Day 30	B27 + AA2P

Section B - Immunocytochemistry (ICC) – further information

DABCO Antifade recipe

Table B.1 - Reagents included in DABCO (SIGMA : D2522) recipe.

Reagentes	Volume
DABCO	0.233g
TRIS-HCl 1M (ph 8.0)	200µl
Sterile H_2O	800µL
86% Glycerol	9mL

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