INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO EM TECNOLOGIAS LABORATORIAIS EM CIÊNCIAS FORENSES

EVALUATING THE EFFECTS OF CANNABINOIDS ON AN ORGANOID-BASED MODEL OF PRENATAL EXPOSURE

Trabalho submetido por

Rita Covas Gomes

para a obtenção do grau de Mestre em Tecnologias Laboratoriais em Ciências Forenses

outubro de 2022

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Trabalho orientado por

Doutora Sandra Tenreiro

e coorientado por

Prof. Doutora Evguenia Bekman, Doutora Luísa de Lemos,
Prof. Doutor Alexandre Quintas

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Declaração de Honra

Certifico que sou responsável pelo trabalho submetido neste relatório e que o trabalho é original, não sendo copiado ou plagiado de outra fonte, excetuando o especificado nas referências. Adicionalmente, declaro que o trabalho em si contido não foi submetido anteriormente para qualquer outro propósito.

Monte de Caparica, 28 de Outubro de 2022

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(Rita Covas)

Resumo

Nos últimos anos, a prevalência do uso de canabinóides tem aumentado rapidamente, inclusive em mulheres grávidas. Fitocanabinóides, substâncias extraídas da *Cannabis sativa*, atuam principalmente pela ligação aos recetores canabinóides 1 e 2 (CB1R e CB2R), exercendo efeitos psicoativos e não psicoativos. Os canabinóides sintéticos são uma classe de Novas Substâncias Psicoativas, e apresentam altas afinidades de ligação aos CB1/2R. Visto que o sistema endocanabinóide é altamente relevante para o desenvolvimento embrionário, temos como hipótese que o consumo de canabinóides durante a gravidez pode causar defeitos no neurodesenvolvimento. Assim, foi utilizado um modelo tridimensional que recapitula os passos iniciais do neurodesenvolvimento. Organoides corticais com 46 dias foram gerados e expostos aos fitocanabinóides Δ^9-tetrahidrocanabinol (Δ^9-THC) e canabidiol (CBD), e aos canabinóides sintéticos THJ-018 e EG-018 por 9 dias, mimetizando a exposição pré-natal aos mesmos. Estes foram também administrados na presença de um antagonista dos CB1R, para determinar se os seus efeitos são mediados por estes recetores. Análises de imunofluorescência, RNA e Western Blot foram realizadas para avaliar alterações em marcadores de diferenciação neuronal, e na expressão dos recetores canabinóides. Foi efetuado *single-cell calcium imaging* para avaliar a funcionalidade celular. Embora preliminares, estes resultados sugerem que o Δ^9-THC pode retardar a diferenciação neuronal e afetar a funcionalidade celular por mecanismos mediados pelos CB1R. O CBD induziu mínimas alterações nestes parâmetros, mas evidenciou a interação complexa entre CB1R e CB2R. Os canabinóides sintéticos THJ-018 e EG-018 apresentaram um impacto mais negativo comparativamente aos fitocanabinóides. Estes induziram uma redução de progenitores neurais e neurónios maduros, e um aumento da apoptose, sugerindo efeitos neurotóxicos. Algumas destas alterações não foram revertidas pelo antagonista dos CB1R, indicando a interação com outros recetores. No entanto, os nossos resultados não obtiveram significância estatística, evidenciando a necessidade de validação adicional. Contudo, este projeto demonstra o potencial dos canabinóides para impactar o neurodesenvolvimento embrionário, evidenciando a importância da investigação científica sobre exposição pré-natal a canabinóides.

Palavras-chave: fitocanabinóides, canabinóides sintéticos, recetores CB1, organoides corticais, diferenciação neuronal.

Abstract

In the last years, the use prevalence of cannabinoids has been rapidly rising, including among pregnant women. Phytocannabinoids, substances extracted from *Cannabis sativa*, act mainly by binding to cannabinoid receptors 1 and 2 (CB1R and CB2R), exerting a range of psychoactive and nonpsychoactive effects. Synthetic cannabinoids are a class of New Psychoactive Substances, and also present high affinities to CB1/2R. Since the endocannabinoid system is highly relevant for embryonic development, we hypothesise that cannabinoids consumption during pregnancy can lead to neurodevelopmental defects. For this purpose, a three dimensional model, recapitulating the first steps of neurodevelopment, was used. 46 days-old dorsal forebrain organoids were generated and exposed to phytocannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), and to synthetic cannabinoids THJ-018 and EG-018 for 9 days, to mimic prenatal exposure to these compounds. They were also administrated in the presence of a CB1R antagonist, to determine if the effects are CB1R-mediated. Immunofluorescence, RNA analysis and Western Blot were performed to evaluate alterations in neuronal differentiation markers, and in CB1/2R expression levels. Single-cell calcium imaging was performed to evaluate cell functionality. Although preliminary, the results suggest that Δ^9-THC might delay neuronal differentiation and affect neuronal functionality by mechanisms mediated by CB1R. CBD induced minimal effects on these parameters, but highlighted the complex interaction between CB1R and CB2R. Synthetic cannabinoids THJ-018 and EG-018 had a more negative impact compared to phytocannabinoids. Their administration led to a reduction of neural progenitors and mature neurons, and increased apoptosis, suggesting neurotoxic effects. Some of these alterations were not reverted by the CB1R antagonist, indicating interation with other receptors. However, our results lack statistical significance, highlighting the need for further confirmation. Nonetheless, this project demonstrates the potential of cannabinoids to impact embryonic neurodevelopment, highlighting the importance of research on prenatal exposure to cannabinoids.

Keywords: phytocannabinoids, synthetic cannabinoids, CB1 receptors, dorsal forebrain organoids, neuronal differentiation.

Outputs ensuring from the present thesis

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2-AG - 2-arachidonoyglycerol

2D – Two dimensional

3D – Three dimensional

Δ^9-THC - Delta^9-tetrahydrocannabinol

AAI - Aminoalkylindoles

AEA – Anandamine

A-P – Anterior-posterior

BDNF – Brain-derived neurotrophic factor

Ca^{2+} - Calcium

cAMP – Cyclic adenosine monophosphate

CB1R – Cannabinoid receptor 1

CB2R – Cannabinoid receptor 2

CBD – Cannabidiol

CD – Chemically defined

CNS – Central nervous system

CYP450 – Cytochrome P450

DAGL – Dyacilglycerol lipase

DSE – Depolarization-induced suppression of excitation

DSE – Depolarization-induced suppression of inhibition

EG-018 - 1-naphthalenyl(9-pentyl-9H-carbazol-3-yl)-methanone

EtOH – Ethanol

FAAH – Fatty acid hydrolase

FGF – Fibroblast growth factor

GABA – Gamma-aminobutytic acid

GDNF – Glial cell line-derived neurotrophic factor

GFAP – Glial fibrillary acidic protein

GPCR - G protein-coupled receptor

GSK3β - Glycogen synthase kinase 3β

hESCs – Human embryonic stem cells

hiPSCs – Human induced pluripotent stem cells

HPLC – High performance liquid chromatography

iPSC – Induced pluripotent stem cells

Ki – Inhibition constant

MAGL - Monoacylglycerol lipase

mTORC1 – Mammalian target of rapamycin complex 1

NAPE – N-Acylphosphatidylethanolamine

NAPE-PDL - N-acyl phosphatidylethanolamine-specific phospholipase D

NGF – Nerve growth factor

NPS – New Psychoactive Substances

NSAID – Non steroidal anti-inflammatory drug

OCT-4 - Octamer-binding transcription factor 4

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PI3K – Phosphatidylinositol 3-kinase

PNS – Peripheral nervous system

PPAR – Peroxisome proliferator-activated receptor

qRT-PCR – Quantitative real-time polymerase chain reaction

ROCKi – ROCK inhibitor

RT – Room temperature

SD – Standard deviation

TGFβ – Transformin growth factor β

THJ-018 - 1-naphthalenyl(1-pentyl-1H-indazol-3-yl)-methanone

TRPV – Transient Receptor Potential Vanilloid

TUJ1 – β-tubulin III

UNODC – United Nations Office on Drugs and Crime

VGCC – Voltage-gated calcium channels

1. Introduction

1.1. Endocannabinoid System

The endocannabinoid system is a group of endogenous signals, receptors and metabolic enzymes that was characterised in the 1980-1990s, after the discovery of the main phytocannabinoids (Cristino et al., 2020; Crocq, 2020). This system is composed by two G protein-coupled receptors, cannabinoid receptors 1 and 2 (CB1R and CB2R, respectively), their endogenous ligands – lipids also referred to as endocannabinoids, being the main ones anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes that synthetize and degrade them, N-acylphosphatidylethanolamine (NAPE), N-acylphosphatidylethanolamine-specific phospholipase d-like hydrolase (NAPE-PLD), fatty acid hydrolase (FAAH), diacylglycerol lipase (DAGL) α and β, and monoacylglycerol lipase (MAGL) (Cristino et al., 2020). This neuromodulatory system is mainly present in the nervous system, playing important roles on its development, synaptic plasticity and the response to endogenous ligands and exogenous mediators (Lu & Mackie, 2016).

CB1R are found essentially in the central nervous system (CNS), and in lesser amounts in the peripheral nervous system (PNS) (Hondebrink et al., 2017). These receptors are typically located presynaptically in excitatory and inhibitory neurons (Cristino et al., 2020) and are one of the most expressed GPCRs in the adult brain. There is a strong evidence that CB1R is related with cognition, memory, motor movement and perception (Gaffuri et al., 2012). CB2R are mainly expressed in the PNS, being predominantly present in cells of the immune system. They are also expressed in specific brain areas, such as the amygdala, brain stem, cerebellum, amongst others, though their expression levels are much lower than CB1R (Hondebrink et al., 2017). Cannabinoid receptors are present at the cell membrane and are coupled to heterotrimeric $G_{i/o}$ proteins. When activated by endogenous or exogenous cannabinoids, they trigger the pathway of inhibition of adenylyl cyclase activity and reduction of cyclic adenosine monophosphate (cAMP) levels (Galve-Roperh et al., 2013). Their activation also reduces neuronal activity by inhibiting voltage-gated calcium (Ca^{2+}) channels (VGCC) and modulating potassium channels (Galve-Roperh et al., 2013; Sarne et al., 2011).

Endocannabinoids are eicosanoids, oxidised derivatives of long-chain polyunsaturated fatty acids, and 2-AG is their main biosynthetic precursor (di Marzo, 2008). They are produced in response to the increase of intracellular Ca^{2+} concentrations by enzymes sensitive to this ion, with NAPE-PDL responsible for synthetising AEA, and DAGLα and β for the synthesis of 2-AG. As to its degradation, AEA and 2-AG are hydrolysed by FAAH and MAGL, respectively. Endocannabinoids have high affinities to cannabinoid receptors and act as local autocrine or paracrine mediators (di Marzo, 2008; Galve-Roperh et al., 2013), being AEA a partial agonist of CB1R and CB2R, and 2-AG a full agonist of both receptors (Rodrigues et al., 2019).

Evidence shows that several elements of the endocannabinoid system are present since early developmental periods, both *in vivo* and *in vitro* (Rodrigues et al., 2019), being its proper functioning highly critical, since it interacts with several other pathways, and regulates aspects such as lineage commitment, neuronal maturation and synaptogenesis (Rodrigues et al., 2019). Further information on the roles of CB1R and CB2R in neuronal differentiation can be found in sections 1.1.2. and 1.1.3., respectively.

1.1.1. Endocannabinoid Mechanism of Action

Endocannabinoids exert their effects mainly through CB1/2R receptors binding, most commonly in a mechanism designated by retrograde signalling (Figure 1.1). The neurotransmitter release in the presynaptic compartment induces an elevation in the Ca^{2+} levels in the postsynaptic compartment, which consequently leads to the synthesis and release of endocannabinoids that will bind to the cannabinoid receptors in the presynaptic compartment and suppress the release of neurotransmitters [γ-aminobutyric acid (GABA) or glutamate]. The existing enzymes in the neuronal membrane will then degrade the endocannabinoids and rapidly end the signal (Kreitzer & Regehr, 2002; Rodrigues et al., 2019). Endocannabinoids are synthetised in both inhibitory and excitatory neurons, and participate in short-term synaptic plasticity. This happens due to their modulation of depolarisation induced suppression of inhibition (DSI) and depolarisation-induced suppression of excitation (DSE). DSI occurs in GABAergic neurons, and consists of a transient decrease in their postsynaptic potentials after a repeated action potential or depolarisation. DSE is associated to a similar process, but in excitatory glutamatergic neurons (Cristino et al., 2020).

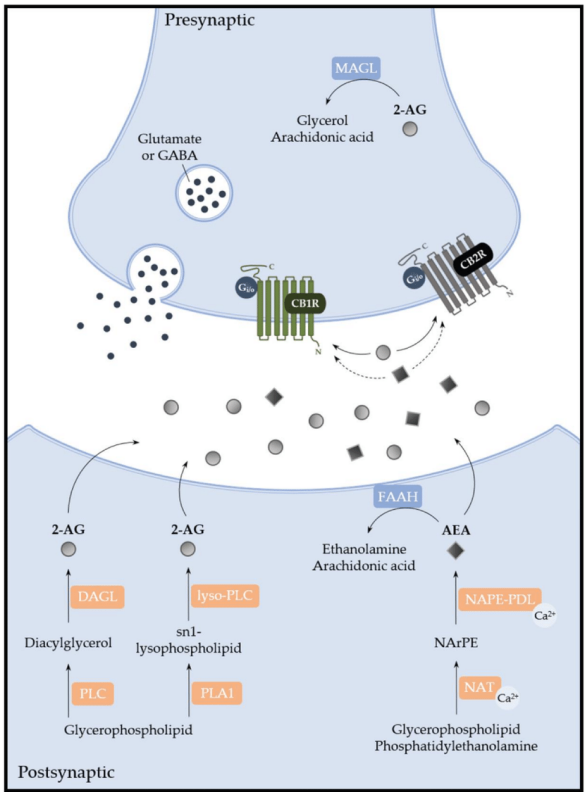

Figure 1.1- Endocannabinoid retrograde signaling and metabolism at neuronal. Endocannabinoids are synthetised in the postsynaptic compartment in response to an increase of intracellular Ca^{2+} levels. Anandamine (AEA) is synthetised from glycerophospholipids and phosphatidylethanolamine, in reactions catalysed by NAPE and NAPE-PDL. 2-arachidoglycerol (2-AG) is synthetised from glycerophospholipids by the action of DAGL and other enzymes. AEA and 2-AG activate CB1R and CB2R located in the presynaptic compartment, causing the suppression of neurotransmitter (GABA or glutamate) release. Endocannabinoids are finally degraded by MAGL and FAAH. Adapted from (Rodrigues et al., 2019).

Besides retrograde signalling, endocannabinoid signalling can also occur by other mechanisms, namely through autocrine signalling, when endocannabinoids bind to CB1R that are located postsynaptically, or by neuron-glia signalling, when endocannabinoids expressed in neurons activate cannabinoid receptors present in the membranes of astrocytes and glia (Rodrigues et al., 2019).

1.1.2. CB1 receptors in neuronal development

As previously said, the endocannabinoid system takes part in the neuronal development of the human brain, where CB1R is considered the most critical element of this system, due to the variety of functions it mediates through brain development (Diaz-Alonso et al., 2012). Its expression increases along neuronal differentiation, induced by neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve

growth factor (Galve-Roperh et al., 2013). CB1R is present in both glutamatergic projection neurons and GABAergic interneurons in the early development of the cerebral cortex and hippocampus. In the fetal brain, these receptors are already highly expressed in the cerebral cortex, caudate nucleus, hippocampus and cerebellum (Diaz-Alonso et al., 2012).

CB1R are expressed since early embryonic stages, in the blastocyst and trophoblast, with functions related to cell proliferation and differentiation, and are also important for embryo implantation (Diaz-Alonso et al., 2012). In embryonic development, studies show that CB1R signalling is deeply involved in determining the fate of neural cells affecting their proliferation, maturation and differentiation (Friedrich et al., 2016).

Regarding cell proliferation, studies show that CB1R (and CB2R) regulate several protein kinase cascades [e.g., phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycin complex 1 (mTORC1) signalling network; glycogen synthase kinase 3β (GSK3β) pathway] related to cell proliferation and survival, and are expressed in cells in the proliferative areas of the developing cortex, such as Cajal-Retzius cells of the marginal zone, and apical and basal progenitors present in the ventricular zone and subventricular zone since gestational week 9 (Diaz-Alonso et al., 2012). CB1R activation also promotes cortical progenitors proliferation (Galve-Roperh et al., 2013).

The action of AEA on CB1R was demonstrated to promote maturation and differentiation of neural stem cells into neurons in mouse embryonic cerebral cortex, evidenced by an increase in the levels of neuronal marker β-tubulin III (TUJ1), and glial marker glial fibrillary protein (GFAP), and a decrease in neural progenitor marker Nestin (Friedrich et al., 2016). CB1R are also highly concentrated in axonal growth cones; their activation also contributes to the dendritic and axonal initial segment development, corroborating the role of these receptors in neuronal maturation (Tapia et al., 2017). Studies indicate that these receptors might regulate Tbr1, Sox5, and other transcription factors that contribute to cortical laminar specification, hence their role in neuronal differentiation (Diaz-Alonso et al., 2012).

At later stages of neuronal development, CB1R signalling is involved in fundamental processes such as the regulation of tangential and radial migration of post-

mitotic neurons, thus contributing to the establishment of the functional architecture of the brain and proper spatial relationships between different types of neurons (Rodrigues et al., 2019). These receptors are also involved in the migration of GABAergic interneurons that are present in different brain areas and contribute to neural circuitry and activity by connecting other types of neurons (Kelsom & Lu, 2013).

1.1.3. CB2 receptors in neuronal development

In mammals, CB2R expression begins in early embryonic stages, in the inner cell mass, playing a role in lineage differentiation and the control of stem cells populations (Diaz-Alonso et al., 2012; Galve-Roperh et al., 2013). Contrarily to CB1R, CB2R expression levels are higher in undifferentiated cells and decrease with the course of neuronal specification (Galve-Roperh et al., 2013) . In the developing brain, CB2R expression is more accentuated in less committed cells and in microglia and macrophage lineages, indicating that these receptors might be involved in the modulation of immune function (Rodrigues et al., 2019). This was already observed in immunocytochemical analysis of human post-mortem samples of the developing brain between gestational weeks 9 to 40, which showed that CB2R was very low or not detectable in healthy samples, being mainly present in samples associated with cortical malformations in a few large dysplastic neurons, and in microglia and macrophages, supporting the role of CB2R in pathological conditions (Zurolo et al., 2010).

As mentioned before, CB2R also participate in the control of cell proliferation and survival, and consequently in progenitor cell fate decisions. In neural progenitor cells, these functions are related to PI3K and Akt pathways, as well as to mTORC1 signalling (Cristino et al., 2020).

1.1.4. Other Cannabinoid receptors

Cases where the activity of previously described cannabinoid receptors could not explain the observed effects, led to the search for other receptors targeted by these compounds. Studies where $CB1R^{-/-}$ and $CB2R^{-/-}$ mice were exposed to cannabinoids, but still exhibited effects characteristic of these compounds also corroborated this hypothesis, suggesting that other GCPRs could be involved in cannabinoids modulation (Brown, 2007). Non-CB1/2R sites are suggested to be essential in the vasculature, on

immune cells and in the CNS (Brown, 2007), but due to the subject of this project, we will focus on the last one.

Orphan GPR119, GPR18 and GPR55, three rhodopsin-like GPCRs whose ligands have yet to be completely elucidated, have been proposed to be novel cannabinoid receptors, being the last two more associated with neuronal functions (Cristino et al., 2020). The role of GPR18 in the CNS has been shown to be mainly related to neuroinflammation, due to its expression in microglia and reports of the existence of CB2R-GPR18 dimers that also support this hypothesis (Cristino et al., 2020). Although endocannabinoid ligands for this receptor are not entirely determined, AEA has been indicated to activate the receptor, and molecular docking studies have also pointed possible binding sites for Δ^9-tetrahydrocannabinol (Δ^9-THC) (Neumann et al., 2020). GPR55 is activated by endocannabinoid AEA, but also by exogenous cannabinoids Δ^9-THC and JWH-015, with effects similar to those of cannabinoid receptors, such as increasing intracellular Ca^{2+} concentration, releasing Ca^{2+} from intracellular stores and inhibiting potassium currents in assays using dorsal root ganglion neurons. Interestingly, CB1R antagonist SR141716A is able to attenuate the effects of Δ^9-THC and JWH-015 in this model, acting also as a GPR55 antagonist; although the same does not occur with the administration of CB2R antagonist SR144528 (Lauckner et al., 2008). It is also suggested that their activation may promote stimulation of hippocampal neurons, and be related to glutamate excitotoxicity in conditions such as epilepsy (Cristino et al., 2020). Little is known about how endocannabinoid signalling through these receptors contributes to embryonic and neonatal brain development.

Another possible cannabinoid receptor is transient receptor potential vanilloid 1 (TRPV1), found in GABAergic and glutamatergic terminals and neuronal somata in the cerebellum and hippocampus (Cristino et al., 2020). TRPV1 binds AEA and 2-AG, and it is thought to be involved in synaptic plasticity, affecting motor skills, mood, memory, etc., and also being responsible for increasing excitability of central neurons and glutamatergic transmission through microvesicle release from microglia during neuroinflammation (Cristino et al., 2020).

The peroxisome proliferator activated receptors (PPARs), a family of receptors typically linked to lipid metabolism, are also speculated to be targeted by cannabinoids (Galve-Roperh et al., 2013; Lu & Mackie, 2016). PPARγ is activated by AEA at

micromolar concentrations and by some oxidation products of 2-AG, and PPARα by other endocannabinoid-like lipids, such as palmitoylethanolamide. In the brain, PPARγ and PPARα have anti-inflammatory and neuroprotective properties in cases of acute and chronic neuroinflammatory events, and the last one is also thought to be involved in neuronal differentiation (Cristino et al., 2020). Studies have shown that transcriptional activation of PPARγ can be a mechanism by which AEA promotes adipocyte differentiation and increases glucose uptake, having a similar action to insulin (Galve-Roperh et al., 2013).

Recently, it was proposed the concept of endocannabinoidome to simultaneously describe the components of endocannabinoid system and all the mediators and contributors of other pathways and metabolic processes that interfere with the endocannabinoid system, being therefore a term that describes this complex network (Cristino et al., 2020). In addition to the cannabinoid receptors, endocannabinoids and interfering enzymes, the endocannabinoidome involves these other cannabinoid receptors - PPARα, Orphan GPR18, 119 and 55, TRPV1, but also calcium channels, $GABA_A$ receptors, N-acylethanolamines, N-acyl-aminoacids, endocannabinoid oxidation products and some enzymes (Cristino et al., 2020).

1.2. Cannabinoids

There are five different classes of identified cannabinoid compounds, based on their different chemical structures and subsequent action on the cannabinoid receptors: classical cannabinoids– such as Δ^9-THC or Δ^8-THC-dimethylheptyl (HU210); byciclic cannabinoids (e.g., CP-55,940); indole-derived cannabinoids (e.g., WIN55,212) – being these last two classes of synthetic cannabinoids; eicosanoids, meaning the endogenous ligands (e.g., AEA, 2-AG); and antagonist/inverse agonists (e.g., SR141716A and SR145528, for CB1R and CB2R, respectively) (Howlett & Abood, 2017).

This study is focused on phyto- and indole-derived synthetic cannabinoids, due to their high prevalence. A great part of these compounds displays a high affinity to cannabinoid receptors. Additionally, their structural differences from endocannabinoids lead to endogenous enzymes failing to identify and degrade them. Both of these factors lead to an extended modulation of CB1/2R, and consequently to a dysregulated endocannabinoid system response and long-lasting effects.

Despite of cannabinoids popularity, the molecular causes and the pathways involved in the effects these compounds on specific groups such as pregnant women are still yet to be elucidated, hence the main focus of this study.

1.2.1. Phytocannabinoids

Phytocannabinoids are C_{21} terpeno-phenols extracted from Cannabis genus plants (Cristino et al., 2020). Although these were only characterised in the 1960s, there are reports of these substances being consumed for medical and recreational purposes for thousands of years (Cristino et al., 2020). Currently, more than 120 phytocannabinoids have been identified, and the most abundant in the cannabis plant include: Δ^9-THC, Δ^8-tetrahydrocannabinol, cannabinol, cannabidiol (CBD), cannabigerol, cannabichromene, Δ^9-tetrahydrocannabivarin, cannabivarin and cannabidivarin. These compounds can be classified as psychotropic or non-psychotropic, based on whether they are mainly agonists of CB1R or CB2R, respectively (Morales et al., 2017).

An interesting property of phytocannabinoids is their high lipophilicity, which allows them to easily pass through physiological barriers and accumulate in the brain and adipose tissue (García-Gutiérrez et al., 2020), and also to cross the blood-placental barrier and pass to breast milk (Lucas et al., 2018). Thus, these compounds can dysregulate the endocannabinoid system, and impact on the functions it plays, affecting embryonic, fetal, and postnatal brain development. Studies show a prevalence of cognitive and behavioural defects in children born from women who consumed cannabinoids during pregnancy, including long-lasting effects in the endocannabinoid system signalling, motor activity, verbal development, nociception, and a predisposition to drug-seeking behaviour and psychiatric disorders (Broyd et al., 2016; Rodrigues et al., 2019).

It is estimated that 3.8% of the world population uses marijuana (2017) (Navarrete et al., 2020), also being the most used illicit drug during pregnancy. A study revealed that between 2009 and 2016 the consumption of marijuana amongst pregnant women increased, from 12.5 to 21.8% in women younger than 18, and from 9.8 to 19% among women aged 18-24 (Young-Wolff et al., 2017).

Being cannabis the most widely used illicit drug in the world (Atakan, 2012), and highlighting the constant increase of recreational and medicinal use of Δ^9-THC and CBD, we decided to focus on these two compounds in further sections.

1.2.1.1. Δ^9 – Tetrahydrocannabinol

Δ^9-THC (Figure 1.2) was one of the first components of *Cannabis sativa* to be discovered, in 1964 by Gaoni and Mechoulam (Gaoni & Mechoulam, 1964), and it is the main psychoactive compound of the plant. Currently, its metabolism, properties and effects are already fairly well characterised in several animal and *in vitro* models.

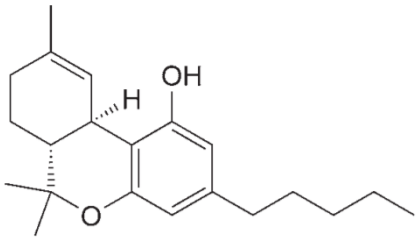

Figure 1.2- Chemical structure of Δ^9-THC. Adapted from (Mechoulam et al., 2007).

The most common route of administration of Δ^9-THC in recreational use is smoking, but due to the variables associated with inhalation, it is still difficult to estimate the typically consumed range of concentrations. Plasma concentrations of the compound peak within about 10 minutes after inhalation, and it is rapidly distributed into well-vascularised organs, having a bioavailability between 10 and 35%. Δ^9-THC is metabolised by cytochrome P450 (CYP450) enzymes CYP2C9, CYP2C19 and CYP3A4 to 11-hydroxy-THC (which also has psychoactive properties) and 11-carboxy-THC, and although the metabolism is mostly hepatic, it can additionally occur in the small intestine and the brain, since these tissues also express CYP450 enzymes. Regarding its elimination, the drug has a rapid initial half-life of 6 minutes. However, the residual concentration can remain in the body for up to 22 hours. These values can increase among heavy users (Lucas et al., 2018).

Δ^9-THC exerts its effects mainly by binding to CB1R and CB2R, being a partial agonist of both, but having a stronger affinity to CB1R (Table 1.1). Studies show that it can also modulate other endocannabidiome related receptors, such as GPC55 and

TRPV2, 3 and 4, and other types of receptors, such as serotonin receptors (namely the 5-HT_{3A} subunit) (Morales et al., 2017) .

Table 1.1- Binding affinities of Δ^9-THC and CBD to CB1R and CB2R. Values for the inhibition constants (Ki) of Δ^9-THC and CBD to human CB1R and CB2R, obtained by radioligand binding assays (Hess et al., 2016; Schoeder et al., 2018).

	Ki to CB1R	Ki to CB2R
Δ^9-THC	3.87 nM	71.6 nM
CBD	3.3 μM	34 nM

In humans, Δ^9-THC is known to cause a paraphernalia of effects, from feelings of well-being, anxiolysis, to euphoria and mood changes. Adverse effects also occur and are more common in chronic users, including panic and anxiety, disturbing changes in perception, hallucinations and delusions, though long-term effects in cognition are still to be confirmed (Carlini, 2004).

Besides recreational use, Δ^9-THC is often sought for therapeutic purposes since it is considered anti-emetic, an appetite stimulant, anxiolytic, antispasmodic, analgesic and hypnotic. These properties can improve symptoms of conditions such as AIDS, cancer and Tourette Syndrome and present as alternatives to conventional treatments for asthma, glaucoma and epilepsy. Additionally, it has been demonstrated that reduces acute, chronic and neuropathic pain when administrated in low doses, increasing symptoms when in high concentrations (Rock & Parker, 2021).

Δ^9-THC is classified as Schedule I Substance in the United Nations Convention in 1961, since it was considered to have a high potential for abuse and dependence, with limited medical and therapeutic value. Nevertheless, the World Health Organization has recently requested it to be rescheduled to IV since the substance has been further characterised and determined to have significant addictive properties (European Monitoring Centre for Drugs and Drug Addiction, 2019). Regarding their legal status, in Portugal cannabis-derived products are allowed for consumption in medical cases, according to Decreto-Lei n.º8/2019.

1.2.1.2. Cannabidiol

Although when discovered CBD was thought to be responsible for the psychoactive effects caused by Cannabis, is now known to be the main non-psychotomimetic compound produced by the plant (de Oliveira et al., 2019). In the last years, the search for CBD for its therapeutic properties has been rapidly increasing, being currently sold in form of over-the-counter supplements, cosmetics, prescription pharmaceuticals, amongst others (Nelson et al., 2020). However, the mislabelling of these products is becoming a recurrent problem – a recent study that tested 80 CBD-containing products showed that only 43 were accurately labelled (within a 10% margin of error), with other products' concentrations ranging from <90% to >110% of the designated dose, highlighting the possible problems of over administration, undesired side effects and drug-drug interactions (Johnson et al., 2022).

Regarding its pharmacokinetics, CBD has a similar profile to Δ^9-THC in peak plasma concentrations, bioavailability and distribution. It is mainly metabolised in the liver, by CYP2C19, CYP3A4 and to a less extent, CYP1A1, 1A2, 2C9 and 2D6; and is a potent competitive inhibitor of CYP2C and CYP3A, possibly leading to drug-drug interactions when administrated with other substances that are metabolised by these enzymes. The main metabolites of this phytocannabinoid are still being studied to evaluate their possible effects. Compared to Δ^9-THC, CBD has a longer elimination half-life, of around 31 hours after inhalation, and reaching 2 to 5 days in cases of chronic oral intake (García-Gutiérrez et al., 2020). Literature indicates that CBD has a safe tolerability profile in acute and chronic administration in a wide range of doses (from a single 6 g dose up to 3.5 g/day for 3 months) (Monti et al., 2020).

Although CBD (Figure 1.3) has a similar chemical structure to Δ^9-THC, the differences between their spatial conformations translate into distinct interactions with CB receptors, and consequently, different binding affinities (Table 1.1) and subsequent effects. Several studies have confirmed it to be a negative allosteric modulator of CB1R, when administrated in concentrations lower than 10 μM (Jakowiecki et al., 2021; Tham et al., 2019). Jakowiecki *et al.* demonstrated, through molecular modelling and ligand docking, that its allosteric binding site involves both the extracellular ends of two CB1R's helices and residues of the N-terminal domain (Jakowiecki et al., 2021). When bound to this site, CBD is able to influence orthosteric binding of other ligands to CB1R, for example, decreasing the potency and efficacy of Δ^9-THC and the

endocannabinoid 2-AG (Jakowiecki et al., 2021), and antagonising the effects of WIN-55212 and CP 55940, two potent CB1R agonists, at concentrations below its orthosteric binding affinity (Pertwee et al., 2002). This is a plausible explanation to why CBD decreases psychoactive and memory-impairing effects caused by Δ^9-THC in humans (Gülck & Møller, 2020).

Studies show that this substance also interacts with other types of receptors, including 5-HT_{1A}, GPR55, TRPV1, ENT1, adenosine and glycine receptors, opioid receptors, among others. Connection with the serotoninergic system may explain why the compound appears to be effective in anxiety and depression (García-Gutiérrez et al., 2020).

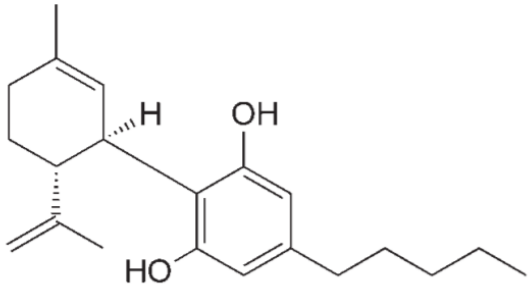

Figure 1.3- Chemical structure of CBD. Adapted from (Mechoulam et al., 2007)**.**

CBD has mainly non-psychoactive properties, due to its low affinity to CB1R orthosteric binding sites (Lucas et al., 2018), binding preferably to CB2R (Table 1.1). The effects of CBD consumption on the human body are yet to be fully elucidated. Actually, the literature is not consistent. Though, there is a consensus that CBD may have an anti-inflammatory and neuroprotective role, related to its ability to modulate pro-inflammatory cytokines and BDNF expression (Campos et al., 2016; Mechoulam et al., 2007). On the toxic side, both animal and *in vitro* studies demonstrate that it can also lead to embryo-fetal mortality and developmental toxicity, including neurotoxicity (Huestis et al., 2019; Miranda et al., 2020).

Therapeutic properties of the phytocannabinoid include anti-emetic and antinausea effects manifested in *in vivo* models of type-1 diabetes and anxiety (Mechoulam et al., 2007). Recently, medical CBD has been proposed for treatment-resistant epilepsy (Gülck & Møller, 2020) and autism, and is already administrated in

doses as high as 30% CBD oil (also containing 1.5% Δ^9-THC) daily for several months (Bar-Lev Schleider et al., 2019).

Consumption of CBD-containing products is legal in Europe since 2019, when The World Health Organization's Expert Committee on Drug Dependence concluded that the substance has no potential for abuse and to produce dependence, and therefore should not be considered a drug in the terms of the 1961 Convention (World Health Organization, 2019). Nonetheless, the easy access and lack of dose control in over-the-counter products highlight the importance of thorough characterisation of this compound.

1.3. New Psychoactive Substances

According to the United Nations Office on Drugs and Crime (UNODC), New Psychoactive Substances (NPS) are defined as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health treat" (United Nations Office on Drugs and Crime, 2021). Having started as legal alternatives to controlled substances – frequently referred to as "legal highs", NPS can be natural products, but are mostly synthetic analogues of previously existing illicit drugs, designed to mimic the actions and psychoactive effects of these substances (Feng et al., 2017; Shafi et al., 2020), and are categorised in eight main groups, namely aminoindanes, synthetic cannabinoids, synthetic cathinones, phencyclidine-type substances, phenethylamines, piperazines, plant-based substances, tryptamines and other substances (United Nations Office on Drugs and Crime, 2021). These compounds can also be organised by the effects they induce, in the following classes: stimulants, synthetic cannabinoid receptor agonists, classic hallucinogens, synthetic opioids, sedatives/hypnotics, dissociatives and others (or not yet assigned) (United Nations Office on Drugs and Crime, 2021).

According to UNODC, one or more NPS was reported by 134 countries and territories, and 1,124 substances have been reported up to December 2021 (United Nations Office on Drugs and Crime, 2021). Thus, NPS are nowadays considered a "growing worldwide epidemic". New substances are created and produced every day, representing a large number of substances categorised as NPS. Additionally, their rapid entrance and exit in the drug markets, and the lack of information on their potency,

effects and other characteristics make drug monitoring, surveillance, and public health response extremely difficult (Peacock et al., 2019). NPS are also growing on popularity, being used for similar purposes of other illicit drugs, such as recreational and therapeutic effects. However, the novelty of these substances, their availability and prices, lack of information about their adverse effects, and specially their legal status and not being detectable in drug screening tests, are factors that translate in a strong motivation for NPS use. Young people, people who are incarcerated and people who inject drugs or regularly use drugs are amongst the main groups of risk for NPS use (Peacock et al., 2019).

Regarding the legal status of these substances, it varies between countries, and it's still a challenge to implement control measures due to their transitory character. In the European Union, many countries, including Portugal, have already adapted their legislation to incorporate NPS. Besides that, in 1997 the European Council has implemented a system to identify, exchange information and rapidly respond to the risks of NPS, to better account with the emergence of these substances (Varì et al., 2020). Even so, scientific research about their mechanisms of action, properties and effects is mandatory and extremely important to characterise and to be able to properly inform the population about the risks of these drugs.

1.3.1. Synthetic Cannabinoids

Being first identified and reported to the European Monitoring Centre for Drugs and Drug Addiction in 2008, synthetic cannabinoids, or synthetic cannabinoid receptor agonists refer to synthetised analogues of the main psychotropic compound in cannabis, Δ^9-THC. Although, these compounds were initially produced to study possible therapeutic effects and cannabinoid receptor pharmacology in the 1960s, they emerged in the mid-2000s as an alternative to cannabis-derived products, with intents to avoid detection in forensic drugs testing procedures. Currently, it is estimated that between 0.2 and 4% of the world population uses synthetic cannabinoids (Cohen & Weinstein, 2018), being the most commonly abused NPS, according to UNODC (Chung et al., 2021).

These compounds are sold in herbal preparations named Spice or K2, and are often presented as dried vegetable matter sprayed with synthetic cannabinoids. They are

mainly marketed on head shops and on the internet, being regularly advertised as air refreshers, bath additives or potpourris to avoid being targeted by authorities (Zawilska & Wojcieszak, 2014).

Synthetic cannabinoids mechanism of action is yet to be completely characterised, but studies show that these compounds have high affinities to CB1R and CB2R, being generally full agonists of these receptors. Some of their matabolites also target CB1R, contributing to longer-lasting effects (Schifano et al., 2017). Such as with phytocannabinoids or other drugs, these effects can be modulated in the presence of other compounds that modulate the same receptors (Schifano et al., 2017). Recently, other possible target molecules of synthetic cannabinoids are being investigated, namely their action on the enzyme monoamine oxidase and on serotoninergic, muscarinic, dopaminergic and $GABA_A$ receptors (Asaoka et al., 2016; Fišar, 2010; Wiley et al., 2016; Yano et al., 2020).

Although some effects are similar to those experienced by cannabis users, reports indicate that the use of higher doses of these substances can also lead to hallucinations and anxiogenic effects associated with a tendency to continued intake. Synthetic cannabinoids induce effects faster, but with a shorter duration of action compared with cannabis use (Schifano et al., 2017). Acute intoxications have already been reported, associated with the following symptoms: high blood pressure, visual and auditory hallucinations, agitation and anxiety, hyperglycaemia, tachypnea, nausea, etc., and have already been associated with cases of chronic abuse and death (Schifano et al., 2017). Though synthetic cannabinoids are structurally different to phytocannabinoids, they are also highly liposoluble, also having possible adverse effects in neurodevelopment (Zawilska & Wojcieszak, 2014).

1.3.1.1. Aminoalkylindoles

Aminoalkylindoles (AAI) are a novel series of cannabinoid receptor ligands, initially designed by the Pfizer Central Research to create a non-opioid, non-aspirin-like analgesic (non- non-steroid anti-inflammatory drugs (NSAIDs)), based on the therapeutic but not psychoactive properties of Δ^9-THC (Eissenstat et al., 1995). Although this study was ended due to undesired side effects associated with the use of levonantradol in early clinical trials, the Sterling Research Group and the Howlet

Laboratory continued to study similar compounds that did not fit NSAIDs properties. The main therapeutic property of these substances was their antinociceptive ability, which was confirmed to be related to their binding to CB1R through radioligand binding assays (Howlett et al., 2021).

Years later, some of the most potent AAI got in the drug market as constituents of Spice. 1-pentyl-3-(1-naphthoyl) (JWH-018) was one of the most used AAI in Spice preparations, being present in 76% of these in 2010. Besides its high affinity to cannabinoid receptors, this compound has nine metabolites, and some present a significant affinity to CB1R and CB2R (Howlett et al., 2021).

In this study, we focused on two emerging AAI: 1-naphthalenyl(1-pentyl-1H-indazol-3-yl)-methanone (THJ-018) and 1-naphthalenyl(9-pentyl-9H-carbazol-3-yl)-methanone (EG-018). These compounds are both derivatives of JWH-018, being categorised as 2nd and 3rd generation synthetic cannabinoids, respectively. Comparing to phytocannabinoids, these compounds have higher affinities to human CB1R and CB2R, as seen in Table 1.2, in values obtained through radioligand binding assays (Schoeder et al., 2018).

Table 1.2- Binding affinities of THJ-018 and EG-018 to CB1R and CB2R. Values for the inhibition constants (Ki) of THJ-018 and EG-018 to human CB1R and CB2R, obtained by radioligand binding assays (Hess et al., 2016; Schoeder et al., 2018).

	Ki CB1R	**Ki CB2R**
THJ-018	5.84 nM	4.57 nM
EG-018	7.17 nM	2.22 nM

Information regarding these substances is scarce, with only a small number of case reports and few studies focused on their characterisation, binding affinities to cannabinoid receptors and metabolites. It is important to refer that for both substances some serious complications were registered. THJ-018, at a plasma concentration of 0.04 ng/mL, was associated with uncontrollable tremors and cognitive impairments such as slurred speech, problems with balance and concentration 24 hours after consumption (Hess et al., 2017); and EG-018 (plasma concentration of 0.14 ng/mL) was present in a lethal intoxication case, although there were other drugs present in both cases (Halter et al., 2019).

Fundamental characteristics of these compounds, such as their mechanism of action, properties and effects are yet to be completely elucidated. However, it is important to refer a pilot-study by Miranda et. al, that demonstrated that at a concentration of 10 μM, the exposure of hiPSCs-derived neurons to THJ-018 and EG-018 for 37 days led to premature neuronal and glial differentiation, abnormal functioning of VGCC, and consequently, to the development of functionally impaired neurons (Miranda et al., 2020).

1.4. Effect of Cannabinoids on Neural Differentiation

In vitro studies have shown that Δ^9-THC induces neuronal maturation, downregulation of cannabinoid receptors 1 (CB1R) and impaired neurite growth (Ao et al., 2020). In human-induced pluripotent stem cells (hiPSC)-derived cerebral organoids, the compound increased the number of deep-layer neurons and reduced the number of upper-layer neurons, suggesting that it might affect neurogenesis; and increased neuronal activity and maturation (Paraíso-Luna et al., 2020). Interestingly, there are still discrepancies on whether these compounds are neurotoxic or neuroprotective, with data supporting both options. These opposite effects are more associated with CBD, which has been shown to reduce oxidative stress in hippocampal neurons *in vitro*, at a 5 μM concentration, although it has a neurotoxic effect at higher concentrations (Nisticò et al., 2008). This cannabinoid also promoted differentiation in a neuroblastoma cell line, in a concentration-dependent manner as well (Blando et al., 2022).

Previous studies aiming to understand the effects of these compounds on neurodevelopment are mainly focused on phytocannabinoids. However, synthetic cannabinoids emerged in the last decades and its effects seems stronger than its natural counterpart. Recently, the exposure of human cell lines to synthetic cannabinoids has demonstrated that these substances can induce DNA fragmentation, morphologic changes and cell membrane damage, reduce neuronal maturation, impair neurite growth, and downregulate the main receptors of the endocannabinoid system (Ao et al., 2020; Coccini et al., 2021; Miranda et al., 2020; Notaras et al., 2021).

The pilot-study previously developed by our group highly contributed to elucidating the effects of synthetic cannabinoids in a chronic exposure prenatal model. The administration of THJ-018 and EG-018 for several weeks to developing neurons

affected neuronal differentiation, decreased cell viability, and altered cannabinoid receptors expression levels. In this study, the effects of Δ^9-THC and CBD were also analysed, having the synthetic cannabinoids-exposed neurons exhibited stronger effects than the Δ^9-THC-exposed ones; with contradictory effects of CBD facing what is known in the literature, that need to be further elucidated (Miranda et al., 2020).

1.5. Modelling neuronal development with Cortical Organoids

The complexity of the human brain has led to years of research to perfect a model that allows to mimic and study its development, functions and connectivity. Whilst in the past most of the information was obtained through the study of the post-mortem brain (Shou et al., 2020), or of animal models such as mice, ethical issues and the disparities between the mouse and human brain have led two-dimensional (2D) cell cultures and three-dimensional (3D) *in vitro* systems using human-derived neural cells to increasingly gain popularity in the neurobiology field (Qian et al., 2019). In the next sections, we discuss the embryonic development *in vivo*, and how these novel models are suitable to recreate them, as well as their advantages and applications.

1.5.1. Embryonic neuronal development

Early human embryonic development is characterised by the formation of the blastocyst, which consists of a small population of cells: the inner cell mass, surrounded by trophoblast that will generate extraembryonic tissues including the placenta. The inner cell mass that will generate the embryo proper, gives rise to the epiblast, the pluripotent primary lineage that originates the three germ layers (endoderm, mesoderm and ectoderm) through a process designated gastrulation. By the 3rd week of development, concerted action of transforming growth factor beta (TGFβ) inhibitors, fibroblast growth factor (FGF), Sonig hedgehog and retinoic acid signalling lead to neural induction, a process that will generate the neural plate. This is followed by neurulation, the folding of the neural plate into the neural tube, that will form brain vesicles in its anterior parte and will eventually form the brain and spinal cord, composing the CNS (B & Muzio, 2019; Sadler, 2005).

Neural tube is patterned along the anterior-posterior (A-P) axis in a way that at each specific A-P location a different brain structure is formed. At the level of each of

these structures (forebrain, midbrain, hindbrain, spinal cord) occurs progenitor expansion, neuron production and migration (Figure 1.4). Expansion of the neuroepithelium generates radial glial cells, creating the ventricular zone, that afterwards divide and generate intermediate progenitors and neurons. While intermediate progenitors are located in the subventricular zone, a neurogenic area that forms adjacent to the ventricular zone, neurons migrate through the intermediate zone to occupy the different layers of the cortical plate (Lancaster et al., 2013).

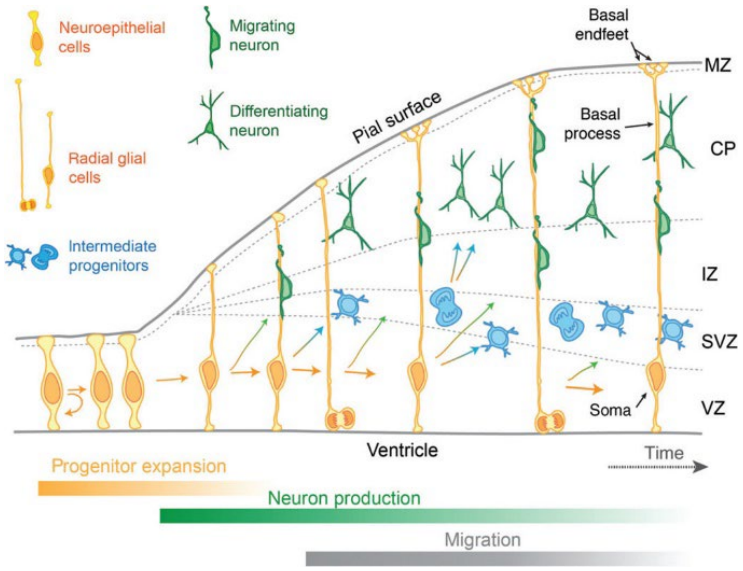

Figure 1.4- Schematic representation of human cortical development. The three populations of neural progenitors (neuroepithelial cells, radial glial cells and intermediate progenitors) are represented, as well as neurons (migrating and differentiated). Neural progenitors of the VZ go through self-renewal division, originating new progenitors (curved arrow) and division to generate either progenitors or neurons (straight arrow). In corticogenesis, progenitors originate intermediate progenitors and neurons. Latter will then migrate and form the several cortical layers. VZ – ventricular zone; SVZ – subventricular zone; IZ – intermediate zone; CP – cortical plate; MZ – marginal zone. Adapted from (Pilaz & Silver, 2015).

1.5.2. Human induced pluripotent stem cells (hiPSC)

An important advance in science occurred in 2006/2007, when, just one year after developing induced pluripotent stem cells (iPSC) from mouse fibroblasts, Takahashi and Yamanaka succeeded in generating human iPSCs. By reprogramming fibroblasts and other types of somatic cells with retroviral transduction of the transcription factors octamer-binding transcription factor 4 (Oct-4), sex determining region Y-box 2 (Sox2), Kruppel-like factor (Klf4) and c-Myc, they produced hiPSCs that were similar to human embryonic stem cells (hESC), cells that are isolated from the inner mass of the blastocyst, in characteristics such as morphology, proliferation,

pluripotency markers [e.g. T cell receptor alpha locus (TRA-1-60), Sox2, Nanog, Oct-3/4], gene expression and *in vitro* differentiation. A great advantage of hiPSCs is that they, similarly to hESC, can self-renew (meaning that they can go through several cell division cycles and maintain their undifferentiated state) and differentiate into any cell type of the three germ layers (Takahashi et al., 2007). hiPSC bear two additional advantages over hESC, they are patient-specific and ethical issues-free (Shi et al., 2017).

Among the various applications of hiPSCs are the generation of donor-derived healthy and diseased lines, that allow disease modelling, drug screening and toxicity studies, drug development and cell-based therapy. The use of hiPSCs also avoids the differences between human cells and animal models, and allows the generation of diseased lines, which was not possible with hESC (Huang et al., 2019; Takahashi et al., 2007).

1.5.3. Brain Organoids

One of the unique characteristics of pluripotent cells is their ability to self-organise in cell aggregates that are able to recapitulate embryonic development and give rise to different organ-like structures called organoids (Shi et al., 2017).

Recently, to find a more accurate model that recapitulates the developmental, architectural, and species-specific aspects of the human brain, 3D systems also designated as brain organoids were developed (Sloan et al., 2018). Brain organoids can be derived from hiPSCs and can be generated through two types of methodologies: unguided, using minimal external factors and relying on self-organisation and spontaneous morphogenesis; and guided, which benefit from the use of specific supplementation and other factors to promote differentiation towards a certain lineage (Qian et al., 2019).

The first 3D structures created to recapitulate the brain were cerebral organoids developed by Eiraku *et al.*, 2008 (Eiraku et al., 2008). Later, Lancaster *et al*. showed that through self-organisation, these organoids recapitulated the main mechanisms of mammalian neurodevelopment, including several regions of the brain and expressing markers characteristic of the forebrain, midbrain and hindbrain, although structures as

the choroid plexus and immature retina were also present (Lancaster et al., 2013). This approach can have disadvantages like the unpredictability and irreproducibility of each lineage and cell types in different experiments, consequently generating variability in quantitative studies (Qian et al., 2019).

New protocols have been established to develop brain organoids, but following a guided differentiation approach, in order to generate region-specific brain organoids. These methods take advantage of the use of patterning factors (supplements, inhibitors, small molecules and growth factors) to generate 3D structures that contain cell types of just a specific brain region, such as the dorsal or ventral forebrain, the cerebellum, etc. These organoids surpass the challenges associated with cerebral unguided organoids, having minimal heterogeneity and increased reproducibility, and although they do not recreate more intricate, whole brain connectivity/circuitry, present themselves as a promising platform to model human neurodevelopmental and neurodegenerative disorders, as well as drug screening (Paşca, 2019).

This project is focused on dorsal forebrain organoids, which resemble the region that generates the cerebral cortex, the most affected area of the brain when there is substance abuse (Paşca, 2019). Cortical organoids are the most well characterised type of patterned organoids, and it has been demonstrated that they exhibit cortical structures with layers that resemble, at a molecular, cellular and structural levels, the ventricular zone, subventricular zone and the cortical plate (Qian et al., 2019), mimicking the development of the neural tube and subsequent proliferation and production of cell lineages of forebrain identity, namely glutamatergic neurons and at later stages, astrocytes (Figure 1.5) (Tao & Zhang, 2016).

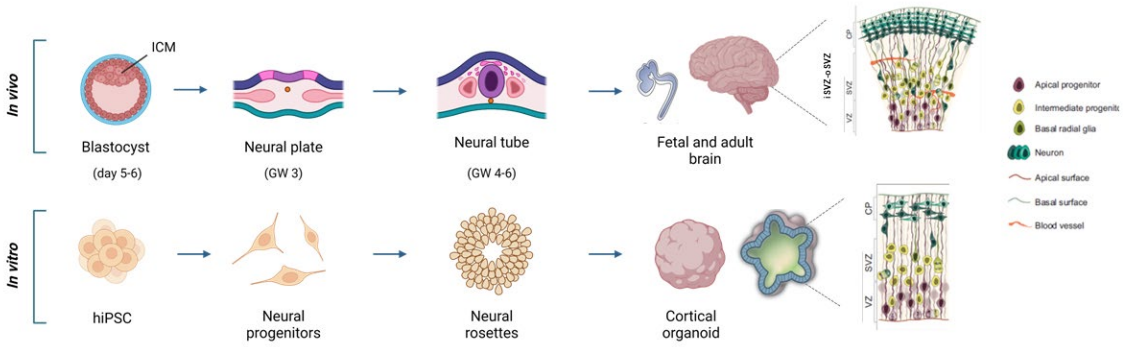

Figure 1.5- Corresponding stages of human neuronal differentiation *in vitro* and *in vivo*. Blastocyst is formed around day 5-6 of gestation, containing the inner cell mass (ICM) that will generate

the embryo and extraembryonic tissues. Neural plate is formed by gestational week (GW) 3, and during GW 4-6, its folding originates the neural tube. Neural tube contains tissues that will, with the gestational progress, create the CNS. *In vitro* differentiation starts by using hiPSC, and with the addition of medium supplements that promote neural differentiation, generate neural progenitors. These organize in neural rosettes, resembling neural tube structure. Neural progenitors and neurons of cortical organoids organize in layers similar to those of the cerebral cortex. VZ – ventricular zone; SVZ – subventricular zone; iSVZ – inner subventricular zone; oSVZ – outer subventricular zone; CP – cortical plate. Created with BioRender.

Cortical organoids have already been used successfully to study exposure to cannabinoids, displaying a functional endocannabinoid system. Exposure of 30 days-old cortical organoids to 1 μM Δ^9-THC for 3 days, through a microfluidic platform, led to decreased expression levels of *CN1R*, the gene that encodes CB1R, inhibited neurite outgrowth and spontaneous neuronal activity, and also altered the ventricular and subventricular zone distribution (Ao et al., 2020). Administration of 10 μM of the indole-derived cannabinoid WIN 55,212-2 to forebrain organoids for 7 days demonstrated that this compound led to increased DNA fragmentation, induction of DNA damage and apoptosis, and a strong depletion of the number of new-born neurons (Notaras et al., 2021).

1.6. Hypothesis and Aim

Since the endocannabinoid system is highly relevant for embryonic development, we hypothesise that the consumption of cannabinoids during pregnancy can lead to altered neurodevelopment, mediated by their interaction with CB1R; using dorsal forebrain organoids to recapitulate embryonic development of the cerebral cortex.

Therefore, the aims of this study are to:

1. Optimise a model of dorsal forebrain organoids to generate a proper platform for screening of cannabinoids;
2. Determine the effects of phytocannabinoids Δ^9-THC and CBD and synthetic cannabinoids THJ-018 and EG-018 on neuronal development, endocannabinoid system signalling, and cell functionality;
3. Determine if the neuronal effects caused by cannabinoids are mediated by CB1R, by administrating an antagonist of these receptors.

2. Materials and Methods

2.1. Purification of Cannabinoids

Phytocannabinoids and synthetic cannabinoids were purified at Egas Moniz Cooperativa de Ensino Superior, in the Molecular and Forensic Pathology Lab. Compounds were isolated from commercial samples through High-Performance Liquid Chromatography (HPLC) and solubilised in ethanol (high purity grade >99%, Sigma) or Chemically Defined (CD) Lipid Concentrate (Gibco). CB1R antagonist SR141716A was also solubilised in these vehicles.

2.2. hiPSC culture

Gibco ® Human Episomal iPSC line derived from CD34+ cord blood (iPSC6.2 (Burridge et al., 2011)) was cultured in Matrigel (1:100 in DMEM, Corning) coated 6-well plates (Corning) and grown in mTeSR plus medium (StemCell Technologies) supplemented with 10 μM ROCK inhibitor (ROCKi, Y-27632, StemGent) after the thawing process to prevent cellular death. The medium was changed every day or every two days, and the cells were passaged in a split ratio of 1:3 or 1:4 when they reached about 80% of confluence by incubation with 0.5 mM EDTA dissociation buffer (ThermoFisher Scientific) for 5 minutes at room temperature (RT). Cells were incubated at a humidified incubator at 37 ºC and 5% CO_2, simulating physiological conditions, and were routinely tested for mycoplasma.

2.3. 3D Induction and maintenance of dorsal forebrain organoids

The development of dorsal forebrain organoids was based on the modified Dual SMAD inhibition differentiation protocol, consisting of using a neural induction medium, TGFβ signalling inhibitors, and a proper system that promotes the development of cortical progenitors and neurons.

When hiPSCs were at around 90% of confluence were incubated with accutase (Sigma) for 6-7 minutes at 37 ºC, then washed and resuspended in mTeSR plus medium supplemented with 10 μM ROCKi. Subsequently, the cells were counted, by dilution in Trypan Blue (Gibco), and plated at a density of 1.5 million cells per well of the AggreWell plate (AggreWell^{TM}800, StemCell Technologies), previously prepared

according to manufacturer's instructions. To assure aggregates formation, the plate was centrifuged for 3 minutes at 1000 rpm in both directions. Cells were incubated at 37 ºC with 5 % CO_2, as previously described.

24 hours after the seeding, the plates were inspected under the microscope, for the presence of a small cell aggregate with well-defined round edges in each microwell. At this timepoint, half of the medium was changed to mTeSR plus without ROCKi. If the aggregates were around 250 – 300 µm in diameter, the neural induction medium was started in the next day, which was defined as day 0.

By days 0, 3 and 5 half of the medium was changed to neuronal medium N2B27 without vitamin A (Table 2.1), supplemented with 2 µM dorsomorphin (Sigma) and 10 µM SB-431542 (Sigma). SB-431542 is a TBFβ-receptor blocker, added to inhibit mesenchymal differentiation and promote neuroectodermal differentiation (Muguruma et al., 2015). Dorsomorphin is a protein kinase inhibitor that regulates cell fate decisions in hiPSCs, supressing mesoderm, endoderm and trophoectoderm by inhibiting TGFβ receptors and BMP signalling pathway (Zhou et al., 2010).

Table 2.1- Composition of neural induction medium N2B27. N2B27 is a chemically defined differentiation medium, composed by equal parts of N2 and B27 media. N2 medium is composed by DMEM/F12 (1:1) + GlutaMAX™, 1% (v/v) N2 supplement, 1.6 g/L glucose, 10 µg/mL insulin and 1% Penicillin-Streptomycin. B27 medium is composed by Neurobasal ® Medium, 2% (v/v) B27 supplement without vitamin A, 2 mM GlutaMAX™ and 0.5 % Penicillin-Streptomycin.

		Reagent	Brand	Concentration
N2B27 Medium	**50% (v/v) N2 Medium**	DMEM/F12 (1:1) + GlutaMAX™	Gibco	-
		N2 supplement	Gibco	1% (v/v)
		Glucose	Sigma	1.6 g/L
		Insulin	Sigma	10 µg/mL
		Penicillin-Streptomycin	Gibco	1% (v/v)
	50% (v/v) B27 Medium	Neurobasal ® Medium	Gibco	-
		B27 supplement without Vitamin A	Gibco	2% (v/v)
		GlutaMAX™	Gibco	2 mM
		Penicillin-Streptomycin	Gibco	0.5%

By day 7, cells were seeded in Ultra-low attachment 6-well plates (transferring one well of the AggreWell plates to 1 well of the Ultra-low attachment plates). Half of the medium was changed adding 1 μM CHIR99021 (Stemgent), 10 μM SB-431542 (Sigma) and 10 μg/mL Heparin (Sigma). Both CHIR99021 and heparin are inhibitors of GSK3β and activate Wnt signalling, regulating proliferation, differentiation, migration and decreasing apoptosis when added at early induction stage (Bejoy et al., 2018; Delepine et al., 2021). Between day 7 and day 15, half of the medium was changed every 2-3 days, without needing to add any specific supplement.

On day 15, half of the medium was changed to N2B27 with vitamin A [similar composition to what is described in Table 2.1, only altering the B27 supplement (Gibco)]. Until day 40, half of the medium was replaced every 2-3 days, without adding any supplements.

On day 37 we started the administration of phytocannabinoids Δ^9-THC and CBD, synthetic cannabinoids THJ-018 and EG-018 or CB1R antagonist SR141716A, testing the conditions presented in Table 2.2. Some conditions were altered between differentiations, with the aim of optimising the study: the first differentiation was used to determine which concentrations would be more suitable to mimic prenatal exposure to cannabinoids, and a concentration of antagonist compatible with the cells; the second differentiation was used to have a first assessment of cannabinoid-antagonist interactions and effects; and the third one was used to optimise the overall protocol, trying a different solution to vehiculate the compounds, due to previous problems associated with the use of ethanol (EtOH).

Table 2.2- Conditions tested in each differentiation. In the 1st differentiation two different concentrations of cannabinoids were tested (2 and 5 μM), as well as two concentrations (10 and 30 nM) of CB1R antagonist SR141716A. The compounds were vehiculated in ethanol (EtOH). In the 2nd differentiation the same compounds were tested at 2 μM concentration individually, and in the presence of SR141716A. 3rd differentiation was used for otimization of the vehicle. 2 μM of Δ^9-THC and CBD were administrated individually, and with SR141716A, both vehiculated in EtOH and in chemically defined lipid concentrate (CD lipid concentrate).

1st differentiation	▪ Untreated ▪ Vehicle (0.01% EtOH) ▪ 2 and 5 μM of the following CB: Δ^9-THC, CBD, EG-018 and THJ-018 (administrated individually and without combination with SR141716A)

	▪ 10 and 30 nM of SR141716A
2nd differentiation	▪ Untreated ▪ 0.01% EtOH ▪ Individual administration of 2 μM of Δ^9-THC, CBD, EG-018 and THJ-018 ▪ 10 nM of SR141716A ▪ Simultaneous administration of 2 μM of each cannabinoid with 10 nM SR141716A
3rd differentiation	▪ Untreated ▪ 0.01% EtOH ▪ 0.01% CD lipid concentrate ▪ Individual administration of 2 μM of Δ^9-THC and CBD (both vehiculated in EtOH and CD lipid concentrate) ▪ 10 nM of SR141716A (both vehiculated in EtOH and CD lipid concentrate) ▪ Simultaneous administration of 2 μM of each phytocannabinoid with 10 nM SR141716A (both vehiculated in EtOH and CD lipid concentrate)

From day 40 on, the organoids were cultured in BrainPhys™ medium (StemCell Technologies), supplemented with BDNF, glial cell line-derived neurotrophic factor (GDNF), cAMP and ascorbic acid. Phytocannabinoids, synthetic cannabinoids and CB1R antagonist were continuously added in the same concentrations in every medium change (every 2-3 days), till the end of the differentiation (day 46). The exhausted medium was collected and saved at -80 ºC to posteriorly be analysed by purification and quantitative analysis, to determine the stability of these compounds in the medium and their uptake by the organoids.

By day 46 of the differentiation the organoids were processed according to the following analysis to perform.

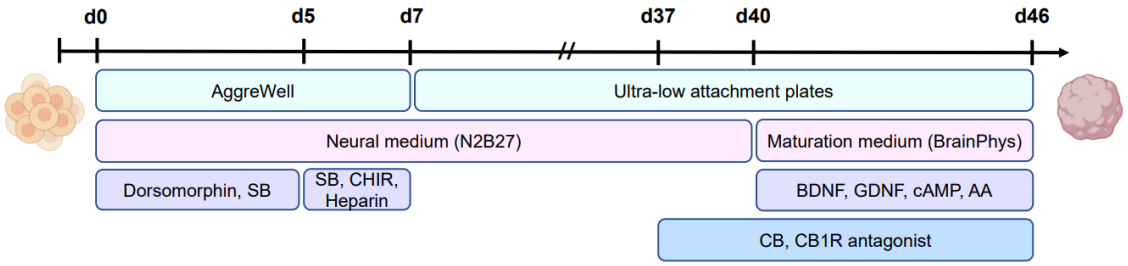

Figure 2.1- Schematic representation of the protocol used to generate dorsal forebrain organoids from hiPSCs. Organoids were seeded in Aggrewell plates, and replated into Ultra-low attachment plates by day 7. N2B27 neural medium was used till day 40, supplemented with dorsomorphin and SB-431542 until day 3, and SB-431542, CHIR99021 and heparin until day 7. Maturation medium BrainPhys was introduced by day 40, supplemented with BDNF, GDNF, cAMP and ascorbid acid (AA). Treatment with cannabinoids (CB) and CB1R antagonist was started by day 37 and continued until the end of differentiation. Created with Biorender.

2.4. Immunofluorescence analysis

2.4.1. Immunostaining in 2D cultures

To prepare cells for immunofluorescence analysis, they were quickly washed with PBS, and fixed with 4% paraformaldehyde (PFA) for 15 minutes at 4 ºC. Then, they were firstly incubated with 0.1 M glycine (Millipore) to remove PFA residues, with 0.1 % Triton-X100 (Sigma) in PBS to permeabilise cell membranes, both for 10 minutes at room temperature (RT), and in blocking solution [10% fetal bovine serum (FBS) in TBST (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20)] to minimise unspecific binding of the primary antibody), for 20 minutes at RT. These steps were followed by incubation with the primary antibody of interest (Table 2.3), diluted in blocking solution, overnight at 4 ºC, and in the next day, after three TBST washes, with the secondary antibody (Table 2.4), also diluted in blocking solution, for 30 minutes at RT. Finally, after a TBST wash, the nucleic acid stain DAPI (4',6-diamidino-2-phenylindole) was added at a concentration of 0.15% (v/v), and incubated for 5 minutes at RT. The cell-coated coverslips were placed in a microscope slide using mounting medium Mowiol (Sigma). Images were acquired in a confocal microscope (Zeiss Laser Scanning Microscope 980) and analysed in ImageJ software (Version 2.3.0).

Table 2.3- Primary antibodies used for immunofluorescence on 2D cells and organoids cryosections. Antibodies and respective references and dilutions used for immunostaining. Nanog and Oct4 antibodies were used in the staining of 2D cultures of hiPSCs. Cleaved caspase 3, MAP2, NESTIN and TUJ1 antibodies were used on organoids cryosections.

Protein of interest	Reference	Species	Dilution (in blocking solution)
Nanog	Abcam (ab173368)	Rabbit	1:100
Oct4	Merk (MAB4419)	Mouse	1:125
Cleaved caspase 3	Cell Signaling (9661)	Rabbit	1:400
MAP2	Sigma (M4403)	Mouse	1:500
Nestin	Invitrogen (MA1-110)	Mouse	1:200
PAX6	Covance (PRB-278P	Rabbit	1:400
TUJ1	Biolegend (MMS-435P)	Mouse	1:400

Table 2.4- Secondary antibodies used for immunofluorescence on 2D cells and organoids cryosections. Antibodies and respective references and dilutions used for immunostaining.

Secondary antibody	Reference	Dilution (in blocking solution)
Alexa 488 goat anti-rabbit IgG H+L	Invitrogen (A11008)	1:500
Alexa 546 goat anti-mouse IgG H+L	Invitrogen (A11003)	

2.4.2. Immunostaining on organoid cryosections

Organoids from different timepoints (day 13 and 46) were washed in PBS, and fixed with PFA for 45 minutes at 4 ºC. After two PBS washes, the organoids were then equilibrated in 15% sucrose overnight at 4 ºC and then in a 15% sucrose and 7.5% gelatine solution for 2 hours at 37 ºC. Organoids were embedded in the previous solution and frozen in -80 ºC isopentane, being kept at this temperature until usage.

The organoids were sectioned in the cryostat Leica CM3050 S (Object temperature: -25 to -30 ºC, chamber temperature: -25 ºC), in 12 µm sections, and placed in SuperFrost™ Plus Adhesion Microscope Slides (Epredia).

The sections were de-gelatinised, by being incubated in pre-warmed PBS at 37 ºC, for at least 40 minutes. After two 5 minutes PBS washes PFA residues were removed by incubating the slides in a solution of 0.1 M glycine in PBS, for 10 minutes at RT, and then permeabilised with 0.1% Triton-X100 in PBS, for the same period of time. Then, they were incubated with blocking solution [10% fetal bovine serum (FBS) in TBST (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20)] for 30 minutes at RT, and later with the primary antibody of choice diluted in blocking solution (Table 5), incubating overnight at 4 ºC. The day after, the organoid sections are incubated with secondary antibody (Table 6) diluted in blocking solution for 30 minutes at RT and 0.15% (v/v) DAPI solution for 5 minutes at RT, doing three TBST washes in between each step. A final wash with PBS for 1 hour was made to minimise background, and finally the slides were prepared adding Mowiol and covering with a coverslip. Samples were observed under a confocal microscope (Zeiss Laser Scanning Microscope 980), acquiring images with 25 and 40 magnitude. Images were processed and analysed with the ImageJ software.

2.5. Western Blot analysis

2.5.1. Protein extraction

By day 46 of the differentiation protocol, the organoids were washed in PBS and kept in a cell pellet, at -80 ºC, till protein extraction.

To proceed to protein analysis, 40 uL of 1X lysis buffer (Cell Signaling) supplemented with Halt™ Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) was added to the samples, followed by 2 rounds of sonication for 3 seconds at 15% intensity in the Branson Digital Sonifier SFX 150 (Emerson). This step aims to break cell membranes, allowing protein extraction and disruption of genomic and cellular nucleic acids, preventing them to interfere with protein purification. The samples were then centrifuged for 10 minutes at 8000g at 4º C to separate membrane fragments, and the supernatant was collected.

The total amount of protein per sample was determined by performing the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and reading the absorbance at 560 nm (Tecan Infinite F200 PRO plate reader).

2.5.2. Sample preparation and Western Blot

Samples were prepared by adding 4x protein sample buffer [250 mM Tris-HCl (pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.02% bromophenol blue] to 20 µg of protein, and being denatured by heating at 95 ºC for 5 minutes.

Eletrophoresis was performed at 90 V after loading the samples in 12% polyacrylamide gels [30% acrylamide, 1.5 M Tris-Base (pH 8.8), 10% ammonium persulphate, N,N,N′,N′-Tetramethylethylenediamine, MilliQ water]. After this step, the proteins were transferred to 0.2 nitrocellulose membranes (BioRad), by semi-dry transfer (Trans Blot ® Turbo ^{TM}, BioRad), followed by a brief incubation in Ponceau S staining solution (0.1% Ponceau S; 5% acetic acid) to confirm the presence of proteins.

To block unspecific binding, membranes were incubated for 1 hour in a solution of 5% milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, distilled water) at RT, in agitation. After three 5 minutes TBS-T washes at RT, the membranes were incubated with the primary antibody of choice diluted in blocking solution (Table 2.5) overnight at 4 ºC with agitation. The next day, after three TBS-T washes, they were incubated with the corresponding secondary antibody (Table 2.6) diluted in 5% milk for 1h at RT, with agitation.

To detect the proteins, membranes were first washed in TBS-T three times, for 10 minutes each, at RT, to remove unspecific binding of the secondary antibody, and after adding ECL^{TM} Prime Western Blotting Solution, the chemiluminescence of the protein bands was measured in the ChemiDoc Imaging System (BioRad). Images were analysed in the ImageLab software (Version 6.1).

Table 2.5- Primary antibody used for Western Blot analysis of protein expression. Antibody and respective references and dilution used for Western Blot.

Protein of interest	Reference	Species	Dilution
SOX2	Abcam (92494)	Rabbit	1:1000

Table 2.6- Secondary antibody used for Western Blot analysis of protein expression. Antibody and respective reference and dilution used for Western Blot.

Secondary antibody	Reference	Species	Dilution (in blocking solution)
ECL Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody	GE Healthcare NA934V	Donkey	1:5000

2.6. mRNA analysis

2.6.1. RNA extraction, purification and cDNA synthesis

To extract RNA from samples (timepoints d0, 13 and 46), we collected the exhausted medium, washed the cells or organoids with PBS, and added 500 µL of NZYol (NZYTech), pipetting up and down several times to lyse cells until having a homogeneous mix. Samples were stored at -80 ºC until next steps.

RNA isolation was performed following the NZYTech NZYol protocol, according to the manufacturer's instructions. The total amount of RNA present in each sample was quantified using NanoVue Plus spectrophotometer (VWR).

To remove contamination with gDNA 5 µg of RNA was diluted in RNase-free water to a final volume of 25 µL, to which was added 25 µL of DNase I mix 2x (10x Buffer, 10 u/µL DNase I, RNase Out (Roche), H_2O), incubating 30 minutes at 37 ºC. Next step consisted of adding 0.1x vol 3 M of sodium acetate (pH 5.5) and 0.5 µL Glycogen Blue (15 µg/µL) to precipitate and stain RNA, respectively. Additionally, we added 3x vol 100% EtOH, and left the samples to precipitate at -80 ºC overnight, due to the small amounts of RNA. After centrifuging (30 min, 13000 rpm, 4 ºC), the supernatant was discarded and the pellet was washed with cold 70% and centrifuged again (5 min, 13000, 4 ºC). Finally, the RNA pellet was air dried for 15 min, resuspended in RNase-free water, and posteriorly quantified in NanoVue Plus.

500 ng of RNA were used as template to synthetize cDNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions.

2.6.2. Real-time quantitative PCR (qRT-PCR)

Relative gene expression was evaluated using 10 ng of cDNA, SYBR ® Green mix (NZYTech) and the primers listed in Table 2.7. All qRT-PCR reactions were done in duplicate, using the ViiA^{TM} 7 RT-PCR Systems (Applied BioSystems). Data was processed in the Design and Analysis software (Applied Biosystems) and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, using *GAPDH* as the reference gene, and normalising the values to the untreated group by day 46 of the differentiation.

Table 2.7- Primers used for qRT-PCR. Nucleotide sequences of forward and reverse primers used for qRT-PCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
hCN1R	GATGCGAAGGGATTGCC	CAGGAGGTCAGTGGTG
hCN2R	AGCTTTTCCCACTGATCCCC	TGATGGGCCTTCCAGAGAAC
hGAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
hGFAP	GTACCAGGACCTGCTCAAT	CAACTATCCTGCTTCTGCTC
hMAP2	CCACCTGAGATTAAGGATCA	GGCTTACTTTGCTTCTCTGA
hMKI67	GAAAGAGTGGCAACCTGCCTTC	GCACCAAGTTTTACTACATCTGCC
hNANOG	GCAGAAGGCCTCAGCACCTA	AGGTTCCCAGTCGGGTTCA
hNESTIN	GAAACAGCCATAGAGGGCAAA	TGGTTTTCCAGAGTCTTCAGTGA
hOCT4	CTGAGGGCGAAGCAGGAGTC	CTTGGCAAATTGCTCGAGTT
hPAX6	GAATCAGAGAAGACAGGCCA	GTGTAGGTATCATAACTCCG
hTBR1	CGTCTGCAGCGAATAAGTGC	AATGTGGAGGCCGAGACTTG
hTBR2 (EOMES)	CACATTGTAGTGGGCAGTGG	CGCCACCAAACTGAGATGAT
hβ-tubulin III	GCCTCTTCTCACAAGTACGTGCCTCG	GGGGCGAAGCCGGGCATGAACAAGAAGTGCAG

2.7. Single-Cell Calcium Imaging (SCCI)

Two days before performing the technique, 44 days-old organoids were gently dissociated with accutase and re-plated on Lab-Tek Chamber Slides (Nunc) previously coated with Matrigel.

On the day of the experiment, cells were preloaded in 5 µM Fura-2 AM (Invitrogen) in Normal Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 nM $CaCl_2$, 6 mM glucose, 10 mM HEPES, $NaHCO_3$, pH 7.4) for 45 minutes, in an

incubator at 37 ºC with 5% CO_2. Fura-2 is a selective fluorescent indicator with a high affinity to Ca^{2+}, altering its excitation peak from 340 to 380 nm when bound to it.

To determine the baseline activity of the cells, they were incubated with Normal Krebs for 4 minutes, followed by KCl Krebs for 2 minutes, to induce a response by mature neurons. After a washout of 3 minutes, immature neurons and neural progenitors were stimulated with 100 µM histamine, for 2 minutes, and a final washout was performed by adding Normal Krebs for 3 more minutes. The emission fluorescence was recorded at 510 nm by a cooled CDD camera (Photometrics CoolSNAP fx), in an inverted microscope with epifluorescence optics (Axiovert 135TV, Zeiss). Cells were excited at 340 nm and 380 nm, using a high-speed switcher (Lambda DG4, Sutter Instrument), and ratio images were acquired every second when the KCl and Histamine stimuli were administrated, and every 10 seconds in baseline and washout times.

Images were analysed using the MetaFluor software (Universal Imaging, version 1.0.93), to determine the percentage of responsive cells to each stimulus.

2.8. Statistical Analysis

Statistical analysis was performed in Graphpad software (version 8.0.2) and the results are presented as mean +- standard deviation (SD). Statistical significance was evaluated between conditions that had 3 replicates. Normal distribution of data was confirmed using Shapiro-Wilk test. When this was verified, we used unpaired parametric Student's t-test to evaluate differences between two groups, and One-way ANOVA test with multiple comparisons for more than two groups. When data did not fit normal distribution, Mann-Witney and Kruskal-Wallis tests were used to compare two groups or more than two groups, respectively. Differences were considered significant when $p<0.05$.

3. Results and Discussion

3.1. Validation of the model

3.1.1 Expression of pluripotency markers by hiPSCs

NANOG and OCT4 proteins constitute a core pluripotency transcriptional network and should be co-expressed in *bona fide* pluripotent cells (Boyer et al., 2005). NANOG is a homeodomain-containing protein, and is essential for early embryonic development (J. Silva et al., 2009). In mouse embryonic stem cells, its expression levels fluctuate from high to low and back, in opposition to the fluctuations in FGF signalling, generating a succession of high NANOG/low FGF stemness state and low NANOG/high FGF differentiation-prone state (Abranches et al., 2014). OCT4 is a core transcription factor essential for the maintenance of the pluripotency state. In the absence of OCT4 stem cells differentiate into trophoblast lineage, while its overexpression induces endodermal differentiation (Niwa et al., 2000). Therefore, monitoring the expression of this protein is crucial, since the cells must still be pluripotent when we begin the differentiation.

Both qRT-PCR and immunofluorescence results indicated the presence of pluripotency markers OCT4 and NANOG by day 0 (Figure 3.1).

It was previously shown that the iPSC6.2 cell line co-expressed these transcriptional factors (T. P. Silva et al., 2020), which goes according to what is observed in Figure 3.1. This validated the pluripotency state of the cells, as they were collected for analysis.

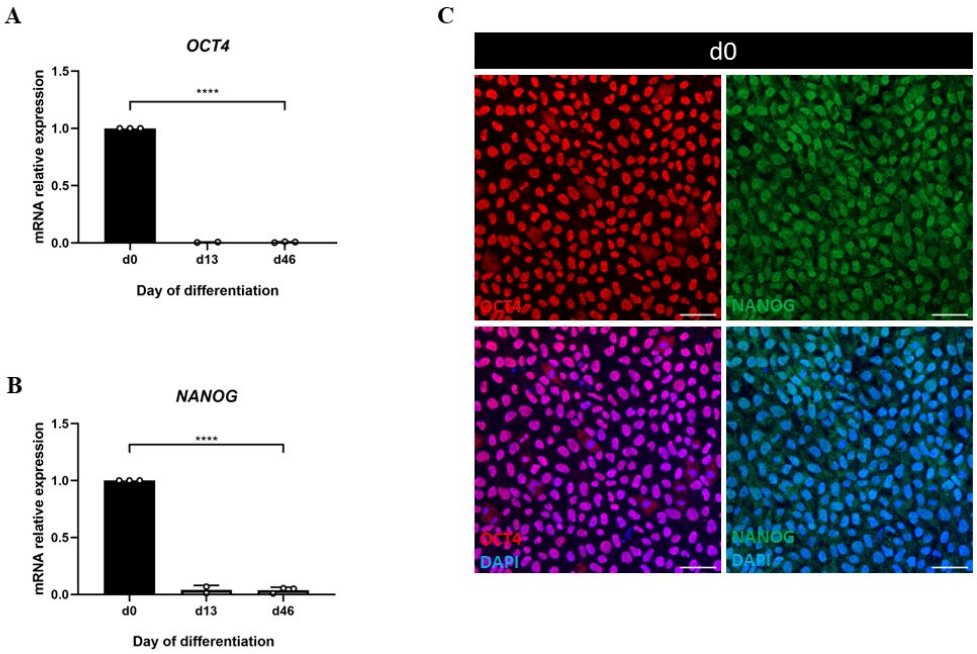

Figure 3.1 - Assessment of pluripotency markers on iPSC6.2 cells (passage 33). qRT-PCR analysis of A) *OCT4* and B) *NANOG* mRNA expression levels relative to *GAPDH* by day 0 of the differentiation. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). Unpaired parametric Students' t-test, ****p<0.001. C) Representative images of immunofluorescence analysis of OCT4 and NANOG in cells at day 0. Scale bars: 50 µm.

3.1.2. Successful neural induction and dorsal forebrain patterning

Dorsal forebrain organoids were generated according to Gomes *et al.* protocol, following dual SMAD inhibition for the acquisition of the dorsal forebrain identity (Gomes et al., 2020). To determine if the neural induction was successful, we evaluated the increase of neural commitment markers throughout the differentiation, which should be accompanied by the absence of pluripotency markers. We also evaluated if the organoids exhibited their typical morphology. The several markers were evaluated by day 13, the first timepoint of neural commitment, and by the end of differentiation (day 46). Additionally, we present brightfield images of organoids to assess shape and size at different timepoints. On day 1 of the differentiation protocol it was possible to observe a small aggregate with defined round edges in a microwell of an AggreWell plate (Figure 3.2 A). By day 7, the organoids were replated to Ultra-low attachment plates, and we observed that they had maintained their shape and that there was an increase in size (Figure 3.2 A). By day 46, it was possible to observe neural rosettes, structures in which neural progenitors organize themselves, resembling the neural tube, in the outer edges of the organoid (Figure 3.2 A).

Proper neural commitment was assessed by mRNA expression levels of *PAX6* and *MAP2*. *PAX6* encodes a transcription factor that is present in neural progenitors of the dorsal forebrain; having a low expression in ventral progenitors. This differential expression contributes to this neuronal specification and dorsal identity (Georgala et al., 2011). *MAP2* is expressed only by mature neurons, being characteristic of later stages of differentiation.

As seen previously in Figure 3.1 A) and B), there was a significant decrease in the expression levels of pluripotency markers *OCT4* and *NANOG*.

By day 13, a timepoint of neural commitment, it was possible to observe an increase in *PAX6* comparatively to day 0, and by day 46, its expression levels were even more accentuated (Figure 3.2 B). Typical morphology was also confirmed by the presence of neural rosettes (PAX6-positive) (Figure 3.2 D, day 13). A gradual increase in *MAP2* expression levels was also observed (Figure 3.2 C). High standard deviations between data are probably related to morphological differences among the organoids of the several differentiations.

Overall, our results confirm the successful neural induction of hiPSC and generation of dorsal forebrain organoids, and are in concordance with other studies with similar protocols (Gomes et al., 2020).

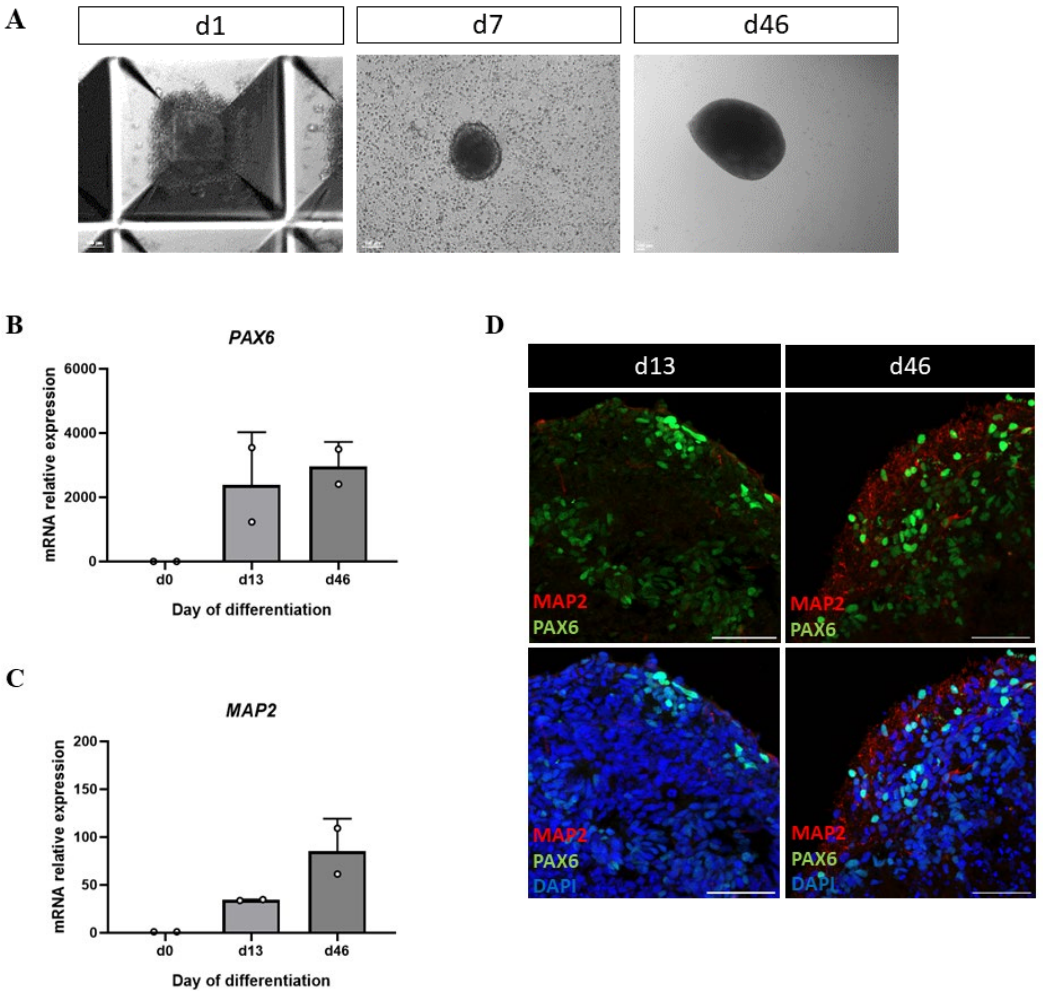

Figure 3.2- Efficient neural commitment of hiPSCs. A) Representative brightfield images of organoids by day 1, day 7 and day 46 of neural differentiation. qRT-PCR analysis of B) *PAX6* and C) *MAP2* mRNA expression levels relative to *GAPDH* by day 0 of the differentiation. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). D) Immunofluorescence staining of neural progenitors marker PAX6 and neuron-specific microtubule-associated protein MAP2 on 13 and 46 days-old organoids cryosections. Scale bars: 50 µm.

3.2. Effects of concentration on neuronal differentiation

The literature presents discrepancies regarding cannabinoids concentrations used in *in vitro* studies, since extrapolation from consumed doses to cellular models remains challenging. Therefore, the first differentiation was aimed to determine the most adequate concentration of cannabinoids and antagonist to use in the next experiments.

The most used concentrations of cannabinoids in similar experiments range between 1 µM and 10 µM, with higher concentrations already being associated with some levels of toxicity (Ao et al., 2020; Miranda et al., 2020). While Δ^9-THC and CBD have been considerably more studied, THJ-018 and EG-018 have only been used in a

similar study at a 10 µM concentration, and having already strong effects on neuronal maturation and functionality (Miranda et al., 2020). Therefore, we chose 2 and 5 µM as the concentrations of cannabinoids to test in this first experiment.

In vitro assays using cell lines administrate SR141716A in concentrations between 3 nM and 1 µM (Klegeris et al., 2003; Molina-Holgado et al., 2007; Papariello et al., 2021; J. P. Silva et al., 2019). Additionally, dose response experiments indicate that 10 nM may be the "optimal" concentration of the compound, since it counteracted neurite length reduction caused by the administration of 2 µM WIN-55, an AAI synthetic cannabinoid, but had the less effects when administered by itself, comparing to other concentrations. This study was performed on iPSC-derived neurons, having an administration period of only 24 hours (Papariello et al., 2021). Likewise, we decided to test 10 and 30 nM of CB1R antagonist SR141716A. We opted to use concentrations on the nanomolar range to prevent its activity as an inverse agonist. Also, at higher (micromolar) concentrations, this substance can have some affinity to CB2R.

The main criteria that we had in mind to choose a concentration was to determine if 2 µM was enough to cause alterations and to not choose a concentration too high that would reduce cell viability to a point that it would compromise the experiments. Regarding SR141716A, an optimal concentration would be the one that has minimal effects when administered individually, but is able to bind CB1R and prevent cannabinoids from binding to it and exerting their effects. However, due to the outline of the experiment (and limited samples), we were only able to test the first parameter. In Figure 3.3 we present the main results of qRT-PCR, with the only biological replicate that we had at the time of deciding the next concentration.

Results suggest that 2 µM Δ^9-THC increased *PAX6* expression levels and decreased *MAP2* levels compared to EtOH-treated cells. At 5 µM it appeared to have an opposite effect on *PAX6*, leading to its downregulation, accompanied by minimal changes in *MAP2* expression levels (Figure 3.3 A and B).

CBD had minimal effects on *PAX6*, but a slight downregulation was observed in the 2 µM treated cells (Figure 3.3 A). Regarding *MAP2*, both concentrations led to downregulation of the gene compared to the vehicle-treated group, although stronger alterations were observed when administered at a higher concentration (Figure 3.3 B).

Synthetic cannabinoid THJ-018, when administrated at 2 µM, seemed to reduce both *PAX6* and *MAP2* expression levels. On the other hand, 5 µM of the compound had minimal effects on *PAX6* and a milder downregulation of *MAP2* (Figure 3.3 A and B).

2 µM EG-018 led to an upregulation of *PAX6*, and had minimal effects on *MAP2* expression levels. At 5 µM the synthetic cannabinoid led to a substantial increase of PAX6 and a decrease in MAP2. Interestingly, it follows a similar pattern to CBD (Figure 3.3 A and B).

The CB1R antagonist appears to reduce both *PAX6* and *MAP2*, with stronger effects associated with the higher concentration (Figure 3.3 A and B).

These results are preliminary and were used only to decide which concentration would be adequate to use in the next differentiations. Data indicate that 2 µM induces changes in the tested markers. Adding 5 µM of the same compound does not seem to promote significant changes when compared to 2 µM, excepting for Δ^9-THC, where at 5 µM there is a strong decrease of *PAX6*. This implies that even low concentrations might have an impact in the developing brain, therefore we opted for 2 µM as the concentration of cannabinoids. Regarding the CB1R antagonist, we decided to use the lower concentration, to minimize the effects of the compound, since a reduction of both neural progenitors and mature neurons markers could indicate some toxicity.

If these trends maintained with a higher number of replicates, we could hypothesise that the tested phytocannabinoids appear to delay neuronal differentiation at 2 µM, but have an opposite effect when administrated at 5 µM. This would support studies that demonstrate dose-dependent effects of cannabinoids (Miranda et al., 2020).

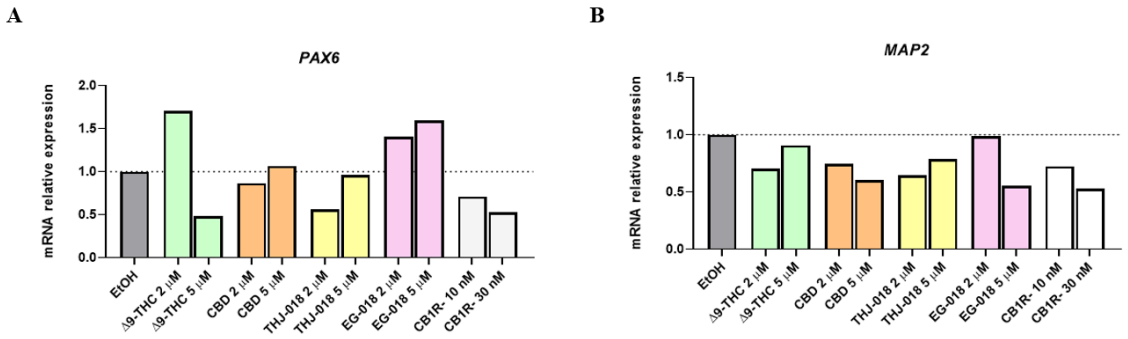

Figure 3.3- Assessment of concentration-dependent effects of phytocannabinoids Δ^9-THC and CBD, synthetic cannabinoids THJ-018 and EG-018 and CB1R antagonist SR141716A. qRT-PCR analysis of A) *PAX6* and B) *MAP2* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH)-treatment. SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-".

3.3. Effects of phytocannabinoids Δ^9-THC and CBD on a model of embryonic neurodevelopment

The potential of phytocannabinoids to impact neuronal differentiation and neurogenesis has been demonstrated in the literature. Additionally, as previously mentioned, CB1R affects neuronal proliferation, differentiation and maturation by regulation of protein kinase cascades (Galve-Roperh et al., 2013). Therefore, we decided to evaluate the effects of Δ^9-THC and CBD on neuronal differentiation, and determine if CB1R binding has a role on it. To do so, we determined the expression levels of genes involved in this process, as well as protein levels of markers characteristic of neural progenitors and markers related to mature neurons.

3.3.1. Effects of Δ^9-THC and CBD on cannabinoid receptors gene expression

As previously mentioned, the endocannabinoid system is highly relevant to neurodevelopment due to functions mediated by CB1/2R (Galve-Roperh et al., 2013). CB1R levels increase with the progress of neural differentiation, contrarily to CB2R. In our organoids there are populations of both neural progenitors, that can still express CB2R, and mature neurons, that preferentially express CB1R. Thus, both cannabinoid receptors' gene expression was evaluated, by determining mRNA levels of *CN1R* and *CN2R*, the genes that encode CB1R and CB2R, respectively.

Results obtained by mRNA expression analysis show that exposure to Δ^9-THC induced minimal effects on the expression levels of *CN1R* and *CN2R*, and a slight upregulation of both genes when administrated in the presence of CB1R antagonist SR141716A (Figure 3.4 A and B).

The administration of CBD did not affect the expression of *CN1R* compared to the vehicle group (0.01% EtOH), and led to a slight upregulation of *CN2R*. Both genes showed a tendency to be upregulated when the compound was administrated in the presence of SR141716A (Figure 3.4 A and B).

The exposure of CB1R antagonist *per se* led to an upregulation of *CN1R*, and minimal effects or a slight upregulation of *CN2R* expression (Figure 3.4 A and B).

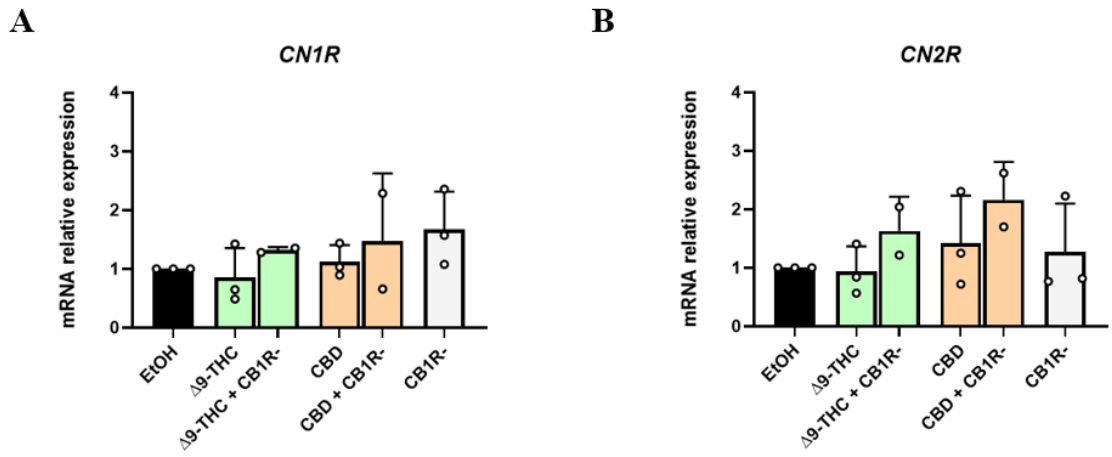

Figure 3.4- Effects of phytocannabinoids Δ^9-THC and CBD on cannabinoid receptors gene expression. qRT-PCR analysis of A) *CN1R* and B) *CN2R* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH)-treatment. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-".

Firstly, we can conclude that these results validate the expression of the genes that encode the main cannabinoid receptors, confirming that our organoids contain the main elements of the endocannabinoid system. This enforces that cortical organoids are adequate to our research aims: to evaluate prenatal exposure to cannabinoids and determine if the effects are related to CB1R.

Although not statistically significant, the results suggest that, in this model, 2 μM Δ^9-THC does not affect cannabinoid receptors gene expression. A downregulation of *CN1R* and *CN2R* was expected, since at the used concentration the compound is bound to both CB1R and CB2R and the overactivation of the receptors would, in theory, lead to a downregulation of the genes as a compensatory mechanism. This was already observed in several models (Ao et al., 2020), and at even lower concentrations of the psychotropic compound (Ao et al., 2020). In our model, the time of exposure may not be enough to induce similar alterations, but as seen by the standard deviation, it should be further evaluated. The slight upregulation of *CN1R* upon simultaneous administration of Δ^9-THC and SR141716A may be due to the antagonist preventing endogenous ligands from binding CB1R. The lack of activation of CB1R by its endogenous ligand would upregulate *CN1R*, by negative feedback.

As SR141716A is specific to CB1R at low nanomolar concentrations, it is not expected to bind CB2R at the used concentration. Therefore, it should not affect the binding of cannabinoids to CB2R, neither the expression levels of the gene that encodes it (*CN2R*). However, it slightly upregulates *CN2R*, comparing to the individual administration of Δ^9-THC. This could indicate that *CN1R* is involved in *CN2R* regulation.

At 2 µM, CBD binds to CB2R, which is expected to cause a downregulation of these receptors, and to CB1R, though not in saturation. By our observations, CBD does not induce significant alterations in *CN1R*, and a slight increase in *CN2R* expression levels. As previously said, we would expect that the overactivation of CB2R would lead to a downregulation of *CN2R*, although a study describes a similar alteration to the one we observed (Miranda et al., 2020).

The administration of CBD and SR141716A appears to lead to an upregulation of both genes. We hypothesise that CBD may be binding to its allosteric site in CB1R, rather to the orthosteric one, due to competitive binding of SR141716A (or vice-versa) (schematic representation in Figure 3.5). This could affect *CN1R* expression, and consequently *CN2R,* supporting our hypothesis. Thus, our results obtained with CBD may help to enlighten its role as an allosteric modulator of CB1R as previously described by molecular docking studies (Jakowiecki et al., 2021; Laprairie et al., 2015).

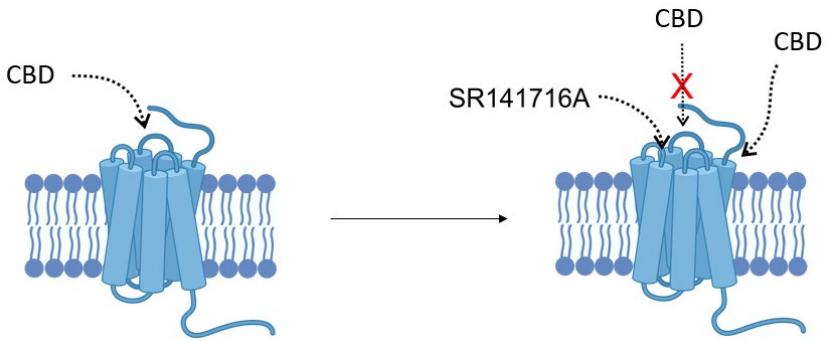

Figure 3.5- Competitive binding of SR141716A to CB1R and consequent allosteric binding of CBD. Due to having a higher affinity to its orthosteric binding site, SR141716A competitively binds to it. Consequently, CBD exerts its effects by binding to an allosteric binding site in CB1R.

Our results present high standard deviations, which are possibly due to the differences in the size of our organoids. In one of the differentiations the organoids were considerably smaller in size, which affects the uptake of cannabinoids by the cells, as

well as the number of cells that are affected. This parameters should be optimized in further experiments, for example by monitoring the number of organoids per well.

3.3.2. Effects of Δ^9-THC and CBD on neuronal differentiation

The use of cannabinoids has increased consistently, including among pregnant women (Young-Wolff et al., 2017). However, evaluating the effects of prenatal exposure to cannabinoids on embryonic neurodevelopment is hardly possible. These effects can only be determined years later, by cognitive and behavioural studies (Scheyer et al., 2019). The *in vitro* model we are using allows us to have an insight of these effects on a molecular level, on neurons and progenitors characteristic of the embryonic neurodevelopment.

3.3.2.1. Neural progenitors

To evaluate the differences between populations of neural progenitors on the dorsal forebrain organoids, we assessed mRNA expression levels of *PAX6* and *Nestin*, and performed immunofluorescence of the proteins these genes encode.

As previously mentioned, PAX6 is a transcription factor present in dorsal forebrain progenitors in the ventricular zone. Absence of this marker affects dorsoventral patterning, progenitors proliferation and morphology, being critical for the CNS development (Georgala et al., 2011).

NESTIN is a cytoskeletal protein present in the intermediate filaments of neural progenitors. It is involved in several functions, such as self-renewal and proliferation, survival, differentiation and migration of neural progenitors. Its expression levels typically decrease with the progress of differentiation (Bernal & Arranz, 2018).

We also tested the levels of *MKI-67*, a general marker for proliferative cells. Immunofluorescence of the protein KI-67 was performed, but almost no cells were stained among the different conditions (data not shown).

Finally, we tested SOX2 levels by western blot. Besides being present in pluripotent stem cells, the transcription factor is present in neuroepithelial cells and radial glia. SOX2 levels, and also the balance of other transcription factors, contribute to neural induction of hiPSC to neural progenitors and maintenance of their properties (Roll et al., 2022).

Results indicate that Δ^9-THC induced minimal effects on *PAX6* expression levels, which was slightly downregulated in the presence of CB1R antagonist SR141716A (Figure 3.6 A). Immunofluorescence analysis showed stronger alterations, namely an increase in the transcription factor, that was counteracted by SR141716A (Figure 3.6 D, G). The phytocannabinoid led to minimal alterations in NESTIN, assessed by both methods; and in *MKI-67* expression levels, and these effects appear to be exacerbated by SR141716A (Figure 3.6 B, C, E, H). Western blot analysis indicates higher levels of SOX2 in cells exposed to the compound; once again reverted by the antagonist (Figure 3.6 F).

CBD did not induce alterations in any of the neural progenitors markers evaluated by qRT-PCR analysis (Figure 3.6 A-C). A slight upregulation of *Nestin* and *MKI-67* was observed in the presence of SR141716A (Figure 3.6 B, C). Immunofluorescence analysis indicates more evident alterations, although also exhibiting higher standard deviations. PAX6 and NESTIN immunofluorescence quantifications show higher levels of the markers compared to the vehicle, being in the first case reverted by SR141716A (Figure 3.6 D, E, G, H). CBD appears to have no effects on SOX2 (Figure 3.6 F).

Individual administration of SR141716A led to minimal changes, both in qRT-PCR results and immunofluorescence quantifications (Figure 3.6).

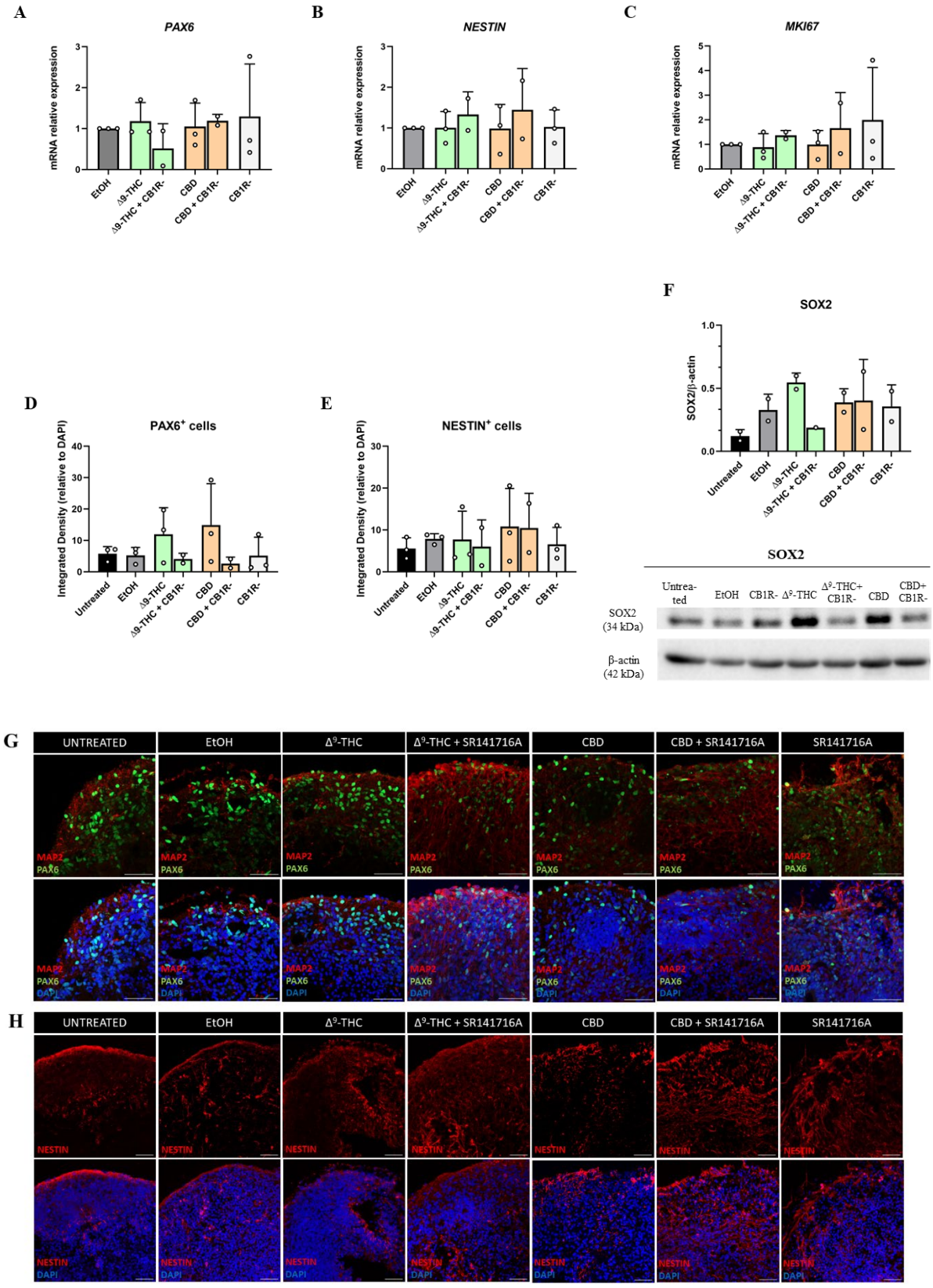

Figure 3.6- Effects of phytocannabinoids Δ⁹-THC and CBD on neural progenitors markers. qRT-PCR analysis of A) *PAX6*, B) *NESTIN* and C) *MKI-67* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH) treatment. Immunofluorescence quantification of neural progenitors D) PAX6 and E) NESTIN, normalized to DAPI.

Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-". F) Protein quantification and representative bands of SOX2 and β-actin. Immunofluorescence staining of G) PAX6 and MAP2, and H) NESTIN on 46 days-old organoids cryosections. Scale bars: 50 µm.

3.3.2.2. Mature neuronal markers

To evaluate neuronal differentiation, we assessed the levels of *MAP2* and *β-tubulin III*, which are typically expressed in mature neurons. MAP2 is present in the microtubules of the cell bodies, but specially of the dendrites of mature neurons. It is only expressed in neurons, and not in neural progenitors, being an indicator of a more advanced state of differentiation. It is related to functions such as regulating microtubules networks (Dehmelt & Halpain, 2004). *β-tubulin III* is typically expressed in post-mitotic neurons of the subventricular zone (Gomes et al., 2020). It encodes TUJ1, a neuronal-specific β-tubulin that is present in the microtubules of dendrites and axons. *GFAP*, typically expressed in astrocytes (Guillemot et al., 2006), was also tested, by qRT-PCR and immunofluorescence, but its levels were almost undetectable. Western blot analysis also did not detect the protein, nor alterations between conditions.

Results obtained by qRT-PCR demonstrated that Δ^9-THC might reduce the expression levels of differentiation markers *MAP2* and *β-tubulin III*, and both are reverted in the presence of CB1R antagonist SR141716A (Figure 3.7 A, B). Immunofluorescence analysis shows similar trends (Figure 3.7 C - E).

CBD appears to have minimal effects on *MAP2* expression levels, but led to a slight upregulation of *β-tubulin III*, in this last case contradicted by CB1R antagonist (Figure 3.7 A, B). Immunofluorescence has similar trends, although with higher variability (Figure 3.7 C - E).

Individual administration of SR141716A increased *MAP2* expression levels, and no alterations *β-tubulin III* expression levels (Figure 3.7 A, B). An opposite trend was observed in immunofluorescence analysis of MAP2 (Figure 3.7 C).

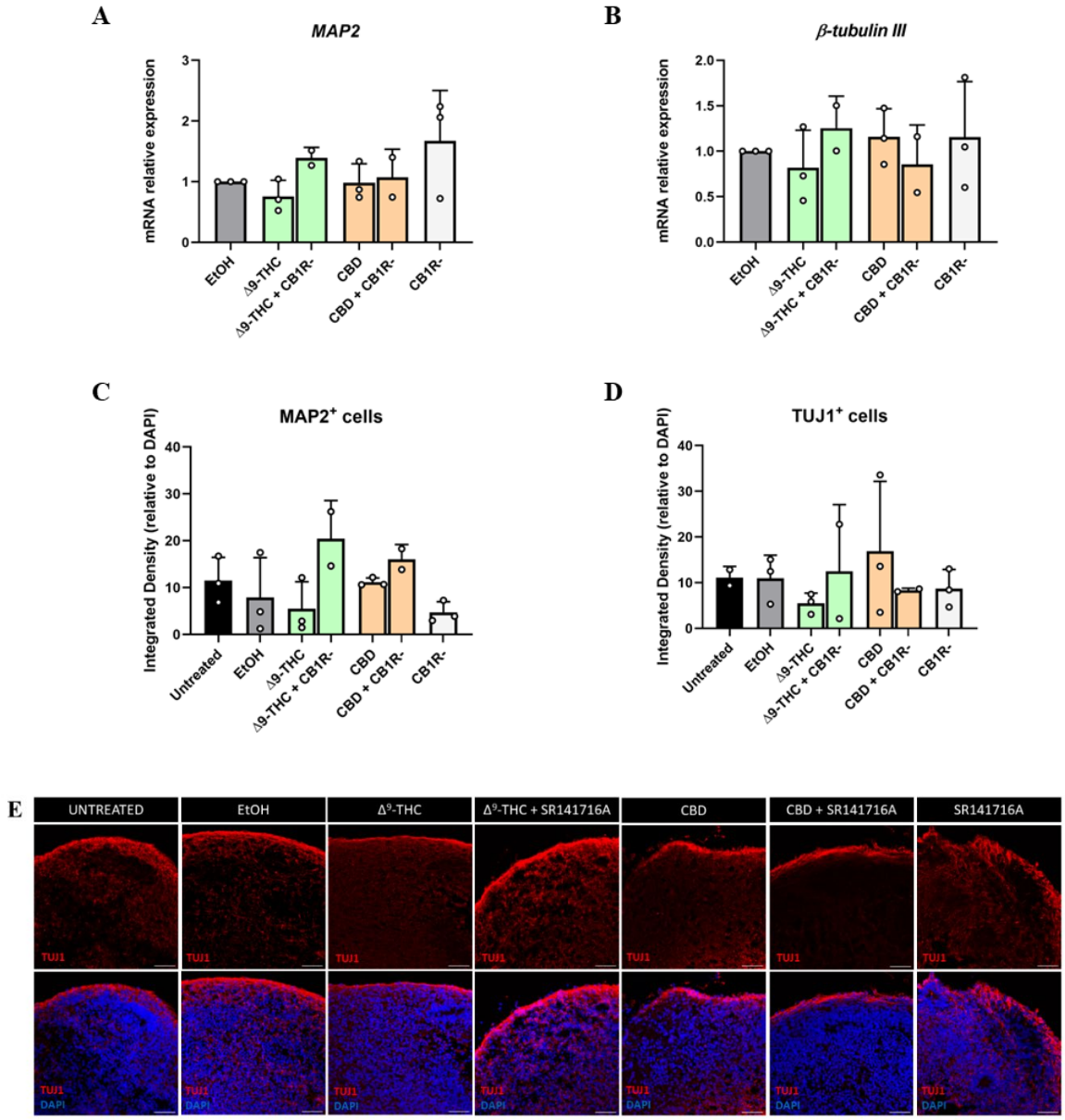

Figure 3.7 - Effects of phytocannabinoids Δ^9-THC and CBD on mature neurons markers. qRT-PCR analysis of A) *MAP2* and B) *β-tubulin III* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH) treatment. Immunofluorescence quantification of neuron-specific markers C) MAP2 and D) TUJ1, normalized to DAPI. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-". E) Immunofluorescence staining of TUJ1 on 46 days-old organoids cryosections. Scale bars: 50 µm.

Cells exposed to the different conditions exhibited minimal differences on markers present in neural progenitors and mature neurons of the dorsal forebrain, demonstrating that exposure to 2 µM of Δ^9-THC and CBD for 9 days caused only slight alterations on neural differentiation.

Despite the lack of statistical significance, our results indicate that Δ^9-THC slightly increased the levels of PAX6 and SOX2, and downregulated *MAP2* and *β-tubulin III*. The increase in neural progenitor markers and decrease in mature neurons markers suggests that the phytocannabinoid might delay neuronal differentiation.

Bearing in mind that our results are preliminary, they corroborate previous literature on the topic. A study demonstrated that the exposure of cortical organoids to Δ^9-THC, but in a concentration of 1 μM, led to significant upregulation of *PAX6* and downregulation of *β-tubulin III*, similar alterations to the ones we observed (Ao et al., 2020). Additionally, since the previous changes are both to be reverted by CB1R antagonist SR141716A, we postulate that Δ^9-THC activation of CB1R and downstream pathways is the mechanism by which it affects neuronal differentiation.

Interestingly, if a higher number of replicates confirms these alterations, they may contribute to supporting evidence of concentration-dependent effects of Δ^9-THC. Several studies indicate that higher doses of the compound can promote neuronal maturation (Miranda et al., 2020; Paraíso-Luna et al., 2020).

Nonetheless, these are minor alterations, and immunofluorescence images show that the organoids still exhibit a typicall morphology, and similar structural organization to the controls. Besides that, our data display high standard deviations, possibly related to differences in the size of the organoids between differentiations, which alter the neural promoting factors distribution but also their uptake of the drugs. We also still have to take into consideration that not all neural progenitor markers exhibit the same trend. For instance, *NESTIN* and *MKI-67* mRNA expression levels are not upregulated, although it could be due to these markers being expressed for longer than PAX6 (Bernal & Arranz, 2018). All of these factors highlight the importance of further validating our results.

Exposure to 2 μM CBD appears to have minimal effects on neuronal differentiation. This could be related to its lower binding affinity to CB1R (3.3 μM, Table 1.1), and the receptors not being in saturation with this concentration. Nonetheless, slight alterations in mature neurons markers *MAP2* and *β-tubulin III* indicate further need to confirm if CBD is harmless to embryonic neurodevelopment, as studies indicate that the phytocannabinoid can promote neuronal differentiation. Once again, this disparity is probably due to the use of different concentrations in other

studies. For instance, the same exposure period to CBD led to no alterations when administrated at 1 μM concentration, but with evidence of neurotoxicity if administrated at 10 μM (Miranda et al., 2020). Although CBD has not caused alterations in the tested markers, morphological changes such as a higher number of neural rosettes were observed in organoids treated with this compound (not quantified). These alterations should be further analysed in the future.

Data obtained from SR141716A-treated cells may support the role of AEA and 2-AG in neural differentiation, by indicating the effects of blocking the binding of the endogenous ligand to CB1R. Studies have shown that AEA inhibits proliferation, which is supported by an increase of *MKI-67* levels after CB1R antagonist administration. By preventing the endogenous to bind CB1R, SR141716A slightly increases *MAP2* expression levels and the organoids exhibit morphological alterations such as a dense neuronal network. We were also expecting an upregulation of *β-tubulin III*, since SR141716A was previously shown to increase neuronal differentiation in mouse-derived cortical neural progenitors. In this study, *β-tubulin III* expression was inhibited by AEA in a CB1R dependent manner (Rueda et al., 2002).

3.3.3. Effects of Δ^9-THC and CBD on apoptosis

Literature indicates that cannabinoids can affect cell viability, triggering several mechanisms including apoptosis (Bilkei-Gorzo, 2012). With this in mind, we evaluated the levels of cleaved caspase 3, an enzyme from the cysteine-aspartic acid protease family typically associated with programmed cell death, including in neuronal cells. While caspase 3 exists in its inactive form, specific signals (e.g.: absence of neurotrophins) lead to its activation via autolytic cleavage. Caspase 3 is an effector caspase, meaning that after its activation, it will cleave specific substrates executing cell death along with other caspases (D'Amelio et al., 2012).

We evaluated the levels of this marker by immunofluorescence, quantifying images of sections with similar cell density, to avoid a bias on the results, since high cellular density is associated with higher levels of apoptosis *per se*. Western blot analysis of cleaved caspase 3 levels was performed, but unsuccessfully.

As observed in Figure 3.8, there is a clear reduction of cleaved caspase 3 between the untreated cells and the EtOH-treated cells (Figure 3.8).

Regarding phytocannabinoids, both Δ^9-THC and CBD lead to a minimal rise in cleaved caspase 3 levels comparing to EtOH. Administration of SR141716A appears to increase these levels, when administrated individually but also when in the presence of phytocannabinoids (Figure 3.8).

A reduction of cleaved caspase 3 upon EtOH administration was not expected, since it has been proven to increase the levels of the marker *in vitro* (Young et al., 2005). However, the high standard deviation between data from untreated results cannot be disregarded. As previously mentioned, this could be due to the strong difference in the size of the organoids in one of the differentiations. This is related to a limitation of the model: high rates of proliferation increase the size of the organoids and difficult the distribution of oxygen and medium supplementation to the "core" of the organoid, increasing the levels of cleaved caspase 3 and resulting in apoptosis. Using a 3D structural model, having less cells may have a beneficial impact since this is contradicted. Consistently, the untreated group in one of the differentiations was associated to high expression levels of *MKI-67*, as well as SR141716A-treated organoids. In both cases, higher levels of cleaved caspase 3 were observed.

As EtOH causes such marked alterations, it could be challenging to determine the effects of cannabinoids in this model without ruling out the fact that they may be more related to the vehicle. Compared to EtOH, it is still possible to observe levels slightly higher with the administration of cannabinoids, although not significant. Nonetheless, a similar study using hiPSC-derived forebrain organoids demonstrated that administration of 2 μM Δ^9-THC for 30 days was also insufficient to cause significant alterations on cleaved caspase 3 levels (Paraíso-Luna et al., 2020).

Overall, the tested phytocannabinoids, Δ^9-THC and CBD, appear not to be associated with neurotoxic effects in the organoids we generated. However, consistent alterations such as a lower number of DAPI-stained nuclei in organoids exposed to CBD should be evaluated in the future, since they can also be an indicator of toxicity.

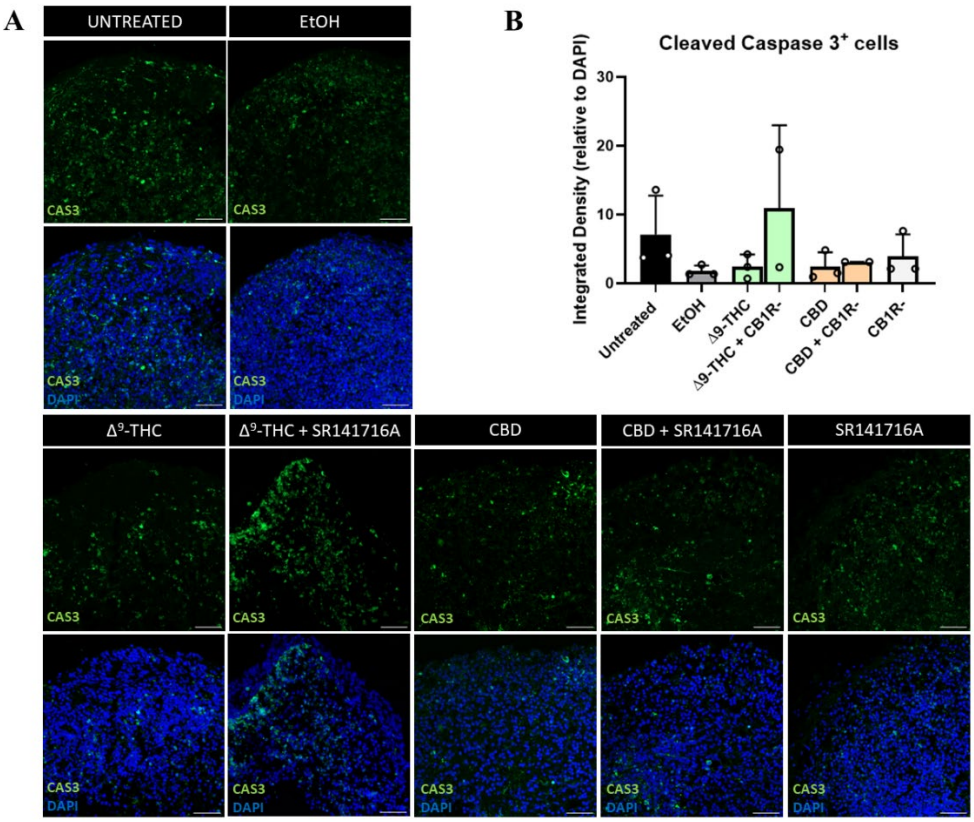

Figure 3.8- Effects of phytocannabinoids Δ^9-THC and CBD on apoptosis. A) Immunofluorescence quantification of apoptosis marker cleaved caspase 3, normalized to DAPI. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-". B) Immunofluorescence staining of cleaved caspase 3 on 46 days-old organoids cryosections. Scale bars: 50 µm.

3.3.4. Effects of Δ^9-THC on cell functionality

Since our results suggest that Δ^9-THC might affect neuronal differentiation, due to an altered ratio of neural progenitors and mature neurons, we decided to evaluate if these cells could also have their functionality affected by assessing if they had a normal response to stimuli. To do so, we performed single-cell calcium imaging, which has been proven to be a reliable method to study cell response and identify distinct types of cells based on their mechanisms of calcium entry, being in this case, related to VGCC in neurons or coupled to histamine receptors in the case of neural progenitors and astrocytes (de Melo Reis et al., 2020). In our model, since we confirmed the absence of astrocytes by *GFAP* mRNA expression levels, we will assume that histamine response is mediated only by neural progenitors. Due to limitations, it was only possible to perform this analysis in one differentiation, nonetheless, we present the preliminary results from this technique.

Untreated neurons exhibited a normal response to KCl stimulus, used to induce depolarization of the membrane and activate VGCC. This increases intracellular Ca^{2+} levels, which binds to the fluorescent indicator and alters its excitation peak, translating into peaks of fluorescence ratio on the plot (Figure 3.9 A). An increase of Ca^{2+} was also observed after histamine solution administration, indicating a response from neural progenitors (Figure 3.9 A). Cells treated with EtOH appear to respond to both stimuli as well. However, they presented lower Ca^{2+} peaks in response to KCl and higher peaks after histamine, comparing to the untreated cells (Figure 3.9 B). Since all the compounds were vehiculated in EtOH, its data will be considered the control group to compare the effects of the other phytocannabinoids.

Regarding the Δ^9-THC-treated cells, the response to both stimuli appears different than the vehicle group. When KCl was administered, intracellular Ca^{2+} concentration peak was higher than the control, also lasting longer, almost until the second stimulus was administered. There is also a lack of response from neural progenitors, marked by the absence of a characteristic Ca^{2+} peak after histamine administration (Figure 3.9 C). Cells that were treated with Δ^9-THC and CB1R antagonist exhibit an opposite behaviour, having a decreased response to KCl and some response to histamine (Figure 3.9 D). However, a low number of cells was tested (Figure 3.9 G), and some of those could not be quantified since they exhibited a high influx of Ca^{2+} in the baseline, which implied cell death. Hence, these results should be further evaluated by repeating the experiment in next differentiations.

CBD results were not presented, since the cells were not properly fixed and moved during the KCl and histamine profusions, which would not allow accurate quantifications. Regardless, we present the data obtained from the cells treated with both CBD and the CB1R antagonist SR141716A. In the ratio plot, it is possible to observe a similar response to KCl compared to EtOH group, but a lower response to histamine (Figure 3.9 E).

SR141716A-treated cells responded to both stimuli, but presented a higher Ca^{2+} peak after the KCl stimulus, and lower peak after histamine, when compared to the vehicle group (Figure 3.9 F).

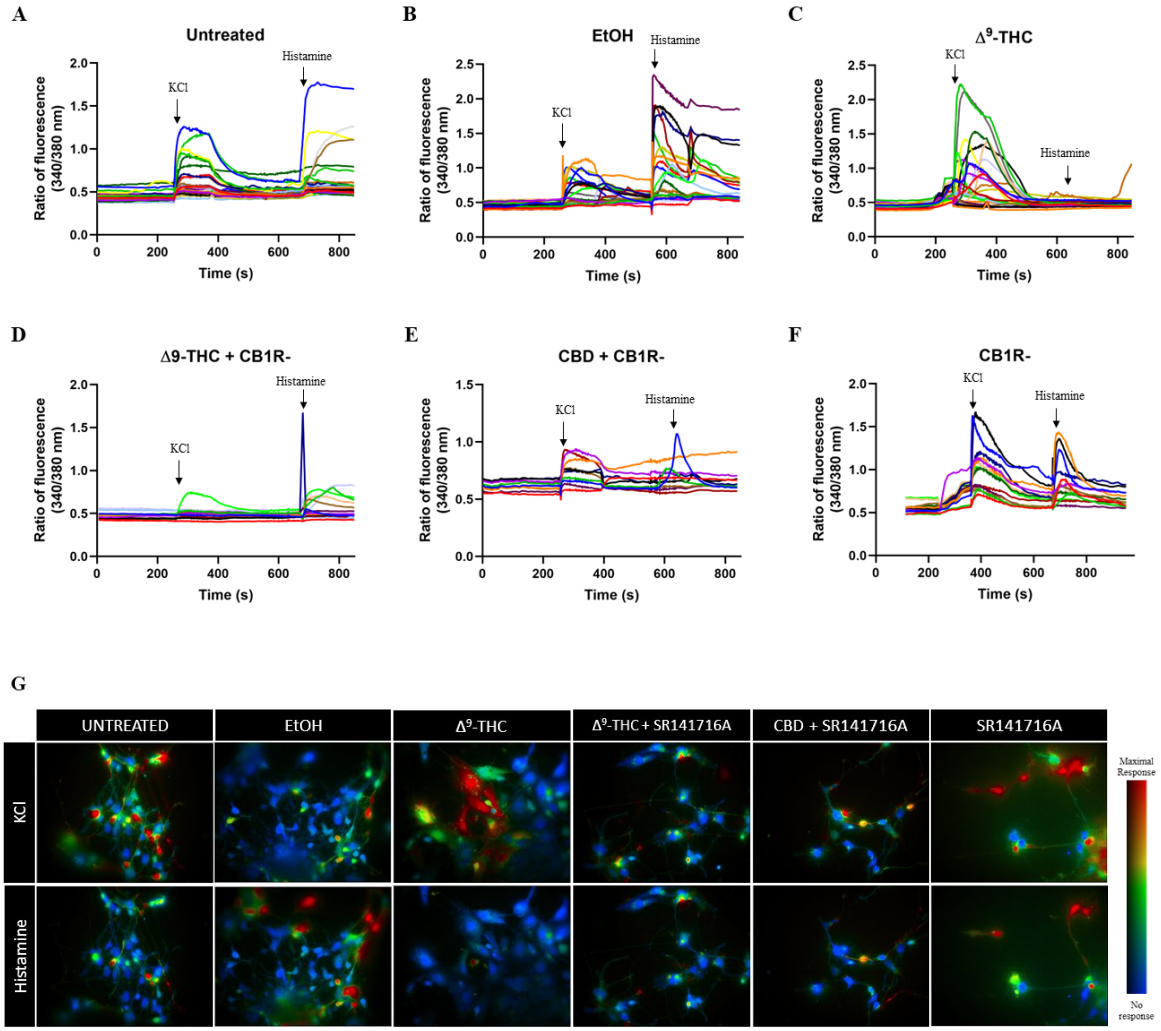

Figure 3.9- Functional assessment of phytocannabinoid-treated cultures on day 46. A-F) SCCI analysis showing the response of different treatment cultures to KCl and histamine stimuli (targeting mature neurons, and immature neurons and neural progenitors, respectively). Data from 1 experiment. SR141716A (CB1R antagonist) treatment was briefly abbreviated to "CB1R-". G) Representative ratio images of the tested population for different culture conditions. Images were taken immediately after each stimulus.

Although preliminary, our results highlight the potential of Δ^9-THC to affect cell functionality. We observed through qRT-PCR and immunofluorescence analysis that the phytocannabinoid might delay neuronal differentiation and the organoids exposed to it present higher levels of neuronal progenitors and lower of mature neurons. However, it is possible that the response of both types of cells might be altered. A study has demonstrated that exposure of 10 µM Δ^9-THC on 2D neurons for 37 days led to a dysregulated response of neurons to KCl, and also a prolonged response, as we observe. These cells did not reach peaks as high as we observed, but this could be related to the higher concentration and period of administration (Miranda et al., 2020). Additionally,

Stanslowsky *et al.* have demonstrated that 10 µM Δ^9-THC administration to hiPSC-derived dopaminergic neurons compromised functional maturation by decreasing voltage-gated sodium and potassium currents, action potential amplitudes and spontaneous synaptic activity by electrophysiology analysis (Stanslowsky et al., 2017). The observed alterations in cell functionality induced by exposure to Δ^9-THC during neural differentiation and formation of functional neuronal circuitry might be related to cognitive impairment observed after prenatal exposure to the compound (Ross et al., 2008). The opposite trend upon Δ^9-THC and CB1R treatment might suggest an influence of CB1R on histamine receptors, which was not previously described in literature (Figure 3.10).

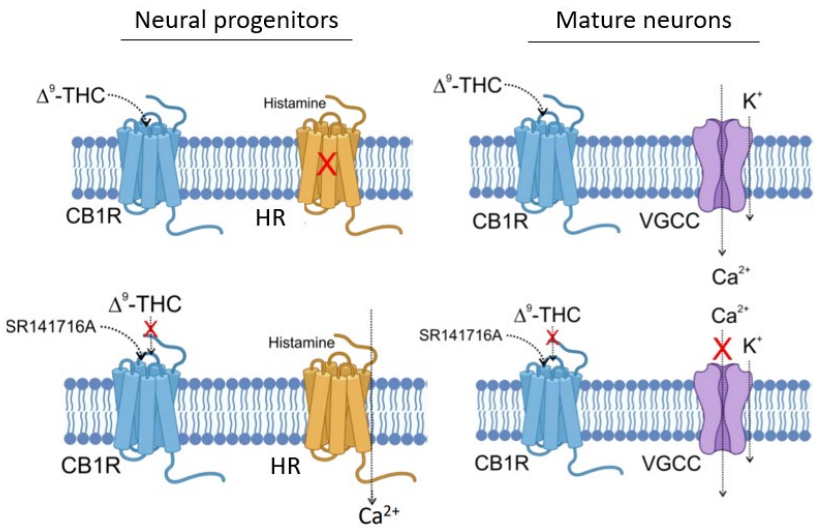

Figure 3.10- Proposed mechanisms by which Δ^9-THC might affect neuronal functionality. Binding of Δ^9-THC to CB1R may modulate the response of histamine receptors (HR) and voltage-gated calcium channels (VGCC) to histamine and K^+ stimuli, respectively.

If the response of histamine receptors is altered in Δ^9-THC-treated cells, the effects of histamine on neural progenitors may also be compromised. Histamine H2 receptors have been demonstrated to be connected to cell proliferation, and histamine H1 receptors to differentiation and reduction of apoptosis levels (Molina-Hernndez & Velasco, 2008). Thus, prolongued exposure to Δ^9-THC could be associated to desensitization of histamine receptors and could consequently negatively impact these parameters.

Results obtained from CBD and SR141716A exposure indicate that the administration of both compounds has only a mild impact on mature neurons. However, minimal response to histamine stimulus suggests an altered response from neural progenitors. Assuming that the response is also mediated by CB1R, such as seen in Δ^9-

THC, it is possible that CBD causes an opposite effect comparing to the psychoactive compound. This is observed in similar studies, that report that exposure to CBD only reduces mature neurons response comparing to control groups (Miranda et al., 2020), and that it is able to block T-type VGCC. These effects may be related to its anti-epileptic properties (Ross et al., 2008).

Once again, it is important to highlight that differences between the number of cells tested in each condition may influence the results to some degree. The observed trends should be confirmed by performing the experiment in a higher number of replicates.

3.4. Effects of synthetic cannabinoids THJ-018 and EG-018 on a model of embryonic neurodevelopment

The overall lack of studies regarding synthetic cannabinoids was the main motivation to test their effects on cannabinoid receptors expression levels, neuronal differentiation and other parameters. Also, lack of information to the population regarding their adverse effects may lead to their misuse, and therefore is critical to determine their effects on embryonic neuronal differentiation. In this section we present the results of exposure of organoids to 2 μM THJ-018 and EG-018, testing the same markers as in previous sections. Untreated, vehicle and SR141716A groups present the same data, so here we mainly focus on the effects of the synthetic cannabinoids in the study, also analysing if they exhibit a similar profile to phytocannabinoids. However, due to the optimization of the model and alteration of conditions between differentiations, the results are still preliminary (below 3 biological replicates).

3.4.1. Effects of THJ-018 and EG-018 on cannabinoid receptors expression levels

As determined by radioligand binding assays, synthetic cannabinoids, including THJ-018 and EG-018, exhibit high affinities to CB1/2R (Hess et al., 2016; Schoeder et al., 2018). We determined the expression levels of the genes that encode them, *CN1R* and *CN2R*, to evaluate possible alterations caused due to overactivation of CB receptors by these exogenous agonists.

THJ-018 had minimal effects on *CN1R* and *CN2R* expression. Administration of CB1R antagonist accentuated its effect on *CN1R* (Figure 3.11 A and B).

The administration of EG-018 did not induce alterations on the expression levels of *CN1R*, but slightly downregulated *CN2R*. Its effects on *CN2R* are reverted by SR141716A (Figure 3.11 A and B).

Our results suggest that these synthetic cannabinoids do not affect *CN1R* expression. This was an expected outcome, since they have lower affinities to CB1R comparing to Δ^9-THC, which was not able to induce significant alterations. EG-018 exhibited a distinct behaviour from the other cannabinoids and led to a slight downregulation of *CN2R*, which can be associated to its higher affinity to the receptor. Interestingly, these results are consistent with what was observed upon chronic administration of 10 µM EG-018 on 2D cultures (Miranda et al., 2020).

Once again, we can hypothesise that the used concentration and time of exposure was related to the absence of strong alterations.

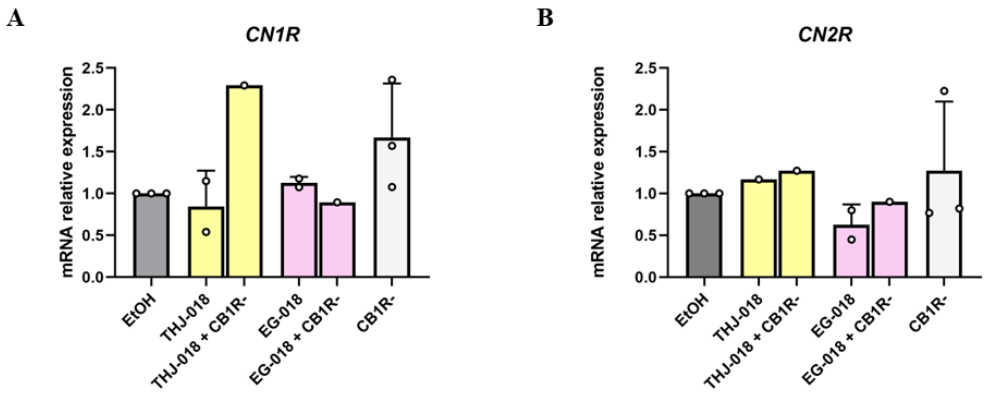

Figure 3.11- Effects of synthetic cannabinoids THJ-018 and EG-018 on cannabinoid receptors gene expression. qRT-PCR analysis of A) *CN1R* and B) *CN2R* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH)-treatment. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-".

3.4.2. Effects of THJ-018 and EG-018 on neuronal differentiation

As previously mentioned, synthetic cannabinoids are often associated to stronger effects than phytocannabinoids, hence the importance of determining their impact in neuronal differentiation.

3.4.2.1. Neural Progenitors

THJ-018 induces a slight decrease in *PAX6* expression levels, corroborated by immunofluorescence analysis. Although we only have data from one replicate, if further results support this trend, the levels of this marker appear not to be reverted in the presence of CB1R antagonist (Figure 3.12 A, D, G). qRT-PCR results also point to a decrease in *NESTIN* and *MKI-67* expression levels, reverted by SR141716A (Figure 3.12 B and C). SOX2 levels were reduced, comparing to the vehicle treatment, and again are intensified by the antagonist (Figure 3.12 F).

EG-018 slightly increased *PAX6* expression levels, which returned to baseline levels in the presence of SR141716A. Immunofluorescence analysis shows an opposite trend, with exacerbated effects upon CB1R antagonist administration (Figure 3.12 A and D). The synthetic cannabinoid reduced the levels of *NESTIN*, both on qRT-PCR results and immunofluorescence (Figure 3.12 B, E, H). Simultaneous administration of CB1R antagonist resulted in even lower levels of the marker. EG-018 also led to a mild downregulation of *MKI-67*, which can to some degree be reverted by SR141716A (Figure 3.12 C). Western blot analysis showed a similar trend to NESTIN, a reduction of SOX2 worsened when by the antagonist (Figure 3.12 F).

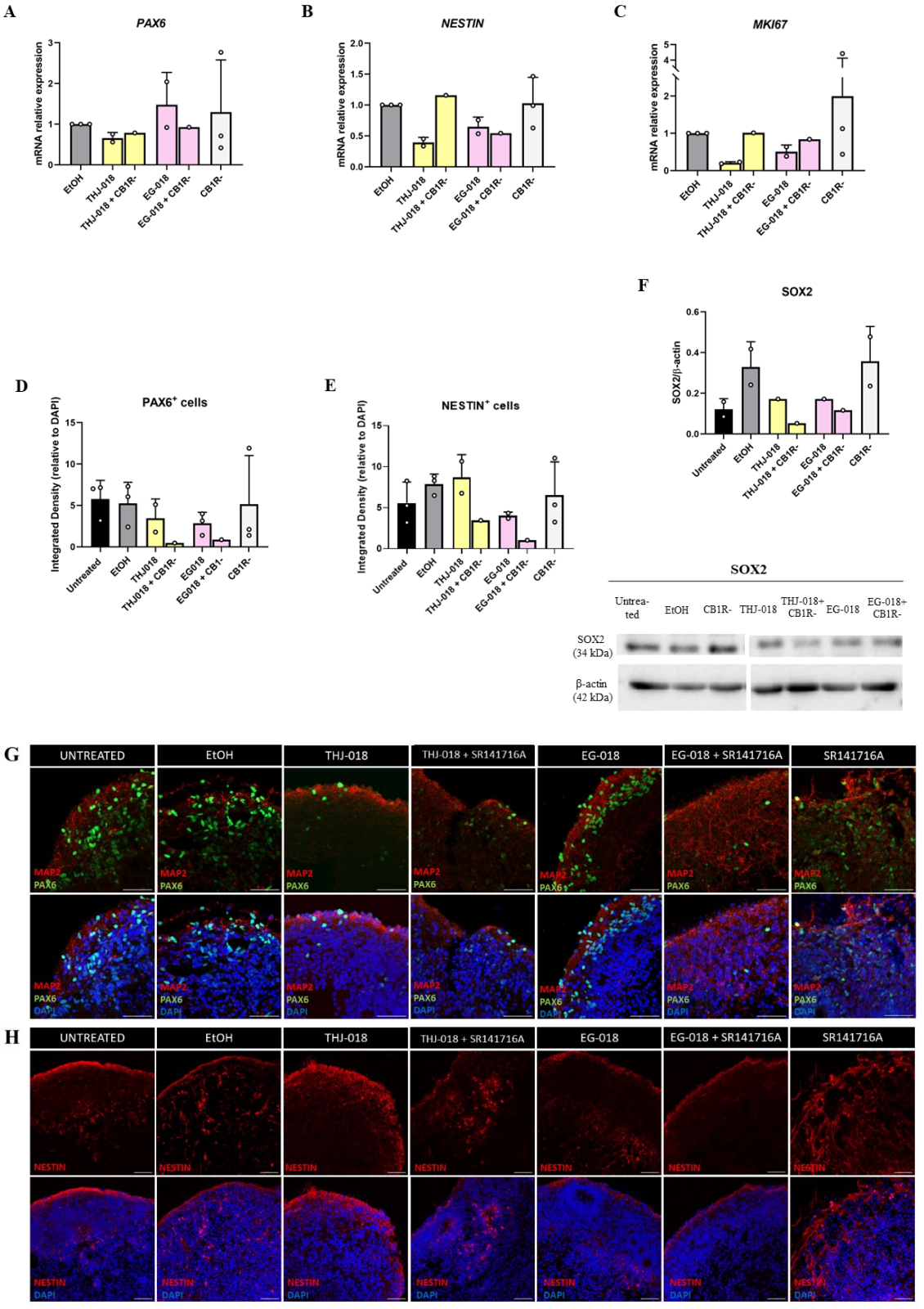

Figure 3.12- Effects of synthetic cannabinoids THJ-018 and EG-018 on neural progenitors markers. qRT-PCR analysis of A) *PAX6*, B) *NESTIN* and C) *MKI-67* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH) treatment. Immunofluorescence quantification of neural progenitors D) PAX6 and E) NESTIN, normalized to DAPI. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-

". F) Protein quantification and representative bands of SOX2 and β-actin. Immunofluorescence staining of G) PAX6 and MAP2, and H) NESTIN on 46 days-old organoids cryosections. Scale bars: 50 µm.

3.4.2.2. Mature neuronal markers

THJ-018 slighly decreased *MAP2* and *β-tubulin III* expression levels, being reverted by CB1R antagonist only in mRNA expression analysis, but not in immunofluorescence analysis (Figure 3.13 A – D).

EG-018 did not affect *MAP2* expression levels, and slightly downregulated *β-tubulin III*. The administration of SR141716A did not alter these values (Figure 3.13 A, B). Immunofluorescence analysis showed opposite trends, but with high standard deviations (Figure 3.13 C, D).

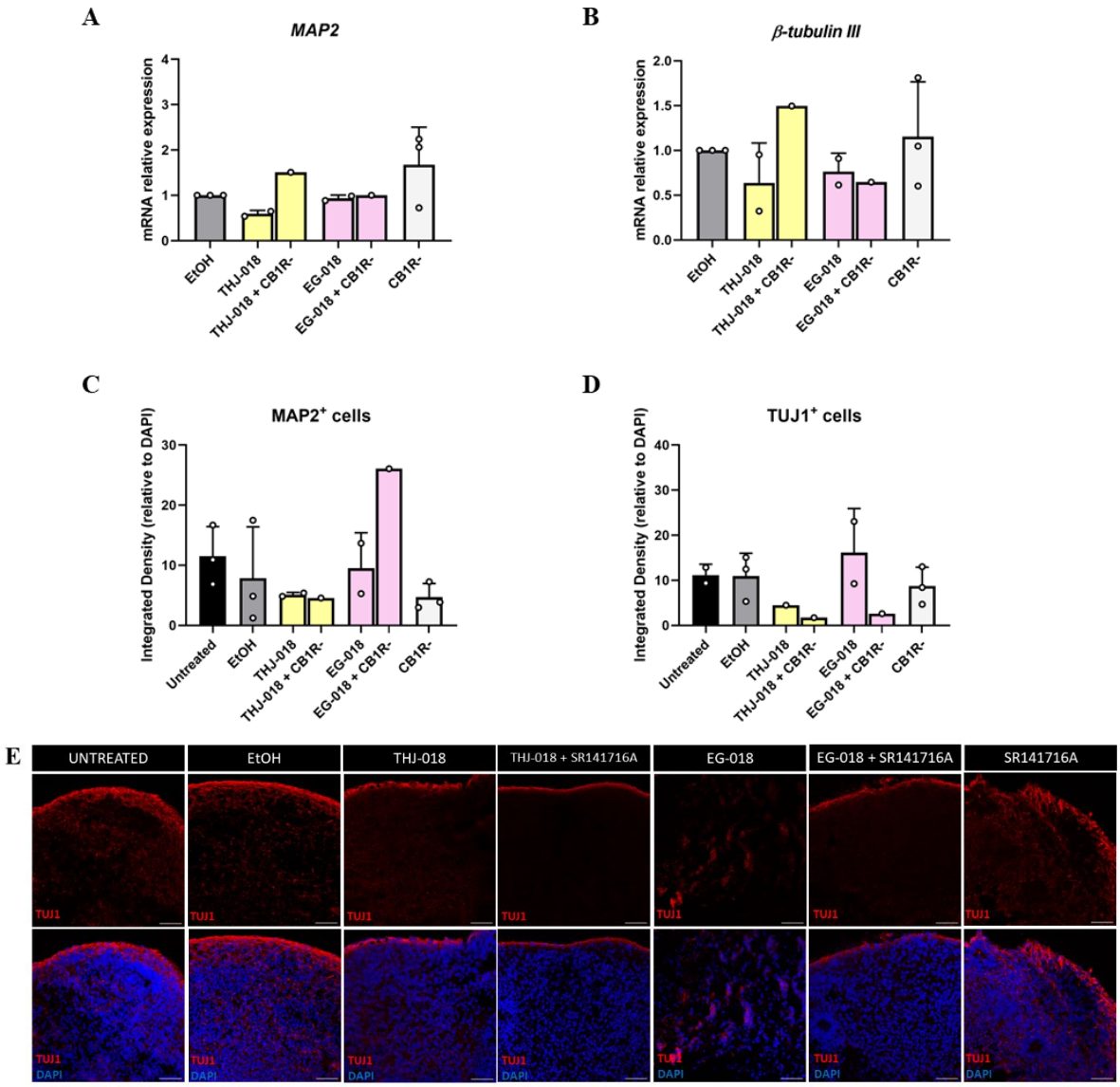

Figure 3.13- Effects of synthetic cannabinoids THJ-018 and EG-018 on mature neurons markers. qRT-PCR analysis of A) *MAP2* and B) *β-tubulin III* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. The values were further normalized to vehicle (EtOH) treatment. Immunofluorescence quantification of neuron-specific markers C) MAP2 and D) TUJ1, normalized to DAPI. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-". E) Immunofluorescence staining of TUJ1 on 46 days-old organoids cryosections. Scale bars: 50 μm.

Preliminary results indicate that THJ-018 and EG-018 cause a downregulation of neural progenitors and proliferation markers, and also mature neuronal markers. Additionally, immunofluorescence images appear to indicate a lower number of cells in organoids treated with EG-018 (similarly to CBD). This could be an indicator that these compounds may have a negative impact on neurogenesis, and could suggest neurotoxicity. Moreover, THJ-018 and EG-018 appear to have a more negative impact comparing to the tested phytocannabinoids, which could be related to worse outcomes from their consumption. Further studies on the functionality of neurons exposed to synthetic cannabinoids could be done in the future, to complement these results.

As previously mentioned, only one paper has been published on the effects of THJ-018 and EG-018 on an *in vitro* model. At 10 μM, these compounds promote precocious neuronal differentiation. Other studies that test the administration of other synthetic cannabinoids obtained similar conclusions, demonstrating that they promote differentiation in a CB1R-dependent manner. Differences between these conclusions and our study reflect differences in the cell model, tested cannabinoids, their concentration and frequency at they are administred.

Not all the effects of THJ-018 and EG-018 appear to be reverted by SR141716A. Sometimes, they are even potentiated by the CB1R antagonist. However, these results are only from one replicate, and therefore cannot be used to confidently make conclusions. Nonetheless, we hypothesise that if the observed trends mantained the same, the effects of THJ-018 and EG-018 might be modulated by other classes of receptors.

Once again, we emphasise that these findings need to be confirmed by a higher number of replicates. Discrepancies between qRT-PCR results and immunofluorescence analysis may be related to the used methods. While by qRT-PCR we obtain results that represent the whole population of cells, immunofluorescence analysis is performed on organoid sections, that can lead to some bias in the quantifications.

3.4.3. Effects of THJ-018 and EG-018 on apoptosis

Synthetic cannabinoids often exhibit stronger effects than phytocannabinoids. These compounds are also related to higher rates of cytotoxicity in vitro (Miranda et al., 2020), hence the importance of evaluating their impact on apoptosis in our model.

Immunofluorescence images show a dispersed staining of cleaved caspase 3 throughout the organoids (Figure 3.14 A). The results indicate higher levels of the marker in organoids exposed to THJ-018 comparing to the vehicle, with these values seeming to be reduced in the presence of CB1R antagonist SR141716A. EG-018 exhibits a similar pattern, although with a milder increase in the marker (Figure 3.14 B).

Results indicate both THJ-018 and EG-018 lead to higher levels of cleaved caspase 3 (Figure 3.14 B), comparing to the vehicle but also to 2 μM Δ^9-THC and CBD (Figure 3.8). This trend further supports the idea of synthetic cannabinoids having more negative effects comparing to phytocannabinoids. In the previous study developed by our group, 10 μM THJ-018 also led to higher levels comparing to Δ^9-THC, followed by EG-018, as seen in our study (Miranda et al., 2020). We can postulate that THJ-018 stronger effects are related to its higher affinity to CB1R, comparing to EG-018.

Additionally, since CB1R antagonist appears to decrease apoptosis back to vehicle levels, we hypothesise that CB1R activation is involved in this cytotoxic effect. This was also observed in another *in vitro* study, but using a different synthetic cannabinoid (as well as different exposure time and higher concentration) (Tomiyama & Funada, 2014).

Overall, these results are consistent with what was observed in other parameters and indicate a possible cytotoxic effect of synthetic cannabinoids. This should be further evaluated, as well as neuronal functionality in these cells, to determine if these compounds can be associated to neurotoxicity and, consequently, to cognitive deficits later in life.

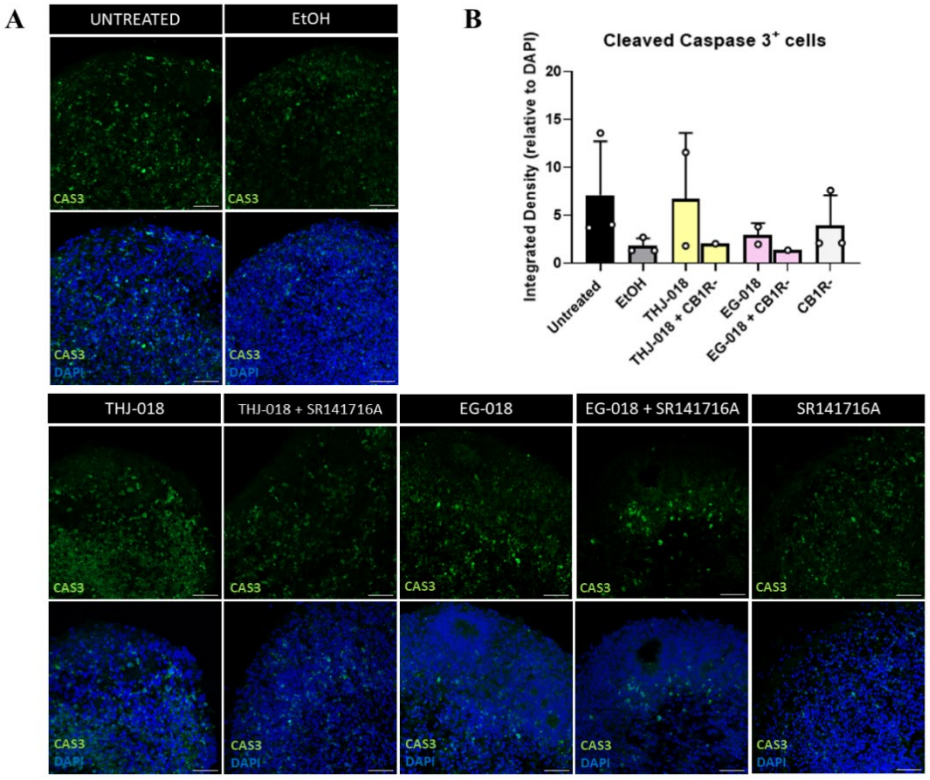

Figure 3.14- Effects of synthetic cannabinoids THJ-018 and EG-018 on apoptosis. A) Immunofluorescence quantification of apoptosis marker cleaved caspase 3, normalized to DAPI. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). SR141716A (CB1R antagonist) treatment was abbreviated to "CB1R-". B) Immunofluorescence staining of cleaved caspase 3 on 46 days-old organoids cryosections. Scale bars: 50 µm.

3.5. Chemically defined lipid concentrate as an alternative to the use of ethanol

EtOH remains one of the most used solvents to vehiculate cannabinoids in *in vitro* studies. Alternatives include dimethyl sulfoxide (DMSO), however, it also has a strong impact on neuronal differentiation. In the next sections we explain why EtOH was not the most suitable option to test some parameters in our model, and our first steps in optimizing the vehicle.

3.5.1. Effects of Ethanol on cortical lamination

Using a 3D model, we also wanted to evaluate the levels of markers that are intrinsically related to cortical lamination, the process by which neurons arrange in the different cortical layers. Therefore, we tested mRNA expression of *TBR1*, which encodes for a transcription factor present in neurons from the cortical plate and suplate,

and new-born neurons from the deep-layer VI (Gomes et al., 2020; Hevner et al., 2001), and *TBR2*, which is expressed in intermediate progenitors and is involved in cortical neurogenesis expansion and cortical excitatory neurons organization (Lv et al., 2019).

Another interesting reason to evaluate this markers is their association with austism-spectrum disorders. Autism can be induced by genetic mutations, epigenetic modifications but also environmental factors. It has been discovered that children whose mothers reported cannabis use during gestation were more likely to exhibit symptoms of autism, such as delayed cognitive skills (DiGuiseppi et al., 2022; Goldschmidt et al., 2000). Several studies indicate that *TBR1* expression alterations are often observed in these conditions (Chan et al., 2020).

mRNA analysis indicated that EtOH caused a substantial decrease in both *TBR1* and *TBR2* expression levels in the first two differentiations, compared to the untreated cells (Figure 3.15 A and B). In the last differentiation, EtOH induced an opposite effect, causing a considerable upregulation of these genes (Figure 3.15 A and B). These results emphasise the variability associated to the use of EtOH. This meant that it was not possible to confidently assess phytocannabinoid effects on these genes since the vehicle itself could influence the observed alterations. This led us to attempt to optimize this model, aiming to evaluate these alterations in the future.

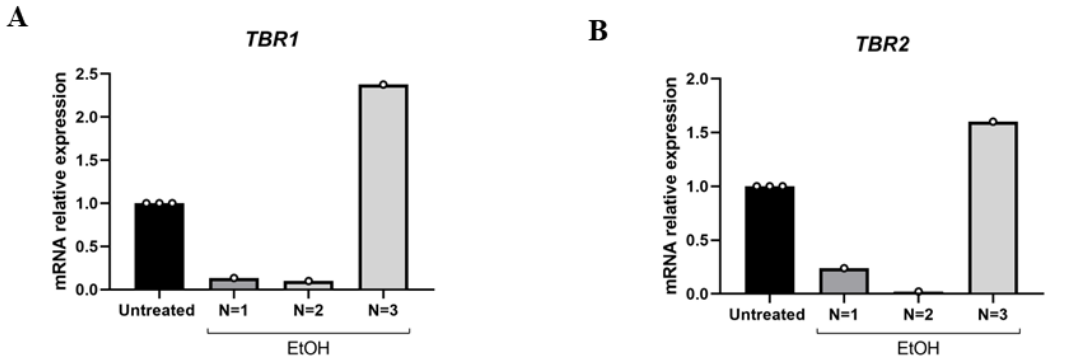

Figure 3.15- Effect of ethanol on cortical markers. qRT-PCR analysis of A) *TBR1* and B) *TBR2* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. Values were further normalized to the untreated group. Each dot represents a biological replica of an independent differentiation.

With this in mind, we tried to find a new alternative that had minimal impact in our protocol, making it easier to evaluate cannabinoid effects. As previously mentioned, cannabinoids are highly lipophilic substances, reducing the options of a solvent to

choose. We decided to determine if CD lipid concentrate would be a more adequate vehicle, by doing the last differentiation in parallel with Δ^9-THC and CBD solubilized in both EtOH and this lipidic solution, and evaluate its effects on neuronal differentiation.

CD lipid concentrate is composed by several lipids, such as arachidonic acid, cholesterol, linoleic acid, among others. The solution is used in several differentiation protocols with different lineage commitments, for example, in protocols to generate retinal, cerebellar and also forebrain organoids (Capowski et al., 2018; Kadoshima et al., 2013; T. P. Silva et al., 2020; Wataya et al., 2008). This meant that CD lipid concentrate probably does not play a critical role in lineage specification, and we hypothesised that it should not have accentuated effects on the organoids we were generating. However, it also contains EtOH, in a percentage that is not disclosed. Assuming that this percentage would be minimal, and still would have lesser effects than using only EtOH by itself, we still tested the effects of CD lipid concentrate in our model.

3.5.2. Preliminary results of CD lipid concentrate use in neuronal differentiation

To determine if CD lipid concentrate would be suitable for this application, we first evaluated if cells trateated with the new vehicle had a normal expression of neuronal markers throughout the differentiation, and similar morphology to our controls. In Figure 3.16 A) and B), we observe that CD lipid concentrate-treated cells exhibit similar levels of *PAX6* and an increase of *MAP2* compared to untreated cells by day 46. Immunofluorescence analysis confirmed the expected morphology (Figure 3.16 C). These results were complemented with single-cell calcium imaging, that showed a similar response to KCl stimulus and a lower response to histamine comparing to untreated cells (Figure 3.16 D-F).

These results are still preliminary and do not allow us to test important parameters such as variability between data. Nonetheless, they are promising, since CD lipid concentrate appears to not induce extreme alterations on neuronal markers and organoids morphology. This could, in the future, allow to use CD lipid concentrate as a valid option vehicle to solubilize cannabinoids.

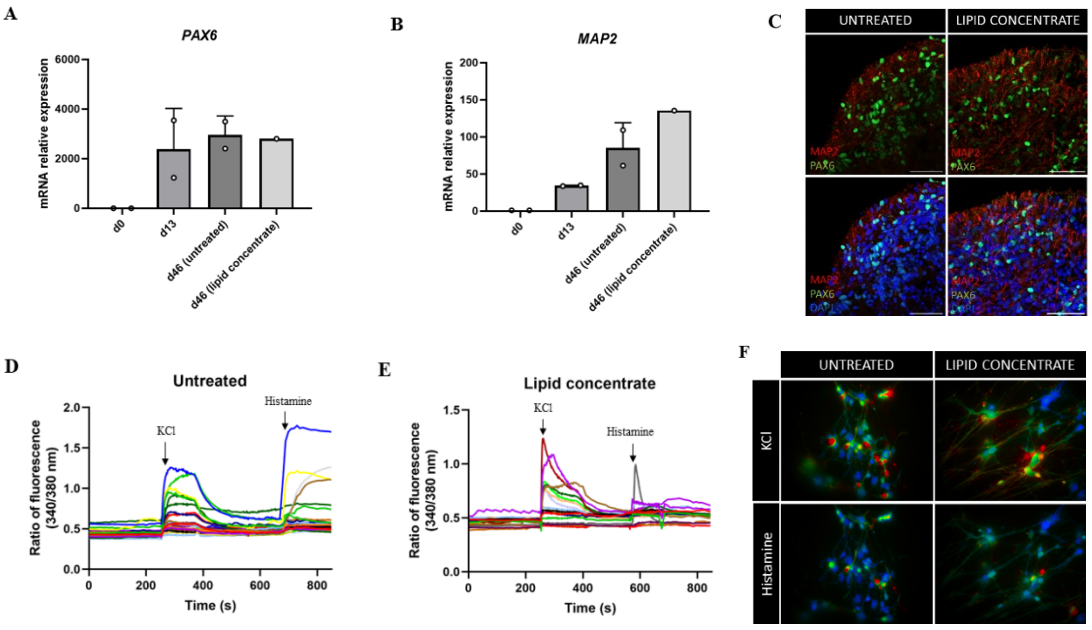

Figure 3.16- Assessment of neuronal markers and functional analysis in chemically-defined lipid concentrate-treated cultures. qRT-PCR analysis of A) *PAX6* and B) *MAP2* mRNA expression levels relative to *GAPDH* and normalized to day 0 of the differentiation. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). C) Immunofluorescence staining of neural progenitors marker PAX6 and neuron specific microtubule associated protein MAP2 on 46 days old organoids cryosections. Scale bars: 50 µm. SCCI analysis showing the response of D) untreated and E) CD lipid concentrate-treated cultures to KCl and histamine stimuli (targeting mature neurons, and immature neurons and neural progenitors, respectively). Data from 1 experiment. F) Representative ratio images of the tested population for the two conditions. Images were taken immediately after each stimulus.

The previous effects of EtOH and the variability it induced in markers such as *TBR1* and *TBR2* were the primary reasons to begin the search for a more adequate vehicle to solubilize cannabinoids. Thus, evaluating the effects of CD lipid concentrate on the expression of these genes was also considered as a parameter for its validation.

As observed in Figure 3.17 A and B, although we only have results from one experiment, CD lipid concentrate appeared to reduce *TBR1* expression levels, and caused an increase of *TBR2* expression comparing to untreated cells. *TBR1* expression levels in the presence of CD lipid concentrate are lower than the values obtained for untreated cells, but are still higher than two of three values obtained with the EtOH. Considering that EtOH either causes an extreme decrease or increase in *TBR1* and *TBR2* expression levels, CD lipid concentrate could still have a milder effect than EtOH.

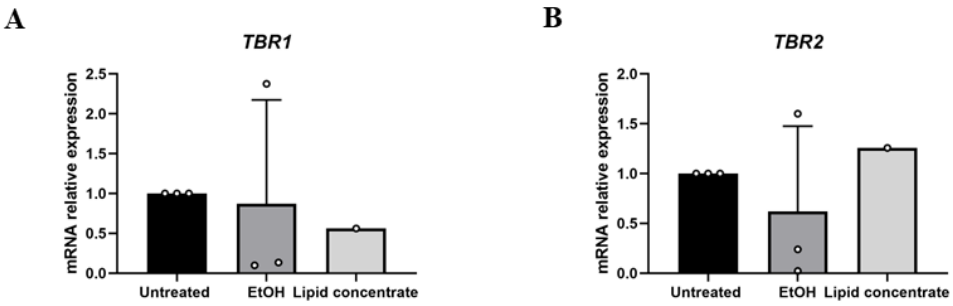

Figure 3.17- Effect of CD lipid concentrate on cortical markers. qRT-PCR analysis of A) *TBR1* and B) *TBR2* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD).

Unfortunately, already after using CD lipid concentrate, we were informed by the manufacturer that it was at only 10% of the designated concentration. When dissolving the cannabinoids, it was noticeable that it was being hard to solubilize them (what was not expected, since they are lipophilic), but now it is understandable due to the altered lipidic content. Although the some of the results are still being presented in the next section, we have to consider that they might be related to differences in the concentration (due to reduced solubilization).

3.5.3. Effects of phytocannabinoids when vehiculated in chemically-defined lipid concentrate

In this section we compare the effects of phytocannabinoids (solubilized in CD lipid concentrate and in EtOH) on mRNA expression levels of *TBR1* and *TBR2*, evaluating if there are similar trends.

When solubilized in CD lipid concentrate, Δ^9-THC induced an upregulation of *TBR1*, which is contradicted by CB1R antagonist (Figure 3.18 A). When administrated with EtOH, it also led to an upregulation of the gene, but accentuated by SR141716A (Figure 3.18 D). The phytocannabinoid led to a slight upregulation of *TBR2*, reduced by CB1R antagonist when in CD lipid concentrate. Solubilization in EtOH induced an opposite effect for the administration of both Δ^9-THC and SR141716A.

CDB led to an upregulation of *TBR1* and *TBR2* when dissolved in CD lipid concentrate, with both trends being contradicted by SR141716A (Figure 3.18 A, B).

When administrated with EtOH, let to minimal effects on the expression of both genes, and SR14716A exacerbated its effects on *TBR2* (Figure 3.18 C, D).

Although using the same phytocannabinoids, at the same concentration, their administration with different vehicles still resulted in distinct trends in gene expression analysis. This highlights the importance of continuing to evaluate the effects of CD lipid concentrate and EtOH on our model, and to determine which one is the most suitable option in the context of this project.

Cortical organoids have already been used to study autism spectrum disorders (even using patient-derived cell lines), which show an overproduction of inhibitory neurons, and a slight upregulation of *TBR1* and *TBR2* (Mariani et al., 2015). If the impact of phytocannabinoids on *TBR1* and *TBR2* observed in these results would be supported by further results, it could help understanding the causes of cognitive deficits and autism-like symptoms detected after prenatal exposure to these compounds.

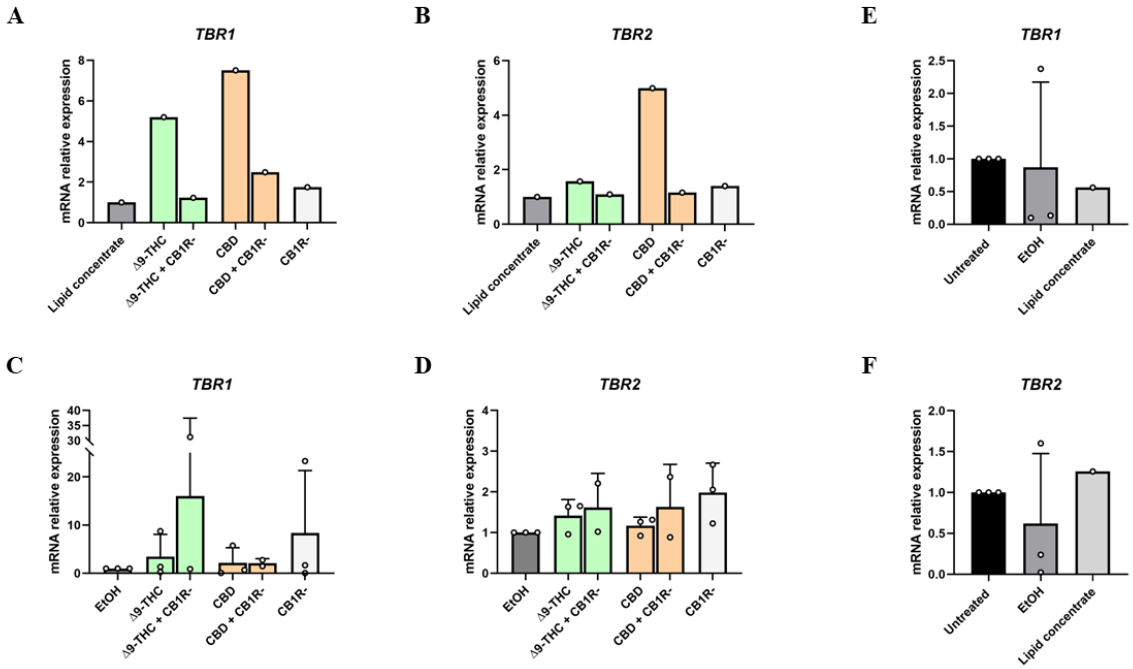

Figure 3.18- Comparison of the effects of Δ^9-THC and CBD on cortical markers when vehiculated in CD lipid concentrate or EtOH. qRT-PCR analysis of A) *TBR1* and B) *TBR2* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. Values were further normalized to CD lipid concentrate treatment. qRT-PCR analysis of C) *TBR1* and D) *TBR2* mRNA expression levels relative to *GAPDH* by day 46 of the differentiation. Values were further normalized to EtOH treatment. Each dot represents a biological replica of an independent differentiation. Error bars represent standard deviation (SD). Values were normalized to CD lipid concentrate. E), F) Data from figure 3.17 was included to allow comparison between untreated and vehicle-treated cells.

Since we only have results from one differentiation, we will need to evaluate if these trends maintain by performing these experiments with a higher number of biological replicates in the future. The use of a different vehicle requires the fulfilment of requirements such as biocompatibility, biodegradability, and adequate particle size to permeate cell membranes, which would imply performing other studies. Currently there are no similar *in vitro* studies that use this solution to solubilize cannabinoids or in a similar context to our project. Regardless, lipid-drug conjugates have been proposed as a promising drug delivery system to increase *in vivo* bioavailability of therapeutic agents, including medicinal CBD. Lipid-drug conjugates allow drugs to cross the blood-brain barrier, and cover requirements as the ones mentioned above (Zielińska et al., 2022). Literature also points to the use of microemulsions to solubilize cannabinoids, including Tween-80 as a surfractant and ethanol as a co-surfractant, two components of CD lipid concentrate (which is also an emulsion) (Park et al., 2021). This indicates the potential of using CD lipid concentrate as a way to vehiculate cannabinoids in *in vitro* models in the future, and lead to a better understanding of their effects on neurdevelopment.

4. Conclusion and Future Perspectives

The use of cannabinoid-based products remains controversial in contemporary times. High rates of use and misuse, even among pregnant women, are a growing concern and highlight the importance of determining the properties, mechanism of action and effects of these substances. With the present work we successfully generated dorsal forebrain organoids, which showed to be a valuable model to study prenatal exposure to cannabinoids.

Although the results are preliminary and need further validation, we can observe trends indicating that Δ^9-THC may delay neuronal differentiation and affect cell functionality, through mechanisms that are associated to CB1R activation. On the other hand, CBD appeared to have minimal effects on neuronal differentiation, but its interaction with the CB1R antagonist indicated the complexity of CB1/2R interplay.

Synthetic cannabinoids THJ-018 and EG-018 induced effects that might be indicative of some neurotoxicity. Though a higher number of replicates is needed to take conclusions, their effects appear to not be reverted by the CB1R antagonist, suggesting that they might be mediated by other classes of receptors.

However, it was possible to observe high standard deviations throughout the results, which led to lack of statistical significance. This was related to differences in organoids morphology and size among differentiations, and highlights the need to increase the number of replicates. Another possibility is both concentration and period of administration were not enough to induce significant alterations. Variability between results may also be related to the chosen vehicle, EtOH, being CD lipid concentrate a promising alternative to solubilize cannabinoids.

In the future, we plan to increase the number of replicates to detect possible outliers, and have stronger trends that support the conclusions behind conditions. To produce size-controlled cell aggregates, we propose alterations such as monitoring the number of organoids per well, the use of dynamic cultures or encapsulation in hidrogels. Dissociation into 2D cultures after day 40 is also an option to allow a uniform distribution of cannabinoids to all of the cells. We also aim to complement this project with electrophysiology studies to evaluate neuronal activity. As mentioned, otimization

of the model is also one of our aims by further testing CD lipid concentrate as a vehicle and possibly test longer periods of administration of cannabinoids.

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