Department of Biochemistry

& Biotechnology

ASSESSMENT OF THE MODULATION OF THE ENDOCANNABINOID SYSTEM BY SYNTHETIC CANNABINOIDS DURING IN VITRO NEURONAL DIFFERENTIATION

Galani Myrto

Larissa, Academic Year 2018/2019

Cofinanciado por:

COMPETE 2020 PORTUGAL 2020 UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Department of Biochemistry

& Biotechnology

ΑΞΙΟΛΟΓΗΣΗ ΤΗΣ ΔΙΑΜΟΡΦΩΣΗΣ ΤΟΥ ΕΝΔΟΚΑΝΝΑΒΙΝΟΕΙΔΟΥΣ ΣΥΣΤΗΜΑΤΟΣ ΑΠΟ ΣΥΝΘΕΤΙΚΑ ΚΑΝΝΑΒΙΝΟΕΙΔΗ ΚΑΤΑ ΤΗΝ ΔΙΑΡΚΕΙΑ IN VITRO ΝΕΥΡΩΝΙΚΗΣ ΔΙΑΦΟΡΟΠΟΙΗΣΗΣ

Γαλάνη Μυρτώ

Λάρισα, Ακαδημαϊκό Έτος 2018/2019

Cofinanciado por:

COMPETE 2020 PORTUGAL 2020 UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Τριμελής Επιτροπή

Δημήτριος Κουρέτας (επιβλέπων): Καθηγητής Φυσιολογίας Ζωικών Οργανισμών-Τοξικολογίας, Τμήμα Βιοχημείας & Βιοτεχνολογίας, Πανεπιστήμιο Θεσσαλίας.

Δημήτριος Στάγκος: Επίκουρος Καθηγητής Φυσιολογίας Ζωικών Οργανισμών, Τμήμα Βιοχημείας & Βιοτεχνολογίας, Πανεπιστήμιο Θεσσαλίας.

Δημήτριος Μόσιαλος: Επίκουρος Καθηγητής Βιοτεχνολογίας Μικροβίων, Τμήμα Βιοχημείας & Βιοτεχνολογίας, Πανεπιστήμιο Θεσσαλίας.

Acknowledgements

Throughout this project, I have received a great deal of support and assistance.

I would like to thank my professor Dr. Dimitrios Kouretas for giving me the opportunity to work in his laboratory, and for all the advices that he gave me these months.

I would also like to express my sincere gratitude to Dr. João Pedro Silva for all the patience, courage, support and guidance through our collaboration in this project. His expertise was invaluable in the formulating of the research topic and methodology in particular.

I would also like to thank Professor Félix Carvalho, for always being by my side, supporting me continuously, prompt to answer to my inquiries, and making me feel at home.

This work was financed by FEDER - European Fund for Regional Development funds through the COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation (POCI), and by Portuguese funds through FCT - Portuguese Foundation for Science and Technology - in the framework of the project POCI-01-0145-FEDER-029584.

Abstract

Synthetic cannabinoids (SCs) represent a variety of psychoactive substances that activate cannabinoid receptors, and imitate the main psychoactive substance of *Cannabis sativa*, Tetrahydrocannabinol (THC). Their toxicological profiles are not completely identified, even though a serious number of acute intoxications and deaths have already been observed following the consumption of these substances. The widespread recreational use of SCs globally is a major health issue, as SCs consumption by pregnant or non-pregnant women in reproductive age may lead to the onset of some neurodevelopment disorders in the offspring. The aim of this work is to investigate how SCs may affect neuronal differentiation *in vitro*.

Neuroblastoma-glia cells (NG108-15) were exposed to some commonly reported SCs (THJ-2201, ADB-FUBINACA and AB-CHMINACA) at a concentration range of 1pM - 1μM, once or in multiple additions. After following neuronal differentiation with forskolin and retinoic acid, with or without SCs, differentiation ratios (number of neurites per total cell number) and total primary neurite length were calculated. Also, a specific CB1 receptor (CB1R) antagonist, SR141716A was added prior to SC addition, to assess the involvement of the inhibition of CB1 receptor in neuronal differentiation process. The inhibitors methyl arachydonyl fluorophosphonate (MAFP) and tetrahydrolipstatin (THL) were also added prior to SC exposure, in order to assess the effects of the endocannabinoid synthesis inhibition.

THJ-2201, ADB-FUBINACA and AB-CHMINACA promoted NG108-15 cell differentiation, as the differentiation ratios were similarly increased, after a single or multiple additions of these SCs. In all the SCs tested, the presence of SR141716A alone significantly enhanced the differentiation, in both one and three additions, compared to the control cells. Moreover, all the cells treated with MAFP and THL presented an abnormal morphology and reduced total neurite network, evidenced also by the lower differentiation ratios.

Taken together these observations indicate a potential modulatory effect of these SCs on neuronal differentiation of NG108-15 cells. Although further research is required to clarify the mechanisms involved in these processes.

Keywords: psychoactive substances, synthetic cannabinoids, cannabinoid receptors, neuronal development, neuronal differentiation, differentiation ratio, neurite outgrwoth, pregnancy, neurodevelopment disorders, deaths, acute intoxicantions.

Περίληψη

Τα συνθετικά κανναβινοειδή αντιπροσωπεύουν μια ποικιλία ψυχοδραστικών ουσιών που ενεργοποιούν τους υποδοχείς κανναβινοειδών, και μιμούνται την κύρια ψυχοδραστική ουσιά του Cannabis sativa, την τετραϋδροκανναβινόλη (THC). Τα τοξικολογικά τους προφίλ δεν έχουν αναγνωριστεί πλήρως, παρόλο που έχει ήδη παρατηρηθεί ένας σοβαρός αριθμός οξείων δηλητηριάσεων και θανάτων μετά την κατανάλωση αυτών των ουσιών. Η ευρέως διαδεδομένη ψυχαγωγική χρήση των συνθετικών κανναβινοειδών σε παγκόσμιο επίπεδο αποτελεί ένα μείζων ζήτημα υγείας, καθώς η κατανάλωση συνθετικών κανναβινοειδών από έγκυες ή μη έγκυες, σε αναπαραγωγική ηλικία γυναίκες μπορεί να οδηγήσει στην εμφάνιση ορισμένων νευροαναπτυξιακών διαταραχών στους απογόνους. Ο σκοπός αυτής της εργασίας είναι να διερευνήσουμε πως τα συνθετικά κανναβινοειδή μπορεί να επιδράσουν στη νευρωνική διαφοροποίηση in vitro.

Neuroblastoma-glia κύτταρα (NG-108-15) εκτέθηκαν σε κάποια κοινώς αναφερόμενα συνθετικά κανναβινοειδή (THJ-2201, ADB-FUBINACA και AB-CHMINACA) σε ένα εύρος συγκέντρωσης 1pM-1μM, με μία ή με πολλαπλές προσθήκες. Μετά από ακόλουθη νευρωνική διαφοροποίηση με φορσκολίνη και ρετινοϊκό οξύ, με ή χωρίς συνθετικά κανναβινοειδή, υπολογίστηκαν οι λόγοι διαφοροποίησης (αριθμός νευρώνων ανά ολικό αριθμό κυττάρων) και τα συνολικά μήκη πρωτογενών νευρώνων. Επίσης, ένας ειδικός ανταγωνιστής CB1 υποδοχέα (CB1R), SR141716A προστέθηκε πριν από την προσθήκη των συνθετικών κανναβινοειδών, για να εκτιμηθεί η εμπλοκή της αναστολής του CB1 υποδοχέα στη διαδικασία νευρωνικής διαφοροποίησης. Οι αναστολείς methyl arachydonyl fluorophosphonate (MAFP) και tetrahydrolipstatin (THL) προστέθηκαν επίσης πριν από την έκθεση σε συνθετικό κανναβινοειδές, προκειμένου να εκτιμηθούν τα αποτελέσματα της αναστολής της σύνθεσης ενδοκανναβινοειδών.

Τα THJ-2201, ADB-FUBINACA και AB-CHMINACA προώθησαν την διαφοροποίηση NG108-15 κυττάρων, καθώς οι λόγοι διαφοροποίησης αυξήθηκαν παρομοίως μετά από μία ή πολλαπλές προσθήκες αυτών των συνθετικών. Σε όλα τα συνθετικά που εξετάστηκαν, η παρουσία του SR141716A αύξησε σημαντικά τη

διαφοροποίηση, τόσο σε μία όσο και σε τρεις προσθήκες, σε σύγκριση με τα κύτταρα control. Επιπλέον, όλα τα κύτταρα που εκτέθηκαν σε MAFP και THL παρουσίασαν μια ανώμαλη μορφολογία και μειωμένο ολικό δίκτυο νευρώνων, όπως αποδεικνύεται και από τους χαμηλότερους λόγους διαφοροποίησης.

Λαμβανόμενες μαζί αυτές οι παρατηρήσεις υποδεικνύουν μια δυνητική ρυθμιστική επίδραση αυτών των συνθετικών κανναβινοειδών στη νευρωνική διαφοροποίηση των NG108-15 κυττάρων. Ωστόσο, απαιτείται περαιτέρω έρευνα για τη διευκρίνιση των μηχανισμών που εμπλέκονται σε αυτές τις διαδικασίες.

Λέξεις Κλειδιά: ψυχροδραστικές ουσίες, συνθετικά κανναβινοειδή, υποδοχείς κανναβινοειδών, νευρωνική ανάπτυξη, νευρωνική διαφοροποίηση, λόγος διαφοροποίησης, απόφυση νευρώνων, εγκυμοσύνη, νευροαναπτυξιακές διαταραχές, θάνατοι, οξείες δηλητηριάσεις.

Index

Table of figures

Table index

Abbreviations

2-Ara-Gl (2-AG) : 2-Arachidonylglycerol

AEA: Anandamide

BSA: Bovine Serum Albumin

CBD: Cannabidiol

CB1R: Cannabinoid Receptor 1

CB2R: Cannabinoid Receptor 2

DAGL: Diacylglycerol Lipase

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulfoxide

DTT: Dithiothreitol

GPCR: G protein-coupled Receptors

ECS: Endogenous Cannabinoid System

ERK: Extracellular Signal-Regulated kinase

eCBS: Endogenous Cannabinoids

FAAH: Fatty Acide Amide Hydrolase

FBS: Fetal Bovine Serum

HBSS: Hank's Balanced Salt Solution

JNK: JUN N-terminal Kinases

MAFP: Methyl ArachidonylFluorophosphonate

MAGL: Monoacylglycerol Lipase

MAPK: Mitogen-Activated Protein Kinase

MM: Maintenance Medium

NAE: N-acylethanolamine

NAPE: N-acyl-phosphatidyl-ethanolamine

NAPE-PLD: N-acyl-phosphatidyl-ethanolamine-specific phospholipase D

NPS: New psychoactive substances

SCs: Synthetic Cannabinoids

SR141716A: Rimonabant

THC: Tetrahydrocannabinol

THJ-2201: [1-(5-Fluoropentyl)-1H-indazol-3-yl] (1-naphthyl) methanone

THL: Tetrahydrolipstatin

TRPV1: Transient receptor potential cation channel subfamily V member 1

PKA: Protein Kinase A

PBS: Phosphate Buffered Saline

PPARγ: Peroxisome proliferator-activated receptor-γ

WB: Western Blot

Abuse of Synthetic Cannabinoids: A crucial problem

In recent years, the recreational use of psychoactive substances, such as cannabinoids, has dramatically increased. After the ingestion or the inhalation of cannabis, endogenous cannabinoid receptors are activated, leading to the observed psychoactive effects. Nevertheless, cannabinoids may also be used in medicine (e.g. during cancer treatment, to increase the patient's appetite). In 2010, the medical use of cannabis extracts was approved by ten European countries.

The large number of new psychoactive substances (NPS), namely synthetic cannabinoids (SCs) is particularly concerning. SCs comprise a chemically diverse group of substances that imitate the effects of Δ^9-Tetrahydrocannabinol (Δ^9-THC), the main psychoactive substance in cannabis. Generally, SCs represent molecules that contain a mixture of psychoactive compounds and they are easily accessible (Cohen and Weinstein, 2018) and not readily detected in standard urine drug screens (Salani and Zdanowicz, 2015). Also, due to the SCs great binding affinity to cannabinoid receptors, they are able to activate the endogenous cannabinoid system. SCs represent an extensive danger because they cause psychoactive and physiological effects similar, but more intense than THC, often resulting in severe adverse effects and medical emergencies (Castaneto *et al.*, 2014). The most crucial problem of the spreading use of synthetic cannabinoids, is that there is an unawareness concerning their toxicological profiles, since they are chemically diverse and that new types of SCs, comprising unidentified effects, are constantly appearing (EMCDDA, 2018).

Prenatal exposure to cannabinoids, may be associated with abnormal offspring outcomes (e.g. low birth weight) or the occurrence of neurodevelopmental disorders such as schizophrenia and autism spectrum disorder (Brown *et al.*, 2017). In this sense, there is a mandatory need to identify how the endocannabinoid system regulates neuronal development-related processes (e.g. differentiation, proliferation, migration).

2. Cannabinoids

Cannabinoids are diverse chemical compounds that activate endogenous cannabinoid receptors being involved in the neurotransmitter release in the brain. They are a group of biologically active terpenophenols derived from 2-substituted 5-amyl resorcinol. The ligands for the receptor proteins may be endogenous (endocannabinoids), phyto- or synthetic cannabinoids. Endocannabinoids are produced in the animal's body and they represent lipid neurotransmitters that react with the endogenous cannabinoid receptors (CB1R, CB2R). Phytocannabinoids represent a group of cannabinoids produced in cannabis or other plants. They are more than 110 different cannabinoids isolated from cannabis, each having a variety of effects. The main phytocannabinoids are the psychotropic tetrahydrocannabinol (THC), and the abundant non-psychotropic cannabidiol (CBD). Synthetic cannabinoids comprise a variety of molecules, which are manufactured artificially and they act as agonists of cannabinoid receptors. Here, we focused on the endogenous (section 2.3) and synthetic cannabinoids (section 3).

2.1 The Endocannabinoid System

The endocannabinoid system (ECS) is a complex cell-signalling system, which involves three core components: the endogenous cannabinoids (ECs), the cannabinoid receptors (e.g. CB1 and CB2), and the enzymes responsible for ECs biosynthesis and degradation (such as fatty acid amide hydrolase or monoacylglycerol lipase). Noteworthly, the endocannabinoid system is present from the first stages of neuronal development, and it plays a major role in the neuronal differentiation. The ECS further contributes to the regulation of a variety of physiological and cognitive processes including pregnancy (Wang, Xie and Dey, 2006), pre- and postnatal development (Fride, 2004), fertility (Klein *et al.*, 2012), appetite, mood, pain modulation, energy metabolism and in mediating the pharmacological effects of cannabis (Aizpurua-Olaizola *et al.*, 2017; Donvito *et al.*, 2018).

2.2 Cannabinoid Receptors

The cannabinoid receptors are guanine-nucleotide-binding protein (G protein) – coupled receptors (GPCR) that are activated by cannabinoids, and they exist primarily on central and peripheral neurons, and on immune cells. In 1988 (Di Marzo, Stella and Zimmer, 2015), the first cannabinoid receptor (CB1 receptor), which is encoded by gene CNR1 was discovered in the brain. In 1993 (Di Marzo, Stella and Zimmer, 2015), the second cannabinoid receptor (CB2 receptor), which exhibits 48% homology with the CB1 receptor, was predominantly identified in immune cells (e.g. mast cells, B-lymphocytes and macrophages) (Cabral G. A., 2009) with the greatest density in the spleen.

These receptors (CB1,CB2) are common in animals, having been found in mammals, fish, birds, and reptiles. They differ in their sensitivity to specific agonists and antagonists, predicted amino acid distribution, signaling mechanisms, and tissue distribution. CB1 receptor is the most abundant GPCR in the brain regions, like cortex, basal ganglia, hippocampus, cerebellum, also in both male and female reproductive systems, liver, heart, bones, lung and it is expressed in inhibitory – GABAergic – and excitatory – glutamatergic– presynaptic terminals (Castaneto *et al.*, 2014)(Di Marzo, Stella and Zimmer, 2015). The binding affinity of the cannabinoids to the CB1R and CB2R, define the effects evoked by these ligands. For instance, a higher affinity to CB1R may induce a psychotropic effect if the density of this receptor is higher in neuronal cells, and a higher affinity towards CB2R may result in chronic pain reduction, but not in psychotropic effects (Anand *et al.*, 2009).

CB1 and CB2 receptors are usually coupled to G protein type $G_{i/o}$ and they activate mitogen-activated protein kinases (MAPK), like JUN N-terminal kinases (JNKs), p38 MAPKs, extracellular signal-regulated kinase 1 (ERK1) and ERK2. Through this coupling, they also inhibit adenylyl cyclase and protein kinase A (PKA). Activation of the CB1 receptor in particular inhibits L-, N- and P/Q-type voltage-activated Ca^{2+} channels, and it also modulates potassium channels. As a result, there is a reduction of neurotransmitters (e.g. GABA, acetylcholine, noradrenaline, glutamate). Activation of the CB2 receptor, modulates immune cell migration and cytokine

release, within and outside the brain. They are also found in post-synaptic localizations in neuronal cells (e.g. neurons, glia, endothelial brain cells).

Several polymorphisms in the genes that lead to the expression of CB1 (*cnr1*) and CB2 (*cnr2*) receptors have been linked to disorders such as schizophrenia, and depression (for CNR1) and postmenopausal osteoporosis (for CNR2).

2.3 Endogenous Cannabinoids

The endogenous cannabinoids (ECs) were discovered in 1995, shortly after the discovery of CB1 and CB2 receptors, as a group of lipid neurotransmitters produced intracellularly, which act as endogenous agonists of cannabinoid receptors (Fiar, 2009). They consist of derivatives of the non-oxidative metabolism of the polyunstaturated fatty acid, arachidonic acid (Di Marzo, Stella and Zimmer, 2015). Although there appears to be several endocannabinoids, only two of these endogenous mediators have been studied so far: N-arachidonoyl-ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). Despite having similar structures, AEA and 2-AG differ in many ways, namely regarding their localization, biosynthesis, binding affinity and efficacy at the receptors (receptor selectivity), and their levels in brain cells (Pertwee *et al.*, 2010; Zou and Kumar, 2018).

Anandamide was discovered in the porcine brain, but it is present in human brain and it has the ability to bind to the CB1R with high-affinity and low-efficacy (Di Marzo, Stella and Zimmer, 2015). Generally, it is a partial agonist for both receptors (CB1R, CB2R), but it has lower efficacy at CB2 receptors. It derives from the hydrolysis of N-arachidonoyl phosphatidylethanolamine by a phospholipase D-like enzyme (NAPE-PLD) (Di Marzo, 2008). Levels of anandamide in unstimulated brain cells around 29 pmol/g of tissue in rat hippocampus (Felder *et al.*, 1996), which are much lower compared to levels of 2-AG. Of note, anandamide is a mediator that also shows further functional flexibility because it activates non-cannabinoid receptors such as TRPV1 channel and possibly the PPARγ receptors (Di Marzo, Stella and Zimmer, 2015).

The endogenous 2-monoglyceride, termed 2-arachidonyl glycerol (2-AG) was discovered in the canine gut, and it is synthesized through the action of an sn-1-diacylglycerol lipase on sn-2-arachidonate containing diacylglycerols (Di Marzo, 2008). It is an agonist for CB1R and CB2R, with similar affinities for both receptors. In comparison to anandamide, 2-AG elicits more significant responses at both CBRs. Levels of 2-AG have been detected up to 4 nmol/g of brain tissue (Sugiura *et al.*, 1995), much higher than anandamide. Intracellularly, anandamide and 2-AG can be rapidly degraded by enzymatic hydrolysis, via fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine, and by monoacylglyerol lipase (MAGL) into arachidonic acid and glycerol, respectively (Deutsch and Chin, 1993; Di Marzo *et al.*, 1994).

The discovery and the whole research on these endogenous cannabinoids revealed their significant role on the function of the endocannabinoid system, due to their various intracellular regulatory effects like the inhibition of the release of excitatory (e.g. acetylcholine, glutamate) and inhibitory (e.g. GABA, glycine) neurotransmitters in central and peripheral nervous system, rendering them as a potential alternative targets for therapeutic use (Howlett, 2002). Endogenous cannabinoid activity may also trigger other cellular responses such as the enhanced release of other neurotransmitters (Waksman *et al.*, 1999),downstream inhibition of NO release in microglial cells when a CB1R agonist is present, enhanced NO release from monocytes following exposure to 2-AG, or inhibition of T-helper cell activation in a CB2R-associated way.

2.4 Enzymes responsible for Endocannabinoid Synthesis and Degradation

Endogenous cannabinoid biosynthesis or degradation are catalyzed by specific enzymes (Di Marzo, 2008).When intracellular Ca^{2+} is increased, and after activation of metabotropic $G_{q/1}$-coupled receptors or neuron depolarization, the ECs are produced from phospholipid precursors. Generally, AEA is a low-efficacy, high-affinity CB1 agonist, while it elicits almost no response from CB2 receptors. On the

other hand, 2-AG is a full agonist at both CB1 and CB2 receptors, however it displays moderate-to-low affinity for both receptors (Di Marzo, Stella and Zimmer, 2015; Zou and Kumar, 2018).

The production of the N-acylethanolamine (NAE) anandamide occurs through the hydrolysis of an appropriate N-acyl-phosphatidyl-ethanolamine (NAPE). In the intracellular membranes, pre- and postsynaptically, N-acylphosphatidylethanolamine (NAPE)-specific phospholipase D (NAPE-PLD) is able to catalyse this process in a single step. Concerning the degradation of AEA, fatty acide amide hydrolase 1 (FAAH) is an enzyme located postsynaptically that is able to hydrolyze the eCBs that they are taken up by cells (membrane carriers, intracellular carrier proteins or endocytosis). FAAH cleaves anandamide into arachidonic acid and ethanolamine. It may be also oxidized by the enzyme cyclooxygenase 2 (COX 2 – prostaglandin G/H/ synthase 2) (Di Marzo, Stella and Zimmer, 2015).

2-AG and other 2-acylglycerols are biosynthesized in a single step by the hydrolysis of diacylglycerols (DAGs), via diacylglycerol lipases ($DAGL_{\alpha}$ or $DAGL_{\beta}$). The degradation of 2-AG is similar to AEA' s, but the reaction is catalysed by monoacylglycerol lipase (MAGL), which cleaves 2-AG into arachidonic acid and glycerol. 2-AG may be also hydrolysed by ABHD6 (α,β hydrolase 6), ABHD12 and FAAH (Di Marzo, Stella and Zimmer, 2015).

2.5 Importance of the endocannabinoid system to the regulation of the neuronal function (neurodevelopment)

Brain development is a highly complex process, requiring the concerted action of morphogens and extracellular signals that regulate and orchestrate functional neurogenesis (Galve-Roperh *et al.*, 2009). This is achieved through a multitude of steps, including the proliferation of progenitor neuronal cells, the differentiation and maturation of various neuronal cell types, including the establishment of appropriate synapses. Endocannabinoid ligands, their receptors and their downstream signaling pathways are active during neurodevelopment at all stages (from embryonic to the adolescent brain). Thus, cannabinoid signaling plays a major role in neuronal

homeostasis and in modulating cellular plasticity, as well as neuronal proliferation and apoptosis in the immature brain (Sagredo *et al.*, 2018).

High CB1 receptor expression levels have been observed in selective areas (such as the mesolimbic dopaminergic system) during the early developmental stages of nervous system, as well as in the adult brain (Fernández-Ruiz *et al.*, 2004) (Galve-Roperh *et al.*, 2013). In embryonic rat brains, both CB1R and CB2R have been detected around the 11^{th} day of gestation (Buckley *et al.*, 1997).The endogenous cannabinoids interact with these receptors, preventing excessive synaptic activity (Galve-Roperh *et al.*, 2009). During the fetal stage, concentrations of 2-AG are much higher (almost 1000-fold) than those of the anandamide (Fernández-Ruiz *et al.*, 2000).

Recent studies provided interesting insights regarding the potential of cannabinoids and their receptors in the regulation of proliferation and pyramidal specification of neural progenitors.

More specifically, regarding neuronal proliferation, a recent study utilizing both selective CB1R (Arachidonyl-2'-chloroethylamide) and CB2R agonists (HU-308), as well as a non-selective CB1R and CB2R agonist (WIN55,212-2), showed that simultaneously activation of CB1R and CB2R increased the proliferation rate in early postnatal Sprague-Dawley rats (Rodrigues *et al.*, 2017). Experiments in a mouse model showed that hippocampal CB1R activation, modulates neural progenitor cell fate by favoring proliferation instead of neuronal differentiation, an effect that was diminished following the exposure to a CB1R antagonist (SR141716) (Aguado, 2006; Harkany *et al.*, 2007; Mulder *et al.*, 2008). Furthermore, Aguado et al. (2005) reported that WIN-55,212-2 enhanced neuronal progenitor cell proliferation in a CB1R-dependent way, since proliferation levels were not affected in cells lacking CB1R (Aguado *et al.*, 2005). The same study concluded that neuronal progenitor cell proliferation may be regulated through the endocannabinoid system. In addition, the endocannabinoid system may promote neuronal migration as shown by Díaz-Alonso *et al.* (2016) (Díaz-Alonso *et al.*, 2017). According to the authors, acute silencing of CB1R impaired radial migration and altered neuronal morphology in mice.

In another study, exposure of PC12 cells to exogenously added anandamide inhibited neuronal differentiation in these cells, via CB1R in a Rap1/B-Raf/ERK-dependent way (Rueda *et al.*, 2002), while the same compound also inhibited neurogenesis in adult rats (Rueda *et al.*, 2002).

CB2 receptors are mostly known by their ability to modulate neuroinflammation by diminishing inflammatory cytokines, innate immunity and the infiltration of peripheral immune cells (Turcotte *et al.*, 2016). According to previous studies, CB2R agonists may prevents HIV-associated inhibition of hippocampal neurogenesis in adult transgenic mice (Avraham *et al.*, 2014).

Cannabinoids may also affect neuronal differentiation by post-translationally regulating a key transcription factor (Pax6), as observed following exposure to HU-210 (Bromberg *et al.*, 2008). This SC led to the modulation of the PI3k/Akt pathway, thus promoting Pax6 phosphorylation in differentiating neuroblastoma cells. The observed phosphorylation resulted in the activation of kinase signaling, thus promoting neuronal outgrowth. Generally, via autocrine signaling, cannabinoids contribute to the primary growth of axons and in rat brain, the axonal navigation and the positioning can be affected by cannabinoids through phosphorylation of ERK1 and ERK2 (Berghuis *et al.*, 2007).

Therefore, the potential of cannabinoids to modulate neurodevelopment-related pathways has been demonstrated, shedding light on their importance in the fine-tuning of this particularly complex neurodevelopmental process.

3. Synthetic Cannabinoids

SCs represent an heterogeneous group of molecules chemically designed to activate cannabinoid receptors (Carroll *et al.*, 2012). They interact with CB1 and CB2 receptors and elicit effects similar to Δ^9-THC. The first synthetic compound, Δ^9-THC, was designed in 1965 by Mechoulam et al. (Mechoulam and Gaoni, 1965). Initially, SCs represented research tools to explore the endocannabinoid system and have also been used as potential therapeutics (Castaneto *et al.*, 2014). SCs have been

marketed since the early 2000s as herbal incense or “herbal smoking blends” (Shevyrin and Morzherin, 2015). Their most common brand names were Spice, K2 and most of them had the ability to escape detection by standard screening tests (Castaneto *et al.*, 2014). Herbal smoking blends, or mixtures, may not contain cannabis, but they may produce similar effects when smoked (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2017). They promote much more potent pharmacological effects than THC, such as anti-inflammatory, analgesic, weight-loss and anti-cancer growth effects (Castaneto *et al.*, 2014). Most of the times, SC users smoke the dried material, but SCs can also be sold as liquids for vaporization and inhalation (e.g. e-cigarettes), included in food (e.g. cakes) or brewed in tea (UNODC, 2011).

The classification of SCs, varies among different authors, but they may be generally classified as: a) classical cannabinoids, which are dibenzopyran derivatives (e.g. isomers of THC, HU-210), b) nonclassical cannabinoids, which are synthetic cyclohexylphenol derivatives (e.g.CP47497, CP55940), c) hybrid cannabinoids (e.g. AM-4030), d) aminoalkylindoles, which are subdivided into naphtoylindoles (e.g. JWH-122), phenylacetylindoles (e.g. JWH-250), benzoylindoles (e.g. AM-694), and naphtylmethylindoles (e.g. JWH-175), e) eicosanoids (e.g. anandamide), and f) other compounds, such as naphthoylpyrroles, diarylpyrazoles (e.g. SR144528, SR141716A) and naphthylmethylindenes (Shevyrin and Morzherin, 2015).

Although severe SC toxicity has been reported, the daily SC use still remains at a high level, resulting in withdrawal and dependence (Cooper, 2016). Adverse effects related to acute intoxication and withdrawal have been reported after daily use of SCs, and the suggested treatment was emphasized in pharmacotherapies for managing detoxification (Cooper, 2016). Crucially alarming is the risk pertaining to the unpredictable and severe nature of acute intoxication. Moreover, the effects of their long-term chronic use of SCs also need to be identified through studies on their behavioural and physiological impact (Cooper, 2016). There are also reports indicating that SCs may be significantly more addictive than marijuana due to a strong compulsion to re-dose and persistent cravings that last up to a week after the consumption (Abouchedid *et al.*, 2016). These severe effects of SCs are thought to

stem from the fact that SCs are full agonists to the cannabinoid receptors, while THC is only a partial agonist (Fantegrossi *et al.*, 2014).

3.1 Health impact of SC use

Synthetic cannabinoids represent an evolving challenge to drug policies. Up to two years ago, the substances appearing on the drug market were increasing, with about one additional SC being reported every week in Europe (EMCDDA, 2018). In the past two years, these numbers seem to have stabilized as a result in legislation changes (EMCDDA, 2019). Generally, risk assessments predict whether a new psychoactive substance is liable to similar abuse and produces similar ill effects as the most reported drugs; whether it has the capacity to produce dependence and stimulation of the central nervous system or depression, resulting in hallucinations and disturbances in motor function, mood, behavior, thinking, and perception; and if a substance has been abused so as to constitute a public health and social threat that needed to be under international control (EMCDDA, 2010).

The adverse effects of SCs include panic attacks and cardiovascular problems like hypertension and tachycardia (Znaleziona *et al.*, 2015). They may also cause red eyes, palpitations, vomiting, and nausea (Shevyrin and Morzherin, 2015; Abouchedid *et al.*, 2016). Between 2014 and 2015, the Centers for Disease Control and Prevention (CDC) recorded, a tripled number of deaths from SC use (Abouchedid *et al.*, 2016). Of note, deaths caused by SC use are usually linked to myocardial complications (Mir *et al.*, 2011). The health effects resulting from the action of synthetic cannabinoids in the human brain can be unpredictable and dangerous due to their strong binding ability to the cell receptors. Brain effects reported after SC use include an elevated mood, symptoms of psychosis (disordered or delusional thinking detached from reality) and also psychotic effects such as paranoia, hallucinations, confusion, poor coordination or extreme anxiety.

3.2 Cannabinoid use in pregnancy

With prenatal exposure to SCs being extremely high, there is an urgent need to identify the impact of the use of SCs during pregnancy, and on neonatal development (Grant *et al.*, 2018). Cannabinoids, namely THC, have the ability to cross the placenta and reach the fetus, and cannabinoid receptors have already been identified in the fetal brain and placenta (Grant *et al.*, 2018). As a result, prenatal consumption of cannabinoids could potentially have a detrimental impact on fetal development and generally on the child's brain, by causing an imbalance in neurotransmitter release (Castaldo *et al.*, 2010; Friedrich *et al.*, 2016). Also, in experiments using rats, the altered migration of interneurons in early postnatal hippocampus, induced by the prenatal stimulation of the CB1 receptor from THC, has shown to cause the deterioration of cognitive functions (Mereu *et al.*, 2003; Berghuis *et al.*, 2005). Due the complex function of the nervous system in the mature versus developing brain, long-term effects of fetus exposure to cannabinoid are more impactful than long-term implications to the mother (Pinky *et al.*, 2019).

The use of cannabis during pregnancy may lead to the disruption of developmental processes, including a risk of fetal growth restriction, adverse obstetrical outcomes, miscarriage or low birth weight (Grant *et al.*, 2018). Prenatal exposure to cannabis also seems also to be associated with adverse behavioral effects, such as deficiencies in attention, learning, memory, and executive function in the offspring (Pinky *et al.*, 2019). This can be attributed to the increased susceptibility of the neurotransmitter systems during the developmental stage(Pinky *et al.*, 2019). Additionally, the expression of genes encoding for neuron-glia cell adhesion molecules may also be affected by cannabinoids (Fernández-Ruiz *et al.*, 2004).

Pre-clinical and clinical research has concluded that the use of cannabis during pregnancy is associated to constant modifications concerning the psychological well-being and higher-level cognition (Grant *et al.*, 2018; Pinky *et al.*, 2019). The use of preclinical models is thus important to better understanding the complex mental, cognitive, and emotional disturbances associated with the use of SCs during and/or before pregnancy (Green, Gabrielsson and Fone, 2011).

3.3 Effects of SCs on neuronal development

The effects of SCs on neuronal development remain largely unknown. However, there are some studies that show that SC may modulate the pathways that take part in the stages of the developing nervous system (e.g. differentiation, proliferation, migration) (Tojima *et al.*, 2000; Jiang *et al.*, 2005). For example, Jiang et al.(Jiang *et al.*, 2005) reported that chronic treatment of cultured embryonic hippocampal neural stem cells with the synthetic cannabinoid HU-210 promoted the embryonic and adult neurogenesis, especially their proliferation and not differentiation, by activating CB1 receptors, $G_{i/o}$ proteins and the ERK pathway. Palazuelos *et al*. (Palazuelos*et al.*, 2012) reported that HU-308 was able to stimulate neurogenesis in ratHiB5 hippocampal progenitor cells, through the modulation of CB_2R and the $PI_3K/Akt/mTOR_1$ pathway, which is activated by the CB_2R. However, these studies present some limitations, including the lack of reproducibility between different studies, or the high heterogeneity of the models used, with many different cell types at different stages of differentiation (Jiang *et al.*, 2005; Jensen and Parmar, 2006).

The table below lists major studies based on the neurodevelopment alterations noticed both *in vivo* and *in vitro* following treatment with different synthetic cannabinoids (Prenderville, Kelly and Downer, 2015). Some SCs may play pivotal roles, at least in some of the phases of neurogenesis, but further research is required.

Table 1: Assessments concerning the *in vivo* and *in vitro* effects of synthetic cannabinoids in neurogenesis.

Treatment	Model	Measurement	Observation	Reference
HU-210	*In vivo* (Rats)	Cell proliferation in the dentate gyrus	Intensified	(Jiang *et al.*, 2005)
HU-210/AEA	*In vitro*	Proliferation of embryonic hippocampal neural progenitor/neural stem cells	Intensified	(Jiang *et al.*, 2005)
HU-308	*In vivo* (Mice)	Hippocampal progenitor proliferation	Intensified	(Palazuelos *et al.*, 2012)
HU-308	*In vitro*	Proliferation of HiB5 neural progenitor cells	Intensified	(Palazuelos *et al.*, 2012)
HU-308	*In vitro*	Proliferation of cortical progenitors in organotypic	Intensified	(Palazuelos *et al.*, 2012)

		cultures		
AEA / ACEA	*In vitro*	Differentiation of embryonic murine neural precursors that come from the cortex	Intensified	(Compagnucci *et al.*, 2013)
WIN55,212-2	*In vivo* (Rats)	Dorsal hippocampal neurogenesis during adolescence	Decreased	(Abboussi *et al.*, 2014)
WIN-55,212-2/URB597/ AEA/2-AG	*In vitro*	Number of $GFAP^+$ cells after differentiation of postnatal neuronal stem cells for 48 hours	Intensified	(Aguado, 2006)
AM1241	*In vitro*	Differentiation/proliferation of human neuronal stem cells in presence of Gp120	Intensified	(Avraham *et al.*, 2014)

4. Aims of the experimental work

The emergence and use of synthetic cannabinoids globally represents a critical challenge for public health. The major problem concerning these new SCs is that their toxicological profiles are not completely identified, even though a serious number of acute intoxications and deaths have already been observed following the use of these substances (Trecki, Gerona and Schwartz, 2015; Shanks and Behonick, 2016). In particular, the use of SCs by pregnant or non-pregnant women in reproductive age is also alarming.

Accordingly, the aims of this work were:

1) to assess the *in vitro* effects of 3 different, and commonly reported, synthetic cannabinoids (THJ-2201, ADB-FUBINACA, and AB-CHMINACA) on neuronal differentiation. These SCs were tested at different concentrations, in a neuroblastoma-glioma hybrid NG108-15 cell line, and parameters like total neurite length and differentiation ratio (number of neurites per total number of cells) were analyzed.

2) to evaluate if such effects could be mediated by the endocannabinoid system. For this purpose, the effects of inhibitors of CB1 receptor and inhibitors of the synthesis of endogenous cannabinoids on neuronal development were tested in cell differentiation conditions. Also, the expression of some neuronal differentiation-related proteins was analyzed, by Western-Blot.

5. Materials & Methods

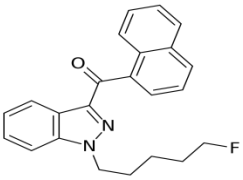
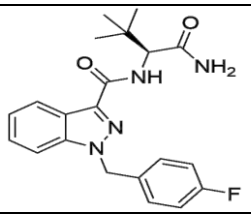
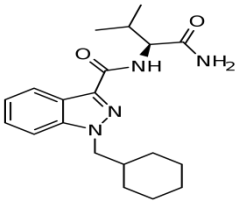
5.1 Chemicals

The synthetic cannabinoid THJ-2201 ([1-(5-Fluoropentyl)-1*H*-indazol-3-yl](1-naphthyl)methanone) is a naphtoylindole, structurally similar AM-2201, but compromising an indazole group instead of the central indole ring. ADB-FUBINACA (N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide) features a carboxamide group at the 3-indazole position, being a specific agonist of CB1 receptor. AB-CHMINACA (N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide) is also an indazole-based synthetic cannabinoid, and a potent agonist of CB1 and CB2 receptor. THJ-2201 was kindly supplied by Dr Ana Santos Carvalho (Center for Neurosciences and Cell Biology, University of Coimbra, Portugal). The other two SCs (ADB-FUBINACA and AB-CHMINACA) represent a kind gift from TicTac Communications Ltd. (UK).

The chemical structures and other information (e.g. binding affinities) concerning these SCs are represented in Table 2. SR141716A (rimonabant), an antagonist of the CB1 receptor, was acquired from Tocris Bioscience (Bristol, UK). A 10 mM stock of SR141716A was prepared in dimethylsolfoxide (DMSO). This stock was further diluted in Hank's Balanced Salt Solution (HBSS) before cell exposure to attain a final inhibitor concentration of 500 nM in a final DMSO concentration below 0.5%. This DMSO concentration was previously described as being non-toxic to NG108-15 cells. Methyl arachydonyl fluorophosphonate (MAFP) and tetrahydrolipstatin (THL) inhibit N-acylphosphatidylethanolamine-PLD (NAPE-PLD) and diacylglycerol lipase (DGL), respectively, which represent the enzymes that synthesize anandamide and 2-arachidonoylglycerol, respectively. As a result, they inhibit the endocannabinoid synthesis. MAFP was acquired from Cayman Chemicals and THL was acquired from Sigma-Aldrich (St Louis, Missouri). The final concentrations of MAFP and THL in the wells were 100µM and 1µM, respectively. Heat-inactivated fetal bovine serum (FBS), phosphate buffered saline (PBS), 0.25% trypsin/EDTA, antibiotic (10000 U/ml penicillin, 10000 µg/ml streptomycin) and Hank's balanced salt solution (HBSS) were purchased from Gibco Laboratories

(Lenexa, Kansas, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, Missouri, USA), unless stated otherwise.

Table 2: Structures, chemical names, cannabinoid class and binding affinities to CB1 and CB2 receptors of the synthetic cannabinoids tested in this study.

Name	Structure	Cannabinoid Class	Ki – CB1	Ki– CB2
THJ-2201 ([1-(5-Fluoropentyl)-1H-indazol-3-yl](1-naphthyl)methanone)		naphtoylindoles	1.34 nM	1.32 nM
ADB-FUBINACA (N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide)		1-alkyl-1*H*-indazole-3-carboxamides(Banister *et al.*, 2016)	0.36 nM	---
AB-CHMINACA (N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide)		1-alkyl-1*H*-indazole-3-carboxamides(Banister *et al.*, 2016)	0.78 nM	0.45 nM

5.2 Cell culture

The mouse neuroblastoma x rat glioma hybrid NG108-15 cell line was used as a model for all the experiments. In an undifferentiated state, these neuronal cells are usually flat and round, presenting a diameter between 10 to 100 µM. They represent a well-characterized model of neurite outgrowth and synapse formation, under stressed conditions, such as a state of limited nutrients. This cell line represents a well-characterized model to study the morphological and adhering modifications occurring during neuronal differentiation and has many advantages over primary cultured neurons since they display an homogenous cell type identity, thus they are not influenced by other cells in the culture, and they grow faster in culture medium compared with primary cultures (Campanha, Carvalho and Schlosser, 2014). These cells express several neuronal-related proteins and neurotransmitters, and also express mouse and rat CB1 receptor, but not the CB2 receptors (Tojima *et al.*, 2000)(Ho and Zhao, 1996).

The NG108-15 cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, United Kingdom). Cells were cultured in 75 cm^2 flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and a solution of 100 U/ml penicillin and 100 µg/ml streptomycin, henceforth termed Maintenance Medium (MM). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Upon reaching about 80% confluence, the cells were subcultured by trypsinization with a 0.25% trypsin/EDTA solution.

5.3 Neuronal differentiation

Differentiation of NG108-15 cells was induced, according to Chao CC *et al.* (Chao *et al.*, 2014). Cells were seeded at a density of $1.5x10^4$ cells/ml in 96-well plates and allowed to adhere overnight at 37°C (at 5% CO_2). The Maintenance Medium was replaced by Differentiation Medium (DM), which consisted of DMEM supplemented with 1 % FBS and the differentiation factors 10 µM Retinoic Acid and 30 µM forskolin. To assess the effects of SCs on neuronal differentiation, the above-mentioned SCs, were added either once (1 single addition, performed on day 0, right after replacing the maintenance medium), or three times (3 multiple additions, performed at every 24h). The cells were incubated at 37°C, 5% CO_2, and cell differentiation followed up to 72h. As negative controls, cells were maintained in DM, in the presence of no more than 0.5% DMSO.

To assess the involvement of the CB1 receptor in the SC-mediated neuronal differentiation, NG108-15 cells were incubated with 500 nM SR141716A, a specific CB1R antagonist, 20 min prior to SCs addition, according to the procedure described by Silva et al. (2018) (Silva, Carmo and Carvalho, 2018). The effect of SR141716A on cell differentiation was also considered.

Inhibition of endocannabinoid synthesis, was attained by incubating NG108-15 cells with 100 µM MAFP and 1 µM THL for 20 min, before SCs addition (Bisogno *et al.*, 2006). A control in which MAFP and THL were added to the cells in the absence of SCs was also considered.

After 72h of incubation, neurite outgrowth was imaged using bright field with the Lionheart™ FX Automated Microscope (Bio-Tek, Winooski, Vermont, USA). Total cell number and total primary neurite length were determined in the images taken using the ImageJ software (Image J 1.52a; Java 1.8.0_112, National Institutes of Health, USA). The differentiation ratio was calculated by assessing the number of newly-formed neurites per number of cells. We considered neurites as every outgrowth extending longer than 20 µM from the soma, in accordance to Campanha H et al. (Campanha, Carvalho and Schlosser, 2014).

5.4 Western-Blot

5.4.1 Total protein extraction

NG108-15 cells were seeded at a density of $1.5x10^5$ cells/ml, in 6-well plates and the differentiation protocol performed as described above (Section 5c). Treatment with specific synthetic cannabinoids was tested by adding once the cannabinoids in 1pM and 1nM concentration. After 72 h, the cell culture medium was collected into 15ml tubes placed on ice. The cells were then scrapped (with a cell scraper) in the presence of 1 ml of HBSS, and collected into the previous tubes. The cell suspensions were centrifuged at 1000 g for 5 min, 4°C, and the supernatants were discarded. The pellets were rinsed with 1 ml HBSS and the suspension was centrifuged again at 1000g for 5 additional minutes. The supernatants were again discarded and the pellets were resuspended in 100 µl of collecting buffer (20 mM HEPES/NaOH pH 7.5, 250 mM Sucrose, 10 mM KCl, 2 mM $MgCL_2$, 1 mM EDTA pH 7.5) supplemented with 2 mM dithiothreitol (DTT), 100 µM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (containing 1 ug/ml of leupeptin, chymostatin, antipain and pepstatin A). The pellets were further disrupted by sonication with three pulses of 10 seconds, intercalated with 30 seconds on ice. The amount of total protein in each sample was quantified using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction, and using Bovine Serum Albumin (BSA) as standard. The samples were stored at -80°C until used.

5.4.2 Western-Blot analysis

The expression of five proteins (Acetylcholinesterase, β3 Tubulin, PSD-95, CB1 receptor polyclonal and Synaptophysin), which are associated with the neuronal differentiation, was determined by Western-Blot analysis on the total protein extracts (obtained in Section 5.d.i). The samples were denatured at 90°C for 5 min, following their dilution in 4x SDS Sample buffer (1:3 v/v). The sample loading buffer consisted of 0.25 M Tris-HCl, 10 % SDS, 50 % glycerol, 0.2 M DTT, and 0.001% Bromophenol Blue.

The samples, containing 50µg of protein, were then separated in 7.5% polyacrylamide gels by SDS/PAGE electrophoresis and transferred into polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pennsylvania, USA). Membranes were blocked in 5% skim milk for 2 h at room temperature, washed three times with TPBS (0.05% Tween 20 in phosphate buffered saline, pH 7.4), and incubated overnight at 4°C, with each of the following primary antibodies: mouse anti-AChE (A11) (1:250, Santa Cruz Biotechnology, California, USA), mouse anti-β3 Tubulin (2G10) (1:250, Santa Cruz Biotechnology, CA, USA), mouse anti-PSD-95 (6G6) (1:250, Santa Cruz Biotechnology, CA, USA), mouse anti-SYP (4H255) (1:250, Santa Cruz Biotechnology, CA, USA) and rabbit anti-CB1 Receptor Polyclonal (1:200, Cayman Chemicals,Michigan, USA). To normalize the results per the amount of protein loaded, after stripping the membranes, blots were also probed for mouse anti-β-actin (1:5000, Sigma-Aldrich, St Louis, Missouri, USA). All the primary antibodies were diluted in 1% BSA prepared in TPBS, and 0.05% NaN_3. After the overnight incubation with the primary antibodies, the membranes were washed three times in TPBS and further incubated with Horseradish Peroxidase-linked anti-mouse lgG (1:10000, GE Healthcare, USA), or in the case of the CB1 Receptor Polyclonal Antibody, anti-rabbit lgG (1:2000, GE Healthcare, Pennsylvania, USA). The secondary antibodies were diluted in 1% BSA prepared in TPBS. After three washes in TPBS, immune reactive bands were detected by labeling the blots with the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) for 5min. The bands were visualized using a molecular imager Chemi-Doc XRS system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using Image Lab 6.0 (Bio-Rad, Berkeley, CA,

USA). All band intensities were normalized against the β-actin control. Results from SC treatments were expressed as fold-change relatively to the vehicle control.

5.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, California, USA). To determinate the normality of the distributions prior to each analysis, Dunnett's multiple comparisons test, and Sidak's multiple comparison test were used, and the acceptability of skewness and kurtosis values were also taken into account. Based on the normality tests results, One-way ANOVA, followed by a Dunnett's comparison post-test, was performed. The number of independent experiments, as well as the number of replicates performed per experiment, if any, are detailed in the figure legends. A value of $p < 0.05$ was considered to represent a statistically significant difference.

6. Results

6.1 Modulation of Neuronal Differentiation by Synthetic Cannabinoids

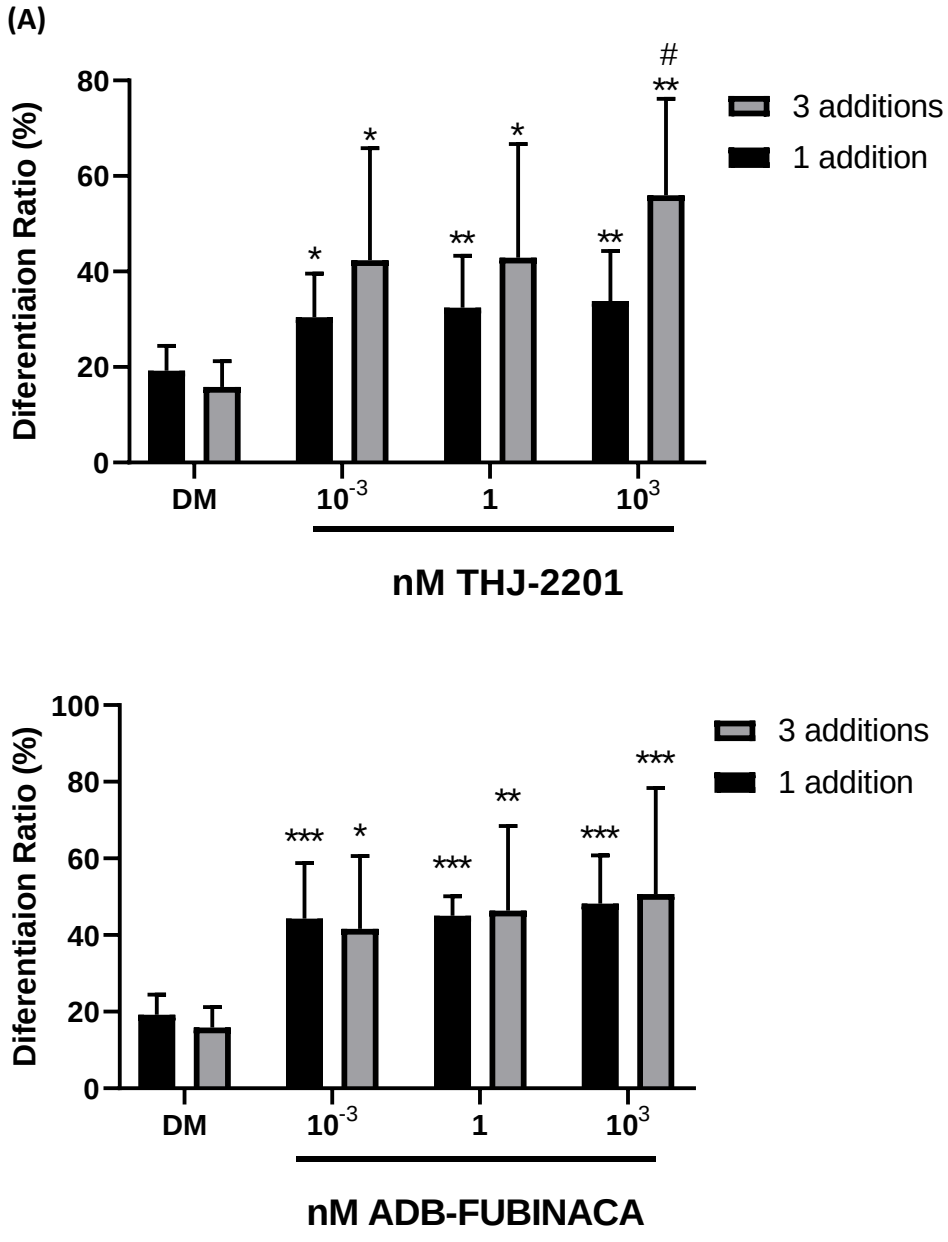
Neuronal cell differentiation was assessed in NG108-15 cells by measuring the differentiation ratios and the total primary neurite length of cells exposed to the different SCs (THJ-2201, ADB-FUBINACA, AB-CHMINACA). As observed in the Figure 1(a), in the control cells (DM only), the differentiation process was triggered and a complex system of neurite outgrowth was developed following the administration of differentiation factors.

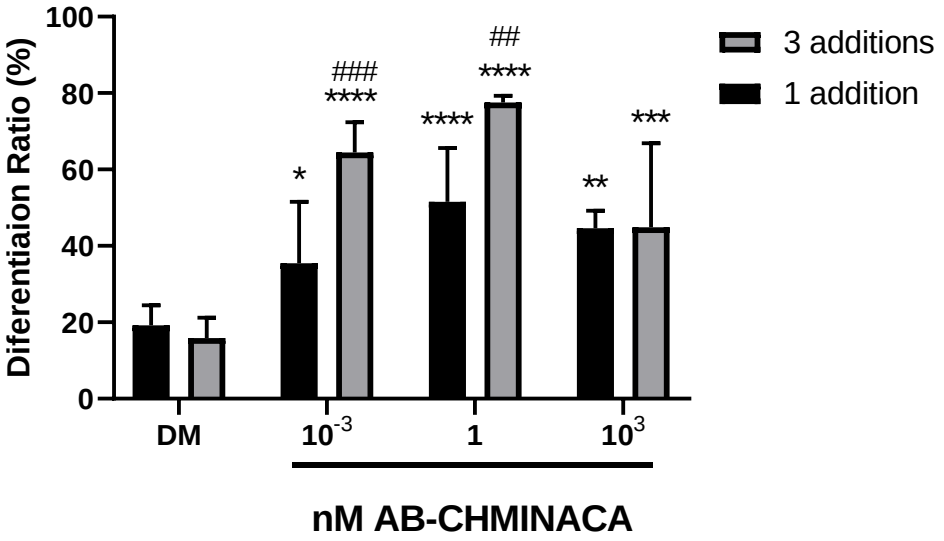
The same figure shows that neuronal differentiation ratios increased (between 1.5 and 3.5-fold) following both a single and multiple additions of the THJ-2201, compared to the Differentiation Medium alone. This increase was more accentuated at the highest concentration 1μM of THJ tested. A significant difference of 1.6-fold in the differentiation ratio was also noticed between the two types of treatments (1 and 3 additions), with the latter displaying a higher ratio. The total neurite length was more extensive following a single addition (compared with multiple additions) at 1 nM and 1 pM THJ-2201, as observed in the Figure 1(a).

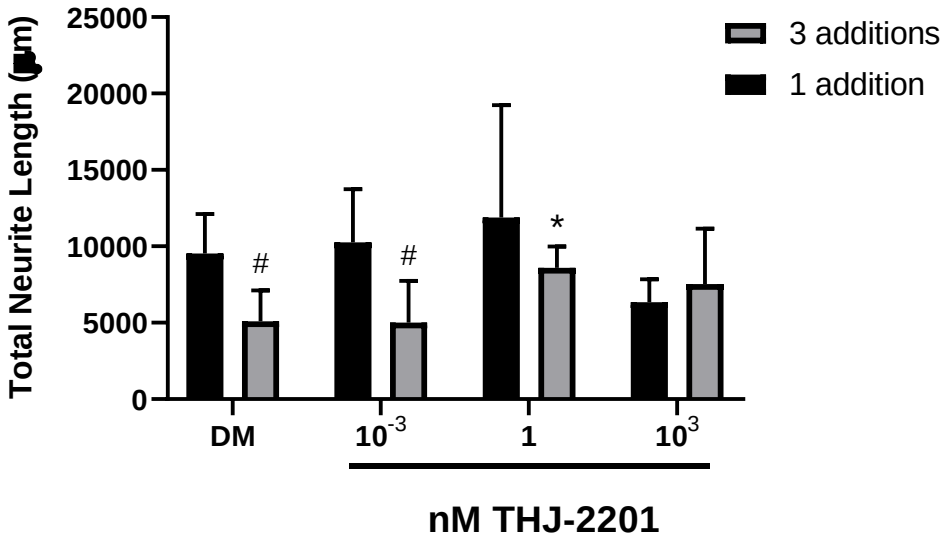
Figure 1(a) also displays a specific trend on differentiation ratios for the cells treated with the cannabinoid ADB-FUBINACA. In particular, increased neurite outgrowth (about 2.6- to 3.1-fold) was observed in all the concentrations tested with 1 and 3 additions, compared to the vehicle-treated controls. Regarding the total neurite length, no significant difference was observed between controls and the cannabinoid-treated cells, with the exception of 3 additions of 1pM, in which a 1.9-fold increase was evident.

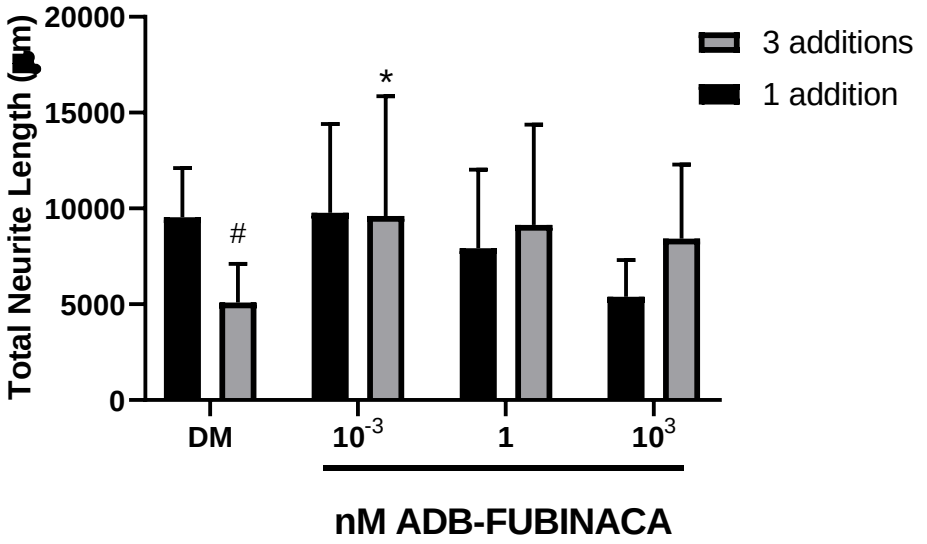
Moreover, after exposure of the cells to AB-CHMINACA (either following a single or multiple additions), significant 1.8-fold to 4.8-fold increases were noticed in the differentiation ratios at all the tested concentrations (Figure 1a). This increase confirmed that the synthetic cannabinoid AB-CHMINACA promoted NG108-15 cell differentiation. Also, a rise in differentiation ratio was observed in the multiple additions condition, compared to a single addition, at 1 pM and 1 nM. As also

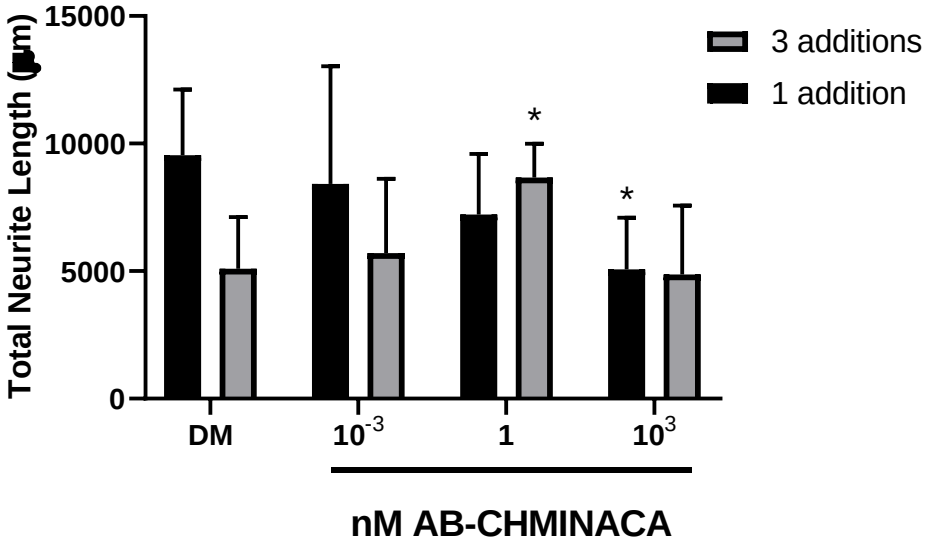
displayed in the representative images of Figure 1(b) and further represented in the graphical representations (Figure 1(a)), the total neurite length in the cells concerning 1 addition experiments were more complex and developed (there are more and larger neurites) than in the cells following 3 additions. In the other concentrations tested, the total primary neurite length was not affected by the exposure to AB-CHMINACA, except at 1 nM multiple additions and 1 μM at single addition, in which a 1.7-fold increase and a 1.9-fold decrease were noticed, respectively.



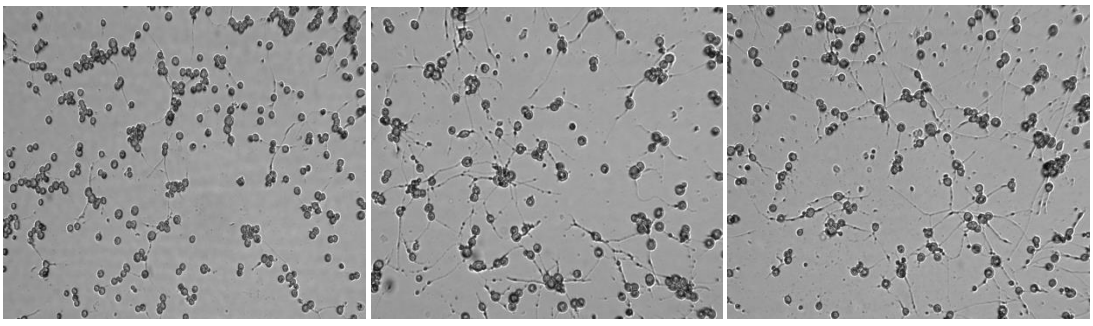

Diferentiaion Ratio (%)
100
80
60
40
20
0
DM
10-3
1
103
nM AB-CHMINACA
3 additions
1 addition
*
###
****
****
##
****
**
***

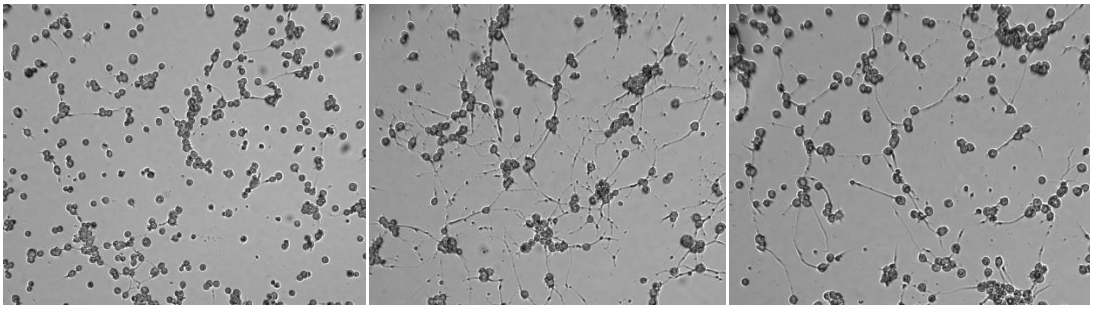

Total Neurite Length (µm)
25000
20000
15000
10000
5000
0
DM
10-3
1
103
nM THJ-2201
3 additions
1 addition
#
#
*


Total Neurite Length (μm)
20000
15000
10000
5000
0
3 additions
1 addition
*
#
DM
10-3
1
103
nM ADB-FUBINACA

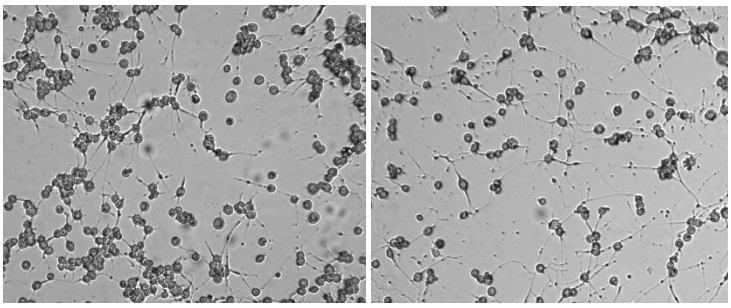

Total Neurite Length (μm)
15000
10000
5000
0
3 additions
1 addition
*
*
DM
10-3
1
103
nM AB-CHMINACA

(B)


DM (1 addition)
THJ-2201 (1 addition)
ADB-FUBINACA (1 addition)



DM (3 additions) **THJ-2201 (3 additions)** **ADB-FUBINACA (3 additions)**



AB-CHMINACA (1 addition) **AB-CHMINACA (3 additions)**

Figure 1:Effects of THJ-2201, ADB-FUBINACA and AB-CHMINACA in different dose treatments on neuronal differentiation.

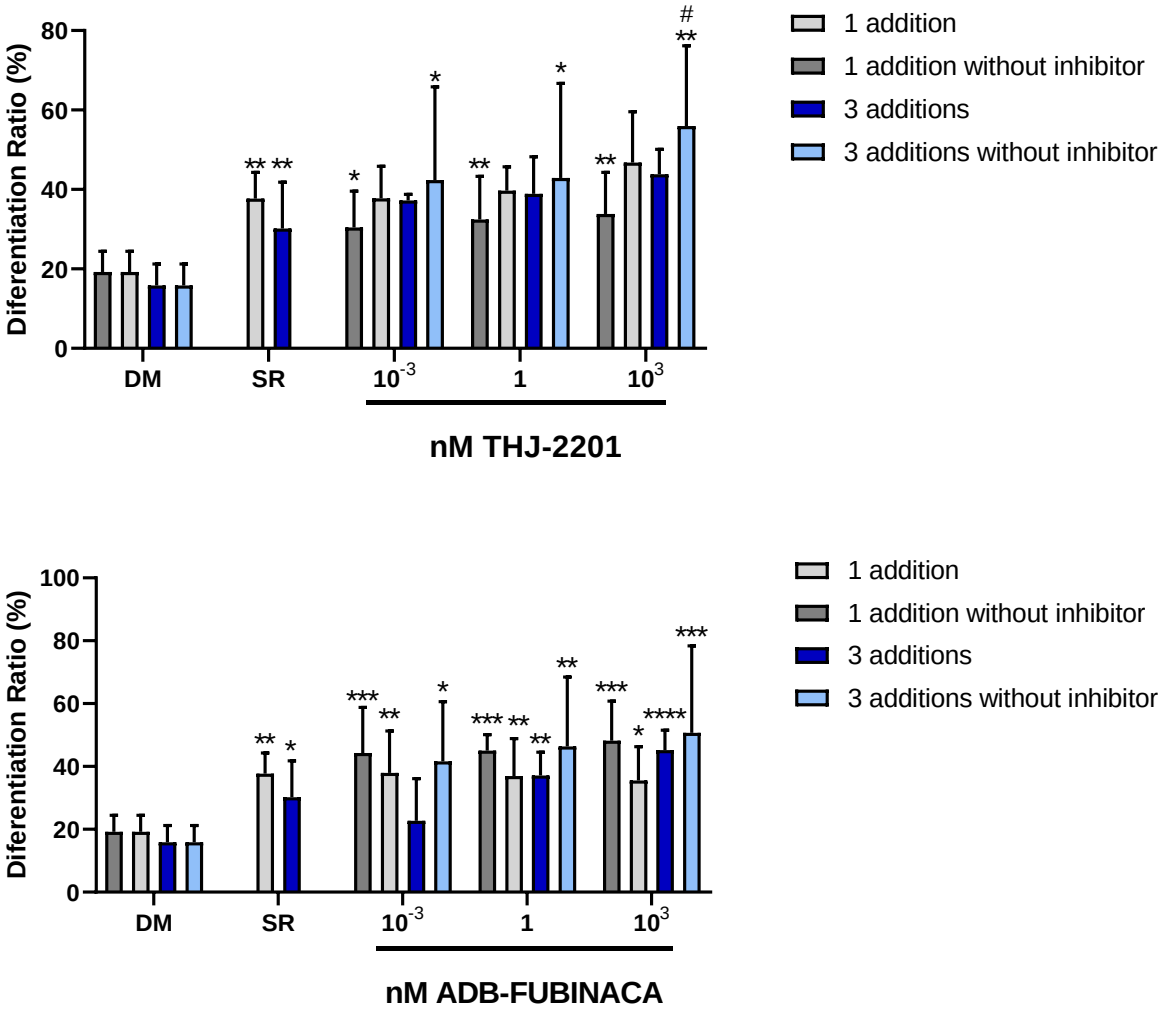
*(A) Graphical representations of the differentiation ratio and total neurite length of NG108-15 cells, which were incubated with increasing THJ-2201, ADB-FUBINACA and AB-CHMINACA concentrations ranging from 1pM to 1μM. Neuronal differentiation was induced as previously described, and significant changes in neurite outgrowth were observed. In all the experiments, the synthetic cannabinoids were added once, or three times. Each bar represents the mean ± S.E.M. for four independent experiments (n=4), performed in duplicate. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 compared to the control (DM). # p≤ 0.05, ## p ≤0.01, ### p ≤0.001 compared to a single addition. (B) Representative images of NG-108-15 cells treated with THJ-2201 (1 and 3 additions), ADB-FUBINACA (1 and 3 additions), AB-CHMINACA (1 and 3 additions).*

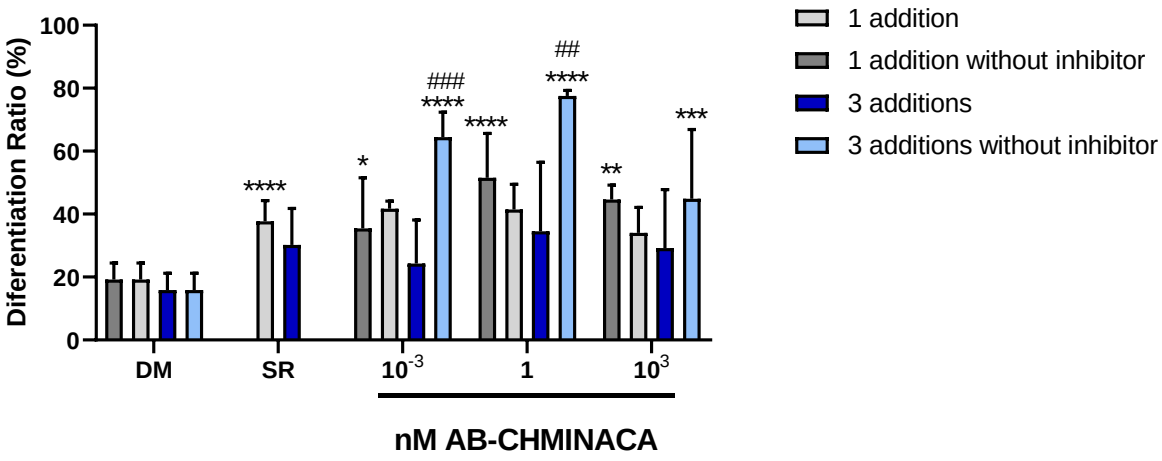
6.2 Assessment of the Involvement of the CB1-receptor in SC-mediated Neuronal Differentiation

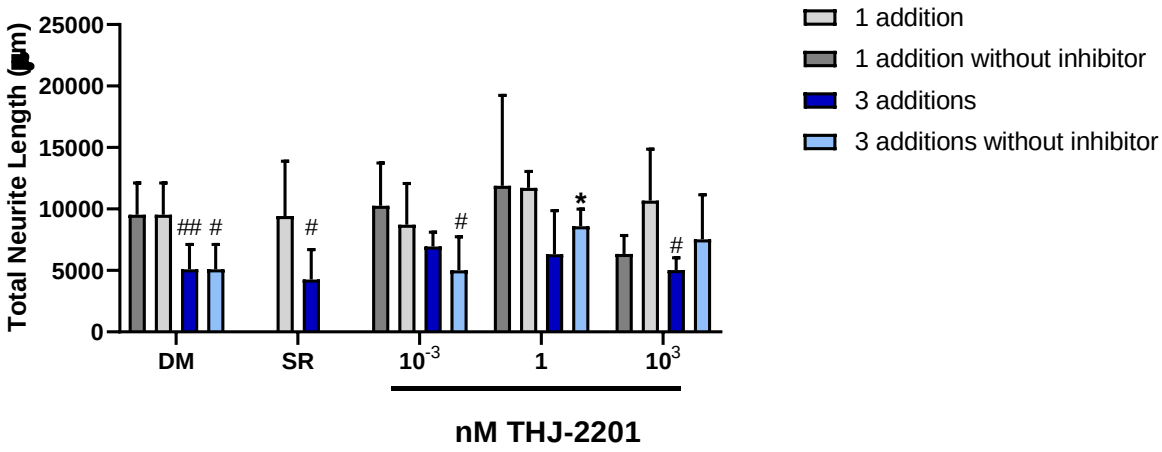
The role of CB1 receptor in the enhanced differentiation mediated by the tested SCs was assessed by incubating the cells with a CB1R inverse agonist (SR141716A), 20 minutes prior to SCs addition. Results in Figure 2(a) show that a significant increase of the differentiation ratio is occured in cells treated with SR1417167A alone, compared to the controls (Figure 2a). Noteworthly, this increase was similar to the ones observed in cells treated with the SCs alone. The presence of the CB1R inhibitor also did not affect the differentiation ratios induced by the SCs. However,

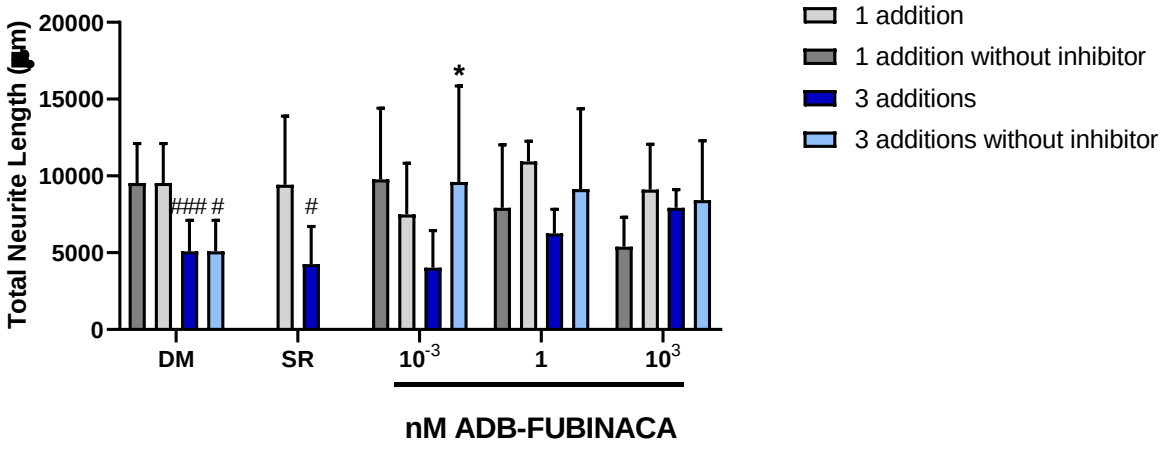
total primary neurite length was not affected by the presence of SR141716A, compared to the respective control. No other statistical significant results were noticed in the length neurite network of cells treated the SCs in the presence of SR141716A.

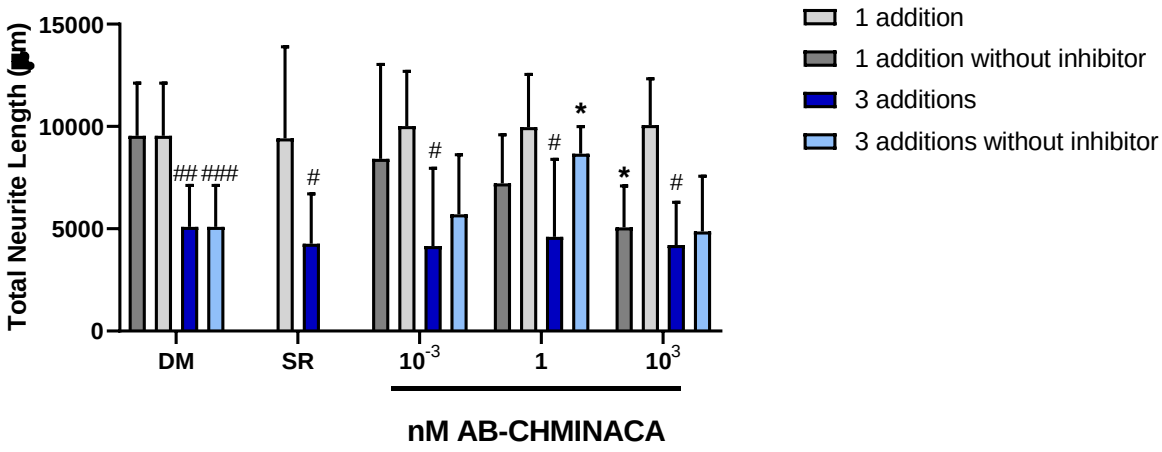
(A)




Diferentiation Ratio (%)
100
80
60
40
20
0
DM
SR
10-3
1
103
nM AB-CHMINACA
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor


Total Neurite Length (µm)
25000
20000
15000
10000
5000
0
DM
SR
10-3
1
103
nM THJ-2201
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor


Total Neurite Length (µm)
20000
15000
10000
5000
0
DM
SR
10-3
1
103
nM ADB-FUBINACA
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor



(B)

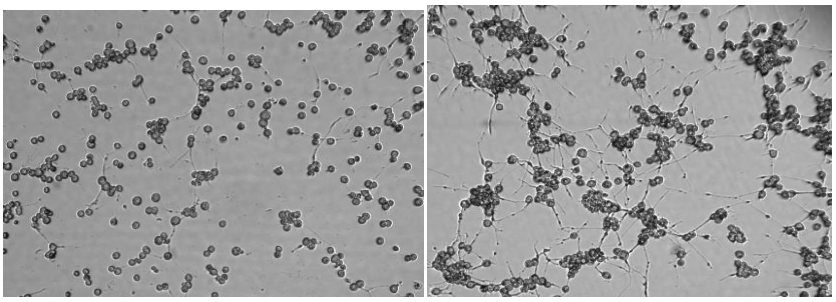


Figure 2: Assessment of CB1R involvement in neuronal differentiation in the presence and absence of SCs.

*(A) Graphical representations of the differentiation ratio and total neurite length of NG108-15 cells, which were incubated with increasing THJ-2201, ADB-FUBINACA, AB-CHMINACA concentrations ranging from 1pM to 1µM. To ascertain a possible effect in the endocannabinoid synthesis, NG108-15 cells were also incubated with 500nM SR141716A (SR), which inhibits CB1 receptor, 20 min prior to THJ addition. In all the experiments, the synthetic cannabinoids were added once, or three times. Each bar represents the mean ± S.E.M. for four independent experiments (n=4), performed in duplicate. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001, compared to the control (DM). # p≤ 0.05, ## p ≤0.01, ### p ≤0.001 compared to a single addition. (B) Representative images of control NG108-15 cells and cells treated in the presence of SR141716A alone.*

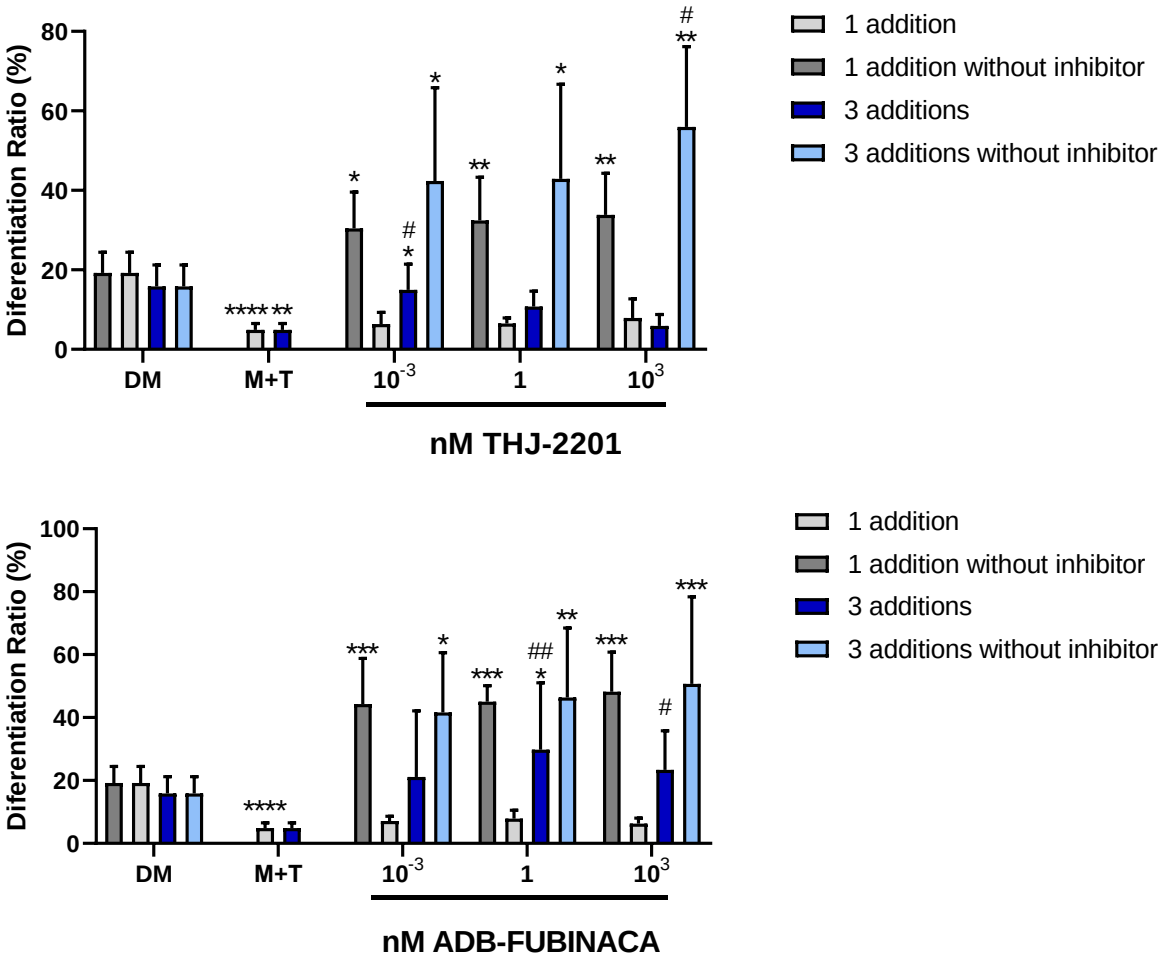
6.3 Role of the Endocannabinoid System in SC-mediated Neuronal Differentiation

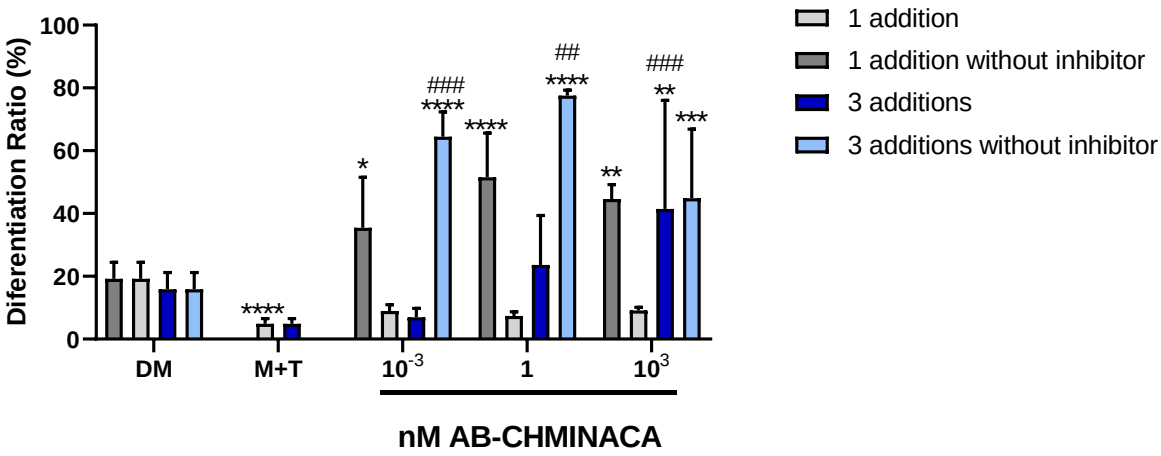
The potential involvement of the endocannabinoids was tested by adding methyl arachydonyl fluorophosphonate (MAFP) and tetrahydrolipstatin (THL), 20 and 15 min, respectively, prior to the SCs addition. As displayed in the representative images of Figure 3(b), all the cells treated with these inhibitors alone present an

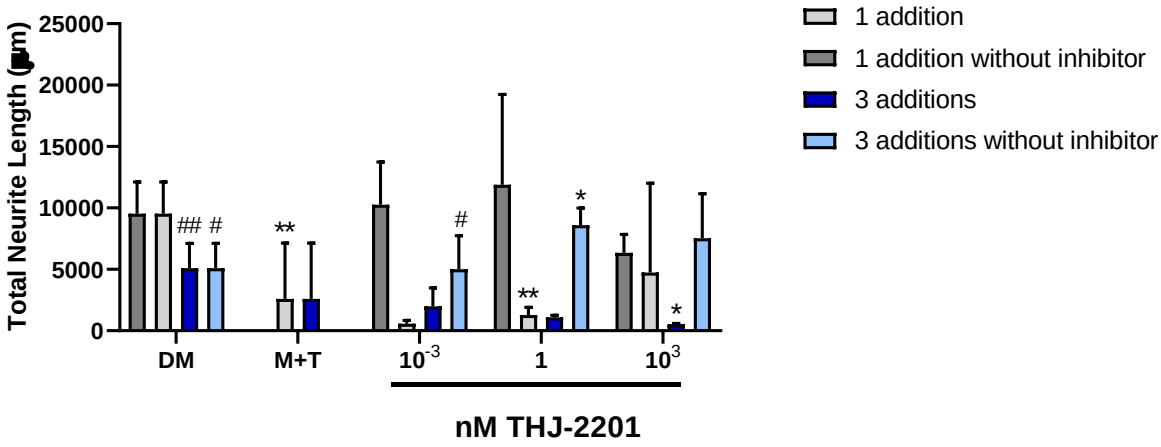
abnormal morphology (i.e. they were round-shaped, seemed smaller and showed few or no neurites) compared to the controls without the inhibitors MAFP and THL and for all the concentrations of the cannabinoid tested, compared to the control without inhibitors.

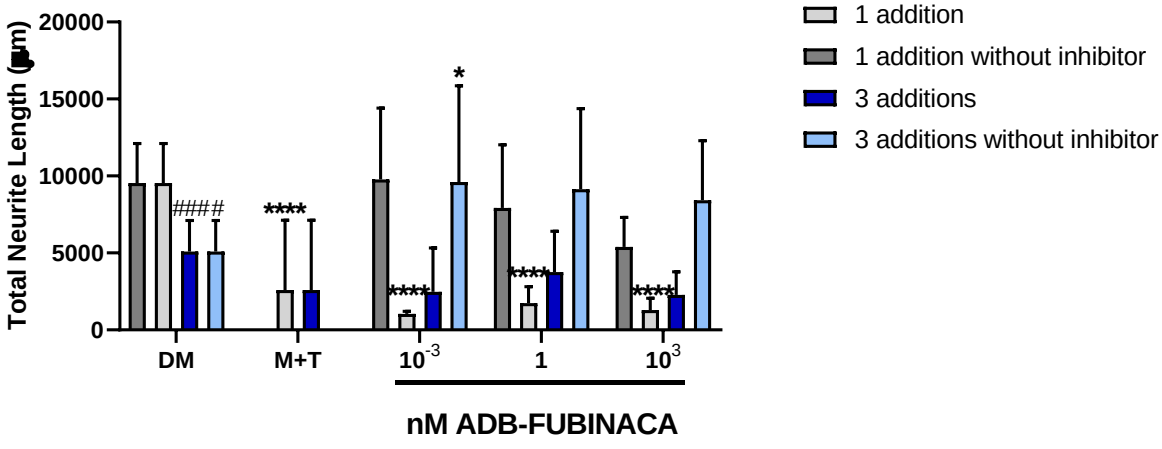
The results depicted in the Figure 3(a) show that there is a more than three-fold decrease in differentiation ratios of cells treated with MAFP and THL alone, compared to the cells without inhibitors. Cell treatment with MAFP and THL also resulted in a decreased total primary neurite length. However, this effect seemed to be partly reversed by multiple additions of 1µM THJ-2201.

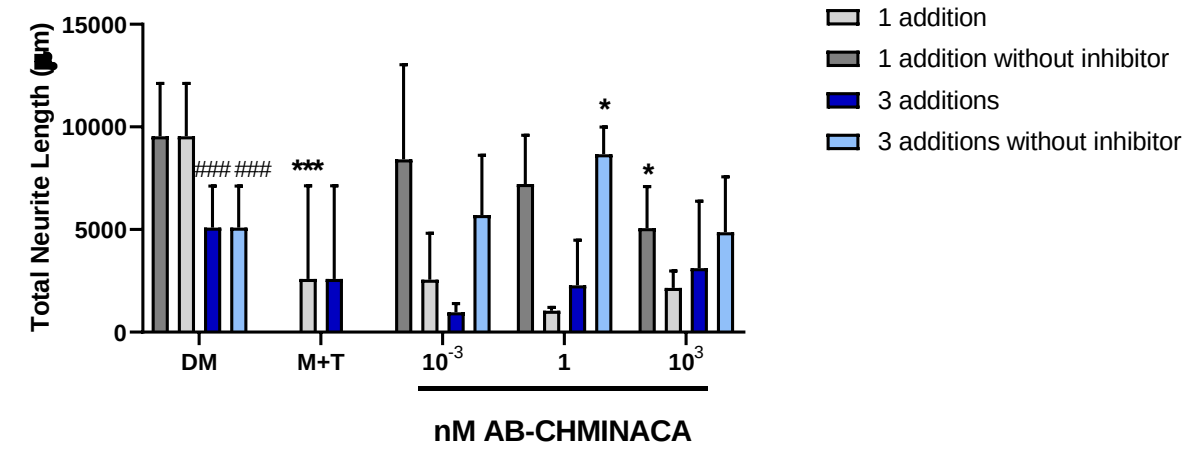
(A)



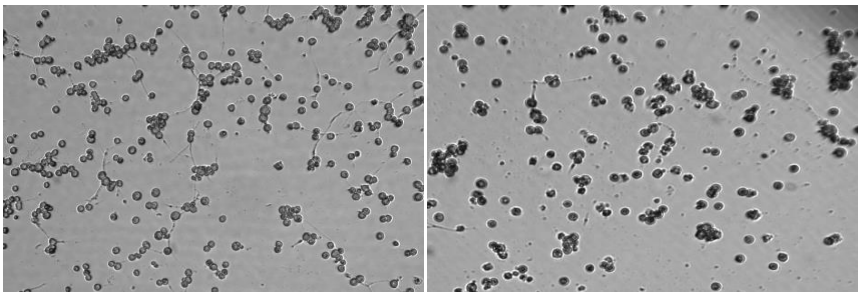

Diferentiation Ratio (%)
100
80
60
40
20
0
DM
M+T
10-3
1
103
nM AB-CHMINACA
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor


Total Neurite Length (µm)
25000
20000
15000
10000
5000
0
DM
M+T
10-3
1
103
nM THJ-2201
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor


Total Neurite Length (µm)
20000
15000
10000
5000
0
DM
M+T
10-3
1
103
nM ADB-FUBINACA
1 addition
1 addition without inhibitor
3 additions
3 additions without inhibitor



(B)



DM (1 addition) **M+T (1 addition)**

Figure 3: Assessment of the involvement of endocannabinoids on neuronal differentiation. *(A)Graphical representations of the differentiation ratio and total neurite length of NG108-15 cells, which were incubated with increasing THJ-2201, ADB-FUBINACA, AB-CHMINACA concentrations ranging from 1pM to 1µM. To ascertain a possible effect in the endocannabinoid synthesis, NG108-15 cells were also incubated with 100µM MAFP and 1µM THL (M+T), which inhibit the endocannabinoid synthesis, for 20 and 15 min, respectively, prior to THJ addition. In all the experiments, the synthetic cannabinoids were added once, or three times. Each bar represents the mean ± S.E.M. for four independent experiments (n=4), performed in duplicate. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001, compared to the control (DM). # p≤ 0.05, ## p ≤0.01, ### p ≤0.001 compared one and three additions. (B) Representative images of control NG108-15 cells and cells treated in the presence of M+T alone.*

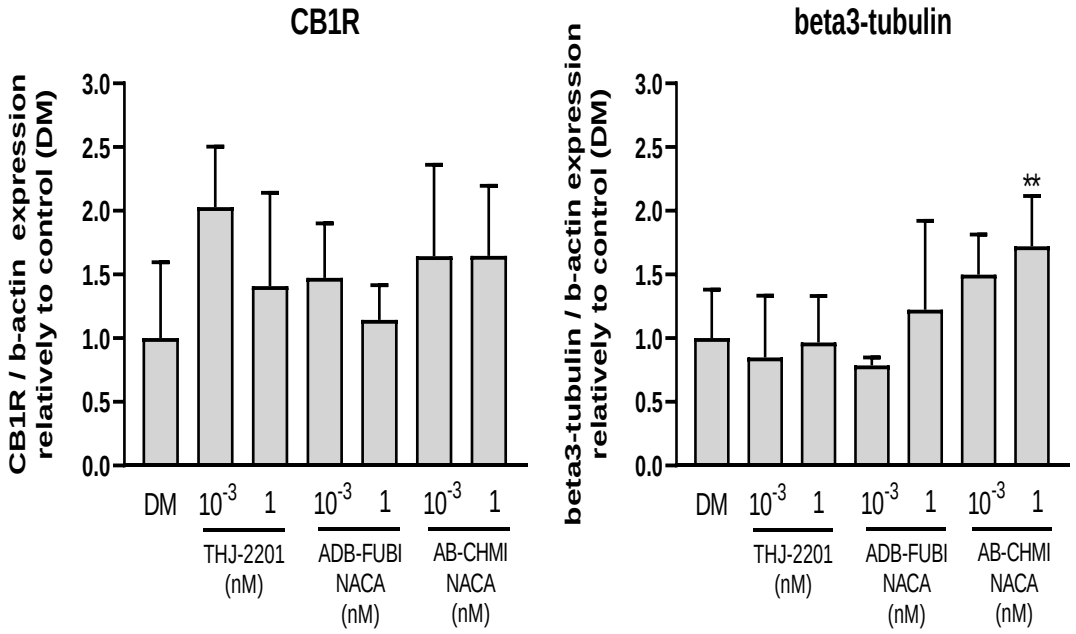
6.4 Analysis of the Expression of Proteins Involved in Neuronal Differentiation

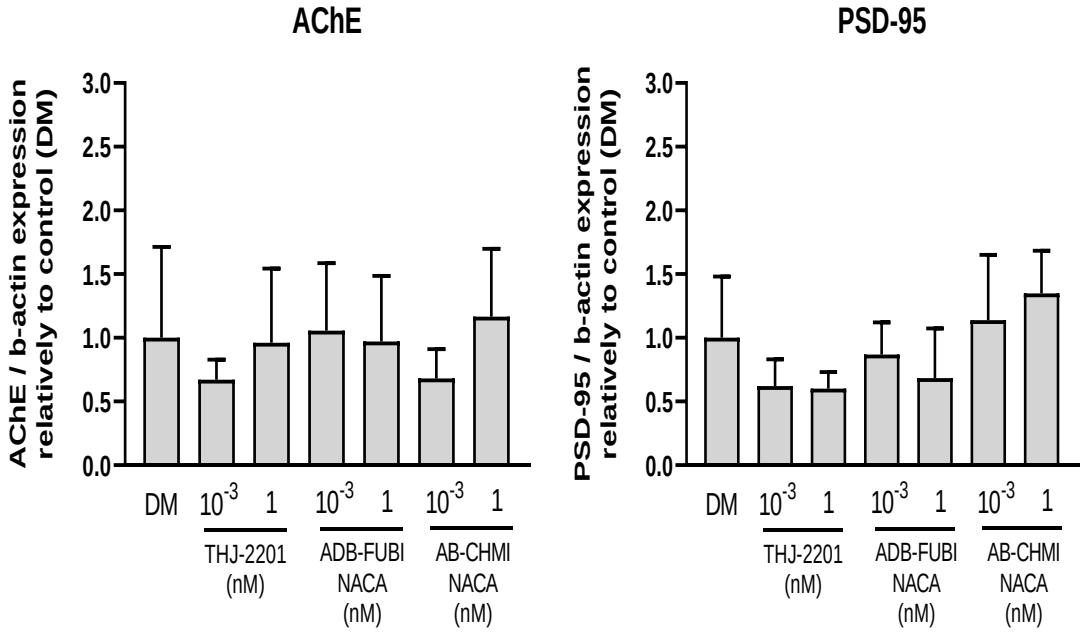
The expression of some neuronal markers was assessed by Western Blot in differentiating NG108-15 cells, in the presence of each of the three SCs, at two different concentrations, 1 pM and 1 nM. Four proteins associated with neuronal differentiation and representative of distinct differentiation stages and processes were tested.

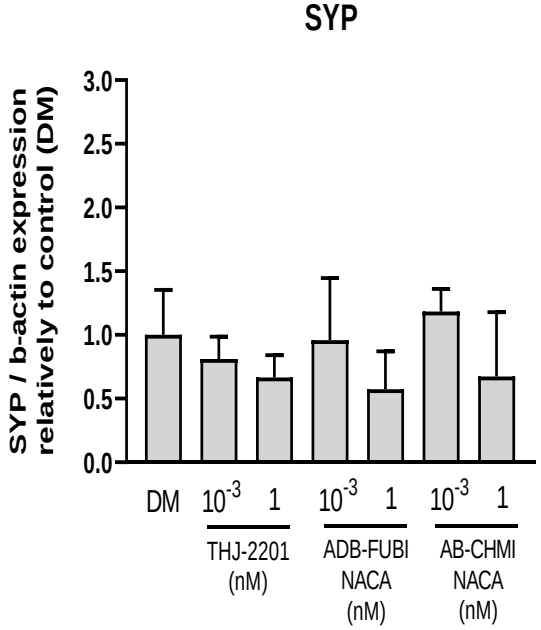
The results depicted in the Figure 4 show that the levels of the proteins CB1R, AChE, PSD-95 and SYP did not significantly change following treatment with the synthetic cannabinoids, relatively to the vehicle-treated controls. These results suggest that SCs are not involved in the processes in which these proteins participate.

Interestingly, the results depicted in the Figure 4, evidence the increased β3-tubulin expression (about 1.7-fold increase) relatively to the control (vehicle-treated DM) following a single addition of 1 nM AB-CHMINACA.

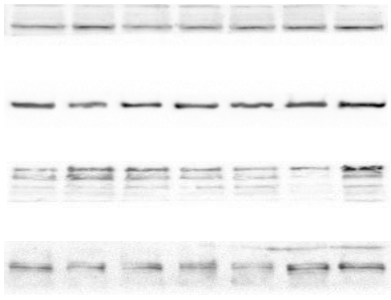
(A)







(B)



60 KDa **CB1 Receptor Polyclonal Antibody**

50 KDa **β3-tubulin**

68/71 KDa **Acetylcholinesterase**

95 KDa **PSD-95**

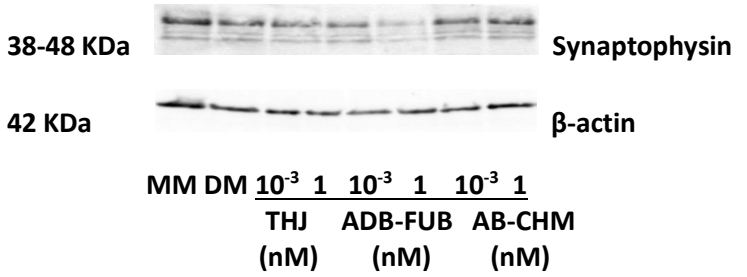


Figure 4: Expression of proteins involved in neuronal differentiation and SC treatment.
*(A)Graphical representations of band intensities, expressed as the percentage of protein expression relatively to the control. These proteins are present in cholinergic, immature, or mature neurons, and their expression was assessed by Western-Blot. NG108-15 cells were incubated with 1pM and 1nM of each synthetic cannabinoid for 72h, according to the procedure described in Materials and Methods. SYP was identified as bands between 38-48KDa, β3-tubulin and CB1 receptor polyclonal antibody as bands of 50 and 60KDa, respectively. AChE was identified as bands of 68 and 71KDa, and PSD-95 as band of 60KDa. Their expression was normalized by the amount of β-actin in each lane. Each bar represents the mean ± S.E.M. for at least four independent experiments (n=4). ** p ≤ 0.01, compared to the control (DM). (B) Representative protein bands following cell treatment with a single addition of THJ-2201 (1pM, 1nM), ADB-FUCINACA (1pM, 1nM), AB-CHMINACA (1pM, 1nM).*

7. Discussion

According to the latest EMCDDA report (EMCDDA, 2019), the health-related problems, intoxications and deaths associated with SCs consumption are increasing and, their impact on public health is elevated. The toxicological profiles of many of these new psychoactive substances are still unidentified. However, there have been reports implying the disturbance of neuronal development by SCs. These problems appear to be more profound in adolescent and generally younger populations, since the central nervous system is not fully developed. Additionally, reproductive-aged women (either pregnant or not) consist another high risk due to the hazardous and adverse potential of SCs to be transferred and accumulated in the offspring.

In this experimental work, *in vitro* neurotoxicological evaluation of three commonly reported SCs (THJ-2201, ADB-FUBINACA and AB-CHMINACA) was assessed, especially concerning their potential involvement during neuronal differentiation in NG108-15 cells. Also, the potential involvement of the endocannabinoid system in the effects caused by the SCs was tested.

Generally, from the early stages of neuronal development, the endocannabinoid system seems to be well established and the cannabinoids are able to modulate various stages of neurodevelopment (Biegon and Kerman, 2001). According to our neurodifferentiation experiments, the differentiation ratios were similarly increased for all the SCs tested, compared to the controls without SCs, clearly indicating a modulatory effect of SCs on neuronal differentiation of NG108-15 cells upon exposure to 1pM, 1nM and 1μM of THJ-2201, ADB-FUBINACA and AB-CHMINACA. Even a single dose of each SC was able to significantly increase neurite outgrowth, although the administration of three doses (every 24h) exhibited similar effects. Also, Alexandre et al (Alexandre *et al.*, 2018) observed a 2-fold increased differentiation of NG108-15 cells following a single addition of 1 pM THJ-2201. It is worth noting that at 1pM and 1nM of AB-CHMINACA, differentiation ratios in the multiple additions condition were higher, compared to a single addition, indicating a dose-dependent modulation of neuronal differentiation by this SC. The total primary neurite length was higher following three additions of 1nM THJ-2201, multiple

additions of 1pM ADB-FUBINACA, single or multiple additions of 1µM and 1nM AB-CHMINACA, respectively. No significant differences were observed in any of the other conditions.

Previous studies show that some SCs are able to modulate neuronal differentiation, and some of them do not have the ability to play a role in this process, due to the different mechanisms that SCs may trigger as reviewed in (Alexandre *et al.*, 2019).

To ascertain if the effects on neuronal differentiation induced by the SCs could be mediated by the endocannabinoid system, we also assessed the levels of cell differentiation in the presence of an inhibitor of CB1 receptor (SR141716A) or following exposure to inhibitors of the endocannabinoid synthesis (MAFP and THL). Rimonabant (SR141716A) inhibits the thioesterase domain of an enzyme involved in the proliferation of cancer cells, but not normal cells (FAS, fatty acid synthase). MAFP and THL inhibit N-acylphosphatidylethanolamine-PLD (NAPE-PLD) and diacylglycerol lipase (DGL), respectively, which partake in the synthesis of AEA and 2-AG.

The presence of the inhibitor SR141716A alone significantly modified the differentiation ratios in both conditions (single and multiple conditions) tested of THJ-2201, ADB-FUBINACA, and AB-CHMINACA, compared to the vehicle-treated cells. In particular, there is an increase of the differentiation ratio in the presence of the inhibitor alone, which may be explained by the suppression of a profitable effect of ECs, by THJ-2201, ABD-FUBINACA, AB-CHMINACA and CBR antagonists. Calvey and Williams (Calvey and Williams, 2008) mentioned that in the presence of a full agonist, a partial agonist compete for the occupancy of the receptor and decrease its net activation, which means that it may act as a full agonist. As a result, endocannabinoids which are partial agonists of cannabinoid receptors may be suppressed or act as antagonists upon binding of a full agonist SC to CBRs. Also, regulation of ADB-FUBINACA-mediated effects on neuronal differentiation appeared to be dependent on the activation of CB1R, as the inhibition of CB1R increased the neurite outgrowth of ADB-FUBINACA treated cells in both single and multiple conditions, compared to the controls. For all the SCs tested, co-incubation with SR141716A did not induce any significant differences in the total primary neurite

length. Interestingly, Jian et al. (Jiang *et al.*, 2005) described that treatment of cultured embryonic hippocampal neural stem cells with the SC HU-210 promoted only cell proliferation in the dentate gyrus of rats, and not differentiation, by activating the CB1 receptor.

Cells treated with MAFP and THL produced a significantly reduced neurite network compared to the DMs without inhibitors, evidenced by the lower differentiation ratios in the cells treated with these inhibitors, compared to the vehicle-treated cells. Indeed, in the presence of these inhibitors, the cells displayed abnormal morphology, being round-shaped, smaller, with fewer or no neurites, indicating a severe negative impact of these inhibitors in the differentiation process. Similarly, the total primary neurite length was diminished compared to the control cells. Taken together these observations indicate a potential modulation of the neuronal differentiation by the endocannabinoids. Nevertheless, the specific mechanisms involved require further clarification. Also, using *in vitro* models, Forlenza et al. (Forlenza *et al.*, 2007) observed that inhibition, with MAFP, of phospholipase A2, an enzyme involved in developmental processes in the early stages of the nervous system, reduced the neurite outgrowth, as well as the neuronal viability. Moreover, Schaeffer et al. (Schaeffer and Gattaz, 2015) also reported that the *in vivo* chronic inhibition of phospholipase A2 (also by using MAFP) in the hippocampus of adult rats, decreased the newborn and mature neurons in the ventral dental gyrus and ultimately impaired the neuronal survival and maturation.

In addition, the expression of different neuronal markers involved in neuronal differentiation were examined. In particular, we evaluated the expression of different neuronal differentiation markers, including: Acetylcholinesterase (AChE), which is a 68/71KDa protein, present in cholinergic neurons and it is responsible for the termination of the impulse transmission at cholinergic synapses through hydrolysis of acetylcholine and it was previously shown that it enhances neurite outgrowth (Tojima *et al.*, 2000); PSD-95 which is a rat synaptic protein, with a molecular weight of 95KDa, that plays a crucial role in intracellular signal transduction and in regulation of growth; Synaptophysin (SYP) which is a N-glycosylated integral membrane protein detected between 38-48 KDa, that it is

present in mature neurons and endocrine cells, it is involved in synaptic vesicle exocytosis in differentiated cells (Tojima *et al.*, 2000) and it is a marker for presynaptic terminals; CB1 receptor which binds Δ^9-tetrahydrocannabinol; and β3-tubulin, which is a 50KDa cytoskeleton protein which is present in immature differentiating neurons of mammalian tissues (Cao *et al.*, 2017).

We observed that the effects of 1 nM AB-CHMINACA on neuronal differentiation process led to an overexpression of β3-tubulin. β3-tubulin was highly expressed in cells exposed to differentiation medium compared to the control (maintenance medium), as expected, considering its localization in neurons of brain tissue and its involvement in neurodevelopmental processes. This result is in line with previous published data, which showed that β3-tubulin's expression correlates with the earliest phases of neuronal differentiation from multi potent progenitors, having been used as an early biomarker of positive neuronal identity in many research studies (Glaser *et al.*, 2014). In this sense, it further supports the evidence that AB-CHMINACA enhances neuronal differentiation.

Interestingly, there were no significant fluctuations in the other neuronal markers, following incubation of the differentiating cells with the three SCs at the concentrations tested. Thus, further research is required to clarify the mechanisms involved in the regulation of neuronal differentiation by the SCs.

This study presents some interesting data concerning the role of specific SCs during neuronal differentiation process. Further research is required to clarify the potential mechanisms and pathways that are involved in the function of SCs, since different additions of these SCs achieved almost similar results, ruling out a dose-dependent effect, and also considering that the expression of common neuronal differentiation biomarkers was not affected. The study concerning the modulation of the neuronal development by SCs is at an early stage, and it could be interesting to further evaluate if SCs are able to interact with the endocannabinoid metabolism. Nevertheless, the data presented in this work already provides evidence that the endocannabinoids may play an important role during neuronal differentiation.

As mentioned before, MAFP and THL partake in the synthesis of AEA and 2-AG. Based on our results, in the future, it would be interesting to assess whether the effects of MAFP and THL on cellular morphology and neurite outgrowth could be reversed upon administration of the endocannabinoidanandamide (AEA) and/or 2-AG. If the normal cell phenotype was rescued, this would confirm the role for endocannabinoids in neuronal development.

8. Bibliography - References

1. Abboussi, O. *et al.* (2014) 'Chronic exposure to WIN55,212-2 affects more potently spatial learning and memory in adolescents than in adult rats via a negative action on dorsal hippocampal neurogenesis', *Pharmacology Biochemistry and Behavior*. doi: 10.1016/j.pbb.2014.02.014.
2. Abouchedid, R. *et al.* (2016) 'Acute Toxicity Associated with Use of 5F-Derivations of Synthetic Cannabinoid Receptor Agonists with Analytical Confirmation', *Journal of Medical Toxicology*. doi: 10.1007/s13181-016-0571-7.
3. Aguado, T. *et al.* (2005) 'The endocannabinoid system drives neural progenitor proliferation', *The FASEB Journal*. doi: 10.1096/fj.05-3995fje.
4. Aguado, T. (2006) 'The Endocannabinoid System Promotes Astroglial Differentiation by Acting on Neural Progenitor Cells', *Journal of Neuroscience*. doi: 10.1523/jneurosci.3101-05.2006.
5. Aizpurua-Olaizola, O. *et al.* (2017) 'Targeting the endocannabinoid system: future therapeutic strategies', *Drug Discovery Today*. doi: 10.1016/j.drudis.2016.08.005.
6. Alexandre, J. *et al.* (2018) 'Synthetic cannabinoids enhance neuronal differentiation in neuroblastoma cells at in vivo relevant concentrations', *Toxicology Letters*. doi: 10.1016/j.toxlet.2018.07.025.
7. Alexandre, J. *et al.* (2019) 'Synthetic cannabinoids and their impact on neurodevelopmental processes.', *Addiction biology*. United States, p. e12824. doi: 10.1111/adb.12824.
8. Anand, P. *et al.* (2009) 'Targeting CB2 receptors and the endocannabinoid system for the treatment of pain', *Brain Research Reviews*. doi: 10.1016/j.brainresrev.2008.12.003.
9. Avraham, H. K. *et al.* (2014) 'The cannabinoid CB(2) receptor agonist AM1241 enhances neurogenesis in GFAP/Gp120 transgenic mice displaying deficits in neurogenesis.', *British journal of pharmacology*. England, 171(2), pp. 468–479. doi: 10.1111/bph.12478.
10. Banister, S. D. *et al.* (2016) 'Pharmacology of Valinate and tert-Leucinate Synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and Their Analogues', *ACS Chemical Neuroscience*. doi: 10.1021/acschemneuro.6b00137.
11. Berghuis, P. *et al.* (2005) 'Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0509494102.
12. Berghuis, P. *et al.* (2007) 'Hardwiring the bain: Endocannabinoids shape neuronal connectivity', *Science*. doi: 10.1126/science.1137406.
13. Biegon, A. and Kerman, I. A. (2001) 'Autoradiographic study of pre- and postnatal distribution of cannabinoid receptors in human Brain', *NeuroImage*. doi: 10.1006/nimg.2001.0939.
14. Bisogno, T. *et al.* (2006) 'Development of the first potent and specific inhibitors of endocannabinoid biosynthesis', *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. doi: 10.1016/j.bbalip.2005.12.009.

15. Bromberg, K. D. *et al.* (2008) 'Design logic of a cannabinoid receptor signaling network that triggers neurite outgrowth', *Science*. doi: 10.1126/science.1152662.
16. Brown, Q. L. *et al.* (2017) 'Trends in marijuana use among pregnant and nonpregnant reproductive-aged women, 2002-2014', *JAMA - Journal of the American Medical Association*. doi: 10.1001/jama.2016.17383.
17. Buckley, N. E. *et al.* (1997) 'Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat', *Neuroscience*. doi: 10.1016/S0306-4522(97)00348-5.
18. Cabral G. A., G.-T. L. (2009) 'Emerging Role of the CB2 Cannabinoid Receptor in Immune Regulation and Therapeutic Prospects', *Expert Reviews in Molecular Medicine*. doi: 10.1017/S1462399409000957.Emerging.
19. Calvey, T. N. and Williams, N. E. (2008) *Principles and Practice of Pharmacology for Anaesthetists*, *Principles and Practice of Pharmacology for Anaesthetists*. doi: 10.1002/9781405194853.
20. Campanha, H. M., Carvalho, F. and Schlosser, P. M. (2014) 'Active and peripheral anionic sites of acetylcholinesterase have differential modulation effects on cell proliferation, adhesion and neuritogenesis in the NG108-15 cell line', *Toxicology Letters*. doi: 10.1016/j.toxlet.2014.03.012.
21. Cao, S. *et al.* (2017) 'PAX3 inhibits β-Tubulin-III expression and neuronal differentiation of neural stem cell', *Biochemical and Biophysical Research Communications*. doi: 10.1016/j.bbrc.2017.02.086.
22. Carroll, F. I. *et al.* (2012) 'Designer drugs: A medicinal chemistry perspective', *Annals of the New York Academy of Sciences*. doi: 10.1111/j.1749-6632.2011.06199.x.
23. Castaldo, P. *et al.* (2010) 'Altered regulation of glutamate release and decreased functional activity and expression of GLT1 and GLAST glutamate transporters in the hippocampus of adolescent rats perinatally exposed to Δ9-THC', *Pharmacological Research*. doi: 10.1016/j.phrs.2009.11.008.
24. Castaneto, M. S. *et al.* (2014) 'Synthetic cannabinoids: Epidemiology, pharmacodynamics, and clinical implications', *Drug and Alcohol Dependence*. doi: 10.1016/j.drugalcdep.2014.08.005.
25. Chao, C. C. *et al.* (2014) 'Protection of differentiated neuronal NG108-15 cells from P2X7 receptor-mediated toxicity by taurine', *Pharmacological Reports*. doi: 10.1016/j.pharep.2014.01.005.
26. Cohen, K. and Weinstein, A. M. (2018) 'Synthetic and Non-synthetic Cannabinoid Drugs and Their Adverse Effects-A Review From Public Health Prospective', *Frontiers in Public Health*. doi: 10.3389/fpubh.2018.00162.
27. Compagnucci, C. *et al.* (2013) 'Type-1 (CB1) Cannabinoid Receptor Promotes Neuronal Differentiation and Maturation of Neural Stem Cells', *PLoS ONE*. doi: 10.1371/journal.pone.0054271.
28. Cooper, Z. D. (2016) 'Adverse Effects of Synthetic Cannabinoids: Management of Acute Toxicity and Withdrawal', *Current Psychiatry Reports*. doi: 10.1007/s11920-016-0694-1.
29. Deutsch, D. G. and Chin, S. A. (1993) 'Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist', *Biochemical Pharmacology*. doi: 10.1016/0006-2952(93)90486-G.

30. Díaz-Alonso, J. *et al.* (2017) 'Loss of Cannabinoid CB1 Receptors Induces Cortical Migration Malformations and Increases Seizure Susceptibility', *Cerebral Cortex*. doi: 10.1093/cercor/bhw309.
31. Donvito, G. *et al.* (2018) 'The Endogenous Cannabinoid System: A Budding Source of Targets for Treating Inflammatory and Neuropathic Pain', *Neuropsychopharmacology*. doi: 10.1038/npp.2017.204.
32. EMCDDA (2010) *Risk assessment of new psychoactive substances Operating guidelines*, *European Monitoring Centre for Drugs and Drug Addiction Risk*. doi: 10.2810/2428.
33. EMCDDA (2019) 'European Drug Report Trends and Developments', *European Drug Report*. doi: 10.2810/191370
34. EMCDDA (2018) 'European Drug Report Trends and Developments', *European Drug Report*. doi: 10.2810/688395.
35. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2017) 'PERSPECTIVES ON DRUGS: Synthetic cannabinoids in Europe', *Perspectives on Drugs*. doi: 10.2810/32306.
36. Fantegrossi, W. E. *et al.* (2014) 'Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: Mechanism underlying greater toxicity?', *Life Sciences*. doi: 10.1016/j.lfs.2013.09.017.
37. Felder, C. C. *et al.* (1996) 'Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat', *FEBS Letters*. doi: 10.1016/0014-5793(96)00891-5.
38. Fernández-Ruiz, J. *et al.* (2000) 'The endogenous cannabinoid system and brain development', *Trends in Neurosciences*. doi: 10.1016/S0166-2236(99)01491-5.
39. Fernández-Ruiz, J. *et al.* (2004) 'Cannabinoids and gene expression during brain development', *Neurotoxicity Research*. doi: 10.1007/BF03033314.
40. Fiar, Z. (2009) 'Phytocannabinoids and Endocannabinoids', *Current Drug Abuse Reviewse*.
41. Forlenza, O. V. *et al.* (2007) 'Inhibition of phospholipase A2 reduces neurite outgrowth and neuronal viability', *Prostaglandins Leukotrienes and Essential Fatty Acids*. doi: 10.1016/j.plefa.2006.10.002.
42. Fride, E. (2004) 'The endocannabinoid-CB 1 receptor system in pre- and postnatal life', *European Journal of Pharmacology*. doi: 10.1016/j.ejphar.2004.07.033.
43. Friedrich, J. *et al.* (2016) 'The grass isn't always greener: The effects of cannabis on embryological development', *BMC Pharmacology and Toxicology*. doi: 10.1186/s40360-016-0085-6.
44. Galve-Roperh, I. *et al.* (2009) 'The endocannabinoid system and the regulation of neural development: potential implications in psychiatric disorders.', *European archives of psychiatry and clinical neuroscience*. Germany, 259(7), pp. 371–382. doi: 10.1007/s00406-009-0028-y.
45. Galve-Roperh, I. *et al.* (2013) 'Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation', *Progress in Lipid Research*. doi: 10.1016/j.plipres.2013.05.004.
46. Glaser, T. *et al.* (2014) 'Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor', *PLoS ONE*. doi:

10.1371/journal.pone.0096281.
47. Grant, K. S. *et al.* (2018) 'Cannabis use during pregnancy: Pharmacokinetics and effects on child development', *Pharmacology and Therapeutics*. doi: 10.1016/j.pharmthera.2017.08.014.
48. Green, A., Gabrielsson, J. and Fone, K. (2011) 'Translational neuropharmacology and the appropriate and effective use of animal models', *British Journal of Pharmacology*. doi: 10.1111/j.1476-5381.2011.01361.x.
49. Harkany, T. *et al.* (2007) 'The emerging functions of endocannabinoid signaling during CNS development', *Trends in Pharmacological Sciences*. doi: 10.1016/j.tips.2006.12.004.
50. Ho, B. Y. and Zhao, J. (1996) 'Determination of the cannabinoid receptors in mouse x rat hybridoma NG108-15 cells and rat GH4C1 cells', *Neuroscience Letters*. doi: 10.1016/0304-3940(96)12792-0.
51. Howlett, A. C. (2002) 'International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors', *Pharmacological Reviews*. doi: 10.1124/pr.54.2.161.
52. Jensen, J. B. and Parmar, M. (2006) 'Strengths and limitations of the neurosphere culture system', *Molecular Neurobiology*. doi: 10.1385/MN:34:3:153.
53. Jiang, W. *et al.* (2005) 'Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects', *Journal of Clinical Investigation*. doi: 10.1172/JCI25509.
54. Klein, C. *et al.* (2012) 'Circulating Endocannabinoid Concentrations and Sexual Arousal in Women', *Journal of Sexual Medicine*. doi: 10.1111/j.1743-6109.2012.02708.x.
55. Di Marzo, V. *et al.* (1994) 'Formation and inactivation of endogenous cannabinoid anandamide in central neurons', *Nature*. doi: 10.1038/372686a0.
56. Di Marzo, V. (2008) 'Endocannabinoids: Synthesis and degradation', *Reviews of Physiology, Biochemistry and Pharmacology*. doi: 10.1007/112-0505.
57. Di Marzo, V., Stella, N. and Zimmer, A. (2015) 'Endocannabinoid signalling and the deteriorating brain', *Nature Reviews Neuroscience*. doi: 10.1038/nrn3876.
58. Mechoulam, R. and Gaoni, Y. (1965) 'A Total Synthesis of dl-Δ'-Tetrahydrocannabinol, the Active Constituent of Hashish', *Journal of the American Chemical Society*. doi: 10.1021/ja00952a624.
59. Mereu, G. *et al.* (2003) 'Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0537849100.
60. Mir, A. *et al.* (2011) 'Myocardial Infarction Associated With Use of the Synthetic Cannabinoid K2', *PEDIATRICS*. doi: 10.1542/peds.2010-3823.
61. Mulder, J. *et al.* (2008) 'Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0803545105.
62. Palazuelos, J. *et al.* (2012) 'CB 2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M111.291294.
63. Pertwee, R. G. *et al.* (2010) 'International Union of Basic and Clinical Pharmacology.

LXXIX. Cannabinoid receptors and their ligands: Beyond CB1 and CB2', *Pharmacological Reviews*. doi: 10.1124/pr.110.003004.

64. Pinky, P. D. *et al.* (2019) 'Prenatal cannabinoid exposure and altered neurotransmission', *Neuropharmacology*. doi: 10.1016/j.neuropharm.2019.02.018.
65. Prenderville, J. A., Kelly, Á. M. and Downer, E. J. (2015) 'The role of cannabinoids in adult neurogenesis', *British Journal of Pharmacology*. doi: 10.1111/bph.13186.
66. Rodrigues, R. S. *et al.* (2017) 'Interaction between cannabinoid type 1 and type 2 receptors in the modulation of subventricular zone and dentate gyrus neurogenesis', *Frontiers in Pharmacology*. doi: 10.3389/fphar.2017.00516.
67. Rueda, D. *et al.* (2002) 'The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M206590200.
68. Sagredo, O. *et al.* (2018) 'Cannabinoid signalling in the immature brain: Encephalopathies and neurodevelopmental disorders', *Biochemical Pharmacology*. doi: 10.1016/j.bcp.2018.08.014.
69. Salani, D. A. and Zdanowicz, M. M. (2015) 'Synthetic cannabinoids: The dangers of spicing it up', *Journal of Psychosocial Nursing and Mental Health Services*. doi: 10.3928/02793695-20150422-01.
70. Schaeffer, E. L. and Gattaz, W. F. (2015) 'Chronic inhibition of brain phospholipase A2 in adult rats impairs the survival of newborn mature neurons in the hippocampus', *Journal of Neural Transmission*. doi: 10.1007/s00702-014-1305-0.
71. Shanks, K. G. and Behonick, G. S. (2016) 'Death after use of the synthetic cannabinoid 5F-AMB', *Forensic Science International*. doi: 10.1016/j.forsciint.2016.03.004.
72. Shevyrin, V. A. and Morzherin, Y. Y. (2015) 'Cannabinoids: Structures, effects, and classification', *Russian Chemical Bulletin*. doi: 10.1007/s11172-015-1008-1.
73. Silva, J. P., Carmo, H. and Carvalho, F. (2018) 'The synthetic cannabinoid XLR-11 induces in vitro nephrotoxicity by impairment of endocannabinoid-mediated regulation of mitochondrial function homeostasis and triggering of apoptosis', *Toxicology Letters*. doi: 10.1016/j.toxlet.2018.01.023.
74. Sugiura, T. *et al.* (1995) '2AG A possible endogenous cannabinoid receptor ligand in brain', *Biochemical and Biophysical Research Communications*.
75. Tojima, T. *et al.* (2000) 'Acquisition of neuronal proteins during differentiation of NG108-15 cells', *Neuroscience Research*. doi: 10.1016/S0168-0102(00)00110-3.
76. Trecki, J., Gerona, R. R. and Schwartz, M. D. (2015) 'Synthetic cannabinoid-related illnesses and deaths', *New England Journal of Medicine*. doi: 10.1056/NEJMp1505328.
77. Turcotte, C. *et al.* (2016) 'The CB2 receptor and its role as a regulator of inflammation', *Cellular and Molecular Life Sciences*. doi: 10.1007/s00018-016-2300-4.
78. UNODC (2011) *Synthetic cannabinoids in herbal products*, *Drugs*.
79. Waksman, Y. *et al.* (1999) 'The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells.', *The Journal of pharmacology and experimental therapeutics*.
80. Wang, H., Xie, H. and Dey, S. K. (2006) 'Endocannabinoid signaling directs

periimplantation events', *AAPS Journal*. doi: 10.1208/aapsj080250.
81. Znaleziona, J. *et al.* (2015) 'Determination and identification of synthetic cannabinoids and their metabolites in different matrices by modern analytical techniques - a review', *Analytica Chimica Acta*. doi: 10.1016/j.aca.2014.12.055.
82. Zou, S. and Kumar, U. (2018) 'Cannabinoid receptors and the endocannabinoid system: Signaling and function in the central nervous system', *International Journal of Molecular Sciences*. doi: 10.3390/ijms19030833.