2017/2018

Mestrado em Toxicologia Analitica
Clínica e Forense

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Synthetic Cannabinoids in the regulation of neuronal differentiation

João André da Costa Alexandre

Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Toxicologia Analítica Clínica e Forense

Trabalho realizado sob a orientação do Doutor João Pedro Silva, Professora Helena Carmo e Professor Félix Carvalho

Setembro de 2018

anciado por:
COMPETE 2020
PORTUGAL 2020
UNIÃO EUROPEIA
Fundo Europeu
de Desenvolvimento Regional

É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC.) APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Acknowledgements

I would first like to thank my supervisor Dr. João Pedro Silva for all the guidance, support and, sometimes, patience throughout this dissertation. I have nothing but admiration and respect for him, and can't thank him enough for all the knowledge passed onto me, for always pushing me to do my best, for the trust he had in my work and for never leaving a text message or e-mail unanswered.

I would also like to thank my co-supervisors Professor Félix Carvalho and Professor Helena Carmo, for always guiding me through this dissertation and making sure I was producing and presenting the best work possible. Their guidance was invaluable and I can't thank them enough for always having their door open for any questions I had.

To the Portuguese Foundation for Science and Technology for partially financing of this work (PTDC/SAU-TOX/29584/2017).

To TICTAC Communications Ltd. (UK), for providing one (MDMB-CHMICA) of the synthetic cannabinoids here studied.

To everyone, and I mean everyone, at the Toxicology department. You all made me feel at home since the very beginning and the comradery I found there was unmatched. I would never have been able to finish this work without all your help.

To my friends, who were always there to support me and help me in every way they could. You were always there in moments of need, even if just by sharing workspace and making long hours funnier.

À minha minha família (Primos, Tios, Padrinho e Avós). Não vou dizer todos os vossos nomes porque, como sabem, somos muitos e vocês sabem que é a vocês que me dirijo. Não tenho palavras para vos agradecer a todos por todo o apoio que me deram e por estarem lá sempre para mim. Desde palavras de apoio, até mesmo à vossa presença em alturas de festa permitiram que eu conseguisse aqui chegar.

Por fim, gostaria de agradecer especialmente ao meu irmão, Pai, Mãe e à Tatiana. Penso que não tenho de explicar o porquê de vos referenciar em destaque, já que sem vocês nada disto seria possível por inúmeras razões. Obrigado por sempre me apoiarem, ajudarem e acreditarem em mim. Sabem que não sou de muitas palavras pelo que espero que um OBRIGADO seja suficiente para fazer chegar a minha intenção.

Resumo

Canabinóides sintéticos (CS) são novas substâncias psicoativas que se ligam e ativam pelo menos um recetor canabinóide, com maior potência do que o tetrahidrocanabinol (THC), a principal substância psicoativa da *Cannabis sativa* L. O consumo destas substâncias por mulheres grávidas ou em idade fértil é preocupante, devido à possibilidade da ocorrência de disfunções durante o neurodesenvolvimento fetal. Este trabalho teve como principais objetivos: 1) avaliar o potencial neurotóxico de 5 CS comummente reportados; e 2) avaliar se esses CS afetam a diferenciação neuronal *in vitro*. Para tal, células de neuroblastoma (NG108-15) foram expostas a uma gama de concentrações entre 1pM e 100μM de 5F-PB22, JWH-122, MDMB-CHMICA, THJ-2201 e XLR-11. Analisaram-se vários parâmetros toxicológicos, incluindo a viabilidade celular, proliferação celular, potencial de membrana mitocondrial e níveis intracelulares de energia. Determinaram-se os rácios de diferenciação na presença (a concentrações com relevância *in vivo*) ou ausência de CS, em meio promotor de diferenciação (suplementado com forscolina e ácido retinóico, na presença de 1% FBS). Os CS foram adicionados uma única vez (no dia 0) ou três vezes (uma a cada 24 h, até 72 h). Tanto o 5F-PB22 (após três adições de 1pM e 1nM) como THJ-2201 (uma adição de 1pM e 1nM) promoveram o aumento da diferenciação neuronal, sendo que ensaios na presença de 500 nM SR141716, um antagonista do recetor CB_1, sugerem a modulação desse efeito por esse recetor. O aumento na diferenciação modulado por 5F-PB22 foi acompanhada por um aumento significativo da expressão de β3-tubulina e p73, duas proteínas envolvidas no processo de diferenciação neuronal. No entanto, o mesmo não se verificou para o THJ-2201, ocorrendo mesmo uma diminuição da expressão de p73. Nenhum CS diminuiu a viabilidade celular (atividade metabólica, retenção lisossomal de Vermelho Neutro ou integridade da membrana celular) para concentrações até 10 μM. Com exceção do 5F-PB22, todos eles levaram a um aumento da acumulação mitocondrial de TMRE após 24 h, sem, contudo, alterarem os níveis intracelulares de ATP. Em conclusão, os SC testados não apresentam níveis significativos de toxicidade para as células NG108-15 em concentrações com relevância *in vivo* (<1μM), embora aparentem interferir com a função mitocondrial. No entanto, o 5F-pB22 e o THJ-2201 levaram a um aumento significativo da diferenciação neuronal, num processo mediado pelo recetor CB_1. Contudo, é necessário elucidar os mecanismos envolvidos neste processo.

Palavras-Chave: canabinóides sintéticos, novas substâncias psicoativas, recetores canabinóides, desenvolvimento neuronal, diferenciação neuronal

Abstract

Synthetic cannabinoids (SCs) are new psychoactive substances that bind and activate at least one cannabinoid receptor, with higher potency than tetrahydrocannabinol (THC), the main psychoactive substance of Cannabis sativa L. The consumption of such substances by pregnant woman and of child-bearing potential is alarming due to the possible occurrence of disfunctions during fetal neurodevelopment. This work had as two main objectives: 1) evaluate the neurotoxic potential of 5 SCs commonly reported; and 2) evaluate if such SCs affect neuronal differentiation in vitro. As such, neuroblastoma cells (NG108-15) were exposed to a range of concentrations between 1pM and 100μM of 5F-PB22, JWH-122, MDMB-CHMICA, THJ-2201 and XLR-11. Various toxicological parameters were analyzed, including cell viability, cell proliferation, mitochondrial membrane potential and intracellular levels of energy. Ratios of differentiation were determined in the presence (at in vivo relevant concentrations) or absence of SCs, in differentiation inducing medium (supplemented with forskolin and retinoic acid, in the presence of 1% FBS). SCs were added once (at day 0) or three times (one time at every 24 h, until 72h). Both 5F-PB22 (after three additions of 1pM and 1nM) and THJ-2201 (one addition of 1pM and 1nM) promoted the increase in neuronal differentiation, while assays in the presence of 500nM SR141716, a CB_1R antagonist, suggest a modulation of such effects by this receptor. The increase in neuronal differentiation modulated by 5F-PB22 were followed by a significant increase in the expression of both β3-Tubulin and p73, two proteins involved in the process of neuronal differentiation. However, the same was not seen for THJ-2201, even being observed a decrease in p73 expression. No SC decreased cell viability (metabolic activity, lisossomal NR retention or cellular membrane integrity) for concentrations up to 10 μM. Every SC, except 5F-PB22, resulted in increased mitochondrial accumulation of TMRE after 24h, without, however, changing intracellular levels of ATP. In conclusion, tested SCs did not show significant levels of toxicity to NG108-15 cells at in vivo relevant concentrations (<1μM), despite appearing to interfere with the mitochondrial function. However, 5F-PB22 and THJ-2201 lead to an increase in neuronal differentiation, in a CB_1R mediated process. Nonetheless, it is necessary to clarify the involved mechanisms in this process.

Keywords: synthetic cannabinoids, new psychoactive substances, cannabinoid receptors, neuronal development, neuronal differentiation

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Abbreviations

2-Ara-Gl: 2-Arachidonylglycerol

5F-PB22: 1-pentyfluoro-1H-indole-3-carboxylic acid 8-quinolinyl ester

AEA: Anandamide

ATP: Adenosine Triphosphate

CB1R: Cannabinoid Receptor 1

CB2R: Cannabinoid Receptor 2

DMEM: Dulbecco's Modified Eagle's Medium

FBS: Fetal Bovine Serum

JWH-122: (4-methyl-1-naphthyl) -(1-pentylindol-3-yl) methanone

LDH: Lactate dehydrogenase

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NPS: New psychoactive substances

NR: Neutral Red

SCs: Synthetic Cannabinoids

SRB: Sulforhodamine B

THC: Tetrahydrocannabinol

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

TMRE: Tetramethylrhodamine, ethyl ester

WB: Western-Blot

XLR-11: (1-(5-fluoropentyl)–1H–indol–3–yl) (2,2,3,3-tetramethylcyclopropyl) methanone

1. Synthetic cannabinoids: a rising threat

Psychoactive substances have been used for centuries. In particular, cannabinoids have been used in several different settings, including for recreational purposes, as well as in medicine and war [1, 2]. These substances represent a group of molecules that activate endogenous cannabinoid receptors [3], possibly resulting in psychoactive effects such as those experienced after the consumption of cannabis [3]. Use of new psychoactive substances (NPS) continues to represent a major challenge for public health and policy-makers due to the health and social consequences of their fast-paced emergence [4, 5]. Among these, the widespread use of synthetic cannabinoids (SCs), a diverse group of substances that mimic, with higher potency, the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of cannabis, constitute a particular concern since they display a substantial harm-causing potential. Although primarily aimed at therapeutic applications (*e.g.* nabilone, dronabinol) [6, 7], SCs' popularity as recreational drugs has been increasing among drug users [8], as their intensified psychoactive effects, compared to THC, had regular consumers of cannabis (mainly comprising young people) turn into SCs [9]. In fact, SCs currently represent 45 % of all NPS seizures and have become increasingly chemically diverse, having 179 new SC structures been detected since 2008 within the European Union (EU) [10]. In general, prevalence of SCs is low, but it has been increasing in specific settings (e.g. prisons, nightlife) [5].

There is currently scarce research data that may help predict the short- and long-term toxicologic consequences of SC use [11]. Moreover, the various reports of severe intoxications and deaths following SC use [12, 13] have further contributed to turn their use into a major public health issue. These reports have further triggered the European Union to join efforts with the World Health Organization (WHO) to implement a strong legislative response aimed at controlling these substances [5]. Noteworthy, prevention and treatment of substance abuse has been set as one of the United Nations 2030 Agenda's Sustainable Development Goals (3.5) to achieve by 2030 [14].

Among cannabinoid consumers, pregnant women and women of child-bearing potential (WOCBP) represent particular risk groups, due to the possible adverse consequences to the exposed fetus, namely the onset of neurodevelopment disorders (e.g. schizophrenia, autism spectrum disorders, attention deficit and hyperactivity disorder) [15, 16]. Understanding the role played by cannabinoids and the cannabinoid system in the regulation of the neuronal function, in particular during neuronal

development, thus assumes critical relevance. In the following sections, we review the mechanisms involved in cannabinoid-mediated regulation of neuronal development, particularly focusing on how these processes may be modulated by synthetic cannabinoids.

2. The endocannabinoid system

The endocannabinoid system (ECS) is composed of endogenous cannabinoids (ECs), the enzymes responsible for EC biosynthesis and degradation, and by cannabinoid receptors and transporters [17]. This system plays a major role in central and peripheral nervous systems by controlling the release of neurotransmitters, thus being involved in different processes, including pain modulation [18], vasodilation-mediated thermoregulation [19] and energy metabolism [20]. In addition, the ECS is present since the early stages of neuronal development, being especially relevant during neuronal differentiation [21], since the CB_1 receptor has been found to play a key role in neuronal progenitor cells proliferation, pyramidal specification, axon patterning[22] and promotion of neuronal differentiation in the presence of endocannabinoids [23] .

2.1 Cannabinoid receptors

The groundbreaking publication of the chemical structure of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of cannabis, by Gaoni and Mechoulam in 1964 [24] aroused the scientific community's interest in the active compounds of cannabis. Moreover, studies on the effects of these substances within the human body further resulted in the identification and characterization of an endogenous G protein-coupled receptor in the brain [25], later referred as CB1 receptor.

There are at least two main receptors for cannabinoids, commonly defined as type 1 (CB_1R) and type 2 (CB_2R). These G protein-coupled membrane receptors play different roles and are present in several tissues [26, 27]. Although CB_1 receptors prevail in central and peripheral neuronal cells and CB_2 receptors are mainly present in cells from the immune system [28, 29], they are not exclusively present in one specific type of cells or tissues. Instead, they are widely distributed throughout various organs and tissues, with distinct tissues displaying different densities of each receptor type [30, 31].

Both CB_1R and CB_2R are linked to the inhibition of adenylate cyclase and cAMP pathways [29, 32], although only the CB_1R has been shown to modulate calcium and potassium channels [29, 33]. CB_1R is predominantly present in presynaptic terminals and in brain areas that coordinate numerous key functions, like the hippocampus. In this sense, binding of cannabinoids to CB_1R alters brain functions such as cognition, motor coordination, memory and autonomic function [34]. Moreover, CB_1R is also found in basal ganglia, skeletal muscle mitochondria [35] and in post-synaptic sites [36].

Cannabinoids bind and regulate the action of this receptor by inhibiting the release of neurotransmitters such as GABA [18], glutamate [37], acetylcholine [38] and noradrenaline [39].

The CB_2 receptors are found at a higher number in cells that compose the immune system (e.g. B-lymphocytes, mast cells and macrophages), playing a major role in its regulation [29]. Specifically, activation of CB_2Rs in both T- and B-cells results in a reduced immune response [40]. These receptors are also present in the central nervous system, namely in microglia and neuronal (mostly in post-synaptic) cells [41, 42] and in other sites, like the intestine [43], adipocytes [44] and spleen [45]. Although this receptor has been related with the regulation of non-neuronal cells of the CNS and neuronal cells' proliferation and differentiation [46], its exact functions in the nervous system still remains unclear.

Cannabinoids' binding affinity towards CB_1R and CB_2R determines the type and intensity of the effects elicited by these ligands [47]. For example, a higher affinity towards CB_1R induces a stronger psychotropic effect due to the higher density of this receptor in neuronal cells. On the other hand, higher affinity to CB_2R may instead result in, for example, chronic pain reduction, but with fewer or absent psychotropic effects [47]. Nevertheless, most cannabinoids are able to bind both cannabinoid receptors, making the outcome of its binding unpredictable to a certain extent.

In addition, cannabinoids may act as ligands for other receptors. In fact, cannabinoids also modulate the activity of Transient Receptor Potential Vanilloid 1 (TRPV1) channels [48], nuclear receptors from the peroxisome proliferator-activated receptor (PPAR) family [49] and G-protein coupled receptors 55 (GPR55) and 18 (GPR18) [50, 51]. The effects of cannabinoid binding to these targets have been reviewed elsewhere [52, 53].

2.2 Endogenous cannabinoids

Cannabinoids may be classified into one of three main categories, according to their nature: endogenous (endo-), phyto- and synthetic cannabinoids [7]. Endocannabinoids comprise a group of lipid neurotransmitters produced intracellularly that react with the endogenous cannabinoid receptors (CB1R or CB_2R) [54]. Phytocannabinoids, on the other hand, are cannabinoids produced in plants such as *Cannabis Sativa* which includes, most notably, Δ9-THC [54]. Synthetic cannabinoids

represent a group of molecules chemically designed to activate cannabinoid receptors [55].

In 1992, Devane and co-workers [56] described an arachidonic acid derivative (N-arachidonylethanolamine, commonly referred as anandamide) present in porcine brain with the ability to bind to the CB_1R. Later, Mechoulam et al. [10] reported the presence of an endogenous 2-monoglyceride, termed 2-arachidonyl glycerol (2-Ara-GI), in the canine gut. These findings represented important hallmarks in the study of the endocannabinoid system, particularly by presenting it as a potentially interesting target for medicinal use, due to its numerous intracellular regulatory roles, such as the inhibition of the release of both inhibitory and excitatory neurotransmitters (in CNS and peripheral nervous system) [26, 28]. Such neurotransmitter regulation can further lead to a downstream enhancement of the release of other neurotransmitters [57], inhibition of helper T cell activation in a CB_2R related way [58], inhibition of NO release in rat microglial cells after exposure to a full CB_1R agonist [59] and enhancement of nitric oxide release from human monocytes after exposure to the endocannabinoid 2-Ara-Gl [60].

Anandamide derives from the hydrolysis of N-arachidonoyl-phosphatidylethanolamine by a phospholipase D-like enzyme, while 2-Ara-Gl is synthesized through the action of an sn-1-diacylglycerol lipase on sn-2-arachidonate-containing diacylglycerols[61]. The biosynthesis of anandamide and 2-Ara-Gl precursors occurs via a phospholipid-mediated pathway in the central nervous and immune systems [62] and is dependent on Ca^{2+}-activated phospholipid remodeling[61]. Moreover, it should be noted that their release from brain neurons is also Ca^{2+}-dependent [61, 63, 64]. Anandamide has also been documented to be synthesized by an alternative pathway when the above-mentioned pathway is compromised. Studies performed in mouse brain and RAW264.7 macrophages showed that N-arachidonoyl-phosphatidylethanolamine may be cleaved by a phospholipase C (PLC)-catalyzed reaction, producing phosphoanandamide that can then be dephosphorylated by phosphatases such as PTPN22 [65]. Intracellularly, anandamide has been found to be rapidly degraded into arachidonic acid, and 2-Ara-Gl into ethanolamine, or glycerol, by enzymatic hydrolysis (e.g. by monoacylglyerol lipase (MAGL), fatty acid amide hydrolase (FAAH) or serine hydrolases) [63, 66].

Levels of anandamide in unstimulated brain cells have been found to range between 29 pmol/g of tissue in rat hippocampus [67] to being undetectable in homogenized rat brain [68]. However, levels of 2-Ara-Gl have been observed to be much

higher compared to anandamide, having been detected up to 4 nmol/g of brain tissue [69, 70].

Binding affinity for CBRs differs among endocannabinoids. For example, anandamide is a partial agonist for both CB_1R and CB_2R, with stronger affinity towards CB_1R. It thus shows similar effects to Δ^9-THC in mice [71] and may even prevent 2-Ara-Gl-induced CB_2R activation [72]. By its turn, 2-Ara-Gl has similar affinities for both receptors, generally displaying higher intrinsic activity at both CBRs in comparison to anandamide[73].

2.3 Cannabinoid-mediated regulation of neuronal function

The effects of cannabinoids in the neuronal system were firstly studied by Howlett and Flemming in 1984 [74]. These authors showed, even before cannabinoid receptors were identified, that Δ^9-THC inhibited adenylate cyclase present in plasma membranes of neuroblastoma cells. More recent studies reported that the administration of exogenous cannabinoids significantly affected neuronal function [75-77].

The CB_1 receptor is known to modulate ion channels in brain cells, in particular by inhibiting Ca^{2+} channels and activating K^+ channels, resulting in membrane depolarization and exocytosis. This results in a pre-synaptic CB_1R activation and subsequent inhibition of the release of neurotransmitters, such as glutamate [78], dopamine and GABA, as depicted in Figure 1. This figure further displays the modulation that occurs upon post-synaptic release of CB_1R agonists, like endocannabinoids. As the neurotransmitter activates its receptors in the post-synaptic neurons, the intracellular Ca^{2+} concentration rises, leading to the cleavage of a cannabinoid precursor present in the post-synaptic neuronal membrane. This precursor is then turned into an active cannabinoid, acting as a retrograde messenger to the pre-synaptic neuron. This cannabinoid-mediated signaling is halted by a membrane-transport system in the post-synaptic neuron that still remains to be identified [79].

Cannabinoids also play an important role in the control of glutamatergic synaptic transmission and Ca^{2+} homeostasis. For example, in cases of ischemia, cannabinoids decrease the release of pre-synaptic glutamate, which is elevated during such condition [37]. This is achieved by inhibiting the Ca^{2+} channels and activating the K^+ channels by

blunting the cell membrane's depolarization and also by inhibiting the adenylyl cyclase–cyclic AMP (cAMP)–protein kinase A pathway [79].

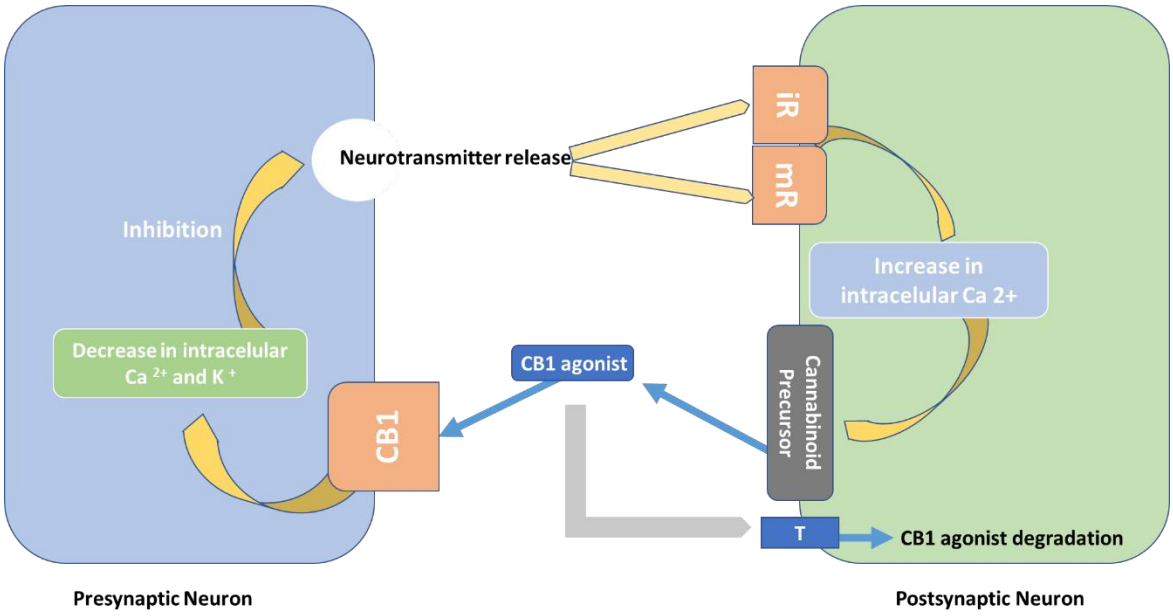


Figure 1 - Effect of cannabinoids on Ca^{2+} and K^{+} channels and subsequent modulation of neurotransmitter release. The activation of CB_1 by an agonist (e.g. anandamide, 2-ara-GI) results in the decrease in pre-synaptic neuronal levels of Ca^{2+} and K^{+} and also leads to a decrease in the released neurotransmitter. The binding of this neurotransmitter in the post-synaptic neuron through ionotropic (iR) or metabotropic (mR) receptors leads to an increase of Ca^{2+} levels and to the release of an activated cannabinoid precursor. The remaining CB_1 agonists left in the synapse are then transported into the postsynaptic neuron through a transporter (T) and degraded by a family of intracellular enzymes, like the fatty acid amide hydrolase. Adapted from Guzman *et al.* (79).

CB_1 receptor activation is also associated with many other functions in neuronal cells. These include the activation of protein kinase B (PKB, also known as Akt) [80] and assistance in apoptosis induction [81]. Apoptosis mediated by the CB_1 receptor relies on the sustained *de novo* synthesis of ceramide in a process acutely activated by FAN (Factor associated with neutral sphingomyelinase activation), independent of G-protein [79]. The high levels of ceramide then lead to the ERK (extracellular-signal-regulated kinase) cascade activation [81]. Although this cascade leads to cell proliferation, its inhibition may result in cell growth arrest and even cell death [82]. The duration of the stimulus is the key to the end result of the ERK cascade: a short (e.g. a few minutes [81]) stimulus is beneficial to the cellular metabolic regulation, while a

longer stimulus (e.g. extended to a few days [81]) results in the activation of apoptotic pathways [83].

In addition to ERK, other mitogen-activated protein kinases (MAPKs) are stimulated by cannabinoid activation. These include the stress-activated JUN amino-terminal kinase (JNK) and p38 MAPK, which have key roles in cell growth and differentiation [79]. Cannabinoids (Δ^9-THC and an anandamide analogue) have also been shown to activate the phosphatidylinositol 3-kinase (PI3K)–Akt survival pathway in prostate PC-3 cells. In fact, activated Akt was shown to inhibit the expression of pro-apoptotic proteins through the nuclear translocation of forkhead transcription factors [79, 80]. However, Ellert *et al.* (2005) noted that WIN 55,212-2 (a synthetic cannabinoid, agonist of CB_1R) downregulated PI3K/Akt and ERK signaling pathways, which in turn resulted in the activation of the proapoptotic role of Bad in glioma cells [84]. A summary of these CB_1R-mediated mechanisms is summarized in Figure 2.

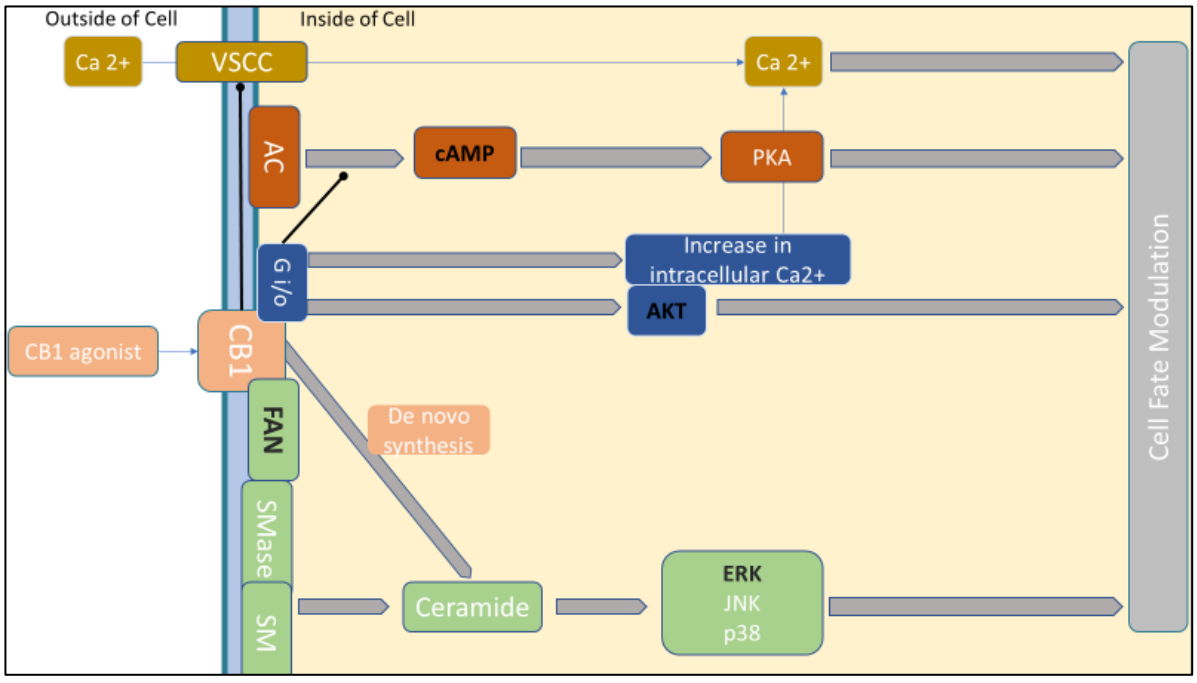


Figure 2 - Downstream mechanisms upon CB_1 activation by cannabinoids. The effects of the activation of the CB_1 receptor range from the inhibition of the adenylyl cyclase(AC)- cyclic AMP- protein kinase A (PKA) pathway, to the activation of mitogen-activated protein kinase cascades, such as extracellular-signal-regulated kinase (ERK), JUN amino-terminal kinase (JNK) and p38. They also include the modulation of the ion mobilization through the inhibition of voltage-sensitive Ca^{2+} channels (VSCC), by the release of Ca^{2+} from intracellular stores and the production of ceramide through FAN-sphingomyelinase (factor associated with neutral sphingomyelinase activation, SMase). Boxes represented in the same color are part of the same cascade of reactions. The names and abbreviations in Black are further detailed in the text. Adapted from Guzman et al (79).

Cannabinoids have also been related with centers of pleasure in the brain. Indeed, the CB_1 receptor is overexpressed in key areas such as the mesolimbic dopaminergic system [85]. The activation of this receptor in this brain region facilitates the reward-associated functions, while its inactivation contributes to a less effective reward function. Moreover, cannabinoids have also been associated with high dopamine levels [86]. This is due to the cannabinoid-induced activation of CB_1 receptor, which inhibits the release of GABA, a neurotransmitter that usually inhibits dopamine release from the mesolimbic system [87]. This process, often termed as "disinhibition", thus results in increased dopamine levels in the nucleus accumbens [18, 87, 88]. Activity of CB_2 receptors in the brain is more associated with their potential to reduce acute pain sensation. However, this effect does not correlate with the presence of psychotropic activity, contrary to an agonist-induced CB_1 activation [89]. This is a very important feature that allows the regular use of CB_2-inducing drugs without the alteration of consciousness [90]. In addition, this receptor is thought to be overexpressed in the CNS in cases of neuroinflammation and a potent anti-inflammatory activity has been reported for CB2-receptor agonists [91]. This indicates a primary role for this receptor in the progression of this condition, turning it into a therapeutic target in inflammatory and neurodegenerative diseases [91, 92] . A summary of various effects induced by cannabinoids at the neuronal level is depicted in Table I.

Furthermore, cannabinoids have been related with other neuronal effects, including: inhibition of nitric oxide production in the brain [93], inhibition of the production of pro-inflammatory cytokines [94] and activation of phosphoinositide 3'kinase/PKB pathway [80].

Table I- Effects originated by cannabinoids in neurons. Adapted from Guzman, *et al.* (95).

System	Model	Cannabinoid	Observed Effects	CB receptor	References
In vivo	Primary rat cortical neurons	Δ^9-THC	Activation of the tumor suppression protein p53 by increasing expression of Bax and promoting Bcl phosphorylation.	CB_1R	Downer *et al* (2007a) [96]
	Primary cultured mouse cerebellum neurons	2-AG	Release of arachidonic acid with cPLA2α activation.	N.D.	Nabemoto *et al* (2008) [97]
	Wistar rat	Δ^9-THC	Increase in JNK and Caspase-3 activation in neonatal, but not adult, cerebral cortex	CB_1R	Downer *et al* (2007b) [98]
In vitro	Rat pheochromocytoma cell line PC-12	2-AG	Activation of cPLA2α dependent on Src phospholipase C-protein kinase.	N.D.	Nabemoto *et al* (2008) [97]
	Rat pheochromocytoma cell line PC-12	Dexanabinol	Inhibition of the apoptosis correlated protein NF-kappaB by stopping NF-kappaB's inhibitor degradation and reducing NF-kappaB's transcriptional activity	Neither CB_1R nor CB_2R	Juttler *et al* (2004) [99]

Δ^9-THC: Δ^9-Tetrahidrocannabinol; 2-AG: 2-arachydonoylglcycerol ;N.D.: not defined;

2.4 The role of the endocannabinoid system in neurodevelopment

Brain development is a highly dynamic and adaptive event that starts at the third gestational week with neuronal progenitor cells' differentiation and stops at late adolescence (arguably throughout the individual's lifespan) [100]. A well-defined endocannabinoid expression pattern is key in neuronal cell survival, proliferation, migration and differentiation. This is true during adulthood, but also in the earliest stages of development, such as embryonal implantation, prenatal neurodevelopment and postnatal suckling [101-103].

It is worth noting that both CB_1R and CB_2R have been detected around day 11 of gestation in embryonic rat brain [104]. After the differentiation of neuronal progenitor cells they are positioned along the rostral-caudal midline of the upper layer of the three-layered embryo, which is referred to as the neural plate (the first stage of neuronal development) [105]. The first signs of a neural tube development are then shown by the appearance of ridges that form on each side of the neural plate. These ridges then fold inward and fuse, over the course of several days, and form the neuronal tube [105]. The neuronal tube is initially a hollow structure that will change its shape with the development and enlargement of the brain, leading to the formation of the ventricular system. After this, a rapid growth will take place and the shape of primitive neurons will change, known as the proliferation stage [100]. This stage results in the arrangement of the three primary brain vesicles: "prosencephalon" (the embryonic precursor of the forebrain), "mesencephalon" (the precursor of midbrain structures) and "rhombencephalon" (the precursor to the hindbrain). Two of these vesicles then subdivide to form the five secondary brain vesicles. The prosencephalon originates the "telencephalon" and the "diencephalon" and the rhombencephalon divides into the "metencephalon" and "myelencephalon". However, the mesencephalon does not subdivide [100]. Recently, Rodrigues *et al.* [106] observed that the simultaneous CB_1R and CB_2R activation increased neuronal proliferation in early postnatal Sprague-Dawley rats. This was attained using selective CB_1R (Arachidonyl-2'-chloroethylamide) and CB_2R agonists (HU-308) and a non-selective CB_1R and CB_2R agonist (WIN55,212-2). The non-selective cannabinoid WIN55,212-2 showed an increase in proliferation which was reverted after exposure to either CB_1R or CB_2R selective antagonists [106]. Following the formation of the five secondary vesicles, neuronal cells start to migrate from ventricular zones to their final location through glial-guided locomotion [107]. This occurs by the attachment of migrating neurons to guide glial cells, which then move along the cortical plate [108]. However, during this stage, a different class of inhibitory cortical interneurons migrate through "tangential migration", as the route of migration passes the developing cortical mantle tangentially. This type of migration uses a specific type of molecules that are produced locally in various regions to direct the neurons' migration [109, 110]. The migration of neurons into the developing neocortex forms a six-layered structure that is ordered by time of migration [111]. Interestingly, Turunen *et al.* (2018) [112] found that the increased endogenous production of 2-Ara-Gl led to bursts in the mobility of neuroblasts regulated by the metabotropic glutamate receptor 5 (mGluR5) or its transducer canonical transient receptor potential channel 3 (TRPC3) in isolated mice neuronal progenitor stem (NPS) cells. Furthermore, Díaz-Alonso *et al.*

(2016) [113] also reported the key role of the endocannabinoid system in promoting a correct neuronal migration. The authors noted that acute silencing of the CB_1R impaired radial migration and altered neuronal morphology in mice. Generally, earlier migrating neurons form the deepest layers, while the latest migrating neurons form the more superficial layers. However, this does not apply to the first neurons to migrate, as these originate a structure named "preplate". The next wave of neurons separates the preplate, creating a new region in the middle of the preplate that originates the marginal zone (MZ) and the subplate (SP) [109, 110]. The MZ contains Cajal-Retzius cells, which control the positioning of neurons into the correct layers of cortex through the production of reelin. This molecule then signals neurons when to stop migrating and assume their position in the cortex [110]. Although the particular signaling pathways that induce differentiation in the progenitor population still remains to be fully understood [114], it is generally agreed that neurons differentiate and produce axons and dendrites after reaching their destination. Activation of CB_1R in the hippocampus has been shown to modulate the fate of neural progenitor cells by promoting proliferation and inhibiting neuronal differentiation, also being noted that after exposure to the CB_1R antagonist SR141716 the effects were not present [22, 115, 116]. Rueda et al. (2002) noted that neuronal differentiation was inhibited after exogenous anandamide exposure through the regulation of the activation of the Rap1/B-Raf/ERK pathway via CB_1 receptors in the PC12 cell line [117]. Additionally, the inhibition of neurogenesis in adult rat hippocampus was also observed after anandamide exposure [117]. In 2005, Aguado *et al.* [118] described that the cannabinoid WIN-55,212-2 promoted the proliferation of neuronal progenitor cells, which was only observed in cells with active CB_1 receptors and not seen in cells lacking CB_1R. Moreover, they also observed that the induction of neuronal progenitor cell proliferation may be regulated through the endocannabinoid system present these cells.

Neurons produce several dendrites and one axon which stretches out of the cell body and has a growth cone on its end [100]. As the axon extends, it scans the local environment in order to find attractive or repulsive molecules [100]. Berghuis *et al.* (2007) have also shown that cannabinoids play an important role in the initial growth of axons through autocrine signaling, while target-derived endocannabinoid signals have shown to control axonal navigation and positioning in rat brain, through the phosphorylation of ERK1 and ERK2 following anandamide exposure, acting as an attractant [119]. As the axon reaches its target, it forms synapses for the transmission of electrochemical information [120]. Bromberg *et* al. (2008) reported that cannabinoids also play a vital role in neuronal differentiation through the post-transcriptional

regulation of Pax6, a key factor in neuronal differentiation. This was observed after exposure to HU-210. These authors found that the cannabinoid-mediated modulation of the PI3k/Akt pathway promotes the phosphorylation of Pax6 in differentiating neuroblastoma cells. Pax6 phosphorylation then results in the activation of kinase signaling and promotes the growth of neurites and neuronal differentiation [21].

While in fetal stage the concentrations of anandamide and 2-Ara-Gl are quite different, 2-Ara-Gl is found at much higher concentrations than those of anandamide (almost 1000-fold) [121]. Moreover, CB_1R and CB_2R mRNA has been detected in fetal rat brain [104]. In fact, even after birth, CB_1R mRNA levels and receptor density have been found to be increased [104, 122]. This has been observed both in rats and in humans, where these increases of mRNA levels and cannabinoid receptors' density were found during brain development [123].

Alongside these events, there are also other two processes that take place during neurodevelopment, namely synaptic exuberance and pruning. These result in excessive connections followed by the systematic elimination of up to 50% of those connections, as well as naturally occurring cell death of 50 % or more of the neurons within a certain brain region [124]. These processes represent naturally occurring events that take place in developing brain. However, most neuronal cell death occurs prenatally. On the other hand, both cell death in glia populations and exuberant production and pruning of connections occur mostly at a post-natal stage [124]. Cell death, either by apoptosis or necrosis, has been proposed to serve as a mechanism for correcting defects in neuronal production or migration [125]. In fact, cell death has been reported to play an essential role in eliminating cell populations that only have a time-limited function in brain development, such as cells of the MZ or SP, as shown by the high rate of death in these cell populations [126, 127]. Not only this but apoptosis has been found to be an important factor in neurodevelopment due to its ability to facilitate morphogenesis, control cell population, aid proper positioning and spacing and avoid inappropriate targeting [128]. The events leading to neuronal development are schematized in Figure 3.

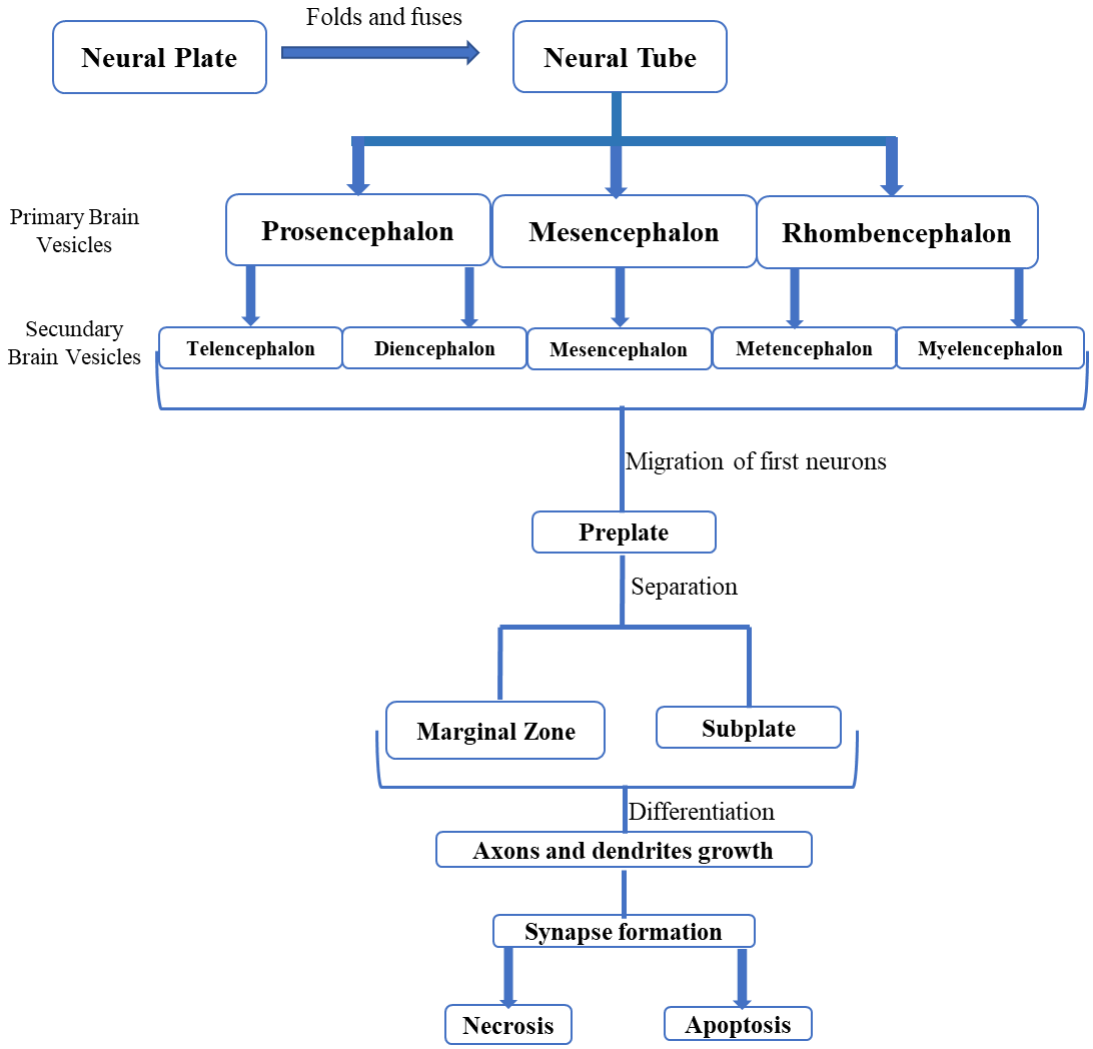


Figure 3- Stages of Neuronal differentiation. The initial Neural Plate folds and fuses to form the Neural Tube. The Neural Tube will divide into the three primary brain vesicles (Prosencephalon, Mesencephalon and Rhombencephalon). The Prosencephalon will subdivide into Telencephalon and Diencephalon, while the Rhombencephalon will subdivide into Metencephalon and Myelencephalon. Following this, neurons will start to migrate and form the preplate, which after being separated forms both the marginal zone and the Subplate. Cells will then start to differentiate by the growth of axons and dendrites which will originate Synapses. The balance of cell poll and other roles will then be maintained through necrosis or apoptosis of neuronal cells.

3. Phytocannabinoids

Phytocannabinoids represent a class of cannabinoids synthesized by plants, specifically by the *Cannabis* genera [129]. Interestingly, parts of these plants (*e.g.* seeds, roots, leaves) have been used for centuries for recreational, religious, warfare and medical purposes (*e.g.* to prevent loss of appetite during chemotherapy), mostly due to their ready availability in nature and relevant biological activities [2, 130, 131]. Nevertheless, the use of cannabis as a recreational drug only soared during the 1960s, particularly in the USA [132]. Due to their high lipophilicity, phytocannabinoids remain in plasma for extended periods, being detected in chronic smokers up to a month of abstinence [133]. One of the most well-studied phytocannabinoids is Δ^9-tetrahydrocannabinol (Δ^9-THC) [24, 134-136]. Δ^9-THC acts mainly as an agonist of the CB_1 receptor, although it also has affinity towards the CB_2 receptor [3]. Upon binding to the CB_1 receptor, Δ^9-THC is able to modulate the release of specific neurotransmitters [137], such as glutamate [78], dopamine [86] and GABA [138], as well as activate ion channels (e.g. Vanilloid Receptor) [139]. After smoking, Δ^9-THC is easily distributed from the lungs into the blood, undergoing oxidative metabolism and originating both the active 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and the inactive metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) [140]. These metabolites and Δ^9-THC are mainly excreted through feces and urine and are usually used as biomarkers for the detection of cannabis use [141]. Although Δ^9-THC metabolism gives place to other active metabolite, the psychotropic effect still associates mainly with Δ^9-THC, due to the reduced affinity towards the CB_1 receptor of 11-OH-THC [142]. Interestingly, the metabolism of Δ^9-THC differs between genders. An almost exclusive metabolization of Δ^9-THC into 11-OH-THC was observed in female rats, while in male rats Δ^9-THC was metabolized into 11-OH-THC and other two metabolites (8a- and 3'-hydroxy-Δ^9-THC) [143].

Other well-studied phytocannabinoids include cannabinol (CBN) and cannabidiol (CBD). The chemical structures of these phytocannabinoids are represented in Table II. CBN has a lower affinity than Δ^9-THC towards the CB_1 receptor with a Ki to CB_1R found to range between 120.2 and 1130 nM [3, 33, 144]. However, CBN is metabolized in the body at a slower rate, compared to Δ^9-THC, thus inducing a much milder, though longer-lasting effect in the body [145]. CBD is not considered to be psychotropic and can actually counter the short- and long-term psychoactive effects of Δ^9-THC by acting as an antagonist of the cannabinoid receptors [36]. In fact, Schubart *et*

al. found that higher concentrations of CBD in smoked products correlated with lesser psychosis-like symptoms [146]. Moreover, Di Forti *et al.* reported that the smoking of cannabis-based products with a lower CBD/THC ratio correlated with earlier psychosis onset as cannabis users showed their first psychosis episode at 28 years old (average) and non-cannabis users only showed their first episode at 31 years old (average) [147]. The metabolism of CBD in the body is similar to that of Δ^9-THC, in the sense that it also undergoes two phases. Phase I occurs in the presence of hepatic enzymes, such as cytochrome P450s: CYP2C9, CYP2C19 and CYP3A4 [145]. Due to the low first-pass effect on smoked CBD, a high percentage of unmodified free CBD is also observed which is then excreted through feces [148]. Phase II is led by UDP-glucuronosyltransferases, originating metabolites that are more hydrophilic, thus being excreted through the urine [149].

Noteworthy, phytocannabinoids and their effects within the human body have already been comprehensively characterized elsewhere [150-152] and will thus not be focused in this review.

Phytocannabinoids have for long been used with medicinal intent and in more recent times these compounds have been isolated and administered according to each situation's need. For example: Sativex® (an oral spray) is comprised of equal parts Δ^9-THC and CBD [153]. This drug is used in the treatment of multiple sclerosis (MS) due to its role in inducing muscle relaxation, which is related to the balance achieved between excitatory and inhibitory neurotransmitters. Ultimately, an increase of intracortical inhibition, a significant reduction of spinal excitability, and improvement in MS associated symptoms after Sativex® administration have been reported [154]. Another example of a phytocannabinoid-based drug is Epidiolex®, which is comprised mainly by CBD. This oral solution is used in the treatment of seizures associated with Lennox-Gastaut syndrome (LGS) or Dravet syndrome and has shown significant reduction of seizure frequency [155, 156]. However, studies on the long-term effects of both these drugs are still missing. Similarly to endocannabinoids, phytocannabinoids also regulate several processes within the CNS. For example, the neuronal-based effects of Δ^9-THC can be divided into four categories [54]: 1) affective (euphoria, happiness); 2) sensory (increased perception of stimuli); 3) somatic (alteration on the perception of falling and floating); 4) cognitive (lack of attention, poor time perception and memory failure). Additionally, other effects that result from Δ^9-THC use include cannabinoid dependence syndrome, disturbance of the ability to organize and integrate complex information and disturbed response time, psychomotor skills and coordination [54].

4. Synthetic cannabinoids

Synthetic cannabinoids (SCs) comprise a diverse group of molecules structurally similar to Δ9-THC that also bind and activate cannabinoid receptors. Noteworthy, the increasing findings demonstrating the biological activity of endo- and phytocannabinoids, as well as their therapeutic potential led to several attempts of synthesizing analogue compounds [157-159]. The first molecule in this group was a synthetic form of Δ9-THC designed in 1965 by Mechoulam, *et al.* [160, 161]. In the early preparations of this molecule, the *D*-isomer was present alongside the *L*-isomer. However, with further studies on Δ9-THC, the *L*-isomer was found to be the active one. A major effort was thus made to improve both the methods of synthesis and purification of such compounds to obtain the active isoform [160]. A synthetic analogue of Δ9-THC is currently marketed under the name of Drabinol® and used in chemotherapy to control nausea and vomiting [162]. Other examples are Rimonabant® (Sanofi-Aventis) and Nabilone (Cesamet®), both used in pain management [6, 163]. In particular, Rimonabant® is a CB_1R antagonist [163] that raised concerns regarding its therapeutic window and due to the fact that the risk of psychiatric disorders in people taking this drug doubled. [164]. As a result, this drug was never distributed in the USA, and while it was being distributed in Europe, it was withdrawn due to its side effects in mood changes (e.g. increased cases of suicidal behavior) [165].

In general, SCs are full agonists of CB_1 and/or CB_2 receptors, contrasting with the partial agonism of Δ9-THC, being more potent (about 100 times) [166]. This higher potency thus results in very strong and longer lasting psychoactive effects. Along with their increased potency and more pronounced psychoactive effects, the easy access to these substances (usually through friends, internet or drugs dealers) [167] and the difficulty in the legislation to keep up with the rapid emergence of these drugs [8, 167, 168] has greatly aroused the interest of drug users in SCs for recreational purposes [167].

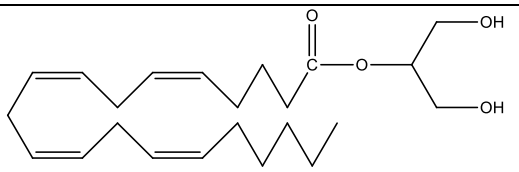
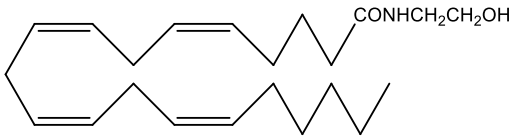
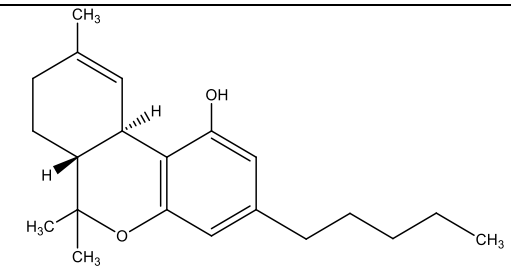
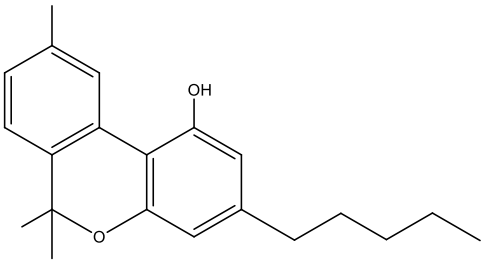
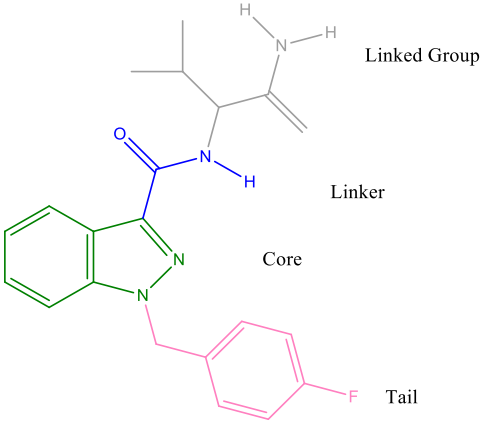
These substances are usually dissolved in an organic solvent (e.g. acetone, ethanol) and sprayed over other materials and substances (*e.g.* herbal extracts, other psychoactive drugs). The mixture is then allowed to dry and marketed in colorful packages or plastic bottles with attractive brand names (e.g. K2, Spice, Black Mamba). However, information like the exact cannabinoid(s) and other substances present in the package, as well as their respective amounts, is usually not specified in the package. Along with a lack of quality control, use of these substances thus becomes a major toxicological safety issue. Usually, SC users smoke the dried material, but these

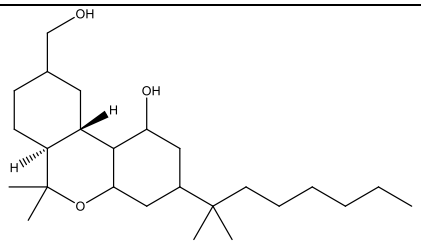
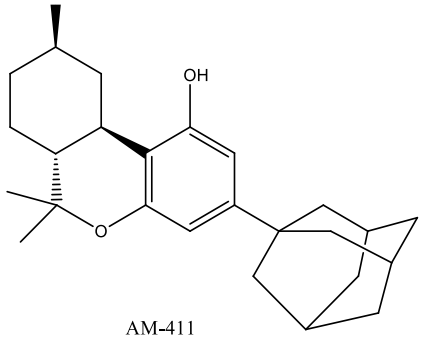
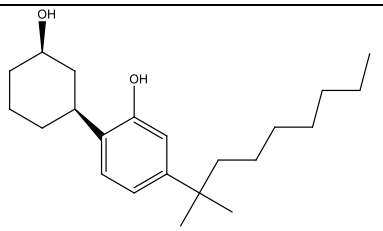
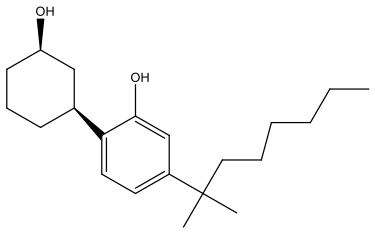
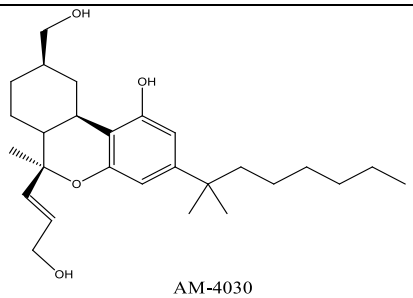
substances can also be sold as liquids for vaporization and inhalation (e.g. e-cigarettes), brewed in tea or added to food (e.g. cakes) [169].

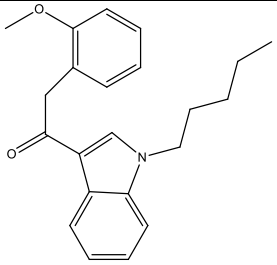
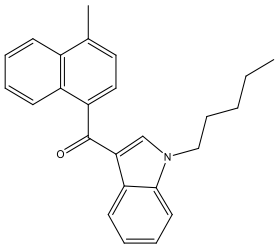
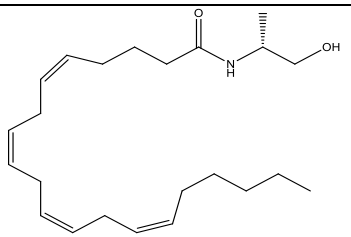
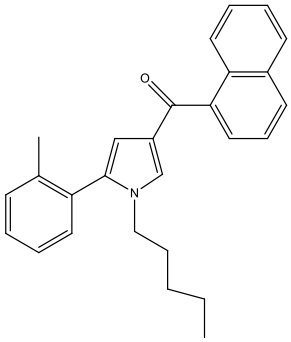
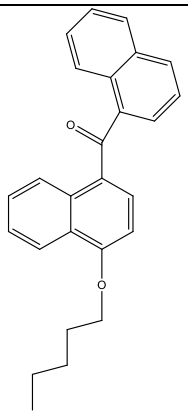
SCs comply with a general structure based on four major groups: the core, which connects to the secondary group through a linker, and the tail. There are several chemical groups that can fill each position, anticipating a high number of possible combinations [170]. Changing one of the components results in modifications in the overall compound's chemistry and reactivity, thus altering its affinity towards cannabinoid receptors and the metabolism pathway for its excretion [170-172]. As a result, several new SCs are identified and reported by the European Monitoring Center for Drugs and Drug Addiction (EMCDDA). In fact, since 2008 there have been 179 SCs detected, with 10 being detected just in 2017 [8]. Moreover, in 2016 SCs were the most seized new psychoactive substance with more than 32 000 seizures reported [8].

There is not a consensus regarding the classification of these substances, as it varies among different authors, although within defined classes [173, 174]. Nevertheless, SCs may be broadly classified into: classical cannabinoids (*e.g.* HU-210); nonclassical cannabinoids (*e.g.* CP-47,497); hybrid cannabinoids (*e.g.* AM-4030); Aminoalkylindoles, which further divide in naphtoylindoles (*e.g.* JWH-122), phenylacetylindoles (*e.g.* JWH-250), naphthylmethylindoles (*e.g.* JWH-175) and Benzoylindoles (*e.g.* AM-694); eicosanoids (*e.g.* methanandamide); additionally, some synthetic cannabinoids display a chemical structure that does not fit within any of the above (*e.g.* JWH-307) [173, 174]. These classes and representative compounds are compiled in Table II.

Table II- Representative chemical structures of different types of cannabinoids, including the distinct broadly-defined classes of synthetic cannabinoids. Representative molecules of each class of synthetic cannabinoids are also depicted.

Classes	Representative Compounds
Endocannabinoids[56, 175]	 2-arachidonyl-glycerol  Anandamide
Phytocannabinoids [176]	 THC  Cannabidiol
Synthetic Cannabinoids **General structure** (AB-FUBINACA is shown as a representative structure) [177]	 Linked Group Linker Core Tail

Classical Cannabinoids (178, 179)	 HU-210  AM-411
Nonclassical Cannabinoids (169)	 CP-47,497-C8  CP-47,497
Hybrid Cannabinoids(180)	 AM-4030

Aminoalkylindoles (169, 181)	 JWH-250  JWH-122
Eicosanoids (182)	 Methanandamide
Undetermined/Other (169, 183)	 JWH 307  CRA-13

An abbreviation can be found in the nomenclature of most of these compounds. This usually refers to their discovery. For example, HU refers to Hebrew University, AM to Alexandros Makriyannis and JWH to John W. Huffman.

4.1 Pharmacodynamics and pharmacokinetics of synthetic cannabinoids

Synthetic cannabinoids have a higher affinity towards the endogenous cannabinoid receptors, when comparing to phytocannabinoids, such as Δ^9-THC [184, 185], being thus able to modulate the endocannabinoid system.

Smoking represents the preferred method of SC use. Inhaled active compounds quickly spread throughout the body and strongly bind to serum proteins. For example, only about 5 % of WIN 55,212-2 is found in free-state after smoking [172].

SCs are metabolized in two phases. Phase I represents the oxidative phase, having been observed alterations such as oxidation, dealkylation, ester hydrolysis, dehydrogenation, mono- and dihydroxylation to the parent molecule [171, 186]. Additionally, dehydrogenation, as well as mono- and dihydroxylation at different positions of the molecule resulting from the ester hydrolysis may also occur, further resulting in various metabolites. Noteworthy, these metabolites may also undergo Phase I metabolism [186].

The P450 cytochrome enzyme superfamily is the main responsible for these reactions during the first phase of the metabolism. primarily resorting to CYP2C9, CYP1A2 and CYP3A4 in various synthetic cannabinoids [187-189].

Phase II is led by enzymes such as uridine 5'-diphosphoglucuronosyltransferase (UGT), which is generally associated with detoxification resulting from the production of glucuronides [134]. Depending on the cannabinoid, these phases will have a different impact on its metabolism and excretion. Both Phase I and II processes are schematized in Figure 4.

The structural differences of synthetic cannabinoids not only determine their binding affinities towards CB_1 and CB_2 receptors, but also affect the way they are metabolized [190]. For example, primary metabolization of JWH-018 by CYP2C9 and CYP1A2 during Phase I metabolism may originate metabolites with different affinities towards the CB_1R. Interestingly, one of such JWH-018-derived metabolites may act as an antagonist towards that same receptor [187, 191].

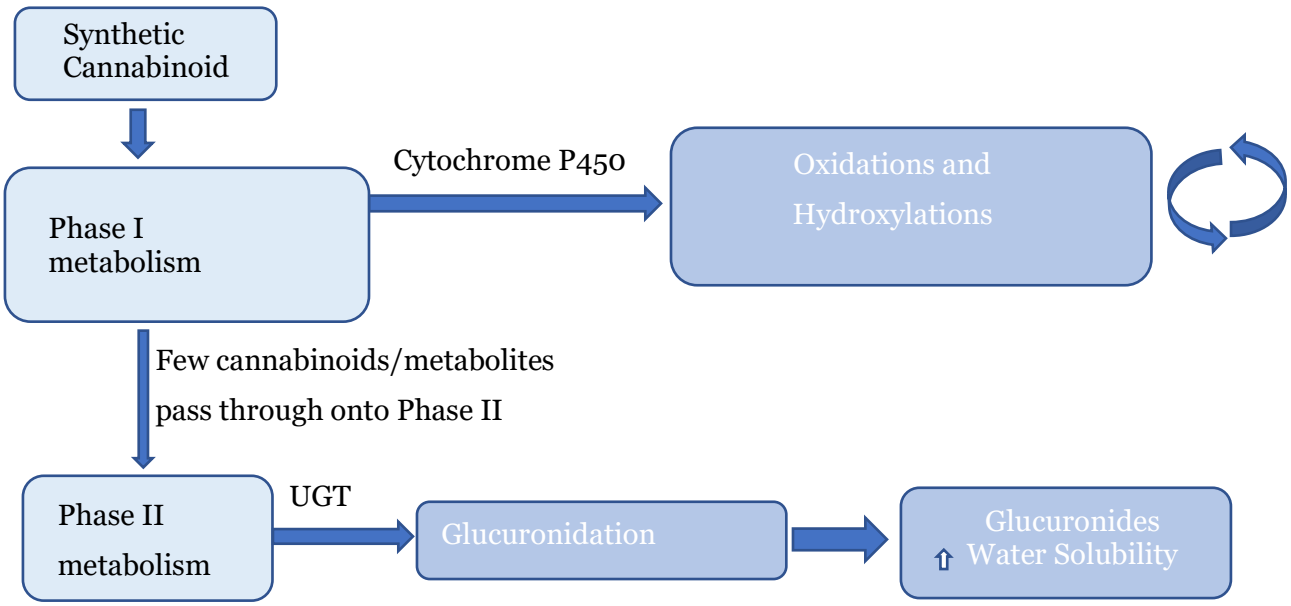
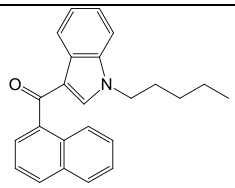
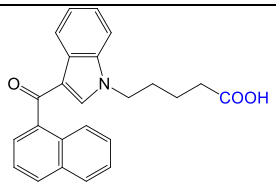
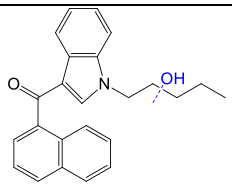
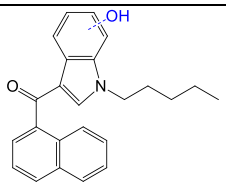
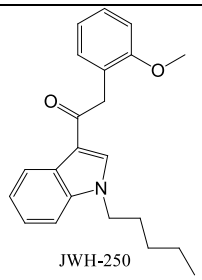
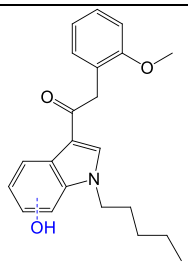
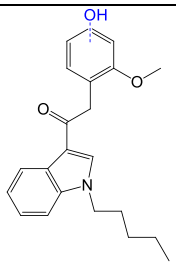
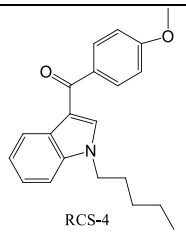
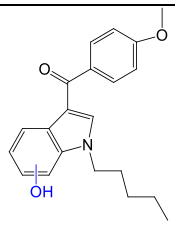
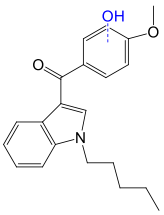


Figure 4-Phases I and II of the metabolism of synthetic cannabinoids in the human body. Phase I is undergone by the activity of the cytochrome P450, mainly by CYP2C9, CYP1A2 and CYP3A4. The metabolites formed from these reactions can also suffer Phase I metabolism and originate new metabolites. Phase II metabolism is mainly done by the activity of UGT in order to create glucuronides which have higher mass and higher water solubility than the original molecules, facilitating their excretion. However cannabinoids mainly undergo Phase I metabolism (175, 192).

Similarly to phytocannabinoids, each SC can undergo several metabolic processes, which may result in a very high number of metabolites that can be found in the body after a drug screening procedure [173]. Several publications have already reported the SC-derived metabolites present in the body after the use of known SCs, as well as the methodologies required for their detection [193, 194]. These studies found that some of the metabolites are similar among different categories of synthetic cannabinoids [194]. Franz *et al.* found thirty metabolites originating from MDMB-CHMICA's Phase I metabolism in urine scans of recurrent SC users [186]. Similarly, Kevin *et al.* revealed that CUMYL-PICA and 5F-CUMYL-PICA undergo extensive oxidative metabolism followed by glucuronidation, which also resulted in a significant number of metabolites for each SC [171]. Detection of specific SC represents a major challenge since these substances are rapidly metabolized, hindering the identification of the parent molecule [186]. Instead, most analyses aim at detecting the SCs' metabolites, since some of them can be found up to 10 days in urine analysis [195]. A few examples of synthetic cannabinoids and their metabolites are represented in Table III.

Table III- Synthetic cannabinoids and some of their urinary metabolites (193).

Cannabinoid	Metabolites
 JWH-018	 A  B  C
 JWH-250	 D  E
 RCS-4	 F  G

In comparison to their original molecule these metabolites suffered carboxylation at N-alkyl chain (A), monohydroxylation at N-alkyl chain(B), monohydroxylation at indole moiety(C), monohydroxylation at the indole moiety (D,F) and monohydroxylation at the phenyl moiety (E,G).

4.2 Legal status

Studies on cannabis and its potential adverse effects resulted in its legal control in 1937 under the Marihuana Tax Act law, which stated that anyone using cannabis would be penalized with a monetary value or imprisonment [196].

As SCs are concerned, mixtures of these substances have been freely sold in "smart shops" or over the internet for several years, without any legal restrictions in many countries. Although it is currently illegal to sell, buy or possess most of these substances, SC manufacturers try to overcome legislation by developing SCs with new chemical formulas. However, there has been a growing effort to revert this situation with the implementation of regulations that are not specifically directed at one single product. At the center of these efforts are organizations such as the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) that monitor and search for information regarding drugs of abuse, such as new detected compounds, overall use per country and age of consumption [8]. Some European countries adopted legislation that cover various substances, their analogues and metabolites, while simultaneously penalizing producers and retailers of new psychoactive substances such as SCs [168]. The most efficient way to regulate all synthetic cannabinoids is through generic legislation which enables simultaneous control over this large group without the need to list them individually. This includes substances that have not yet been reported, potentially preventing their emergence, which is key due to the easy synthesis of new SCs [197]. Moreover, this new legislation is based on the three-step approach against new psychoactive substances that relies on an early warning, followed by risk assessment and application of adequate control measures. To do this, the European Union has strengthened data collection and assessment procedures to assure faster responses [198, 199]. Some countries have even found different approaches, specifically, Portugal has approved a law which states that it is illegal to produce, import, export, publicize, distribute, sell, possess or make available NPS, unless for industrial or pharmaceutical purposes, or authorized by the national regulatory agency (INFARMED) [200] .

Similarly to Europe, the US National Institute on Drug Abuse (NIDA) regulates and proposes legislation to control drug use. Moreover, the US have adopted a more specific approach in the control of NPS, having a list of every substance that is illegal [201, 202]. Most SCs are classified as Schedule I (Class I) substances in the USA by the DEA (Drug Enforcement Agency), meaning that they are illegal due to their high abuse potential, severe safety concerns and no medical use [203].

Other countries have also envisaged strong efforts to regulate SBCs. For example, in Japan, the Pharmaceutical Affairs Law was amended in 2006 in order to establish a new category: "Designated Substances". Noteworthy, by July of 2012 there were 23 synthetic cannabinoids listed in this category [204].

4.3 Health effects of SC use

As stated above, increasing SC use has become a major public health concern. To date, information regarding the toxicological effects of SCs remains scarce. Considering that SCs can produce stronger effects, compared to Δ^9-THC, and that the chemical composition of most SC packages is often unknown, their resulting health effects become unpredictable and potentially harmful.

As full agonists of the same receptors as Δ^9-THC, SCs induce a plethora of symptoms that resemble, but are more intense and longer-lasting, than those induced by that phytocannabinoids [205]. SC users usually look for an elevated mood sensation, relaxation or even increase of appetite. However, several clinical case reports have described a substantial amount of adverse effects, including altered perception (awareness of the surrounding environment), psychotic symptoms (e.g. high anxiety, paranoia, hallucinations), suicidal thoughts, total memory loss or seizures [206-208].

Additionally, there are other physiological symptoms that can arise from the use of synthetic cannabinoids, which are not restricted to the nervous system. These include muscle and thoracic pain, extremely high blood pressure (high risk of stroke), panic attacks, rapid heart rate (possible cause of heart attack), kidney failure and loss of consciousness [205, 208]. Most of these symptoms usually disappear within 4-14 hours. However, some of these effects, such as acute psychosis, may take longer to dissipate and last for several days [205, 207]. Moreover, there have been various cases of deaths directly resulting from the consumption of SCs and that are usually linked to myocardial complications [209-211].

SCs' consumption has not only been correlated with short term effects, but they have also been found to exacerbate psychotic symptoms that were previously stable and even trigger new onset of psychosis [212]. There have been numerous animal trials assessing the effects induced by SC exposure. Hill, *et al.* (2006) [213] noted higher corticosterone levels in rat serum after chronic intraperitoneal exposure to 5-100µg/kg of HU-210, which in turn resulted in increased stress responsivity in adult animals. Furthermore, Lewis, *et al.* (2012)[214] noted that rats starting their sexual maturity, with chronic intraperitoneal exposure to 50µg/kg of HU-210 showed lower kidney and body

weight to the control group. in addition, they also showed a 46 % decrease in sperm production after 7 weeks of exposure to the SC.

Another problem associated with the use of SCs is the development of tolerance and the possibility of withdrawal symptoms. With the increasing use of these synthetic substances, higher doses become required to attain the desired psychoactive effects, thus increasing the toxicological risks. Noteworthy, this tolerance is mainly associated with cannabinoids that interact with the CB_1 receptor. Interestingly, interaction with the CB_2 receptor may originate some of the same CB_1 desired effects, such as pain attenuation, but does not develop tolerance to these substances [215, 216].

Withdrawal is also an issue related with cannabinoids use. Most common SCs' withdrawal symptoms include anxiety, aggressiveness, abdominal pain and sleep disorders [217]. Indeed, Nacca *et al.* reported anxiety and tachycardia in patients from two case studies on SC withdrawal [218]. Noteworthy, withdrawal symptoms improved in one of the patients involved in such studies following the administration of benzodiazepines and in the other after the administration of quetiapine (an anti-psychotic drug) but failed to improve with the administration of benzodiazepines and only improved after quetiapine administration [218]. These results evidence that the uncertainty regarding the composition of SC mixtures poses a major challenge to the application of proper therapeutics.

Synthetic analogues of phytocannabinoids are already being used in various therapies. In fact, Marinol® (also known as Dronabinol®), a synthetic version of Δ^9-THC, has been used in the USA for a long time. This drug is usually administered to patients with Acquired Immune Deficiency Syndrome (AIDS) to treat AIDS-related anorexia and to alleviate nausea and vomiting in cancer patients undergoing chemotherapy-based treatments [7]. Another SC being used in clinical settings is Cesamet® (also known as Nabilone), which is also used in the attenuation of nausea and vomiting in cancer patients undergoing chemotherapy [219]. Both of these drugs are administered orally, in contrast with the usual smoked consumption of SCs [7, 219].

4.4 Cannabinoid use in pregnancy

A recently published US survey reported that 3.9 % of pregnant women and 7.6 % of nonpregnant women in reproductive age reported the consumption of cannabis in the previous month [220]. This is particularly worrisome since cannabinoids have the ability to cross the placental barrier and reach the fetus, causing an imbalance in neurotransmitters release that can lead to the defective development of the child's brain [221]. Additionally, cannabinoids can be secreted into the maternal milk during the post-

birth stage [222]. Moreover, a sustained prenatal stimulation of the CB_1 receptor by Δ^9-THC has been reported to induce altered migration of interneurons in early postnatal hippocampus, which has the potential to impair cognitive functions [223]. Further studies have already shown that THC crosses the placenta and directly affects the exposed fetus, causing impaired neurodevelopment in the offspring that may persist into adolescence [224, 225].

Pre-natal use of cannabis has been further associated with low birth weight, preterm labor and admission in neonatal intensive care units [226]. Moreover, cannabis use during fetal development has also been linked with adverse effects on the growth of fetal and adolescent brains [227], reduced attention, and behavioral problems [228].

Although various studies have been made regarding cannabis use during pregnancy, only few of them address the problem of using synthetic cannabinoids during a pre-natal stage. Mereu *et al.* (2003) [229] reported that after a pre-natal exposure to WIN 55,212-2, neonatal rats exhibited lack of memory retention and hyperactivity. Gilber *et al* (2016) [230] observed dose-dependent ocular malformations after pre-natal exposure to the SC CP-55,940 in mice at concentrations ranging from 0.0625 to 2.0 mg/kg. Additionally, Del Arco *et al* (2000) [231] found that after pre-natal exposure of Wistar Rats to a 25 μg/kg dose of HU-210 there was a reduction in T-helper subpopulation in the spleen.

4.5 Modulation of neuronal development by synthetic cannabinoids

To date, studies regarding the effects of SCs in the developing nervous system remain scarce. Psychoyos *et al.* (2008) [232] found that chick embryos exposed to a concentration range of 0.035 mg/ml to 0.35 mg/ml of O-252 showed malformations in the brain. These defects resulted from the down-regulation of Pax6 expression within the nascent neural tube, which hindered the neural tube's ability to close along the anteroposterior axis of the embryo. Jiang *et al.* (2005) [233] showed that chronic treatment of cultured embryonic hippocampal neural stem cells with HU-210 promoted their proliferation, but not differentiation, by sequentially activating CB_1 receptors, Gi/o proteins and ERK signaling. The SC HU-308 was also found to stimulate the neurogenesis of rat hippocampal HiB5 NP cells, through the regulation of CB_2R and this receptor-mediated activation of the PI3K/Akt/mTOR1 pathway [234].

A summary of the neurodevelopment-related mechanisms found to be modulated by SCs is outlined in Table IV. Nevertheless, it should be noted that the high heterogeneity of the models used in these studies present an important limitation to withdraw general conclusions regarding how SCs affect neurodevelopment. Indeed, the models used comprise many cell types (including proliferating postmitotic neurons and glia), and cells are often at different stages of differentiation [235, 236].

Table IV- Neurodevelopment alterations observed regarding different synthetic cannabinoids in different models.

Synthetic Cannabinoid	Ki ± SEM (nM)		Model	Findings	Altered Pathways	References
	CB1	CB2				
O-2545	1.5 nM	0.32 nM	Pre-neurogenesis chick embryo	-Failure of the presomitic mesoderm to migrate from the primitive streak -Abnormal neural plate formation -Abnormal Neural Tube closing	Pax-6 Downregulation	Psychoyos *et al.* 2008[232]
HU-210	0.061 nM	0.52 nM	Neuro-2A cells	-Neurite outgrowth	Activation of Rap1 through the CB1R mediated degradation of Rap1GAPII	Jordan, *et al.* 2005[237] Howlett, *et al.*1990 [238]
WIN 55,212-2	1.9 nM	-	Rat hippocampal neurons	-Block in the formation of new synapses	CB1R selective inhibition of cAMP formation	Kim, *et al.* 2001[239] Kuster, *et al.* 1993[240]
WIN 55,212-2	1.9 nM	-	40- and 80-day Rat offspring	-Memory impairment	Reduction in hippocampal K+-mediated glutamate release	Mereu, et al. 2003 [229]

5. Aims of the experimental work

The alarming rate at which new SCs are created and distributed worldwide represents a major public health concern, in view of the scarce information regarding their toxicological profiles and taking into account the various reported cases of severe intoxications and deaths directly associated with SC use. The use of SCs by pregnant women or women of childbearing potential represents a particular concern due to the possible onset of neurodevelopmental pathologies (*e.g.* schizophrenia, autism spectrum disorders) in the offspring. Assessing the ability of SCs to modulate neurodevelopment-related processes thus assumes critical relevance. In this sense, this work comprised two main goals:

1) Evaluation of the toxicity of five commonly reported synthetic cannabinoids (5F-PB22, JWH-122, MDMB-CHMICA, THJ-2201 and XLR-11) in a neuroblastoma cell line (NG108-15). This cell line represents a well-characterized model neuritogenesis from a blastoid state under stressed conditions, commonly used to study neuronal development and differentiation. Specifically, different toxicological parameters were analyzed, including cell viability and proliferation, mitochondrial membrane potential and intracellular energy levels, were also analyzed.

2) Assessment of the role played by SCs on *in vitro* neuronal differentiation. NG108-15 cells were exposed at *in vivo* relevant concentrations (< 1μM) of the SCs. In particular, differentiation ratios (assessed by analyzing the number of newly-formed neurites per total number of cells) and the expression of specific neuronal differentiation-related proteins (determined using western-blot) were analyzed. The potential involvement of CB_1 receptor in these SC-mediated processes was ascertained in the presence of a specific CB_1R inverse agonist.

6. Materials and Methods

6.1 Chemicals

The synthetic cannabinoids 5F-PB22 (1-pentyfluoro-1H-indole-3-carboxylic acid 8-quinolinyl ester), JWH-122 ((4-methyl-1-naphthyl)-(1-pentylindol-3-yl)methanone), THJ-2201 ([1-(5-Fluoropentyl)-1H-indazol-3-yl](1-naphthyl)methanone) and XLR-11 ((1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone) were kindly supplied by Dr. Ana Santos Carvalho (Center for Neurosciences and Cell Biology, University of Coimbra, Portugal). MDMB-CHMICA (Methyl (2S)-2-{[1-(cyclohexylmethyl)-1H-indol-3-yl] formamido}-3,3-dimethylbutanoate) was a kind gift from TicTac Communications Ltd. (UK). The chemical structures of these SCs are depicted in Figure 5. SR141716A, a specific antagonist for the CB_1 receptor, was purchased from Tocris Bioscience (Bristol, UK).

Stock solutions of the synthetic cannabinoids and SR141716A were prepared in dimethylsulfoxide (DMSO). These stock solutions were sequentially diluted in Hank's balanced salt solution (HBSS) before cell exposure to attain a final DMSO concentration below 0.5%. This concentration has been previously described as being non-toxic to NG108-15 cells and below the minimum threshold required to promote NG108-15 differentiation, at low serum conditions [241].

Heat-inactivated fetal bovine serum (FBS), antibiotic (10 000 U/ml penicillin, 10 000 µg/ml streptomycin), 0.25% trypsin/EDTA, Phosphate buffered saline (PBS) and Hank's balanced salt solution (HBSS) were acquired from Gibco Laboratories (Lenexa, KS, USA).

All other reagents used in this work were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise.

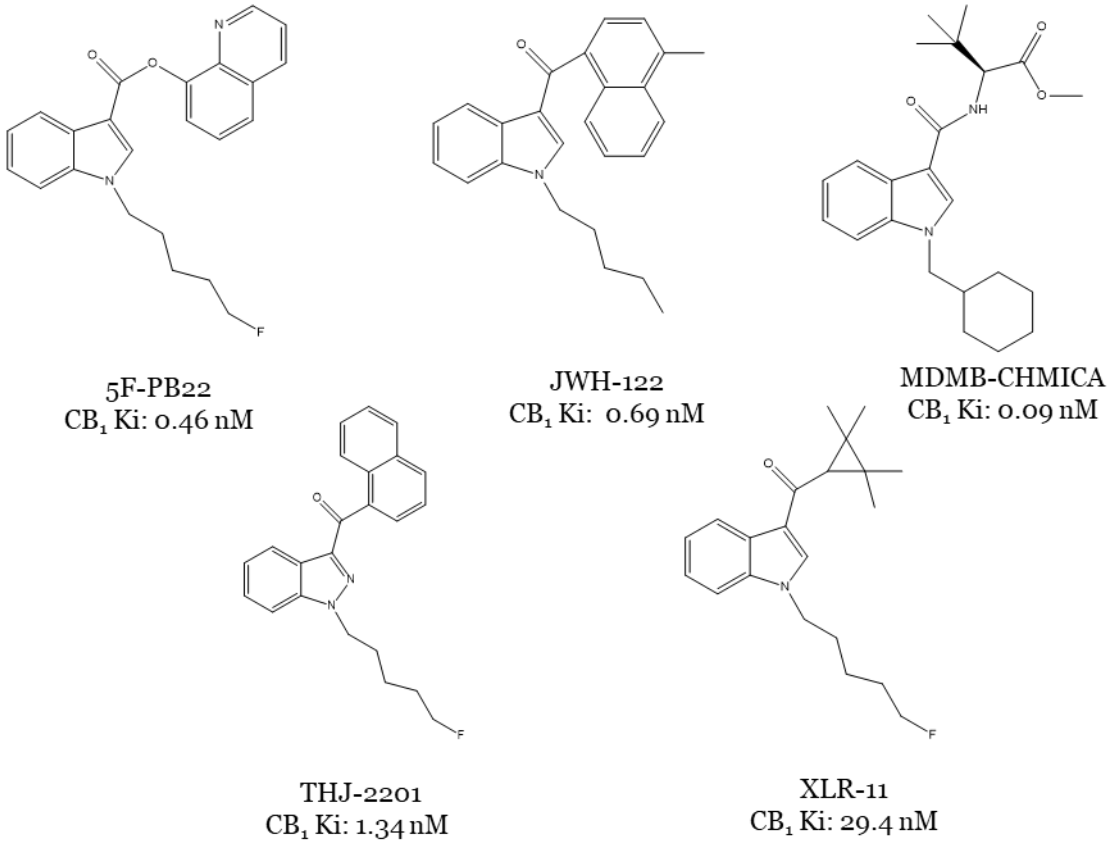


Figure 5- Chemical structures of the Synthetic Cannabinoids used in this study and respective CB1 receptor affinity. [166, 242-244]

6.2 Cell Culture

In this work we used the mouse neuroblastoma clone N18TG-2 x Rat glioma clone C6 BV-1 hybrid cell line NG108-15 as the selected cell model. This cell line represents a well-characterized model of neuritogenesis and synapse formation from a blastoid state under stressed conditions (e.g. nutrient limitation). Differentiated NG108-15 cells present a wide range of voltage-dependent membrane currents, release acetylcholine and express choline acetyltransferase activity, as well as several cell membrane receptors for distinct neurotransmitters. These cells allow a prompt neurotoxicological evaluation and are preferred over primary cells as a model for neuronal differentiation studies, since they: 1) allow assessing the adhesive and morphological alterations that occur during that process; 2) grow more rapidly in culture medium compared with primary cultures; 3) show an homogeneous cell type identity (guaranteeing that the results are not influenced by other cells present in culture) and 4) display synchronous differentiation in culture dishes. They endogenously express CB1 receptor, the CBR

mainly expressed in the brain, representing a further advantage to particularly study SCs neurotoxicity [245-247].

The NG108-15 cell line was acquired from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), and was routinely cultured in 75 cm^2 flasks with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and a 100 U/ml penicillin, 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Once these plates reached 70-80% confluence they were sub-cultured by trypsinization with a 0.25% trypsin/EDTA solution.

6.3 Cell Viability

6.3.1 MTT reduction assay

The cells' metabolic activity was analyzed through the MTT reduction assay, as previously described by Silva *et al.* (2018) [248]. This method relies on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into water-insoluble formazan crystals, in the presence of NAD(P)H-dependent cellular oxidoreductase enzymes [249].

Briefly, NG108-15 cells were seeded at 1.5×10^4 cells/ml density in 96-well plates and exposed to the various SCs at a concentration range of 1 pM-100 µM. Following 24 h of exposure to the SCs, cell culture medium was replaced by 100 µl of a 0.5 mg/ml MTT solution and the plate was further incubated, protected from light, for 90 minutes, at 37°C and 5 % CO_2, to allow the reduction of MTT. After this period, the MTT solution was discarded and formazan crystals were dissolved with 100 µl of DMSO per well. The plate was placed in an orbital shaker for 10 minutes to assure complete dissolution of the crystals and metabolic activity was then measured spectrophotometrically at 550 nm in a Bio-Tek PowerWaveX (Bio-Tek, Winooski, VT, USA) microplate reader. MTT assay was also used to determine cell metabolic activity on differentiated cells, however these cells were seeded at 5×10^4 cells/ml.

Cells were also incubated in the presence of a positive (5 % DMSO) and a negative control (0.5 % DMSO). Results were then normalized by total protein, determined by Lowry protein quantification assay per well and expressed as the percentage of metabolic activity relative to the negative control.

6.3.2 Neutral red inclusion

Neutral red dye permeates the plasma membranes of viable cells and accumulates in lysosomes via nonionic diffusion. This lysosomal uptake occurs due to the presence of a proton gradient between lysosomes and the cytoplasm. The disruption of this gradient, which may result from cell damage or death, hampers neutral red retention in the lysosomes and the dye is eventually removed during the washing steps of this protocol. As a result, it is possible to establish a correlation between the neutral red signal and cell viability [250].

Neutral red uptake was determined as previously described by Arbo *et al.* (2016) [251]. Cells were seeded at the same cell density and exposed to SCs in the same conditions as the ones described for MTT assay. Negative (0.5 % DMSO) and positive (5 % DMSO) controls were also included. Following 24 h incubation, cell culture medium was replaced by 100 µl of a 50 µg/ml neutral red solution prepared in fresh medium and the cells were further incubated for 1 h, at 37º C, 5 % CO_2. Cells were then lysed using a 50 % ethanol/1 % glacial acetic acid solution to extract the dye retained in the lysosomes. Complete dye dissolution was attained by placing the plates on an orbital shaker for 15 minutes (50 rpm) and absorbance was then read at 540 nm in a microplate reader. Results were then expressed as the percentage of neutral red uptake by lysosomes relative to the negative control.

6.3.3 Lactate dehydrogenase (LDH) release

Cell membrane integrity was determined by measuring the release of lactate dehydrogenase (LDH), a membrane leakage marker, into the extracellular medium, as previously described [252]. NG108-15 cells were seeded at the same cell density, and exposed to SC conditions, as reported for MTT assay. A negative (0.5% DMSO) and positive controls (5% DMSO) were also considered. After a 24 h incubation, cell culture medium was collected to quantify the extracellular LDH. Cells were then lysed with 10 mM HEPES (pH 7.4) supplemented with 0.01% Triton X-100 and frozen at −20°C for later quantification of intracellular LDH. Debris from both extra- and intracellular samples were removed by centrifugation at 9 400 *g*, for 10 min in a Heraeus Biofuge Fresco centrifuge (Hanau, Germany). The enzyme's activity was assessed spectrophotometrically in a Bio-Tek PowerWaveX (Bio-Tek, Winooski, VT, USA) microplate reader at 340 nm, by following the rate of conversion of 0.28 mM reduced nicotinamide adenine nucleotide (NADH) into oxidized nicotinamide adenine

nucleotide (NAD+), for 5 min, using 0.32 mM pyruvate (prepared in phosphate buffer, pH 7.4) as substrate. Results were then expressed as the percentage of LDH released relatively to the total (intra + extracellular) LDH activity.

6.4 Sulforhodamine B (SRB) protein binding assay

The effects of SCs on cell proliferation were assessed by measuring the total cellular protein content by the sulforhodamine B (SRB) protein staining assay, according to Silva et al [253] with slight modifications. SRB binds to basic amino acids of cellular proteins and its colorimetric evaluation provides an estimate of total protein mass, which is related to cell number. Evaluation of SRB staining over time provides a reliable indication of cells proliferation [254].

NG108-15 cells were seeded at 3.4×10^4 cells/ml in 24-well plates and incubated in the presence or absence (negative control) of the SCs at concentrations between 1 pM and 1 nM during 24, 48 and 72 h. At each time point, cell culture medium was discarded, cells were fixated in 1 mL of 1 % methanol in acetic acid solution and the plates stored at -80 ºC for later (up to a week) SRB staining quantification.

After thawing, the fixation solution was removed and the plates were allowed to dry at 37 ºC for 15 minutes. Then, 250 µl of an 0.5 % SRB solution in 1 % acetic acid was added to each well and the plates further incubated at 37 ºC for 1.5 h, protected from light. At the end of this period, the plates were thoroughly washed with 1 % acetic acid solution to remove the excess of SRB solution. The plates were allowed to dry in an oven at 37 ºC and 1 mL of a 10 mM Tris solution was then added to each well to dissolve the bound SRB. Two hundred microliters were transferred from each well onto a 96 well plate and the absorbance read at 540 nm in Bio-Tek PowerWaveX (Bio-Tek, Winooski, VT, USA) microplate reader, using a 10 mM Tris solution as blank. Results were then expressed as percentage of SRB binding relatively to the negative control.

6.5 Mitochondrial integrity

Cells' mitochondrial integrity was assessed by measuring the electrophoretical accumulation of the positively-charged tetramethyl rhodamine ethyl ester (TMRE) dye in active mitochondria. TMRE accumulates in mitochondria proportionally to the mitochondrial membrane potential ($\Delta\Psi m$), due to their relative negative charge [255]. The TMRE assay was prepared by seeding the cells at an 8×10^4 cells/ml density in 96-well plates. Cells were incubated for 24h with the SCs at concentrations ranging from 1 pM to 1 µM. After this period, cell culture medium was removed, the wells were washed twice with HBSS and the cells were incubated with 100 µl of a 2 µM TMRE solution (prepared in cell culture medium) for 30 minutes, at 37ºC, 5 % CO_2. The TMRE solution was then discarded by aspiration and the cells rinsed twice with 0.2 % BSA in HBSS. Fluorescence was read in a microplate reader (FLUOstar Optima, BMG Labtech GmbH) using the following settings: 544 nm excitation / 590 nm emission. Fifty µM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was used to confirm the assays was properly functioning. FCCP is an ionophore that uncouples oxidative phosphorylation, eliminating mitochondrial membrane potential and reducing TMRE staining. Results were normalized by total protein, determined by Lowry protein quantification method, per well and then expressed as the percentage of mitochondrial TMRE inclusion comparing to the negative control.

6.6 Intracellular ATP levels

Intracellular net levels of adenosine 5'-Trifosfate (ATP) were determined according to a previously described procedure [248]. This method is based on the emission of bioluminescence derived from the luciferase-catalyzed reaction between luciferin and intracellular ATP. The intensity of bioluminescence is proportional to the ATP levels in the sample [256].

Briefly, cells were seeded at a 1×10^5 cells/ml density in 24-well plates, incubated with SCs for 24h (in a concentration range of 1 pM-1 nM) and washed with HBSS. Following cell exposure to the SCs, cells were precipitated with 200 µl of 5 % perchloric acid and further incubated for 20 min at 4 ºC. Cells were then scrapped and collected into Eppendorf tubes, which were then centrifuged at 6000 *g* for 5 min at 4 ºC. The supernatants were collected into new 1.5 ml tubes and neutralized with 400 µl of 0.76 M $KHCO_3$, while the pellets were resuspended in 0.3 M NaOH and used to determine

the total amount of protein through the Lowry method. The solutions containing the neutralized supernatants were mixed by vortexing and further centrifuged for 1 min at 9400 *g*, at 4ºC. The reaction was then initiated by mixing 75 µl of each supernatant with 75 µl of luciferin-luciferase reagent at a final luciferase concentration of 3 000 000 U/ml in a 50 mM glycine, 10 mM $MgSO_4$, 1 mM Tris, 0.55 mM EDTA and 1 % BSA buffer, at a 7.6 pH.

ATP levels were determined by interpolation from an ATP standard calibration curve, normalized by the total protein amount and expressed in percentage compared to the negative control.

6.7 Neuronal Differentiation

Differentiation of NG108-15 cells was induced according to a previously described procedure [257] with slight modifications. Briefly, cells were plated in 96-well plates at a density of 1.5×10^4 cells/ml and allowed to adhere overnight. Differentiation into mature neurons was then induced by replacing the maintenance culture medium (MM) by DMEM supplemented with 1 % FBS and adding 30 µM Forskolin and 10 µM Retinoic Acid as differentiation factors. This is hereafter referred to as Differentiation Medium (DM).

SCs were added at concentrations ranging from 1 pM to 1 nM either once, right after medium replacement (day 0), or three times (every 24 h for up to 72 h). To analyze the role of CB_1R, 500 nM SR141717, a specific CB1R antagonist, was added 20 min prior to SCs exposure, according to the procedure described by Silva et al. (2018) [248]. A negative control (cells maintained in DM, in the presence of 0.5 % DMSO) and a condition in which NG108-15 cells were maintained in MM after day 0 (thus promoting regular cell growth) were also considered. The latter condition allowed further checking the assay's viability, since cells in this condition continued to proliferate, showing none or reduced differentiation.

After 72 h of incubation (day 3), neurite outgrowth in each condition was imaged using phase contrast with the Lionheart™ FX Automated Microscope. Neurite outgrowth and cell counting was performed using ImageJ software and differentiation ratios were calculated as the number of neurites longer than 20 µm per total number of cells per well [247].

6.8 Western-Blot

6.8.1 Total protein extraction

The expression of specific proteins associated with neuronal differentiation was analyzed by Western Blot in total protein extracts of NG108-15 cells treated in the presence or absence of SCs. Cell differentiation and SC treatment were performed at a density of 1.5×10^5 cells/ml in 6-well plates, in the same conditions described for the differentiation assay (section 6.7.). Following the 72 h incubation, cell culture medium was collected and cells were scrapped in the presence of HBSS, using a cell scraper, and collected into 15 ml tubes. Cell suspensions were centrifuged at 1000 *g* for 5 min and supernatants were discarded. The wells were further rinsed with 1 mL HBSS and the centrifugation step was repeated. Supernatants were once again discarded and the cells were resuspended in 100 µl of collecting buffer (20 mM HEPES, 250 mM Sucrose, 10 mM KCl, 2 mM $MgCL_2$, 1 mM EDTA, pH 7.5) supplemented with 2 mM dithiothreitol (DTT) and 100 µM phenylmethylsulfonyl fluoride (PMSF). The pellets were then disrupted by sonication with three pulses of 30 seconds intercalated with 30 seconds on ice. The samples were then stored at -80ºC until used. Quantification of total protein in the cells extracts was determined using the Bradford assay.

6.8.2 Western-Blot analysis

The expression of specific proteins associated with neuronal differentiation, was analyzed by Western Blot in the total protein extracts of NG108-15 cells treated in the presence or absence of SCs. Briefly, samples containing 40 µg of protein were diluted (1:3 v/v) in 4x SDS Sample Buffer (0.25 M Tris-HCl, 50 % glycerol, 10 % SDS, 0.2 M DTT and 0.001% Bromophenol Blue) and denatured at 90 ºC for 3 minutes. Samples were separated by electrophoresis in 10% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, PA, USA).

Membranes were blocked in 5 % skim milk in TPBS (0.05% Tween 20 in PBS) for 2 h at room temperature, in an orbital shaker. The membranes were then washed three times, for 10 min each, with TPBS and further incubated overnight, at 4 ºC, with the following primary antibodies: mouse anti-β3-Tubulin (1:250, Santa Cruz Biotechnologies, CA, USA), mouse anti-p73 (1:250, Santa Cruz Biotechnologies, CA, USA) and mouse Syntaxin-1 (1:250, Santa Cruz Biotechnologies, CA, USA). Blots were also probed for mouse anti-β-actin (1:4000, Sigma-Aldrich, St Louis, MO, USA) to ascertain equal sample loading. Primary antibodies were diluted in 1 % BSA prepared in TPBS, supplemented with 0.05 % NaN_3). The membranes were again washed in

TPBS (three times, 10 min each) and further incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin (igG, 1:2500, GE Healthcare, PA, USA) diluted in 1 % BSA prepared in TPBS for 1 h, at room temperature under stirring.

The membranes were washed in TPBS (three times, 10 min each) and protein bands were detected by incubating the membranes in Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) for 5 min. The membranes were then imaged using the molecular imager ChemiDoc™ XRS. Band intensities in each lane were quantified using Image Lab (Version 5.1, Build 7, Hercules, CA, USA) and normalized against the intensities of the endogenous control β-actin.

6.9 Protein Quantification

6.9.1 Lowry protein assay

The Lowry protein quantification assay is a simple and sensitive assay that allows the quantification of proteins that are present in solution or precipitated. This is possible due to the measurement of the amount of color produced by the reaction of free Cu^{+} (originated by the oxidation of peptide bonds) with the Folin–Ciocalteu reagent, which correlates with the total protein present in the sample [258].

The protocol used was based on the one reported by Fryer, *et al.* (1986) with slight modifications [259]. At the end of the referred assays, adhered cells were washed to remove any cell culture medium remaining and lysed in 0.3 M NaOH, for at least 30 min at 4ºC. The reactions were started by mixing 50 µl of each sample with 100 µl of a 2%(v/v) Na_2CO_3, 1%(v/v) $CuSO_4$, 2%(v/v) KNa solution for 10 min, protected from light. Then, 100 µl of a 6.6% (v/v) Folin & Ciocalteu's phenol reagent were added and plates were incubated for additional 20 min, protected from light. Absorbance was measured spectrophotometrically at 750 nm using a Bio-Tek PowerWaveX (Bio-Tek, Winooski, VT, USA) microplate reader. Protein concentrations in the samples were interpolated from a standard curve prepared with Bovine Serum Albumin (BSA) standards.

6.9.2 Bradford Protein assay

The Bradford Protein Assay is based on the conversion of the red dye into blue color under acidic conditions while binding to the present proteins in solution (if no proteins are present, a brown color will take place) and it was performed according with the manufacturer's instructions [260]. Briefly, 5 µl of each sample was added to a well in a 96-well plate and incubated for 10 min protected from light with 25 µl of reagent A. After this 200 µl of reagent B were added to each well and plates were incubated for 15 minutes protected from light. Absorbances were measured spectrophotometrically at 750 nm using a Bio-Tek PowerWaveX (Bio-Tek, Winooski, VT, USA) microplate reader. Protein concentrations in the samples were interpolated from a BSA standard curve.

6.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Analysis of the normality of each distribution was assessed using Kolmogorov-Smirnov and Shapiro-Wilk normality tests, and taking into account the acceptability of skewness and kurtosis values. Based on the normality tests results, One-way ANOVA, followed by a Dunnett's post-hoc test, or unpaired two-tailed test were performed, as appropriate. The number of independent experiments, as well as the number of replicates assayed, if any, is detailed in the figures legends.

7. Results

7.1 Cell viability and metabolic activity were not affected by synthetic cannabinoids

The toxicity of the SCs to neuronal cells was assessed using the MTT reduction, LDH release and Neutral Red inclusion assays. Overall, the results depicted in Figure 6 show that none of the tested SCs significantly decreased metabolic activity (MTT reduction, blue bars), lysosomal degradation (Neutral Red inclusion, red bars) or plasma membrane integrity (LDH release, green bars) up to 1 µM. Nevertheless, toxicity levels varied among SCs for concentrations higher than 1 µM. In particular, MDMB-CHMICA decreased the cells' metabolic activity for concentrations starting at 50 µM while none of the other tested SCs significant reduced metabolic activity up to 100 µM. Figure 6 also shows that lysosomal integrity is most affected by MDMB-CHMICA, which induces a significant decrease in neutral red inclusion already at 10 µM. All other SCs only affected this parameter at 50 µM or higher.

In terms of cell membrane integrity, assessed by measuring the lactate dehydrogenase release, Fig. 6 shows that plasma membrane disruption in the presence of MDMB-CHMICA and 5F-PB22 started occurring at 50 µM. while exposure to JWH-122 THJ-2201 and XLR-11 only started affecting this parameter at 100 µM.

These results clearly indicate that at *in vivo* relevant concentrations (<1 µM) none of the tested SCs is toxic to NG108-15 cells.

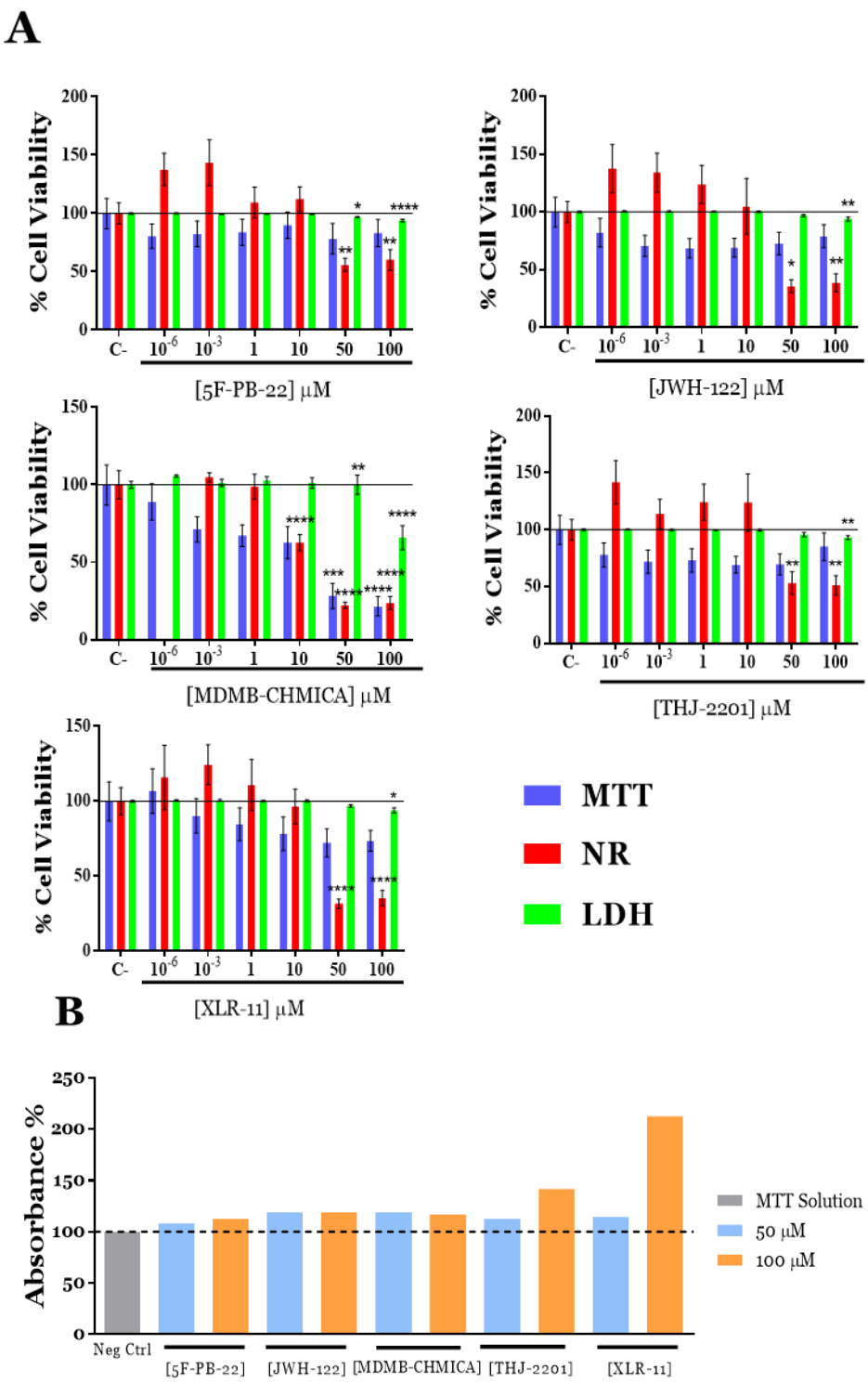


Figure 6- Cell viability (A) Cell viability was assessed by MTT reduction, Neutral Red inclusion and LDH release assays, normalized by total protein per condition. Cells were incubated with different SCs for a 24-hour period with concentrations ranging from 1pM to 100µM. Each bar represents the mean ± SEM (n=7) of the percentage of metabolic activity. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, compared to the control (0.5% DMSO) (B) Absorbance observed by the various Synthetic Cannabinoids at 550nm in a concentration range of 50-100 µM. Each bar represents the value obtained in the assay in percentage compared to a negative control of MTT solution (n=1)

Noteworthy, some SCs promoted higher decreases in cell viability when using the Neutral Red inclusion and LDH assays, in comparison to the MTT reduction assay, usually considered a more sensitive assay. We thus addressed the possibility that the SC absorbance might be interfering with the MTT method. In this regard, we measured the absorbance of the SCs, mixed with a 0.5 mg/ml MTT solution at a 550 nm wavelength, at the highest concentrations tested (50 and 100 μM), in a cell-free plate. Data in Figure 6 (B) shows that at 50 μM none of the SCs interfered with MTT absorbance. At 100 μM, THJ-2201 and XLR-11 displayed some absorbance interference. Nevertheless, it should be noted that at this concentration, the other cell viability assays also indicated high cell death levels, validating the results obtained.

7.2 Mitochondrial integrity was altered after synthetic cannabinoids exposure

The study of the mitochondrial membrane integrity is vital towards understanding the possible toxicological mechanisms that may lead towards cell death. For example, depolarization of the mitochondrial membrane may sometimes trigger apoptosis [261, 262]. It is largely agreed that concentrations above 1 μM may result in off-target cannabinoid-mediated effects [178]. In this sense and to try to ascertain a possible cannabinoid receptor-mediated effect, from this point on we only used concentrations below 1 μM.

Figure 7 shows that after a 24 h exposure, 1 pM JWH-122 (1.38-fold), 1 pM, 0.5 nM and 1 nM MDMB-CHMICA (1.27-, 1.35- and 1.37-fold, respectively), 0.5 nM and 1 nM THJ-2201 (1.25- and 1.32-fold, respectively) and 1 pM XLR-11 (1.29-fold) increased mitochondrial TMRE inclusion. These results thus suggest that these SCs, at low concentrations, enhance the mitochondrial membrane potential. On the other hand, cell exposure to 5F-PB22 did not result in any modifications to mitochondrial TMRE accumulation, at any of the concentrations tested.

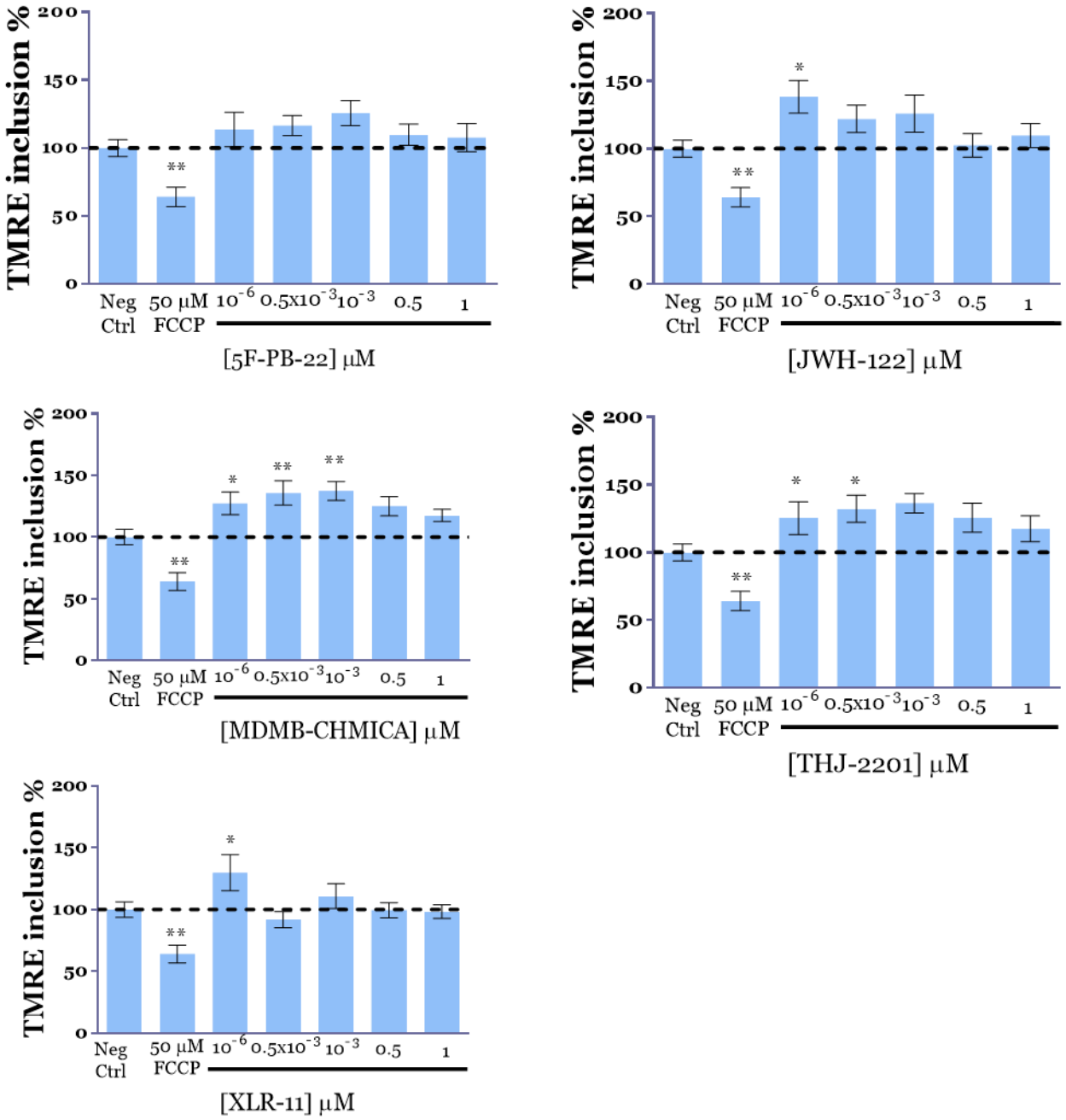


Figure 7- Mitochondrial TMRE accumulation. NG108-15 cells were incubated for 24 h with the synthetic cannabinoids at concentrations ranging from 1 pM to 1 µM. Results were normalized by the total amount of protein and expressed as the percentage relatively to the negative control (0.5% DMSO). 50 µM FCCP was used a positive control. Each bar represents the mean ± SEM (n=4). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, compared to a negative control.

7.3 Synthetic cannabinoids did not change intracellular ATP levels

Intracellular ATP levels are dependent on the balance between ATP production and consumption. In particular, generation of ATP depends on ATP synthase, which uses the electrochemical proton gradient formed by the membrane potential and by the mitochondrial transmembrane proton concentration gradient to convert ADP and phosphate into ATP [263]. In addition, ATP may also be used in various cell mechanisms, from "housekeeping" events to the reversal of ion fluxes through postsynaptic receptors [264, 265] . Figure 8 shows that none of the SCs, at a concentration range of 1 pM-1 nM, altered the net intracellular ATP levels in NG108-15 cells after 24 h. Nevertheless, it should be noted that it is not possible to withdraw any conclusion regarding the effect of the SCs on cellular energy production or metabolism, since this protocol only allows the assessment of the total ATP levels.

7.4 Synthetic cannabinoids did not affect cell proliferation

Cell proliferation is essential for various types of cells. In this particular case, proliferation is a vital stage during neurodevelopment, since alterations on this step can lead to disastrous consequences [266].

The potential role of the SCs on cell proliferation was assessed by the sulforhodamine B assay. As observed in Figure 9, NG108-15 cells incubated with the SCs at the concentrations between 1 pM and 1 nM showed proliferation curves similar to the control (cells growing in the absence of SCs), clearly evidencing the lack of effect of these substances on this parameter.

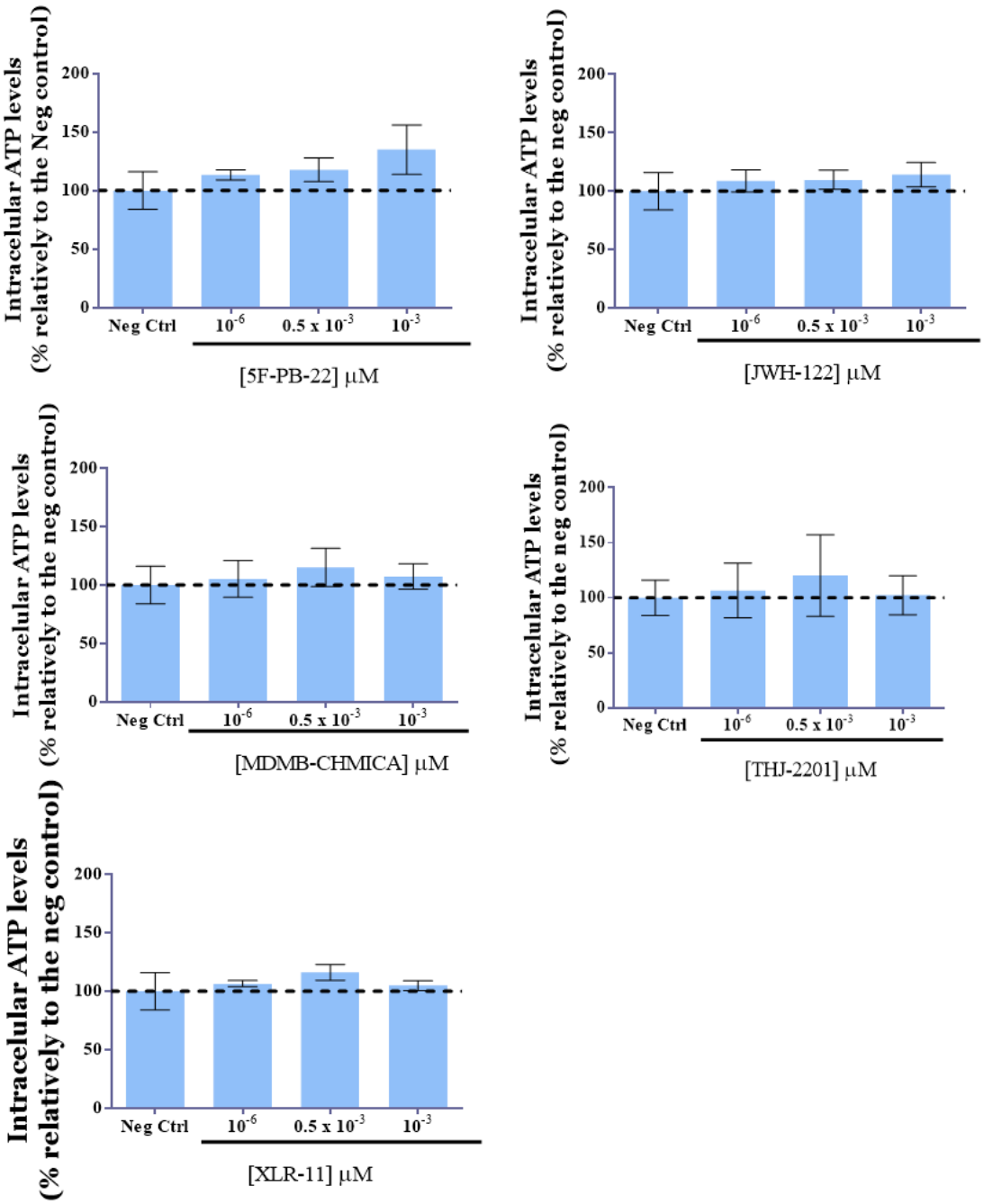


Figure 8- Intracellular ATP levels measured using a luciferin-luciferase-based assay. Cells were exposed to synthetic cannabinoids for 24h at concentrations ranging from 1 pM to 1 nM. Results were normalized by the total amount of protein and expressed as the percentage relatively to the negative control (0.5% DMSO). Each bar represents the mean ± SEM (n=3) of total ATP levels in percentage (0.5% DMSO). * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001, compared to a negative control

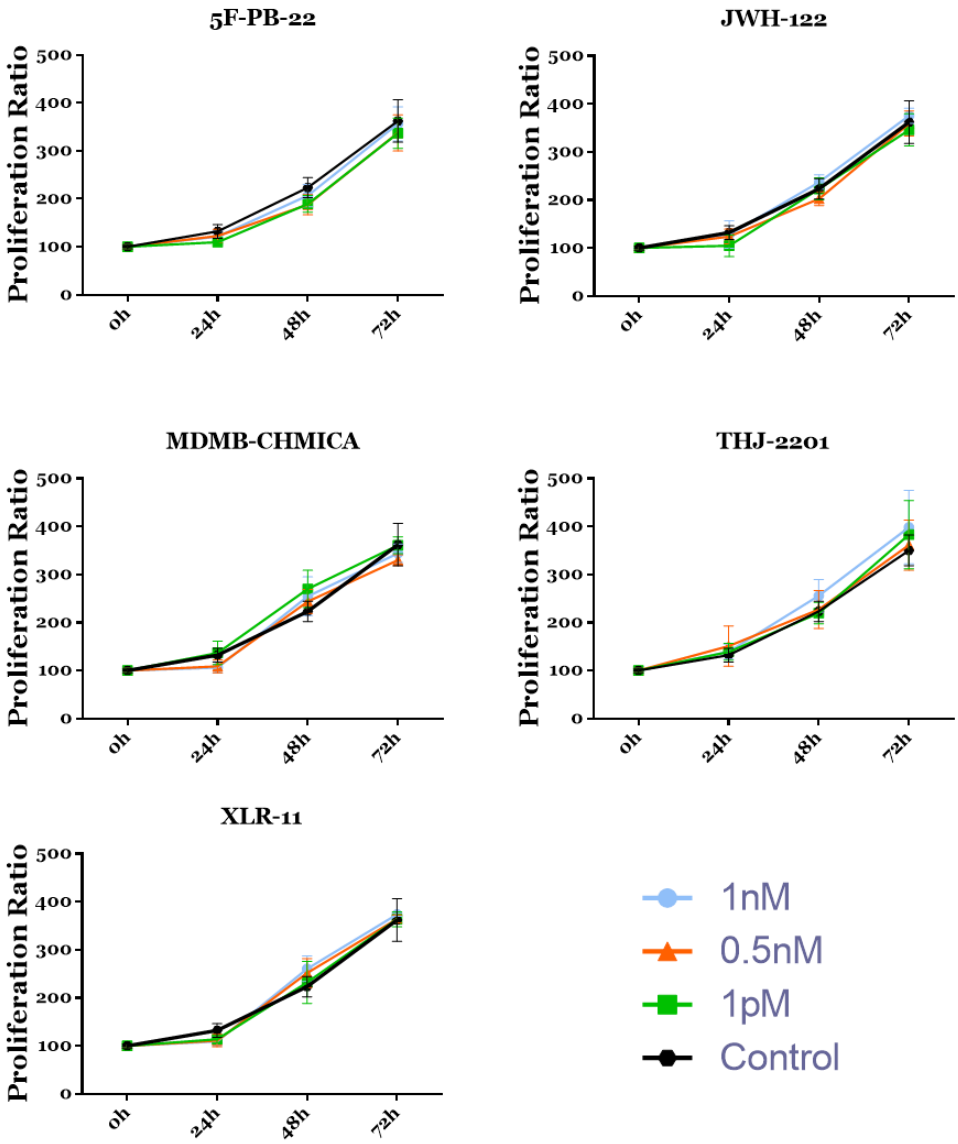


Figure 9- Cell Proliferation determined by the Sulforhodamine B assay. Cells were exposed to synthetic cannabinoids up to 72h, at concentrations ranging from 1pM to 1nM. Each time point represents the mean ± SEM (n=6) of percentage of growth relatively to t=0h. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001, comparing to a negative control at the 0h time point.

7.5 5F-PB22 and THJ-2201 promoted NG108-15 cell differentiation

Neuronal cell differentiation was assessed in NG108-15 cells by measuring the neurite outgrowth of cells exposed to the different SCs in differentiation medium. As observed in Figure 10 A-B, cells cultured in Maintenance Medium (MM) continued to proliferate and showed reduced signs of differentiation, as indicated by the high cell density and low amount of newly formed neurites after 3 days in culture. On the other hand, cells cultured in Differentiation Medium (DM) exhibited clear signs of neurite outgrowth, evidencing the importance of differentiation factors to trigger the differentiation process.

As also displayed in the representative images of Figure 10 A and further represented in Figure 10 B, neuronal differentiation ratios increased following cell incubation with 5F-PB22 and THJ-2201 at concentrations below 1 nM, compared to Differentiation Medium alone. In fact, Figure 10 A shows that those conditions not only promoted the formation of a higher number of neurites compared to the control, but many of these are also longer. These effects were observed following a single (THJ-2201) or multiple (5F-PB22) treatments, indicating that different SCs may require different dosages to induce the same effect. None of the other SCs tested promoted any significant change inNG108-15 neurite outgrowth.

The potential role of the CB1 receptor in the enhanced neuronal differentiation mediated by 5F-PB22 and THJ-2201 was assessed by incubating cells with a specific CB_1R antagonist, SR141716, prior to incubation with the SCs. As observed in the representative images (Figure 11 A) and in the graphical representations (Figure 11 B), the presence of SR141716 alone significantly modified the differentiation ratios. Results in Figure 11 (B) show that the differentiation ratios in the presence of both 5F-PB22 and THJ-2201 are significantly decreased by prior exposure to the CB_1R antagonist. Indeed, the presence of SR141716 decreased about 0.6 and 0.7-fold the neuronal differentiation levels previously induced by three additions of 5F-PB22 (1 pM and 0.5 nM, respectively). Similarly, neuronal differentiation promoted by a single addition of 1 nM THJ-2201 decreased around 0.6-fold in the presence of SR141716. These results thus suggest that the regulation of neuronal differentiation by 5F-PB22 and THJ-2201 seems to be mediated by the CB_1 receptor.

Noteworthy, metabolic activity of differentiated cells was not affected by culture in DM or by the SCs, at concentrations below 1 nM, during the differentiation process, as depicted in Figure 12 . Exposure to SR141716 also showed no variation of metabolic activity in the referred conditions.

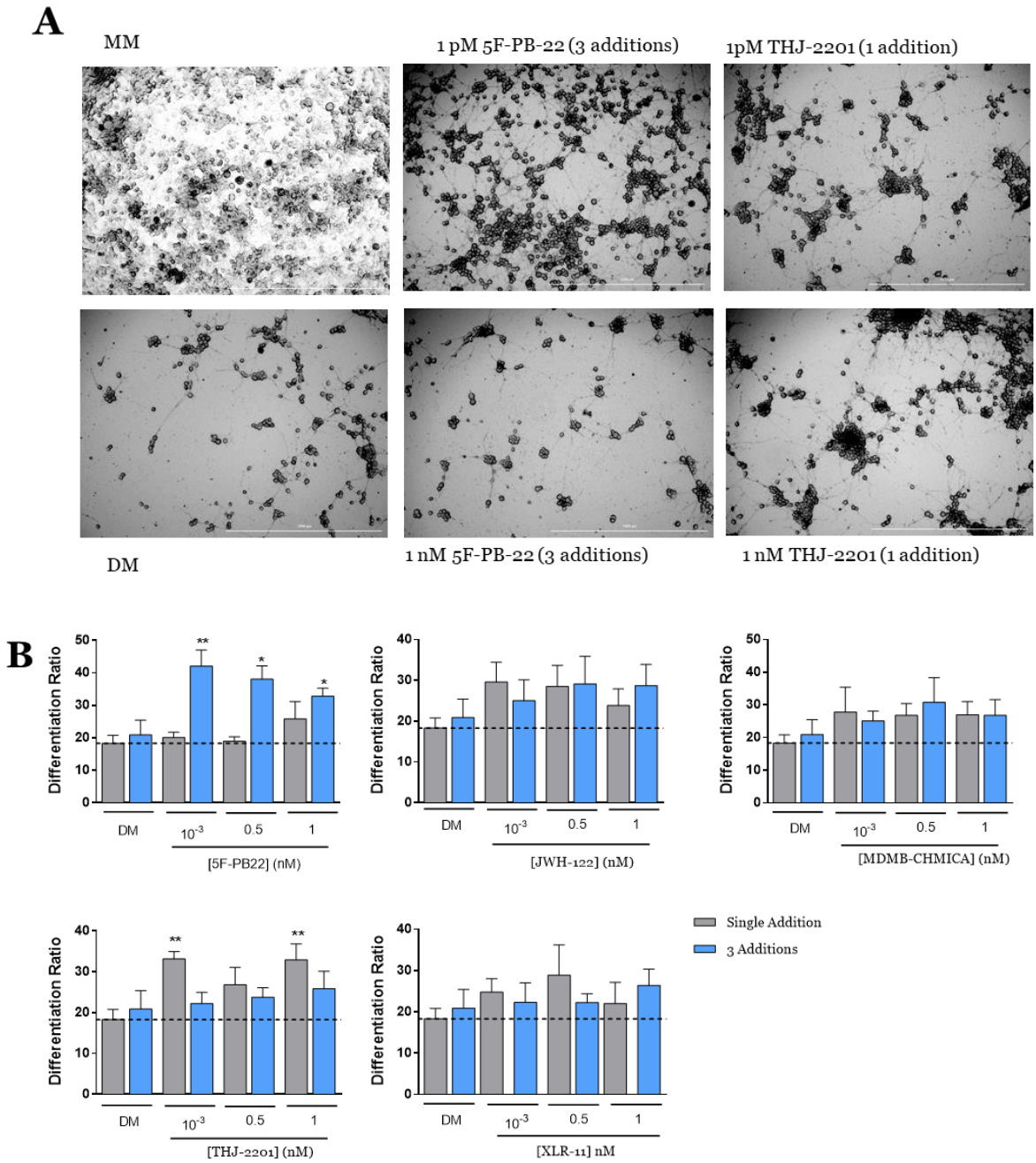


Figure 10- Effects of SCs and different SC dose treatments, on neuronal differentiation. Neuronal differentiation was induced by replacing Maintenance Medium (MM) by Differentiation Medium (DM) in the presence and absence of SCs. SCs were added to NG108-15 cells either once (single addition at day 0) or 3 times (every 24 h up to 72 h). **(A)** Representative images of NG108-15 cells 72 h after seeding in MM or after exposure to 3 additions of 5F-PB22 (1 pM / 1nM) or a single addition of THJ-2201 (1 pM / 1 nM) in DM. **(B)** Differentiation ratios determined by the ratio of neurites with over 20 µm by the total number of cells per well. Bars show the mean ± S.E.M. for four independent experiments (n=4). $*p < 0.05$, $**p < 0.01$, compared to DM.

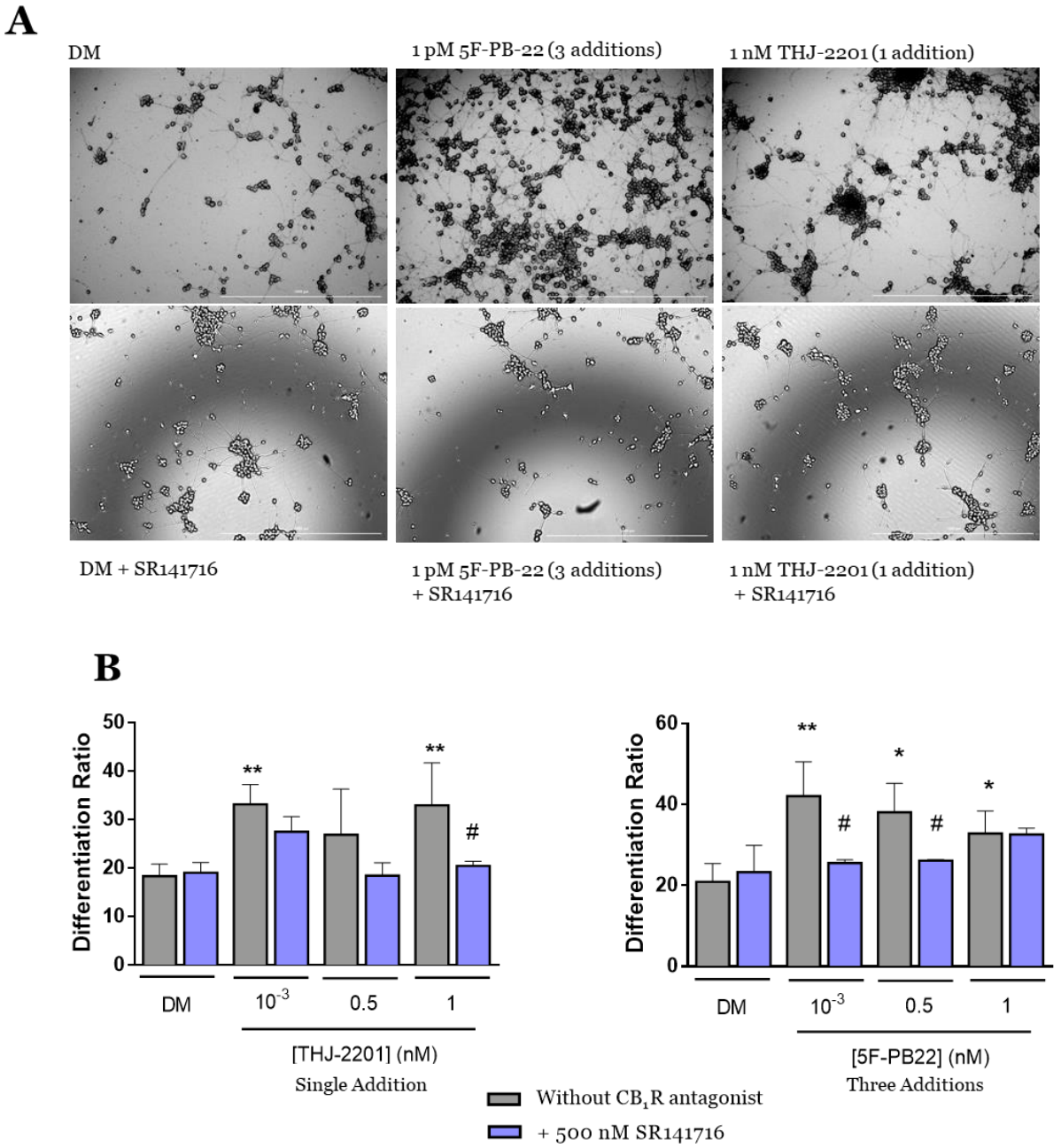


Figure 11 - Assessment of CB_1R involvement in the conditions showing differentiation. Neuronal differentiation was induced as previously described and cells treated with 5F-PB22 or THJ-2201 in the conditions that previously showed significant changes in neurite outgrowth. Before exposure to SCs, cells were incubated with 500 nM of a selective CB_1R antagonist (SR141716). (A) Representative images of NG108-15 cells treated with 5F-PB22 or THJ-2201 in the presence and/or absence of the CB1R antagonist. (B) Comparison of differentiation ratios in the presence and absence of SR141716. Bars show the mean ± S.E.M. for four independent experiments (n=4). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to DM. $^{\#}p < 0.05$, compared to respective SC concentration in the absence of antagonist.

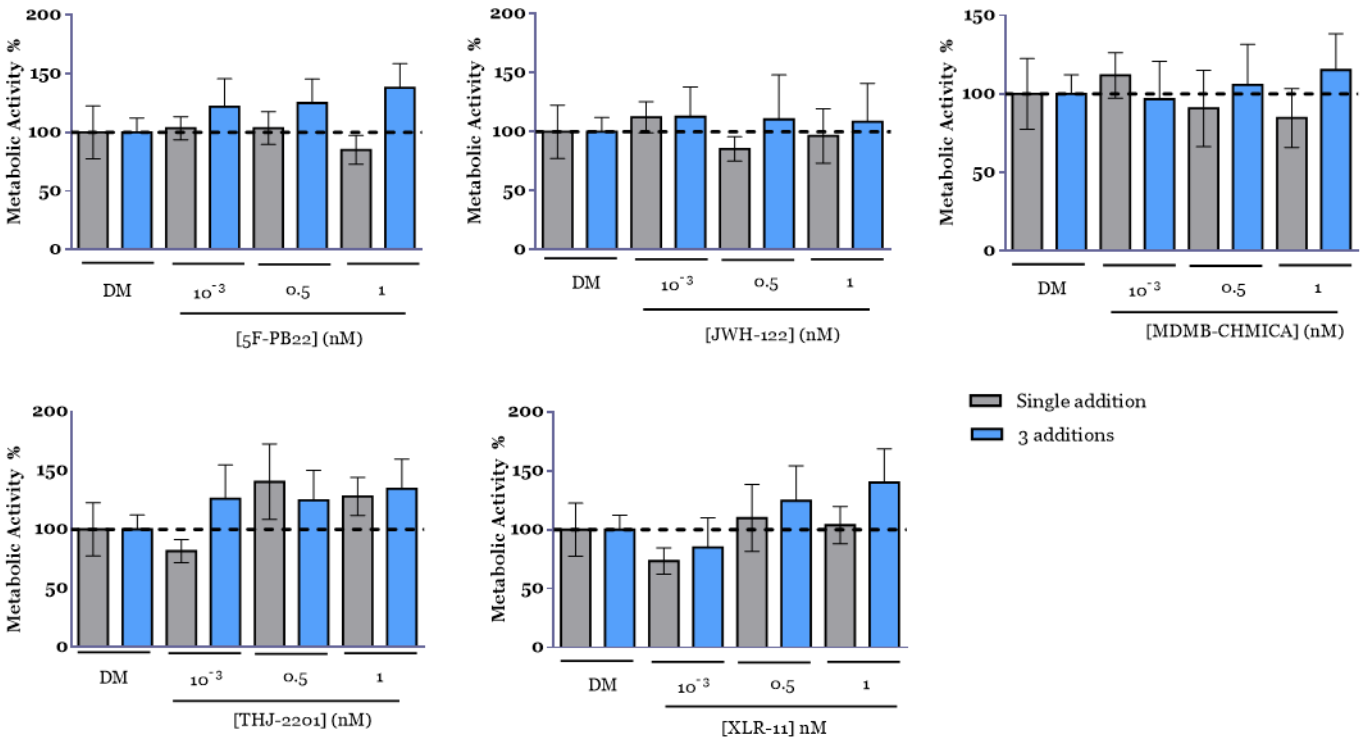


Figure 12– Metabolic activity of differentiated cells assessed by the MTT reduction assay. Neuronal differentiation was induced and cells treated with the SCs as previously described in Materials and Methods. At the end of the differentiation process (day 3), cellular metabolic activity was determined according to the MTT reduction procedure also described in Materials and Methods. Bars show the mean ± S.E.M for at least four independent experiments. No statistically significant changes were observed.

7.6 5F-PB22 increased expression of neuronal differentiation markers

Expression of neuronal markers was further assessed by Western Blot in differentiating NG108-15 cells, in the presence and absence of the SCs that previously demonstrated the ability to promote such process. Three specific proteins associated with neuronal differentiation were analyzed: p73, known to activate the p21 promoter and therefore inducing cell cycle arrest at G1/S transition and also regulates the Glutaminase isoform 2 (GLS2) enzime during retinoic acid-induced terminal neuronal differentiation [267, 268]; syntaxin-1, correlated with the modulation of neurotransmission and neuronal maintenance of already differentiated neurons [269]; and β3-Tubulin, a cytoskeleton protein expressed in differentiated neurons [270].

As observed in figure 13 (A-B), both β3-tubulin and p73 were highly expressed in cells exposed to differentiation medium in comparison to cells growing in maintenance medium, as expected, considering their involvement in this process [271]. Surprisingly, syntaxin-1A was not found to be overexpressed in comparison to MM [272]. Moreover, cell exposure to 1 pM THJ-2201 (single addition) or 1 pM and 1 nM 5F-PB22 (three additions) significantly promoted the overexpression of β3-Tubulin and p73 relatively to MM. Interestingly, these changes only proved to be statistically significant compared to cells cultured in DM following 1 pM 5F-PB22 treatment. In fact, despite having previously shown a significant increase of neurite outgrowth, treatment with THJ-2201 did not enhance the expression of these neuronal markers. In fact, it surprisingly decreased p73 expression, compared to cells maintained in DM.

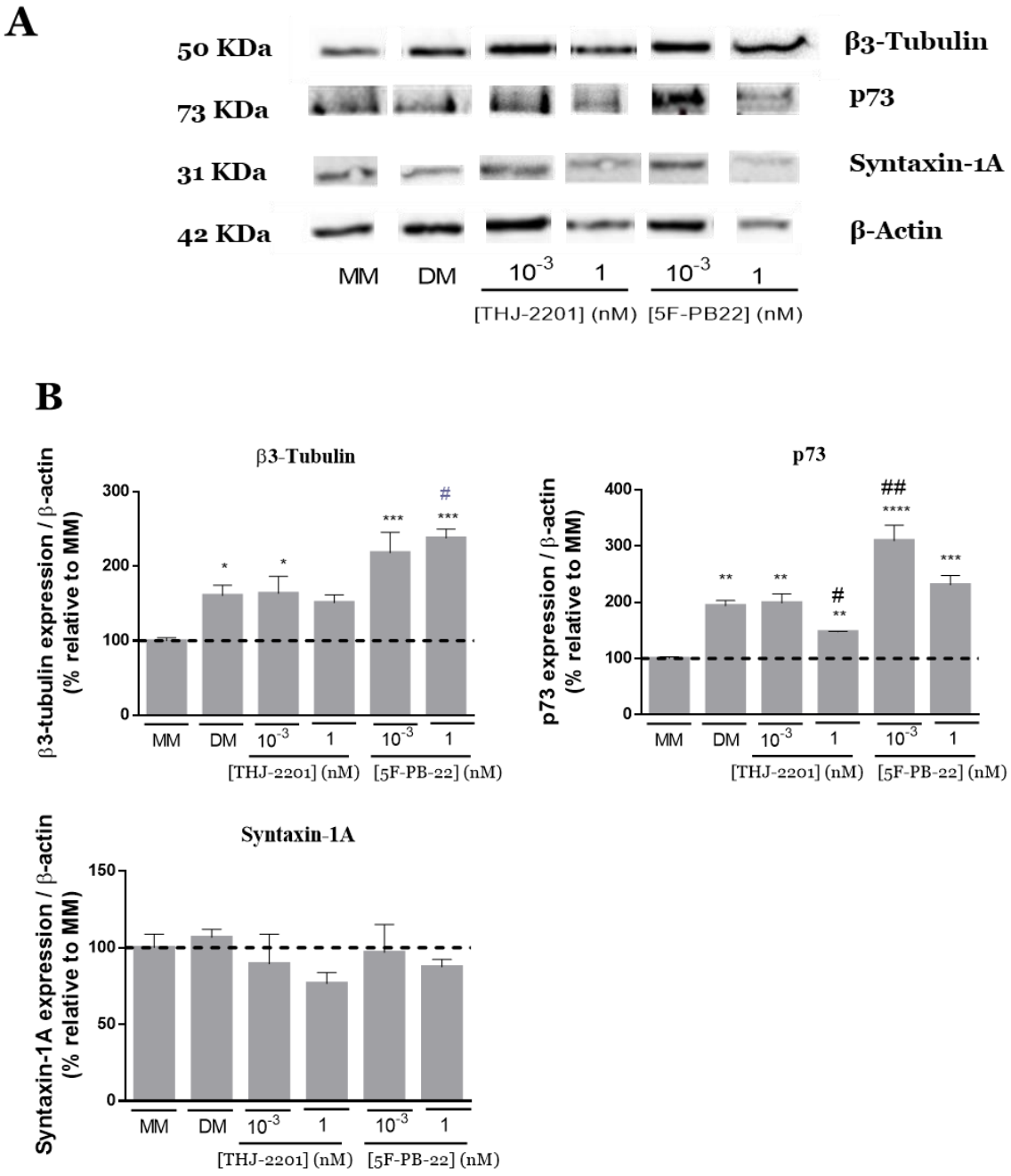


Figure 13- Expression of neuronal markers. Neuronal differentiation and SC treatment was performed according to the procedure described in Materials and Methods. Following cell differentiation for 72 h, the expression of β3-tubulin (50 KDa), p73 (73 KDa) and syntaxin-1A (31KDa) was assessed by Western-blot **(A)** Representative protein bands following cell treatment with a single addition of THJ-2201 (1pM and 1nM) and 3 additions of 5F-PB22 (1 pM and 1 nM). **(B)** Graphical representations of band intensities, expressed as the percentage of protein expression relatively to the control. Their expression was normalized by the amount of β-actin per lane. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, compared to MM. # $p \leq 0.05$, ## $p \leq 0.01$ compared to DM.

8. Discussion

The total amount of SCs reported by EMCDDA has rapidly increased over recent years. Although the emergence of new psychoactive substances belonging to this group has currently stabilized [8], most likely due to the implementation of proper legislation and control measures, the number of health problems and deaths associated with the use of SCs is still growing. In fact, SC-related intoxications were the most common cause of admissions to hospital emergencies associated with NPS use in 2016 [8]. Noteworthy, there is still limited information regarding SCs' neurotoxicological signatures. Moreover, the use of SCs by young people is also concerning since at such ages the CNS is not fully developed. Among such users, pregnant women and women of childbearing potential comprise specific risk groups, due to the harmful potential of SCs to the offspring. In this sense, assessing the neuronal toxicological profile of SCs, especially during neuronal development stages, assumes extreme relevance.

This work aimed at evaluating the toxicological profile of a set of five commonly detected SCs (5F-PB22, JWH-122, MDMB-CHMICA, THJ-2201 and XLR-11), as well as assessing their potential role during neurodevelopment processes, such as *in vitro* neuronal differentiation.

We found that these different SCs, at biologically relevant concentrations (≤ 1 μM), did not significantly changed cell viability parameters, including cell metabolic activity (MTT reduction), lysosomal integrity (Neutral Red inclusion) or plasma membrane integrity (LDH release). However, all SCs except 5F-pB22, promoted the hyperpolarization of the mitochondrial membrane. Similar results have also been reported in renal (HK-2) cells [248], suggesting that SCs may preferentially act at the mitochondria level, modulating the mitochondrial membrane potential, regardless of the cell model used. This mitochondrial involvement in SC-mediated toxicity may not be surprising, since active CB_1 receptors have already been found in neuronal mitochondria membranes [273]. Moreover, such SC-mediated effects on mitochondrial function assumes high relevance, considering that mitochondria are involved in several key mechanisms within the cell. Indeed, mitochondria have been found to be vital in Ca^{2+} handling, from uptake and efflux to storage [274]. It is worth noting that the only SC that did not affect TMRE inclusion was 5F-PB22, despite being one of the two SCs that promoted an increase in neuronal differentiation. This may be associated with the amount of doses administered. In fact, 5F-PB22 was added to NG108-15 cells only once to assess mitochondrial TMRE accumulation, while three additions (every 24 h, up to 72 h) of the same substance were required to attain a significant alteration in

differentiation ratios. Interestingly, no alterations were detected on net intracellular ATP levels. Nevertheless, it should be noted that the protocol used focused on total ATP levels. Further research would be required to determine whether the SCs may modulate ATP generation and/or metabolism independently. Nevertheless, since the total ATP levels were not changed, it is reasonable to state that SC-mediated neurotoxicity is not associated with the regulation of intracellular energy metabolism.

The presence of the endocannabinoid system is well established since the early stages of neurodevelopment [104, 123] and, as previously stated, cannabinoids can influence various stages of neurodevelopment. Here we found that there is a clear modulation of neuronal differentiation after NG108-15 cells' exposure to THJ-2201 and 5F-PB22 at concentrations between 1 pM and 1 nM. Interestingly, a single dose of THJ-2201 was sufficient to significantly increase neurite outgrowth, while three daily doses of 5F-PB22 were required to promote similar effects.

Moreover, regulation of both SCs-mediated effects on neurodifferentiation appeared to be dependent on the activation of CB_1R, as inhibition of this receptor with a selective antagonist hindered the previous 5F-PB22 and THJ-2201-mediated effects on this parameter. The ability of SCs to regulate neurodifferentiation, as well as the importance of CB_1R during this process has been previously reported. Indeed, Compagnucci *et al.* [23] described an increase in differentiation of neural stem cells from mouse embryos after exposure to both anandamide and to the CB1-specific agonist ACEA. Such results were reversed when cells were co-incubated with AM251, an inverse agonist to the CB_1R. On the other hand, Jian *et al* [233] did not find any increased differentiation of primary embryonic and adult hippocampal cells after chronic injections of HU-210 (a SC with high affinity towards CB_1R). Together with the fact that some of the SCs tested in our work did not produce any significant changes in NG108-15 differentiation, these findings further support the idea that not all SCs have the ability to modulate neuronal differentiation, which is most likely related with the different mechanisms they may activate.

In homeostatic conditions, a neuronal stem cell only commits to differentiation when required and within the correct time frame [275, 276]. In this sense, it is reasonable to expect that an unsolicited (e.g. induced by exogenous factors, like SC use) increase in neuronal differentiation, may promote the occurrence of malformations in the CNS during neurodevelopment. In fact, it has been previously observed that fetal exposure to 2.0 mg/kg of the synthetic cannabinoid CP-55,940 induced central nervous system abnormalities, [230] and inhibited proliferation in a rat C6 glioma cell line [277], while,

neuronal stem cell depletion, lack of neuronal stem cell self-renewal and cell-cycle exit have been correlated with loss in brain function [278].

In addition, we further analyzed whether SC-mediated neurodifferentiation was accompanied by the expression of different neuronal markers. In particular, we evaluated the expression of three different differentiation markers: β3-Tubulin, a cytoskeleton protein that is only expressed in differentiating neurons [270]; p73, which induces the expression of neurofilaments and the neural cell adhesion molecule (N-CAM) [267, 268]; and syntaxin-1, which has a vital role in the modulation of neurotransmission and in neuronal maintenance [269]. We observed that the effects of 5F-PB22 on neurodifferentiation were associated with an overexpression of β3-tubulin and p73. These results are in line with the data regarding neurite outgrowth and, taking into consideration that both β3-tubulin and p73 are overexpressed during the differentiation process, they further support the evidence that 5F-PB22 promote neuronal differentiation. Surprisingly, there was no significant β3-tubulin or p73 increases in the THJ-2201 conditions that had previously shown enhanced neurite outgrowth. This suggests that neurons differentiated in the presence of THJ-2201 may already be in a more mature state when compared to the ones differentiated in the presence of 5F-PB22, since β3-tubulin is mostly expressed in periods of maturation and decreases its expression in differentiated cells (such as the CNS) [279, 280].

In addition, we detected no alteration of syntaxin 1 levels following incubation of differentiating cells with the SCs, indicating that the SCs did not affect neurotransmission-related processes. This may be possibly explained by the overactivation of the neurite outgrowth pathway, without any change in cell state. *Kolkova et al.* (2000) [281] previously showed that neurite outgrowth is dependent on protein-kinase-C (PKC) activation and Ras–mitogen activated protein kinase pathway. Moreover, Wang *et al.* (2012) reported that the synthetic cannabinoid WIN 55,212-2 inhibited the activity of the transient receptor potential vanilloid 1 (TRPV1) channel without being a direct agonist, in trigeminal ganglion neurons, with no activation of CB_1 or CB_2 receptors [282]. In addition, they reported that this inhibition was closely related with PKC activation, being that PKC activation leads to a higher TRPV1 inhibition [282]. In this regard, we can presume that PKC activation may be down-activated after SC exposure, independent from CB_1 activation, which in turn will lead to neurite outgrowth. Activation of these pathways could thus possibly comprise one of the mechanisms leading to 5F-PB22 and THJ-2201-mediated enhanced neurodifferentiation and should be further explored in future studies.

Noteworthy, some of the conditions that promoted neuronal differentiation, such as 1 nM THJ-2201, were the same that produced a higher mitochondrial TMRE accumulation, suggesting an underlying effect that could possibly be dependent on mitochondrial function.

Neuronal cell proliferation is a process also found associated with neurodevelopment and prone to regulation by cannabinoids. In fact, WIN-55,212-2 was found to positively modulate neuronal proliferation through CB_1R activation in *in vivo* mice assays [283]. In addition, HU210 was also found to induce proliferation, but not differentiation, of rat cultured embryonic neural stem and progenitor cells after 48 h [233]. However, we did not find any variations in proliferation after SC exposure. Contrarily to this, some SCs have been found to inhibit cell proliferation. In fact, one addition of 3µM of CP 55,940 and JWH015 have been observed to inhibit cell proliferation in a rat C6 glioma cell line [277].

A possible explanation for the different effects observed on this parameter may be associated not only with the different SC used, but also with the different cell models employed. For example, the tested SCs were different, the used model was different and while we used a homogeneous cell line (NG108-15) in terms of cell identity, other studies in which proliferation was affected were performed in mouse olfactory epithelium [283], primary cultured embryonic neural stem and neural progenitor cells [233] possibly explaining the disparities in the outcomes.

In conclusion, we report for the first time the ability of two synthetic cannabinoids, THJ-2201 and 5F-PB22 to promote neuronal differentiation in a CB_1R-dependent way, at concentrations below 1 nM. We have also observed that these two SCs required different dosage regimens to attain similar results and that neurons differentiated in the presence of each of them may be at different maturation states. In addition, we characterized the neurotoxicity profile of a broader group of different SCs, frequently detected in drug seizures and marketed packages. Although we observed no significant changes in most toxicity-related parameters at *in vivo* relevant concentrations, hyperpolarization of mitochondrial membrane was detected, suggesting a possible regulatory action of mitochondrial function. Although this data supports the harmful potential of SC use, especially considering their potentially adverse outcomes to differentiating neurons, further research is required to fully understand the mechanisms involved in the modulation of these parameters.

9. Future Perspectives

This work already presents interesting and promising data towards understanding the role of SCs during neurodevelopment processes. Nevertheless, it is still at an early stage and further research is thus required to explore the underlying mechanisms involved in the observed effects.

Here we found that 5F-PB22 and THJ-2201 promoted neuronal cell differentiation, possibly through different mechanisms, as different dosage regimens were required to achieve similar results and neuronal marker expression also varied between both SCs. In this sense, it could be interesting to further analyze the expression of differentiation related proteins. For example: Protein Kinase C pathway-related proteins, since this pathway has been reported to influence neurite outgrowth; NeuN, a specific marker for differentiated neurons; Neurofilament 200 (NF200), an intermediate filament only expressed in neurons; choline acetyltransferase (ChAT) an enzyme that catalyzes the formation of acetylcholine and coenzyme A; and acetylcholinetransferase (AChE), an acetylcholine-hydrolyzing enzyme that enhances neurite outgrowth.

The endocannabinoid system plays a key role during neurodifferentiation. Considering the potential of SCs to deregulate this system, it could also be important to ascertain if SCs interfere with the intracellular production and/or degradation of endocannabinoids.

Moreover, it would be interesting to further explore the role of the SCs on mitochondrial regulation. For example, by analyzing whether these effects are mediated by mitochondrial cannabinoid receptors or, considering the known effect of cannabinoids in intracellular Ca^{2+} modulation and in Ca^{2+} flow, resorting to fluorescent dyes such as FURA-2AM. In addition, since mitochondrial modulation is taking place, it would also be interesting to analyze mitochondrial dynamics (neuronal trafficking, fusion/fissions and mitophagy) resorting to mitochondrial fluorescent markers such as mito RFP/GFP/YFP.

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