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Evaluation of epigenetic modifications induced by synthetic cannabinoids during in vitro neuronal differentiation.

Telma Marisa Gomes

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Telma Marisa da Conceição Gomes

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Trabalho realizado sob a orientação de:
Doutor João Pedro Martins Soares de Castro e Silva, Investigador Auxiliar, UCIBIO-REQUIMTE Departamento Ciências Biológicas, Laboratório de Toxicologia, Faculdade de Farmácia da Universidade do Porto
Professor Doutor Félix Dias Carvalho, Professor Catedrático, Departamento Ciências Biológicas, Laboratório de Toxicologia, Faculdade de Farmácia da Universidade do Porto

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Abstract

The complex interaction between the endocannabinoid (eCB) system and epigenetic mechanisms has been reported to regulate several processes during neurodevelopment. Considering the importance of the eCB system during neurodevelopment, we hypothesized that the exposure of neuronal cells to synthetic cannabinoids (SCs) could impact neuronal differentiation through the induction of epigenetic modifications.

The effects of two commonly abused SCs, THJ-2201 and ADB-FUBINACA, on the differentiation of NG108-15 neuroblastoma x glioma cells was assessed by measuring differentiation ratios and total neurite length (of primary neurites and branches) after cell exposure to *in vivo* relevant SC concentrations (1 pM – 1 μM). The assessment of the cells' cholinergic phenotype and viability was assessed by measuring acetylcholinesterase activity and the percentage of apoptotic/necrotic cells up to 15 and 12 days, respectively, following induction of differentiation. Based on phenotype and cell viability data, the presence of epigenetic changes, namely DNA methylation, and histone H3 acetylation was evaluated following exposure to both SCs according to 3 exposure settings: A) addition of SCs at the beginning of differentiation and sample collection at the third day; B) addition of SCs at the third day of differentiation and sample collection at the sixth day; C) one addition at the beginning of differentiation and another at the third day, with sample collection at the sixth day.

The results showed a clear modulation of *in vitro* neurodifferentiation by both SCs tested, indicated by an increase of about 2-fold in differentiation ratios. Of note, NG108-15 cells maintained its cholinergic phenotype up to 15 days. However, at day 9 after differentiation, around 53 % of necrotic cells were already observed. Most important, THJ-2201 increased DNA methylation in treatments A and C at 1 μM and 1 pM, respectively, while increasing histone H3 acetylation in treatment B at 1 nM. ADB-FUBINACA increased DNA methylation in treatment A (1 nM), but decreased this parameter in treatments B (1 nM) and C (1 μM). Moreover, 1 pM ADB-FUBINACA decreased histone H3 acetylation in treatment C.

Overall, our data suggested that SC-induced neurodifferentiation may be influenced by changes in global DNA methylation and histone H3 acetylation promoted by these substances at biologically relevant concentrations. However, further research is required to clarify the influence of SCs on epigenetic modifications during neuronal differentiation.

Keywords: New Psychoactive Substances; Cannabinoid receptors; DNA methylation; Histone modifications; neuronal development.

Resumo

A complexa interação entre o sistema endocanabinóide (eCB) e os mecanismos epigenéticos tem sido reportada como podendo regular vários processos durante o desenvolvimento neurológico. Considerando a importância do sistema eCB durante o neurodesenvolvimento, colocámos a hipótese dos canabinóides sintéticos (SC) terem impacto na diferenciação neuronal através da indução de modificações epigenéticas.

Os efeitos de dois SC comummente detetados em apreensões, THJ-2201 e ADB-FUBINACA, na diferenciação de células de neuroblastoma x glioma NG108-15 foram avaliados através da medição dos rácios de diferenciação e do comprimento total de neurites (neurites primárias e ramificações) após exposição das células a concentrações dos SC com relevância *in vivo* (1 pM – 1 μM). A manutenção do fenótipo e a viabilidade celular foram avaliadas pela atividade da acetilcolinesterase e da percentagem de células apoptóticas/necróticas até 15 e 12 dias, respetivamente, na sequência da indução da diferenciação. Com base nos dados do fenótipo e da viabilidade celular, a presença de alterações epigenéticas, nomeadamente a metilação de DNA e a acetilação da histona H3 foram avaliadas após exposição a ambos SC, de acordo com 3 tipos de exposição: A) adição de SC no início do processo de diferenciação e recolha de amostras no terceiro dia; B) adição de SC no terceiro dia de diferenciação e recolha de amostras ao sexto dia; C) uma adição no início da diferenciação e outra adição no terceiro dia, com recolha de amostras no sexto dia de diferenciação.

Os resultados mostraram uma modulação clara da neurodiferenciação *in vitro* por ambos os SC testados, indicados por um aumento de cerca de 2 vezes no rácio de diferenciação. De notar que as células NG108-15 mantiveram o seu fenótipo colinérgico até 15 dias. No entanto, ao dia 9 após a diferenciação, 53 % das células já se encontravam necróticas. Mais importante, o THJ-2201 aumentou a metilação do DNA nos tratamentos A e C a 1 μM e 1 pM, respetivamente, tendo aumentado a acetilação da histona H3 no tratamento B a 1 nM. O ADB-FUBINACA aumentou a metilação do DNA no tratamento A (1 nM), mas diminuiu a acetilação de histona H3 no tratamento C.

De um modo geral, os nossos dados sugerem que a neurodiferenciação induzida por SC pode ser influenciada por alterações na metilação global do DNA e na acetilação da histona H3 promovidas por estas substâncias a concentrações biologicamente relevantes. No entanto, é necessário realizar investigação adicional para clarificar a influência dos SC nas modificações epigenéticas durante a diferenciação neuronal.

Palavras-Chave

Novas Substâncias Psicoativas; Recetores canabinoides; Metilação do ADN; Modificações de histona; Desenvolvimento neuronal.

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Abbreviations

2-AG	2-O-Arachidonyglycerol
5-mC	5-methylcytosine
AEA	N-(2-hydroxyethyl) arachidonamide (anandamide)
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CBR	Cannabinoid receptors
DMC	Differentiation medium control
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
eCBs	Endocannabinoids
FAAH	Fatty acid amide hydrolase
GABA	γ-aminobutyric acid
HAT	Histone acetyltransferase
HMT	Histone methyltransferases
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MM	Maintenance medium
mRNA	Messenger RNA
NAPE-PLD	N-arachidonoyl phosphatidylethanolamine-phospholipase D
PPAR	Peroxisome proliferator-activated receptor
PKA	Protein kinase A
SC	Synthetic cannabinoids
TET	Ten-eleven translocation methylcytosine dioxygenases
THC	trans-Δ^9-tetrahydrocannabinol
TRPV1	Transient receptor potential cation channel subfamily V member 1

Chapter I. Introduction

This chapter is partly based on the following publication: Gomes, T.M., da Silva, D.D., Carmo, H., Carvalho, F., Silva, J.P., Epigenetics and the endocannabinoid system signaling: an intricate interplay modulating neurodevelopment, Pharmacol. Res. 105237 (2020) https://doi.org/10.1016/j.phrs.2020.105237

1. Introduction

Dysregulation of neurogenic processes like neuronal cell proliferation, differentiation, migration, or network integration may result in the onset of disorders fully or partially related to a dysfunction in neurodevelopment-related processes (e.g. Rett syndrome, immunodeficiency, centromeric instability and facial anomalies syndrome, schizophrenia, autism spectrum disorders). The endocannabinoid (eCB) system has been reported to play a crucial role in regulating such processes from the earliest stages of ontogenetic development (e.g. embryonal implantation, prenatal neurodevelopment, postnatal suckling) to late adolescence (Cristino *et al.* 2020; Fride *et al.* 2009; Mata *et al.* 2010). Nevertheless, there is still a sparse understanding of how the eCB system signaling may be disrupted by endogenous stimuli. In particular, the abnormal modulation of the eCB system by epigenetic modifications has been overlooked.

1.1. The endocannabinoid system

The eCB system represents a set of tightly regulated components, which include two main receptors that comprise seven transmembrane domains coupled to the toxin-sensitive G protein, small endogenous lipid ligands (eCBs), and their biosynthesis and metabolic enzymes (Castillo *et al.* 2012; Parsons & Hurd 2015). These elements have distinct subcellular distributions, on pre- and postsynaptic neurons, and interact with each other to modulate several biological processes during neuronal development such as neuronal differentiation, proliferation, and migration, as well as neurotransmitter regulation (Cristino *et al.* 2020).

1.1.1. Endocannabinoids: function, synthesis, and degradation

eCBs are endogenous lipid-derived mediators, including amides, esters, and ethers of long-chain polyunsaturated fatty acids found in the brain and peripheral organs (Battista *et al.* 2012). These mediators are biosynthesized from lipid precursors present in the cytoplasmic membrane, via the activation of specific enzymes, in response to high intracellular Ca^{2+} concentrations (Alexandre *et al.* 2019; Pandey *et al.* 2009). The most bioactive eCBs are *N*-(2-hydroxyethyl)-arachidonamide (anandamide, AEA), and 2-*O*-arachidonyglycerol (2-AG) (Battista *et al.* 2012). AEA is converted from its main precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE) by NAPE-phospholipase D (NAPE-PLD) and depends on elevated postsynaptic Ca^{2+} derived from neuronal depolarization. In turn, 2-AG is synthesized from 2-arachidonate-containing phosphoinositides by diacylglycerol (DAG) lipases (DAGL) (Di Marzo *et al.* 2015; Guzmán 2003). The

biosynthesis of eCBs takes place especially at the postsynaptic level. The enrichment of DAGL type α (DAGLα) in the perisynaptic annulus allows the coupling of postsynaptic metabotropic receptor activation to intracellular Ca^{2+} increase and phospholipase Cβ-dependent 2-AG synthesis. Postsynaptically-produced eCBs may travel backward across the synaptic cleft to inhibit presynaptic neurotransmitter release via activation of cannabinoid receptors (CBRs), a process termed retrograde signaling (Di Marzo *et al.* 2015). 2-AG is then mainly inactivated by presynaptic monoacylglycerol lipase (MAGL) with a contribution from α/β hydrolase domain containing 6/12 (ABHD6/12, also known as MAGL ABHD6/12), as a result of the retrograde signaling. Alternatively, NAPE-PLD may also produce AEA presynaptically, which may act at postsynaptic TRPV1 (transient receptor potential cation channel subfamily V member 1), being inactivated by postsynaptic fatty acid amide hydrolase (FAAH) (**Figure 1**) (Alexandre *et al.* 2019; Maccarrone *et al.* 2014). Of note, transmembrane or intracellular transport mechanisms at the mature synapse [e.g. endocannabinoid membrane transporter (EMT), anandamide intracellular transporter (AIT)] control the availability of eCBs by transporting them for intracellular degradation. In addition, eCBs may undergo oxidative catabolism through palmitoylethanolamide-preferring acid amidase lipoxygenases, cyclooxygenase-2, cytochrome P450, and/or other enzymes with an amidase function (Di Marzo *et al.* 2015; Guzmán 2003).

Neurotransmitters stimulate the release of eCBs in a receptor-dependent way (D'Addario *et al.* 2013; Pandey *et al.* 2009; Wang & Ueda 2009). The release of eCBs may be enhanced through an "entourage effect" that relies on the co-release of other eCBs and endogenous fatty acid derivatives (D'Addario *et al.* 2013; Pandey *et al.* 2009; Wang & Ueda 2009). This entourage effect, described early on by Ben-Shabat and collaborators, is a mechanism through which eCBs act synergistically to modulate their release and effects (Ben-shabat *et al.* 1998). Additionally, AEA may also act as a modulator of other signaling pathways, such as the ones triggered by muscarinic and glutamate receptors, which have allosteric sites for the binding of this eCB, further denoting the extent to which eCBs may modulate the signaling of several neurotransmitters, especially in long-term plasticity (AEA and 2-AG) or short-term plasticity (2-AG) (Battista *et al.* 2012; Mackie 2013).

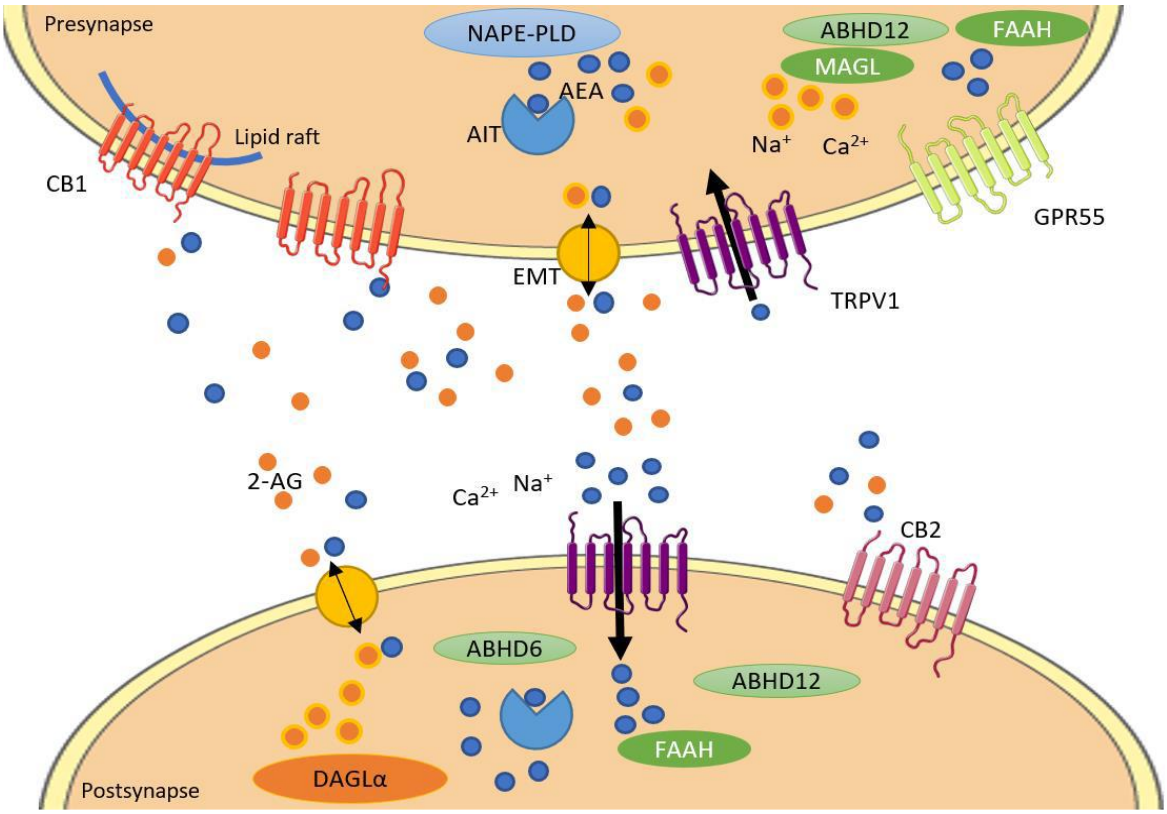


Figure 1. Endocannabinoid system during mature synapse. Endocannabinoids are produced postsynaptically and then transported by diffusion in a retrograde manner across the synapse, activating presynaptic CB_1 to inhibit synaptic neurotransmission. Ultimately, endocannabinoids are inactivated by presynaptic MAGL and FAAH. However, the AEA endocannabinoid can also be produced presynaptically by NAPE-PLD, which then activate TPRV1 and is inactivated by postsynaptic FAAH. Abbreviations: 2-AG: 2-arachidonoylglycerol; AEA: anandamide; AIT: AEA intracellular transporter; ABDH6 & ABDH12: hydrolases; CB: cannabinoid receptor 1 and 2; GPR55: G protein-coupled receptor 55; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D; TRPV1: transient receptor potential cation channel subfamily V member 1. Adapted from Maccarrone *et al.* 2014.

1.1.2. Cannabinoid receptors

The impact of cannabinoids on the central nervous system was evidenced in 1988 by Devane and collaborators after injecting rats with the synthetic cannabinoid CP-55,940 (Devane *et al.* 1992). The authors observed that this cannabinoid was specifically bound to certain structures of the brain, which were later identified as the CBR type 1 (CB_1, encoded by *cnr1* gene). The second main type of CBR was identified in 1992, being labeled as CB_2 (encoded, by *cnr2* gene). *Cnr2* has two known isoforms: CB_2A, predominantly expressed in the human testis; and CB_2B, mainly expressed in the spleen (Onaivi *et al.* 2012). Both CB_1 and CB_2 represent metabotropic receptors belonging to the class of G protein-coupled receptors. The G protein comprises three subunits: αi, β, and γ.

The activation of these receptors promotes their interaction with the G protein, resulting in guanosine diphosphate/triphosphate exchange and subsequent dissociation of the αi and βγ subunits. Of note, the αi subunit is the main responsible for signal transmission, while the βγ subunits are mostly recognized for interacting with second messengers (Galve-Roperh *et al.* 2013).

The CB_1 is predominantly abundant in the brain, mainly in areas such as the hippocampus (associated with memory), basal ganglia and cerebellum (motor coordination), and prefrontal cortex (cognitive and emotional processes). It is responsible for mediating the most psychoactive and neurobehavioral effects of exogenous (phyto- and synthetic) cannabinoids, and participates in the regulation of several physiological and metabolic processes (e.g. feeding, stress response via the interaction of the hypothalamic-pituitary-adrenal and gonadal axes) (Cristino *et al.* 2020; D'Addario *et al.* 2013; Szutorisz & Hurd 2016).

CB_2 is known to regulate various protein kinase cascades involved in cell proliferation and survival, mainly in progenitor cells. Additionally, this receptor is usually downregulated during neuronal differentiation (Di Marzo *et al.* 2015; Galve-Roperh *et al.* 2013). A prominent role for this receptor is the regulation of non-neuronal cells, with key involvement in processes like immune cell differentiation and bone remodeling (Carayon *et al.* 1998; Ofek *et al.* 2006). CB_2s are mainly located on the surface of the immune system cells, influencing the organism's resistance to infectious, allergic, and oncological diseases (Klein 2005). They may be also found in the brain, with a postsynaptic localization (e.g. neurons, glia, endothelial brain cells), although at a lower density than that of CB_1s (Di Marzo *et al.* 2015; Parsons & Hurd 2015). The dysfunctional activation of these receptors may also lead to several immune, cardiovascular, and bone disorders (Galve-Roperh *et al.* 2013). In particular, the overexpression of CB_2 in immune disorders (e.g. myeloid leukemias) suggests its important role in the cellular communication between the nervous and the immune systems, especially during neuronal damage and neuroinflammation (Klein 2005; Palazuelos *et al.* 2006). CB_1 and CB_2 also locate in other peripheral tissues, including adipose tissue, liver, skeletal muscle, reproductive organs, heart, lungs, and gastrointestinal tract (D'Addario *et al.* 2013; Szutorisz & Hurd 2016).

In the nervous system, during the short-term depression, CB_1-mediated inhibition of neurotransmitter release relies on the $G_{\beta\gamma}$ subunit. This inhibition is associated with stress-sensitive Ca^{2+} channel blocking. However, during the long-term depression, the protein kinase A (PKA) activity decreases, and the Ca^{2+}-dependent activity of the protein phosphatase calcineurin, a serine/threonine-dependent protein phosphatase that controls neurotransmitter release by regulating synaptic vesicle dynamics via Rab3-interacting

molecule-1α, increases (Galve-Roperh *et al.* 2013). In turn, the regulatory mechanisms involve the existence of regulatory molecules and second messenger-activated protein kinases and phosphatases expressed in the presynaptic terminal. These elements are capable of regulating the availability of free soluble N-ethylmaleimide-sensitive factor attachment protein receptors, which mediate docking of synaptic vesicles with the presynaptic membrane in neurons, to form the functional fusion machinery. These protein kinases and phosphatases may also substantially affect neurotransmitter release by acting as activators of second messenger molecules (e.g. PKA, protein kinase C, cyclic-dependent kinase 5), and by mediating short- and long-term changes in synaptic efficacy. The presence of these second messengers in presynaptic terminals correlates with decreased neurotransmitter release (Leenders & Sheng 2005).

Targeting of CBRs has been used by pharmaceutical companies for the treatment of several disorders. For example, agonists of CB_1 (e.g. WIN55,212-2) have shown antitumor and cardioprotective properties (Morales & Jagerovic 2019). CB_1 antagonists (e.g. AM251, SR141716A) have been used to treat schizophrenia, and cognitive and memory disorders in neurodegenerative diseases, such as Alzheimer's (Kruk-Slomka *et al.* 2016). CB_2 agonists (e.g. GW405833) display anti-inflammatory and immune-suppressive activities, which are of great interest to inhibit inflammatory states (Parlar *et al.* 2018). Most important, since CB_2 activation does not elicit psychoactive effects, these receptors may represent potential therapeutic targets to modulate the central nervous system without eliciting neurotoxicity (Di Marzo *et al.* 2015; Parsons & Hurd 2015).

In addition to these two well-known CBRs, cannabinoid agonists may also bind to G protein-receptor 55 (GPR55), peroxisome proliferator-activated receptors (PPARs), and TPRV1. The activation of GPR55, sometimes referred to as "CB_3R", has been linked to intracellular Ca^{2+} increase, activation of the small GTPase proteins RhoA, Rac, and Cdc4, and ultimately to extracellular-signal-regulated kinase (ERK) phosphorylation (Battista *et al.* 2012). eCBs may also bind to PPARs, transcription factors that constitute a superfamily of nuclear receptors represented by the subtypes α, δ, and γ. PPARs assemble as heterodimers when associated with a co-activator complex. PPARs bind to DNA sequences termed peroxisome proliferators response elements (PPREs) that are present in the promoter region of various target genes, ultimately leading to the activation or inhibition of transcription (Tyagi *et al.* 2011). The activation of these receptors affects several physiological and pathological processes, such as lipid metabolism, energy balance, appetite, neuroprotection, epilepsy, circadian rhythms, inflammation, addiction, and cognitive functions (Battista *et al.* 2012; Pistis & Melis 2010).

1.1.3. Regulation of neurotransmission by the endocannabinoid system

eCB retrograde signaling is the main mechanism mediating short- and long-term plasticity in both excitatory and inhibitory synapses (Castillo *et al.* 2012). Indeed, binding of postsynaptically-released eCBs to CB_1 on presynaptic terminals, inhibits the release of neurotransmitters such as γ-aminobutyric acid (GABA) and glutamate in a phenomenon described as depolarization-induced suppression of inhibition or excitation (DSI/DSE), respectively (Galve-Roperh *et al.* 2013; Mackie 2013; Szutorisz & Hurd 2016). Regulation of short-term plasticity may occur by DSI, which takes place in the hippocampus, cerebellum, neocortex, substantia nigra, and dentate gyrus; or DSE, which occurs at the hippocampus and cerebellum, as well as at the hypothalamus (Mackie 2013).

The cannabinoid suppression of presynaptic Ca^{2+} influx is mostly the indirect result of activation of presynaptic 4-aminopyridine-sensitive (voltage-dependent) K^+ channels and G protein-coupled, inward-rectifying K^+ channels (Mackie 2013). The Ca^{2+} influx guarantees the correct balance between the activation of Ca^{2+}-dependent processes (e.g. muscle contraction) and neurotransmitter release. CBRs play an important role in the negative response by modulating the activity of the Ca_v2 subfamily of the voltage-dependent Ca^{2+} channels, which are mostly located on neuronal and neuroendocrine cells. The inhibition of Ca^{2+} channels requires free $G_{\beta\gamma}$ dimers, and the cytoplasmic linker between domains I and II of the Ca_v2 αi subunits, which binds to $G_{\beta\gamma}$ dimers. It has been demonstrated that the transfection of either primary neurons or neuronal immortalized cells with $G_{\beta\gamma}$ subunits mimicked CB_1 agonist effects and led to tonic inhibition of the Ca^{2+} current, which could be reversed by a depolarizing pre-pulse, characteristic of voltage-dependent inhibition of these channels (Dolphin 2003; Herlitze *et al.* 1996). The activation of CB_1 promotes a reduction of Ca^{2+} influx into the axon terminals of interneurons, contributing to DSI. Additionally, K^+ channel activation also reduces Ca^{2+} entry due to the increase in K^+ conductance and, therefore, hyperpolarization. In turn, blockade of the K^+ channel results in a reduction of the DSI magnitude. However, this type of regulation is very complex and varies among distinct types of synaptic signaling (**Figure 2A**) (Mackie 2013).

The inhibition of Ca^{2+} channels and the activation of K^+ channels result in the inhibition of the GABA or glutamate transmission, depending on whether these neurotransmitters bind on inhibitory or excitatory synapse, respectively (Mackie 2013). On the other hand, the common form of induction of long-term plasticity depends on *N*-methyl-*D*-aspartate (NMDA) receptor (NMDAR)-mediated Ca^{2+} influx. However, the induction of long-term plasticity by eCB does not depend on postsynaptic NMDARs. The release of eCBs requires the increase in Ca^{2+} levels, and/or the group I metabotropic

glutamate receptor (mGluR-1s) activation in postsynaptic cells that occurs in a similar way to short-term plasticity (**Figure 2B**). This type of regulation takes place in various brain regions, including the nucleus accumbens and the dorsal striatum (designated as homosynaptic long-term plasticity, since it involves only glutamate neurotransmission), hippocampus and amygdala (termed heterosynaptic long-term plasticity, since it involves both glutamate and GABA neurotransmission), and the neo- and cerebellar cortex. Homosynaptic plasticity is triggered by excitatory inputs and requires both postsynaptic mGluR-1 activation and increased Ca^{2+}, which result in decreased presynaptic glutamate release. The activation of this mechanism in the neocortex is promoted by pairing presynaptic stimulations with postsynaptic depolarizations or action potentials, requiring the activation of presynaptic CB_1 and NMDARs. In this case, the eCB release is activated by postsynaptic Ca^{2+} increase. The cerebellar long-term plasticity is regulated differently, as it requires eCBs release evoked by paired activation of climbing and parallel fibers. 2-AG is the main eCB released via a convergent postsynaptic mGluR-1 activation and Ca^{2+} increase (Mackie 2013). Cyclic adenosine monophosphate (cAMP) may contribute to the regulation of neuronal gene expression by CB_1, in a process dependent on the recruitment of complex networks of intracellular protein kinases. The activation of ERK and focal adhesion kinase (FAK) is mimicked by inhibitors of cAMP-dependent kinase and disappears following exposure to cell-permeant cAMP analogs. This suggests that the activation of CB_1 might result in a decrease in intracellular cAMP levels (Piomelli 2003).

The interaction between eCBs and CBRs through the G protein αi subunit leads to the inhibition of adenylyl cyclase, and subsequent decrease of cAMP-dependent PKA, which in turn results in reduced phosphorylation of K^+ channels, as well as Ca^{2+} channels inhibition. The $G_{\beta\gamma}$ subunits are responsible for the stimulation of mitogen-activated protein kinase (MAPK) cascades [specifically ERK, p38 MAPK cascades, and c-Jun N-terminal kinases (JNKs)] and ultimately for the stimulation of additional intracellular pathways through the CB_2 receptors (Battista *et al.* 2012; Pertwee 2006).

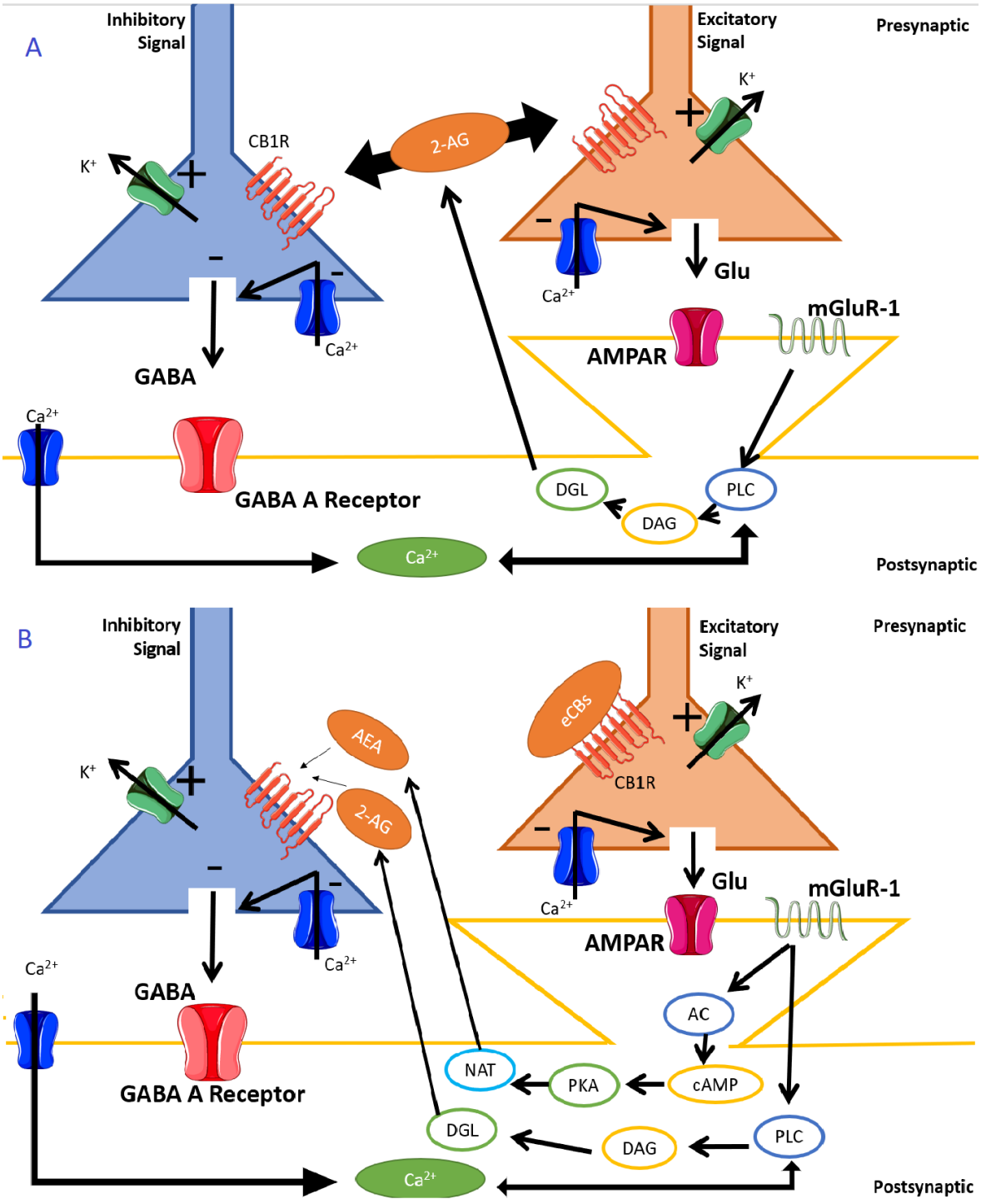


Figure 2. Regulation of synapse transmission by the endocannabinoid system. A) Retrograde signaling in short-term plasticity. Endocannabinoid signaling is triggered by Ca2+ influx through voltage-gated channels as a consequence of postsynaptic step depolarization or action potentials may be then amplified by recruitment of Ca2+ release from intracellular stores. Alternatively, eCB signaling may be triggered by the brief stimulation of excitatory afferents mediated by AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors), which are both glutamate receptors and cation channels, which allows the influx of Ca2+ and on-demand endocannabinoid biosynthesis, and also by a group I mGluR (metabotropic glutamate receptor) activation. Downstream of mGluR, Phospholipase C (PLC) and diacylglycerol lipase (DGL) lead to

the synthesis of 2-AG. Although each pathway can be triggered independently, some degree of cooperativity may exist (e.g. increased intracellular Ca2+ greatly enhances mGluR-induced (mGlu-1) PLC activity). Endocannabinoids then travel to the synaptic cleft and bind to presynaptic CB_1s, resulting in Ca2+channel inhibition, producing a direct effect on the vesicle release machinery and/or K+ channel activation. **B)** Retrograde signaling in long-term plasticity. This process is initiated by glutamate release and mGluR-1 activation, resulting in a decrease in GABA release. Endocannabinoids may be produced by two different pathways in this type of synapse. 2-AG is the result from PLC-DGL activation and takes place in the hippocampus. Contrarily, the AEA release requires the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway, which in its turn will activate the N-acetyltransferase (NAT), an enzyme involve in the synthesis of anandamide precursor, and occur in the amygdala. Adapted from Huganir & Nicoll 2013, and Mackie 2013.

1.1.4. The endocannabinoid system during neurodevelopment

The evidence for the role of the eCB system in neuronal development is based on the observation that 1) its elements are detected in the brain at early stages of fetal development, as evidenced by Romero and collaborators (Fernández-Ruiz *et al.* 2000), who detected CBRs in rat fetuses at gestational day 21; 2) CB_1s have a transient expression, as they may be highly expressed in neurons from some brain regions that play key roles during brain development and that do not express them in adulthood (e.g. the subventricular areas, which are highly involved in neuronal proliferation, migration, and axonal elongation (Fernández-Ruiz *et al.* 2004)). Although this transient expression is not fully understood, it has been supported by different experimental evidence, which has been elegantly reviewed elsewhere (Fernández-Ruiz *et al.* 2000, 2004). For example, the mesencephalic neurons obtained from rat fetuses at gestational day 14, contain CB_1s that colocalize with tyrosine hydroxylase at such fetal stage but do not express them during adulthood. Moreover, while in the adult brain CB_1s are mainly located in neuronal cells, during brain development these receptors may be also found in some subpopulations of glial cells that play a crucial role in neuronal development (Hernández *et al.* 2000). In addition, CB_1 mRNA transcripts have been found in the human fetal brain, but not at later stages (Wang *et al.* 2003); 3) eCB system elements seem to be functional even at an early age. This observation is supported by the fact that the pharmacological activation of CBRs during the developmental period has been associated with several effects, such as the induction of the expression of genes encoding key proteins related to specific neurotransmitters (e.g. the enzyme tyrosine hydroxylase or the opioid precursor proenkephalin), activation of energetic metabolism, arachidonic acid mobilization, and other biological processes possibly related to neural development (Fernández-Ruiz *et al.* 2004; Romero *et al.* 1997).

During neurodevelopment, the eCB system is extensively involved in the regulation of neuronal cell proliferation, migration, differentiation, and survival (Harkany *et al.* 2007;

Maccarrone *et al.* 2014), as summarized in **Figure 3**. Neural progenitor cells (NPC) are known to synthesize eCBs, the catabolic enzyme FAAH, and express functional CB_1s. In particular, the activation of these receptors has been reported to promote NPC proliferation, and differentiation (Harkany *et al.* 2007). However, the first evidence of the active role of CBRs in NPCs derived from the studies on adult neurogenesis regulation involving eCB administration or *cnr1* ablation (Jin *et al.* 2004). Aguado and collaborators (2005) demonstrated that the eCB system interferes with NPC function, by showing that the synthetic non-selective CBR agonist WIN-55,212-2 and the selective FAAH inhibitor URB597 increased both the number and size of NPC neurospheres. By using the selective CB_1 antagonist SR141716A, the same authors further showed the involvement of CB_1 in cell proliferation and the neurons self-renewal.

The activation of the CB_1 leads to the initiation of the eCB signaling cascades, which occurs simultaneously with the commitment of NPCs to generate pyramidal cells and of GABA-containing precursors to undergo tangential migration in the developing neocortex (Wonders & Anderson 2006). Moreover, the eCBs' involvement in the acquisition of neuronal identity has been previously reported (Harkany *et al.* 2007). Noteworthy, the eCB signaling provides a balance between cell proliferation and programmed cell death, assuring the generation of adequate numbers of neuronal cells during brain development. This indicates that the eCB system can modulate various steps in the fate of NPC and supports its key role in the regulation of neuronal function and survival (Aguado *et al.* 2006).

CB_1s are crucial regulators of ion channels' activity, neurotransmitter transporters, metabolic enzymes, and cytoskeletal integrity. The recruitment of second messengers [e.g. proto-oncogene tyrosine-protein kinase Src, ERK1/2, and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)] to this receptor enhances the potential of CB_1 to dynamically regulate the spatial and temporal coordination of NPC proliferation. The antiproliferative action of cannabinoids is mediated by activation of the Raf1-ERK1/2 pathway, which can modulate cell proliferation when transiently or moderately activated. In neuronal cells, the eCB-induced activation of CB_1-mediated epidermal growth factor (EGF)-receptor leads to the simultaneous activation of two independent cascades of events: one includes the sequential activation of Ras, Raf-1, and ERK1/2; and the other one initiates with PI3K activation and triggers the Akt-mediated pathway (Harkany *et al.* 2008). In NPC, CB_1-induced cell proliferation is mediated by the PI3K/Akt/GSK3β (glycogen synthase kinase 3β) signaling, due to a consequence of GSK3β inhibition, and the increased β-catenin nuclear translocation drives cyclin D1 expression (Galve-Roperh *et al.* 2013). Noteworthy,

both ERK 1/2- and Akt-mediated pathways promote cell proliferation (Harkany *et al.* 2008), as summarized in **Figure 3A**.

Cannabinoids can activate different signaling pathways involved in the regulation of cell fate, including the MAPK family (e.g. ERK, JNK, and p38) and Akt. eCBs may directly modulate the differentiation of NPCs by activating ERK1/2 in a process involving the upstream inhibition of Rap-1 and B-Raf (**Figure 3B**). However, neuronal differentiation may be inhibited via CB_1-independent regulation (Rueda *et al.* 2002). This type of regulation is specific to AEA and may allosterically modulate the activity of a broad range of receptors and ion channels affecting neuronal fate (e.g. α7-nicotinic acetylcholine receptors, T-type Ca^{2+}channels, voltage-gated and background K^+ channels and TRPV1) (Van Der Stelt & Di Marzo 2005).

During neuronal migration, the cooperation between eCB- and brain-derived neurotrophic factor (BDNF)-induced migration of cortical GABA-containing interneurons expressing CB_1s, occurs through the cytoplasmic tyrosine receptor kinases (Trks) like Src and Fyn. The proteolytic ectodomain of EGF-like precursors releases active ligands that induce rapid EGF receptor phosphorylation. This transactivation promotes the phosphorylation of Shc (an Src homolog 2 domain) with consequent downstream activation of MAPK pathways (Hart *et al.* 2004). This receptor is also associated with the neurotrophin tyrosine receptor kinase B (TrkB). CB_1 activation in neuronal cells may lead to the activation of the TrkB signaling system, resulting in neuronal migration (**Figure 3C**). In turn, the absence of eCB-induced migration results in the inhibition of TrkB phosphorylation, and consequently in decreased neuronal cell migration (Berghuis *et al.* 2005). Moreover, it should also be noted that eCBs often interact with other signaling systems, such as neurotrophins, growth factors, and inflammatory cytokines, to induce cell migration (Harkany *et al.* 2008).

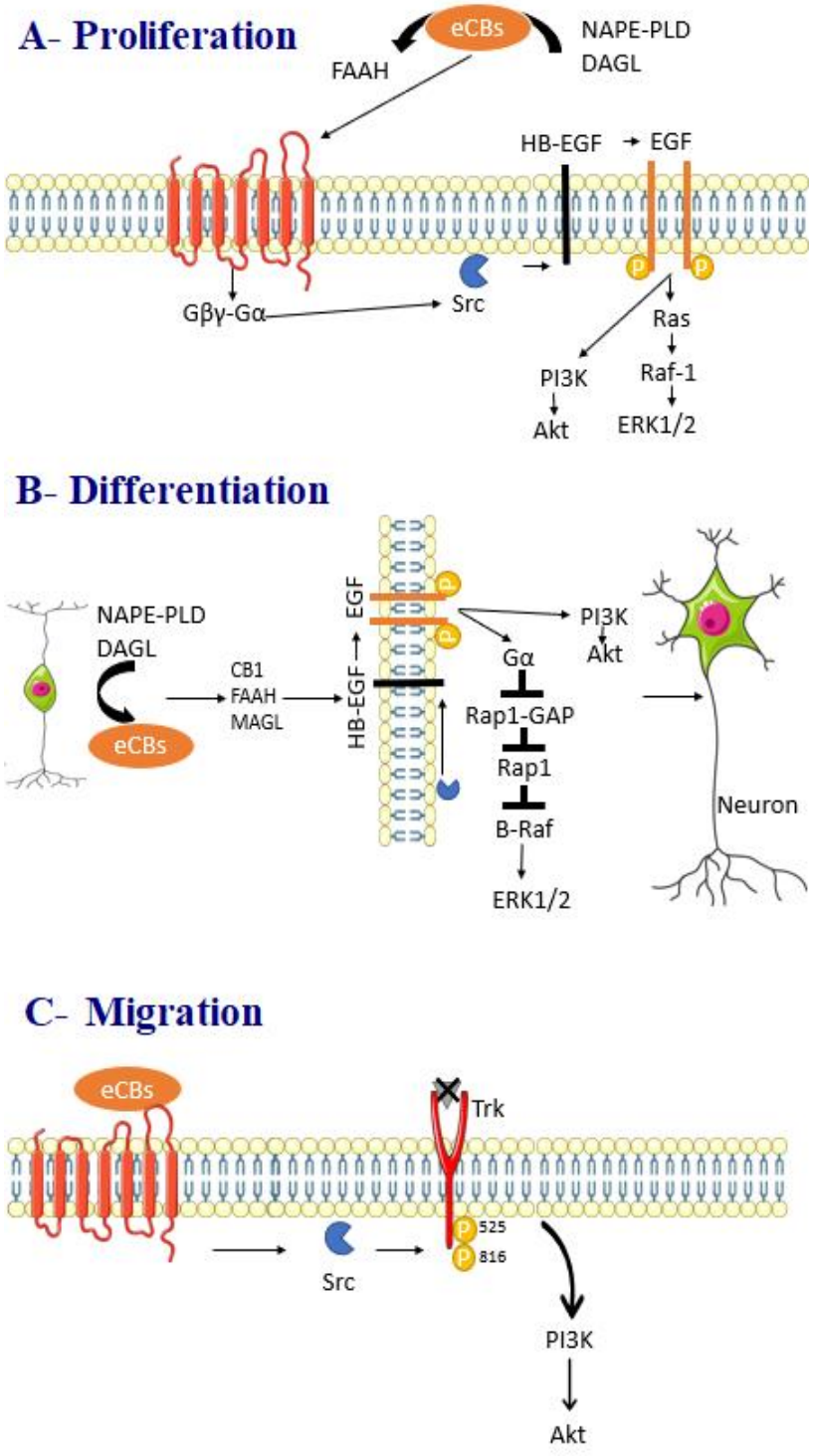


Figure 3. Endocannabinoids action through G-coupled CB_1 during neuronal development-related processes, including: A) Proliferation. Activation of secondary signaling, such as Raf1-ERK1/2 pathway could modulate cell proliferation by CB_1-mediated EGF-receptor transactivation by eCBs, ending with the activation of ERK1/2 and Akt, which in its turn leads to an increase of proliferation. **B) Differentiation**. On the other hand, the blockade of second messengers' cascades such as Rap1 and B-Raf together with the activation of ERK 1/2 induce cell differentiation. **C) Migration**. eCBs activate CBRs that induce Src activation, which in its turn induce the phosphorylation of the Trk receptor and promotes neuronal migration. Abbreviations: Akt: protein kinase B: B-Raf, mitogen-activated protein kinase (MAPK) kinase; DAGL: diacylglycerol lipase;

eCB: endocannabinoid; FAAH: fatty acid amide hydrolase; GAP: GTPase-activating protein; HB-EGF: heparin-binding EGF-like growth factor; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D; PI3K: phosphatidylinositol 3-kinase; Raf-1: MEK kinase; Src: non-receptor tyrosine kinases. Adapted from Galve-Roperh *et al.* 2013, and Harkany *et al.* 2007.

1.2. Epigenetic mechanisms

The epigenetic term broadly refers to the changes in gene function that are mitotically or meiotically heritable and that do not imply an alteration in DNA sequence (Dupont *et al.* 2009). Understanding the epigenetic mechanisms thus allows bridging the gap between genotype and phenotype (D'Addario *et al.* 2013).

Chromatin comprises a structure where the DNA is packed and protected, and in which the nucleosome, comprising short stretches of DNA wrapped around histones, represents the basic unit of chromatin (Michalak *et al.* 2019). This structure is organized into two different levels: euchromatin (active) and heterochromatin (silent). Euchromatin contains most of the genes and is considered to be transcriptionally active. Plasticity of euchromatin maintains DNA open for transcription-related elements, so the genes can be transcriptionally turned on or off. Heterochromatin regions comprise nuclear material, mostly silent, being the main constituent of telomeres and pericentric regions, which tend to be rich in repetitive sequences (Felsenfeld 1978; Williamson *et al.* 2018).

Chromatin replication during cell cycle provides an excellent opportunity for the introduction and propagation of epigenetic modifications (e.g. DNA methylation/demethylation, histone modifications) into the newly synthesized DNA. Failure to properly regulate epigenetic information may lead to catastrophic consequences at the cellular level, such as incorrect gene expression and apoptotic cell death, ultimately resulting in tumor progression, protein malformation, and/or gene silencing (Kim *et al.* 2009).

Epigenetic modifications occur randomly and include mechanisms like DNA methylation (e.g. mostly at cytosine bases), post-translational modification of histones (e.g. acetylation, methylation, and phosphorylation), and nucleosome positioning. In addition, non-coding RNA molecules [e.g. microRNA (miRNA) and endogenous small interfering RNA (siRNA)] may be also involved in epigenetic processes (D'Addario *et al.* 2013; Szutorisz & Hurd 2016; Rangasamy *et al.* 2013). All these epigenetic modifications are summarized in **Figure 4.**

Epigenetic mechanisms play an important role in coordinating DNA transcription, replication, and repair. All these biological tasks require the ordered recruitment of

complex machinery to unfold and modify DNA, as well as to reset it to the correct chromatin state (Kouzarides 2007). Additionally, the cross-talk between the different epigenetic mechanisms may help maintain the proper recruitment of protein factors onto chromatin (Kim *et al.* 2009). The main epigenetic mechanisms are summarized below.

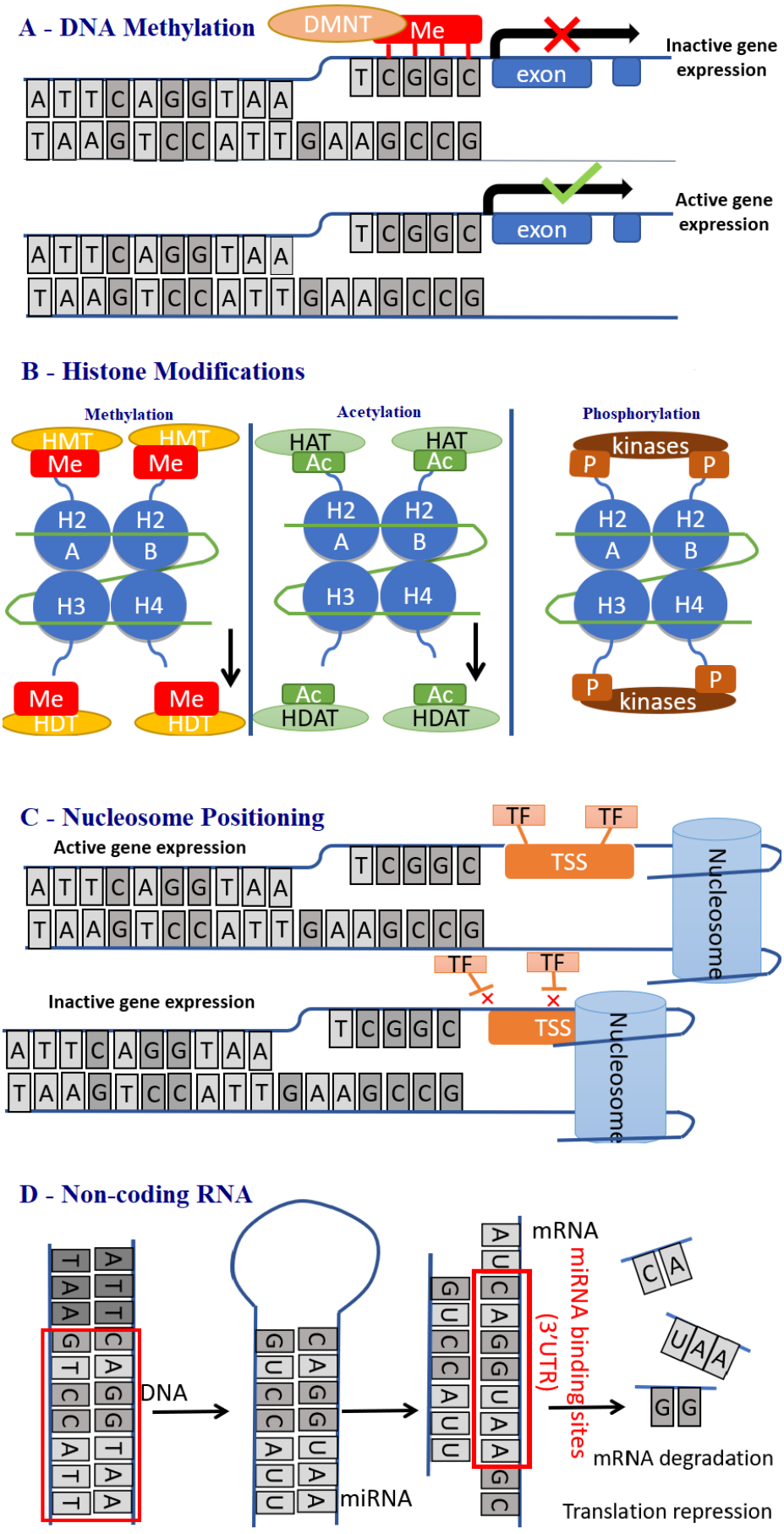

A - DNA Methylation
DMNT
Me
exon
Inactive gene expression
Active gene expression
B - Histone Modifications
Methylation
Acetylation
Phosphorylation
HMT
Me
HAT
Ac
kinases
P
H2A
H2B
H3
H4
HDT
HDAT
C - Nucleosome Positioning
Active gene expression
Inactive gene expression
TF
TSS
Nucleosome
D - Non-coding RNA
DNA
miRNA
mRNA
miRNA binding sites
(3'UTR)
mRNA degradation
Translation repression

Figure 4. Epigenetic modifications. A) DNA methylation, occurring by the action of DNA methyltransferases (DNMTs). Of note, if methylation occurs in the promoter or first exon, gene expression is inhibited. **B) Histone modifications** comprise methylation, involving the action of enzymes such as histone methyltransferases (HMT) and histone demethyltransferases (HDT); acetylation, by the enzymes histone acetyltransferases (HAT) and histone deacetylases (HDAC), which results in the inhibition of gene expression; and phosphorylation involving kinases that causes condensation/decondensation of chromatin; among others modifications. **C) Nucleosome positioning**, which occurs when the nucleosome occupies the transcription start site (TSS), preventing the transcription factors (TF) binding to it, thus repressing gene expression. **D) Non-coding RNA**, which include microRNAs (miRNA), that bind to 3'UTR region of messenger RNA (mRNA) resulting in target degradation and consequently inhibition of translation. Me, methyl group; Ac, acetyl group; P, phosphate group. Adapted from D'Addario *et al.* 2013; Portela & Esteller 2010.

1.2.1. DNA methylation

DNA methylation represents the most studied epigenetic mechanism and mainly consists of the covalent modification of the fifth carbon in the cytosine base (5mC) in the CpG dinucleotides within the genome. As eukaryotic 5mC is mostly located in the context of symmetrical CpG dinucleotides, it has been associated with transcriptional repression. This epigenetic modification occurs along with three phases: establishment (de novo DNA methylation), maintenance, and demethylation (Greenberg & Bourc'his 2019). Methylation is catalyzed by DNA methyltransferases (DNMTs), such as DNMT1, DNMT3A, and DNMT3B, being the latter two the major de novo DNA methylation enzymes. These enzymes methylate the DNA and maintain genomic methylation patterns (Michalak *et al.* 2019). On the other hand, the initial step of active DNA demethylation is catalyzed by ten-eleven translocation methylcytosine dioxygenases (TET), which oxidize the 5-methyl group of 5mC to generate 5-hydroxymethylcytosine, 5-formylcytosine or 5-carboxylcytosine. In DNA sequences, methylation of CpG dinucleotides sometimes appears concentrated in dense pockets called CpG islands, 70 % of which are located in the promoter region of the first exon, and are associated with gene transcription silencing (**Figure 4A**) (Greenberg & Bourc'his 2019; Michalak *et al.* 2019). Although stable methylation of DNA generally occurs in somatic cells, the reprogramming of methylation, such as de-methylation or re-methylation, may be also observed during two developmental steps: in germ cells and preimplantation embryos (Kim *et al.* 2009; Portela & Esteller 2010). Recently, Cardenas *et al.* (2017) showed that moderate mercury exposure during pregnancy can lead to gender-specific epigenetic alterations in the methylation levels of the *pon1* gene [which encodes serum paraoxonase/arylesterase 1, responsible for providing oxidative protection against high-density lipoprotein (HDL)] and in the CpG of *tor4a* (which encodes for the torsin family 4 member A, which is involved in platelet degranulation). The authors found that these changes may persist throughout

childhood and influence the children's cognitive performance, learning, and memory behaviors (Cardenas *et al.* 2017). Of note, the role played by DNA methylation during neuronal development has already been thoroughly reviewed elsewhere (Bruggeman & Yao 2019; Stricker & Götz 2018).

1.2.2. Histone modifications

Histones are proteins that have an essential structural and functional role in the transition between active and inactive chromatin states and are involved in gene regulation and epigenetic silencing (Henikoff & Smith 2015). The structural state of chromatin and its domains regulates DNA replication and gene expression. Moreover, due to histone fold domains and N-terminal tails, histones are vulnerable to post-translational modifications, which are implicated in transcriptional activation, silencing, and chromatin assembly, resulting in changes to DNA and its connected proteins. The nucleosome is the first level of chromatin organization and is composed of two copies of each histone H3, H4, H2A, and H2B, wrapped by a segment of DNA, and joined together by linker DNA and linker histone H1. Due to the presence of a large number of amino acid residues, histones are a common target for various distinct types of modifications, such as methylation, acetylation, phosphorylation, and ubiquitylation, among others (Mariño-Ramírez *et al.* 2005). Some of these modifications are depicted in **Figure 4B.** The main enzymes involved in these modifications comprise histone deacetylases (HDACs) and acetyltransferases (HATs), which catalyze histone deacetylation and acetylation, respectively; lysine- and arginine-specific histone methyltransferases (HMTs) and histone demethylases (HDMs), which catalyze histone methylation and demethylation, respectively; ubiquitinases and deubiquitinases, which catalyze ubiquitylation and deubiquitylation, respectively; arginine deiminases, which are responsible for the hydrolysis of peptidyl-arginine to form peptidyl-citrulline; and kinases which catalyze histone phosphorylation (Marmorstein & Trievel 2009).

Histone methylation may also result either in the activation or repression of gene expression, depending on the sites at which it occurs. Methylation-sensitive transcription factors and methyl-binding proteins are transcriptional complex proteins that bind to specific methyl groups on CpG dinucleotides in association with gene silencing. For example, upon methylation in the promoter region, gene expression is silenced, while its occurrence in another stretch of the DNA sequence may activate the expression of a different gene (D'Addario *et al.* 2013; Kim *et al.* 2009; Kouzarides 2007; Portela & Esteller 2010). While lysine methylation may lead to distinct effects depending on the number of methyl groups and on the position of lysine residues, acetylation, and deacetylation of

lysines relate to chromatin accessibility and transcription (i.e. depends on whether it takes place in euchromatin or heterochromatin). In addition, it is important to note that histone acetylation is exclusively associated with active chromatin (euchromatin) and that it represents the key modulator for transcriptional activation or repression (Kim *et al.* 2009). Arginine methylation may represent an active or repressive mark for transcription. The methylation of arginine is catalyzed by protein arginine methyltransferases (PRMTs), which are involved in a variety of essential cellular processes (e.g. transcription, pre-mRNA splicing, and DNA damage response), and the demethylation is catalyzed by arginine demethylases encoded by the Jumonji gene family, and by proteins such as Tudor, WD40 and PHD (Guccione & Richard 2019).

Multiple types of histone methylations frequently occur during neurodevelopment. NPC and NPC-specific knockout of both *prmt1* and *prmt5* genes leads to post-natal mortality in rats (Jambhekar *et al.* 2019). PRMT5 controls the differentiation and proliferation of NPCs by causing H4R3me2 to downregulate pro-mitotic genes and induce mRNA splicing, further resulting in NPC depletion and reduced number of neurons. However, *prmt1* loss caused a reduction in the number of mature oligodendrocytes, which results in severe hypomyelination in the central nervous system (Jambhekar *et al.* 2019).

The presence of phosphate-binding proteins in the chromatin is often associated with phosphorylation processes, namely histone phosphorylation. These processes also have significant consequences for chromatin condensation/de-condensation during cell replication (Kim *et al.* 2009).

Histone modifications play a crucial role in the recognition and accessibility of damaged sites in response to DNA damage, providing a platform for DNA repair to take place (Kouzarides 2007).

Equally important, these epigenetic changes seem to play an important role in the regulation of neuronal development, as previously reviewed by Lomvardas and Maniatis (2016).

1.2.3. Nucleosome positioning

The nucleosome is the essential unit of chromatin and it is involved in gene regulation by defining access of transcriptional factors to the underlying promoter elements, and by stabilizing protein interactions. Nucleosome positioning in gene promoters or enhancers is associated with gene silencing, through inhibition of binding between transcriptional factors and functional regions (Helbo *et al.* 2017). Moreover, it directly affects cellular processes, such as DNA replication, recombination, and transcription, besides acting as an important target of epigenetic modifications (Liu *et al.*

2018). The nucleosome core particle refers to the regular central part of the nucleosome and consists of a polyanion-polycation complex with a net charge of about 148 electrons, comprising a negatively charged central particle (Korolev *et al.* 2018). Chromatin modifications may directly change its structure by altering the physical properties of individual nucleosomes via neutralization, or through the addition of a positive charge to target residues such as lysine, a modification that occurs in histone acetylation. Consequently, alteration of net charge affects histone-DNA interactions and further opens chromatin or higher-order structures through differential modulation of internucleosomal contacts (Bhaumik *et al.* 2007; Kim *et al.* 2009).

These chromatin modifications may occur in three types of protein complexes: chromatin remodeling complexes, effector proteins with several binding modules for different modifications, and insulator proteins. Of these, chromatin remodeling complexes are the most common and involve energy-driven, multi-protein machinery that allows contacting specific DNA regions or histones by modifying nucleosome position, histone-DNA interactions, and histone octamer positions (Kim *et al.* 2009). For gene expression to occur, transcription factors must bind to DNA, an event that occurs if nucleosomes are missing upstream of the transcription start sites (TSS). On the other hand, gene expression is inhibited if the nucleosome occupies the TSS, thus preventing the binding of the transcription factor, as schematized in **Figure 4C** (D'Addario *et al.* 2013; Portela & Esteller 2010). Noteworthy, nucleosome positioning and DNMTs are strongly related, as the latter preferably target nucleosome-bound DNA (D'Addario *et al.* 2013). The importance of nucleosome positioning during neuronal development has been recently demonstrated by Harwood *et al.* These authors used genome-wide mapping of undifferentiated human-induced pluripotent stem cells and followed the nucleosome positioning dynamics as the cells differentiated into a neuronal progenitor cell stage, noticing an increased positioning during cell differentiation. Moreover, positioned nucleosomes appeared to regulate gene expression by changing the structural conformation of sites involved in chromatin regulation (Harwood *et al.* 2019).

1.2.4. Non-coding RNA

Non-coding RNAs are a family of small 21-25-nucleotide RNAs that negatively regulate gene expression at a post-transcriptional level (He & Hannon 2004). Most of the known non-coding RNA modifications occur at ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs), which are implicated in generic cellular functions such as mRNA translation and splicing, and modification of rRNA (Handy *et al.* 2011; Mattick & Makunin 2006). Non-coding RNAs

were reported to engage in a wider range of functions, including control of chromosome dynamics, RNA editing, translational inhibition, and mRNA degradation (Handy *et al.* 2011; Mattick & Makunin 2006). Moreover, non-coding repetitive RNA may be involved in stress response. They are located at “nuclear stress bodies” that are present in heterochromatin domains that may change their epigenetic status from heterochromatin to euchromatin in response to stress (Brunet *et al.* 2004).

Other relevant small RNAs that may be modified epigenetically include miRNA and siRNA. miRNA is generally 21-25 nucleotides long and derives from larger precursors that form imperfect stem-loop structures. Most of them bind to the target 3’-untranslated region (UTR) with an imperfect complementarity and act as translational repressors (**Figure 4D**). In contrast, siRNA results from RNA interference (RNAi), which is a sequence-specific gene-silencing mechanism induced by the exposure to viral double-strand RNA (dsRNA) that can also act as a defense mechanism against virus infection or parasitic nucleic acids. This dsRNA is processed into siRNA and further incorporated into the RNA-induced silencing complex (RISC). This complex then directs the cleavage of complementary mRNA targets, resulting in post-transcriptional gene silencing (Zamore *et al.* 2000). Similar to miRNA, siRNA may also regulate target transcription. Additionally, miRNAs bind to the target 3’UTRs at multiple sites through imperfect complementarity, ultimately negatively regulating target transcription. In contrast, siRNAs randomly result from long dsRNAs that can be introduced exogenously or form bi-directionally transcribed endogenous RNAs that anneal to form dsRNA. In addition, siRNAs may bind to their target only at one site through perfect complementarity, resulting in direct cleavage of mRNA (He & Hannon 2004; Handy *et al.* 2011).

Recently, the importance of long non-coding RNAs (lncRNAs), representing transcripts longer than 200 nucleotides, in the neuronal development and in the onset of several neurological disorders gained increasing interest (Li *et al.* 2019). The lncRNAs involved in the development of spinal motor neurons were previously summarized by Chen and Chen (2020).

1.3. Interplay between epigenetic mechanisms and the endocannabinoid system

1.3.1. Regulation of the endocannabinoid system by epigenetic mechanisms

The genes responsible for the expression of some eCB system elements, like CBRs, may be regulated by different transcription factors, which may be also involved in DNA methylation and histone post-transcriptional modifications (Lachmann *et al.* 2010).

The epigenetic regulation of CB_1 by DNA methylation/demethylation or histone modifications, represents an important research focus, considering that CB_1 mRNA is found deregulated in different pathological conditions (e.g. obesity, diabetes, colorectal cancer, schizophrenia, Parkinson's, and Huntington's diseases) as a result of the exposure to different drugs (including exocannabinoids) (Laprairie *et al.* 2012). The broad mechanisms by which epigenetic mechanisms like DNA methylation/demethylation or histone modifications regulate the eCB system are summarized in **Figure 5A**.

Börner and collaborators (2012) showed that both *cnr1* and *cnr2* expression may be epigenetically regulated by two modulators: an inhibitor of DNMTs, named 5-aza-2'-deoxycytidine, which results in DNA hypomethylation; and trichostatin A, a histone deacetylation inhibitor that promotes histone hyperacetylation. The authors showed that these molecules regulated the induction of *cnr1* and *cnr2* expression in human neuroblastoma SH-SY5Y cells. Incubation of SH-SY5Y cells, which endogenously express CB_1 but not the CB_2, with these two modulators, individually or combined for 24 h, did not alter the mRNA levels of *cnr1* and *cnr2*. However, the stimulation of the cells with those two modulators resulted in a significant induction of CB_2 mRNA, which is normally silenced. This induction was significantly stronger when both modulators were administered together.

The influence of epigenetic modifications, such as DNA methylation and histone acetylation and deacetylation, on the eCB system, is evident in late-onset Alzheimer's disease (a type of Alzheimer's occurring in individuals over 65 years old). In this pathology, the *faah* gene (responsible for encoding FAAH) is found altered as a result of reduced DNA methylation at its promoter. The lowest levels of methylation were observed in patients with the most severe cognitive impairment, suggesting methylated *faah* as a new potential therapeutic target for Alzheimer's disease (D'Addario *et al.* 2012). In addition, the *faah* gene is the direct target of 17β-estradiol (E_2) in Sertoli cells. The enhancement of the *faah* promoter activity involves the estrogen receptor β (ERβ) and lysine-specific histone demethylase 1 (HDM LSD1), and also requires the assembly of chromatin configuration responsible for transcription (Grimaldi *et al.* 2012). Generally, E_2 induces epigenetic modifications at the *faah* proximal promoter, consistent with transcriptional activation. Specifically, E_2 induces the demethylation of CpG islands and also the demethylation of previously methylated lysine 9 in histone 3 (H3K9me) in the proximal region of the *faah* promoter, resulting in the activation of *faah* transcription (Orth *et al.* 1988).

Chronic unpredictable stress (CUS) induces anxiety- and depression-like behaviors, altered pain perception, and impairment of eCB signaling in rodents. This type of stress

can be reversed by URB597 (FAAH inhibitor), suggesting that CUS impairs AEA signaling by blocking its degradation enzyme. Lomazzo and collaborators (2017) found a decreased expression of *cnr1* in the cingulate cortex of mice with CUS, specifically in low CB_1-expressing neurons. The authors reported that chronic stress-induced reduction in the levels of histone H3K9 acetylation (H3K9ac) associated with the *cnr1* gene could be responsible for causing several impairments at the molecular level. These decreased levels of H3K9ac appeared to be dependent on changes in the function of HDACs, as the HDAC2 protein levels were increased in CUS mice, compared to controls. Of note, the levels of *cnr1*-associated H4K8ac, which are often related to anxiety in rat models, were not altered by chronic stress or an inhibitor of anandamide.

In hippocampal cells, the *cnr1* promoter seems to be mainly repressed by CpG-methylation. Hay and collaborators (2019) cloned the *cnr1* promoter into a CpG-free luciferase vector that cut any CpG dinucleotides and exposed these plasmids to CpG methyltransferase. The authors then transfected these plasmids into primary hippocampal cells to determine the effects of CpG methylation on *cnr1* activity. This procedure confirmed that CpG methylation strongly repressed the *cnr1* promoter, suggesting the promoter's high susceptibility to CpG methylation.

The eCB system is involved in the control of nausea, vomiting, and its homeostatic role extends to the control of intestinal inflammation. CB_1 in sensory ganglia controls visceral sensation and transcription of *cnr1* is modified through epigenetic processes (e.g. DNA methylation/demethylation, histone acetylation/deacetylation) under conditions of chronic stress. Sharkey and Wiley (2016) suggested that chronic stress may induce DNA methylation and subsequently the downregulation of anti-nociceptive *cnr1*, with a concurrent increase in histone acetylation of pro-nociceptive *trpv1* in a region- and cell-specific manner, leading to visceral hyperalgesia. Furthermore, chronic stress was also associated with an increase in DNMT1-mediated methylation of the promoter of the *nr3c1* gene (which encodes for the glucocorticoid receptor, a regulator of *cnr1* transcription) and reduced expression of this gene in nociceptive primary afferent neurons. In visceral primary afferent neurons, the region-specific knockdown of DNMT1 and EP300 gene expression, the levels of DNA methylation and histone acetylation were lower and prevented chronic stress-induced visceral pain.

Due to its important role in the reward circuit, the eCB system is also involved in eating addiction and in the pathogenesis of obesity through epigenetic actions. CB_1 plays an important role in the reinforcement and motivational properties of pleasant food by modulating the glutamatergic excitatory and GABAergic inhibitory synaptic inputs in several regions of the brain. In this sense, the decreased DNA methylation at *cnr1* might

promote an up-regulation of CB_1 on glutamatergic projection terminals resulting in CB_1-dependent inhibition of excitatory glutamatergic transmission, which induces food intake (Mancino *et al.* 2015).

Dogra and collaborators (2016) showed an elevated expression of CB_1 in adult mice exposed to alcohol, which was associated with an increased acetylation of H4K8 (histone 4, lysine 8) at the CB_1 promoter that subsequently led to neurobehavioral abnormalities. Subbanna and coworkers (2015) demonstrated that transcriptional activation of CB_1 followed by widespread neurodegeneration in the neonatal brain involves a specific increase of H4K8 acetylation (associated with active transcription) and H3K9 demethylation (correlated with transcriptional silencing) at exon 1 in the *cnr1* gene.

The *cnr2* gene has two known isoforms, CB_2A and CB_2B. The promoter of CB_2A contains CpG islands and many CCAAT boxes with a binding site for transcription factors related to stress response, such as activator protein 1, heat shock factor (HSF) and stress response element. In opposition, CB_2B does not share these characteristics but has a binding site for transcription factors that bind to the “GATA” DNA sequence (e.g. GATA binding factor-1, also known as erythroid transcription factor), HSF, tinman homolog Ntx2.5 (also known as homeodomain factor) and activator protein 4. The epigenetic regulation of *cnr2* loci via DNA methylation or histone modifications, for example, might play a crucial role in receptor regulation due to the CpG islands found in the promoter regions. As previously reported, CpG islands are frequently methylated by DNMTs. This phenomenon is linked to stress-responsive transcription binding sites and may enable the promoter of CB_2A to be inhibited and activated in some regions of the brain in spatial and temporal modes. The only 5’UTR sequence coded by specific exon 1a and 1b might target CB_2 to specific neuronal regions such as pre- or postsynaptic structures (Onaivi *et al.* 2012).

1.3.2. Regulation of epigenetic mechanisms by endocannabinoid system

In addition to the epigenetic modulation of the eCB system, it has also been reported that the eCB system may regulate epigenetic mechanisms (e.g. DNA methylation, histone acetylation), acting as epigenetic factors. The main mechanisms are outlined in **Figure 5B**.

Endocannabinoids may induce changes in the enzymes responsible for histone modifications (e.g. acetylation, methylation, phosphorylation) at the transcriptional level (Stein & Stein 1984). Moreover, CB_1 activation has been reported to promote changes in the expression of genes that play key functions in various neurotransmitter systems (D’Addario *et al.* 2013). For example, AEA was described to protect neurons from

inflammatory damage by inducing histone H3 phosphorylation of *mpk-1* (the gene encoding for MAPK phosphatase-1) in activated microglial cells, thus regulating *mpk-1* expression and subsequently dephosphorylating ERK1/2 (Upham *et al.* 2003). Mitochondrial CB_1s, in particular, have been implicated in several critical neuronal processes. Cannabinoids may interfere with mitochondrial activity, namely with oxidative phosphorylation, having major epigenetic implications for mito-nuclear balance and trafficking, including mitochondrial stress response, as well as for the supply of metabolic intermediates like acetyl-coenzyme A, which is an absolute requirement for histone acetylation and regular gene activation (Reece & Hulse 2019).

Fetal Alcohol Spectrum Disorders (FASD) are a set of conditions that occur in an individual exposed to ethanol during prenatal development and are characterized by widespread neuropsychological defects. Ethanol exposure is associated with a genome-wide/gene-specific altered pattern of histone modifications, DNA methylation, and long-lasting phenotypes reminiscent of fetal alcohol syndrome at developmental stages. Nagre and collaborators used a mouse model of FASD and demonstrated the mechanism by which fetal exposure to ethanol impairs DNMT1 and DNMT3A, and consequently DNA methylation in the hippocampus and neocortex, two brain regions important for learning and memory. They also demonstrated that ethanol-induced activation of caspase-3 impairs DNA methylation through DNMT1 and DNMT3A in the neonatal mouse brain via CB_1 activation. No significant reduction of DNMT1 and DNMT3A and consequent DNA methylation was observed in CB_1-null mice (Nagre *et al.* 2015).

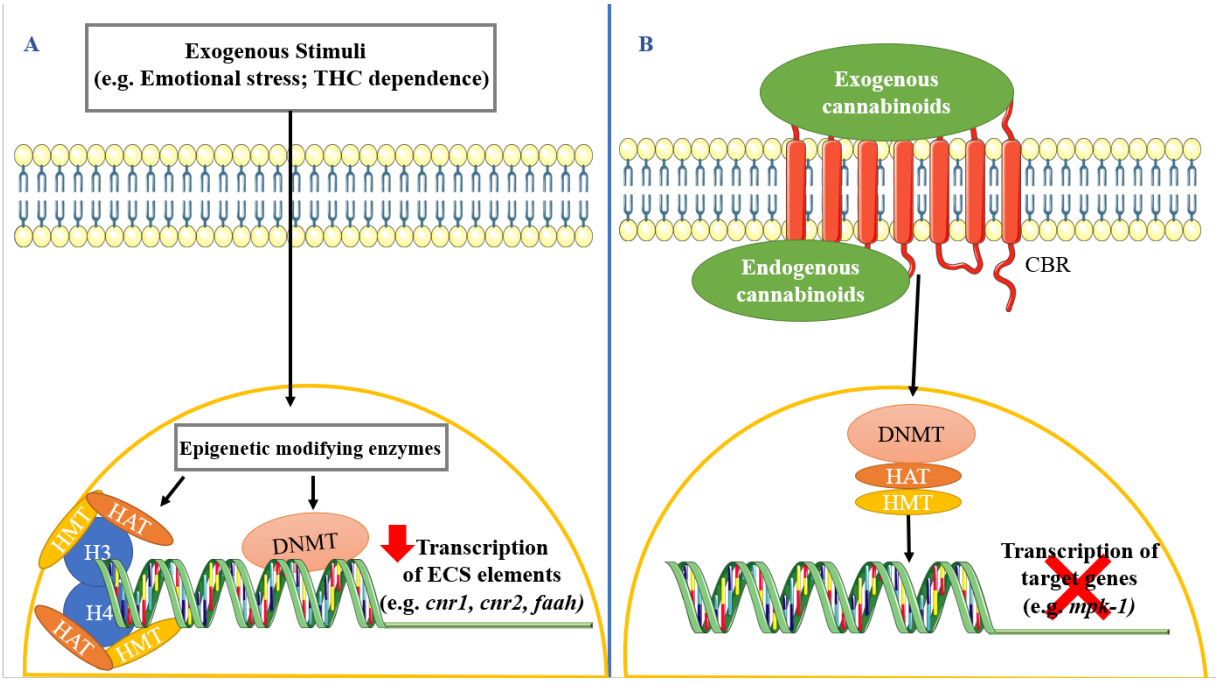


Figure 5. Interactions between epigenetic mechanisms and the endocannabinoid system (ECS). A) Epigenetic regulation of cannabinoid element genes. Hypermethylation of the CB_1 gene (cnr1) promoter by histone methyltransferase (HMT) contributes to downregulation of CB_1 transcription during exogenous stimuli. **B) Epigenetic action of eCB signaling.** Endogenous and exogenous cannabinoids may interact with cannabinoid receptors to reduce gene expression by inducing alterations in epigenetic modifying enzymes. Abbreviations: CBR, cannabinoid receptor; DNMT, DNA methyltransferases; H, histone; HAT, histone deacetylase; Me, methyl group; MeCpG, methylation of CG islands; THC, tetrahydrocannabinol. Adapted from D'Addario *et al.* 2013.

1.4. Implications of epigenetic modifications for neurodevelopmental disorders

Epigenetic modifications (e.g. DNA methylation/demethylation, histone modifications, nucleosome positioning, non-coding RNAs) have been reported to be involved in different neurodevelopment-related events, including neuronal migration and neuronal network formation. Some of these changes may lead to neurological syndromes [e.g. immunodeficiency, centromeric instability and facial anomalies syndrome (ICF), Parkinson's disease] as a result of single-gene mutations in DNA methyltransferases, histone-modifying enzymes, and their 'reader' proteins (Jakovcevski & Akbarian 2013).

Aberrant patterns of DNA methylation can be passed from one generation of cells to another, or remain stable into adulthood, thus providing the mechanisms for early life environment to exert continuing effects on gene expression and phenotype (Weaver *et al.* 2004). For example, autism spectrum disorders (ASD) and attention-deficit/hyperactivity disorder (ADHD) are the most common conditions in which a dysfunction of

neurodevelopmental processes seems to be involved, diagnosed in 1 and 5% of worldwide children, respectively. ASD is characterized by restricted and repetitive behaviors that provoke difficulties in social interaction and communication. ADHD is defined by hyperactivity, impulsivity, reduced attention, and difficulties in organization, usually atypical for the child's age. Dall'Aglio and collaborators (2018) performed a systematic review on the association between DNA methylation and histone modifications and these specific disorders, having identified, in ASD, patterns of methylated DNA in three genes, namely *prrt1*, *c11orf21*/*tspan32*, and *or2li3*, which encode for proline-rich transmembrane protein 1, chromosome 11 open reading frame 21, tetraspanin 32 and olfactory receptor family 2 subfamily L member, respectively. Noteworthy, methylation of these genes is associated with other disorders. For example, DNA methylation of *tspan32* is also associated with Beckwith-Wiedemann syndrome (a disease characterized by macrosomia and hemihyperplasia, which is caused by genetic and epigenetic dysregulation). Regarding histone modifications, trimethylations and acetylation of H3K27 were frequently found in patients with Beckwith-Wiedemann syndrome. In addition, the same authors identified a correlation between hyperactivity symptoms and decreased DNA methylation in *vipr2*, a gene encoding for vasoactive intestinal peptide receptor 2, in ADHD. Interestingly, both *vipr2* (ADHD) and *or2li3* (ASD) are involved in the signaling triggered by G protein-coupled receptors, whose involvement in neurotransmitter signaling has been well-described, suggesting that epigenetic dysregulation of neurotransmission could play a major role in these disorders (Dall'Aglio *et al.* 2018).

Mutations in *dnmt3b* (encoding for DNMT 3 beta) result in defective DNA methylation that can further lead to ICF, a multiorgan syndrome related to mental retardation and defective brain development. Moreover, an imbalance in H3K27me3 (trimethylated lysine 27 at histone H3), H3K4me3 and H3K9ac markings has been demonstrated in ICF at pericentric DNA repeats and promoter regions throughout the genome, which contributes to the deregulated expression of genes that interact with brain development and immune defense (Jin *et al.* 2008).

Mutations in the X-linked gene *mecp2* (which encodes a methyl-CpG-binding protein) results in the onset of Rett syndrome (RTT), a disorder that mainly affects children at an early age and that is associated with developmental impairment, cognitive regression and neurological symptoms, similar to schizophrenia and ASDs. In human *mecp2*-deficient brains, neuronal chromatin is globally disorganized, with linker histone H1 levels above normal values, hyperacetylation of nucleosome core histones, silent DNA repeats, retrotransposons, and repression of transcriptional activity at pericentromeric repeats (Jakovcevski & Akbarian 2013). The *mecp2* gene might bind to two alternatively

methylated forms of DNA: methylated cytosine followed by another nucleotide than guanine (mCH, H=A, C, or T), a repressive mark acting to inhibit gene expression; and hydroxymethylcytosine (hmC), which has been associated with active gene expression. Both are present in human and mouse samples and, contrasting most methylated CpG dinucleotides, these marks accumulate postnatally during neuronal maturation, correlating with an increase in *mecp2* expression. The impact of DNA methylation on transcription is dependent on the location of the mark, leading to inhibition or promotion of the gene expression. The concurrent increase in both MECP2 and its methylated DNA substrate, as well as the role of these marks in transcriptional regulation, it is possible that binding of MECP2 at mCG and hmC regulates gene expression in mature neurons. These two marks are regarded as contributors to the symptoms of RTT. In fact, many of the genes whose transcriptional profiles are altered in mouse models of RTT are enriched for mCH, and also mice that overexpress *mecp2* show an improved MECP2 binding with mCH in genes misregulated, as well as with hmC (Pohodich & Zoghbi 2015).

Schizophrenia (SCZ) is a mental disorder, mostly associated with neurodevelopment impairment, that usually emerges in late adolescence or early adulthood. It is associated with transcriptional mechanisms that depend on the dynamics of chromatin remodeling and genes with dysregulated expression that are in turn associated with epigenetic alterations (Kundakovic & Jaric 2017). Among these epigenetic modifications, methylation of DNA at gene promoters (e.g. *sox10*, which encodes an important transcription factor in development) is the most frequently reported modification. Liu *et al.* (2014) analyzed the DNA methylation at each CpG sites across the genome in 98 SCZ patients and 108 healthy controls. Their findings correlated eleven CpG sites with reality distortion symptoms. Of those CpG sites, seven were hypermethylated and the other four were hypomethylated, these data being consistent with a responsive or protective biological reaction to counteract delusion and hallucination symptoms (Liu *et al.* 2014). On the other hand, altered patterns of histone modifications have also been reported in SCZ. For example, there is evidence of increased expression levels of HMTs in the brains of patients with SCZ. In addition, altered levels of H3K9K14ac in SCZ have been found to correlate with altered expression levels of the affected genes, which include *gad1, htr2c*, and *ppm1e*, encoding for glutamic acid decarboxylase-1, serotonin 2C receptor and protein phosphatase 1 E, respectively (Nestler *et al.* 2016).

The *atrx* gene encodes a multifunctional chromatin regulator (ATRX, for α-thalassemia mental retardation, X-linked) and is responsible for several X chromosome-linked mental retardation syndromes, due to loss-of-function mutations (Jakovcevski & Akbarian 2013). The ablation of this gene results in excessive apoptosis and defective

migration of young neurons. ATRX is found associated with heterochromatic structures, most commonly at heterochromatic, rDNA, and telomeric repeats. Mutations at ATRX are consistent with alterations in the DNA methylation patterns. In association with the death-associated protein DAXX (its binding partner and histone chaperone), telomeres interact with the histone variant H3.3, but in the absence of ATRX, H3.3 is no longer recruited to telomeres. This suggests that ATRX plays a crucial role in the establishment and maintenance of the telomeric chromatin environment, facilitating the replacement of histone with the variant H3.3 (Law *et al.* 2010).

Another neurodevelopment-related disorder associated with mental retardation and intellectual disability is the Rubinstein-Taybi syndrome. It is caused by mutations in *CBp*, which encodes CREB binding protein (CBP) that has intrinsic histone acetyltransferase activity, and is also a transcriptional coactivator. Patients with this syndrome lose the histone-acetylating properties of CBP, which results in deficient recruitment of the transcription factor CREB (Caccamo *et al.* 2010).

Cabezas syndrome is a syndromic form of X-linked intellectual disability characterized by short stature, hypogonadism, abnormal gait, and speech deficits, which occur due to mutations in *cul4b*, which encodes for Cullin-4 ring ubiquitin ligase (Okamoto *et al.* 2017). This ubiquitin ligase degrades WD repeat-containing protein 5 (WDR5), which contributes to histone modifications. The absence of WDR degradation is associated with enhanced H3K4 HMT activity and excessive transcription. Additionally, loss of WDR5 may modify the activity of other epigenetic complexes in which it is incorporated, some notably affecting acetylation and ubiquitinylation (Nakagawa & Xiong 2011).

Sotos syndrome, related to childhood brain overgrowth and intellectual disability, is caused by microdeletions or mutations in the gene *nsd1* that encodes NSD1. NSD1 has histone methyltransferase activity, demonstrated by the use of a recombinant protein containing SET [su(var)3–9, enhancer-of-zeste, trithorax] domain of NSD1, which has the ability to methylate the histone lysine residues H3K36 (lysine 36 at histone H3) and H4K20 (lysine 20 at histone H4). This syndrome has been hypothesized to be the result of missense mutations in the DNA-binding/dimerization domain of the DNA binding protein Nuclear Factor 1-X, leading to the dysregulation of transcription. Berdasco and collaborators (2009) showed that DNA methylation-associated loss of NSD1 in neuroblastoma and glioma cells confirmed the contribution of epigenetic alterations to human tumorigenesis by two mechanisms: DNA hypermethylation at the 5’ regulatory region of the gene and also by the affected target gene, due to NSD1 encoding a histone methyltransferase. Additionally, the hypermethylation of NSD1 CpG island was revealed in primary neuroblastoma and glioma cells. Moreover, it has been reported that epigenetic

silencing of the HMT NSD1 by promoter CpG island hypermethylation contributes to human cancer, establishing a link between the loss of HMT enzymatic activity and the activation of oncogenes.

Kleefstra syndrome, whose main symptom is also intellectual disability, can be caused by a microdeletion in chromosomal region 9q34.3 or a mutation in the euchromatin histone methyltransferase 1(*ehmt1*), which encodes the G9a-related protein (GLP). GLP complexes target non-methylated H3K9 residues to affect H3K9 mono and demethylation and also target DNMTs toward specific genes for methylation-silencing during development (Willemsen *et al.* 2012). The association of the epigenetic changes with neurodevelopment-related disorders is summarized in Table I.

Table I. Epigenetic modifications and their association with neurodegenerative disorders.

Syndromes	Epigenetic Modifications	References
Autism Spectrum Disorder	DNA methylation in *prrt1, c11orf21/tspan32* and *or2li3*	(Dall'Aglio *et al.* 2018)
Beckwith-Wiedemann Syndrome	Trimethylations and acetylation of H3K27 DNA methylation in tspan32	
Attention-deficit/hyperactivity Disorder	Decreased DNA methylation in *vipr2*	
ICF (immunodefiency, Centromere Instability and Facial Anomalies)	Defective DNA methylation (Result due to a mutation in DNA methyltransferase 3 beta) Imbalance in H3K27me3, H3K4me3, H3K9ac markings	(Jin *et al.* 2008)
Rett Syndrome	Mutations in the X-linked *mecp2* (which encodes a methyl-CpG-binding protein) DNA methylation in a cytosine followed by another nucleotide than guanine or DNA methylation in hydroxymethylcytosine Levels of the linker histone H1 above normal values Hyperacetylation of nucleosome core histones	(Jakovcevski & Akbarian 2013)

	Silent DNA repeats Repression of transcriptional activity at pericentromeric repeats Retrotransposons	
Schizophrenia	DNA methylation of *sox10* Hypermethylation and hypomethylation of CpG	(Liu *et al.* 2014)
	Increased expression levels of HMT Altered level of H3K9K14ac and altered expression levels of *gad1, htr2c and ppm1e*	(Nestler *et al.* 2016)
Alpha-thalassemia, X-linked with mental retardation (ATRX)	Replication-independent nucleosome remodeling and histone H3.3 incorporation	(Law *et al.* 2010)
Rubinstein-Taybi Syndrome	Histone Acetylation of *CBp*	(Caccamo *et al.* 2010)
Cabezas Syndrome	Enhanced H3K4 HMT activity and excessive transcription of *cyul4b*	(Nakagawa & Xiong 2011)
Sotos Syndrome	DNA methylation-associated loss of NSD1 DNA hypermethylation at the 5' regulatory region of the gene *nsd1* NSD1 CpG island hypermethylation	(Berdasco *et al.* 2009)
Kleefstra Syndrome	Mutation in the euchromatin histone methyltransferase 1(ehmt1) Microdeletion in chromosomal region 9q34.3	(Willemsen *et al.* 2012)

1.5. Modulation of epigenetic mechanisms by exogenous cannabinoids

Exogenous cannabinoids comprise plant-derived cannabinoids (phytocannabinoids), which are usually partial agonists of CBRs, and synthetic cannabinoids (SCs), which are usually full agonists of the same receptors. Considering that exogenous cannabinoids can interact with the same receptors as eCBs, it is not surprising that they may also be involved in a close interplay with epigenetic mechanisms like DNA methylation/demethylation or histone acetylation/deacetylation. A few studies, detailed below, have already reported how exogenous cannabinoids and epigenetics may interact.

1.5.1. Phytocannabinoids

Brain exposure to cannabis at sensitive periods of its development has been associated with epigenetic disturbances such as altered microRNA, DNA methylation, and histone modifications (Velez *et al.* 2019). For example, prenatal cannabis exposure associated with vulnerability to addiction has been described as inducing the epigenetic regulation of the dopamine D2 receptor gene (*drd2*) in the nucleus accumbens, leading the individual to addiction and possibly to the development of psychiatric disorders. In addition, parental or prenatal exposure to cannabis may activate epigenetic changes that could lead to immunological consequences for offspring and transgenerational effects (Spano *et al.* 2007; Velez *et al.* 2019). Additionally, prenatal brains exposed to tetrahydrocannabinol (THC) showed alterations in the histone modification profile and decreased D2 receptor mRNA, which was associated with increased heroin seeking in adulthood (Szutorisz *et al.* 2014; Velez *et al.* 2019).

Tomasiewicz and collaborators (2012) showed that exposure of adolescent male rats to THC mediates the transcriptional and epigenetic state of proenkephalin (an opioid neuropeptide encoded by *penk*) through repressive regulation of histone H3K9 methylation in the adult nucleus accumbens, an epigenetic effect that represents a pathologic departure from the distinct developmental pattern of histone H3 methylation usually occurs at *penk* in this region, during the transition from adolescence to adulthood.

Prini and collaborators (2018) showed that 2 and 24 h after exposure of adolescent female rats to THC, H3K9me3 (trimethylated lysine 9 at histone H3, related to transcriptional repression) levels in the prefrontal cortex of THC-exposed animals were increased by 25% and 48%, respectively, compared to control. However, 48 h after the last treatment, the H3K9me3 levels returned to basal values, although the H3K9ac (associated with transcriptional activation) increased by 30%. These results indicated the association of THC chronic exposure with transcriptional repression immediately after the exposure, and an association with transcriptional activation at later time points. The authors further hypothesized that the THC-induced H3K9me3 increase observed at 2 and 24 h may represent the main effect promoted by THC exposure on adolescents, whereas increased H3K9ac levels at 48 h might correspond to a homeostatic mechanism required to counterbalance the strong repression initially induced by THC exposure. The same authors also observed that a THC-induced increase in H3K9me3 levels promoted chromatin changes in a set of genes whose expression was downregulated following THC exposure, namely *Homer1*, *Mgll*, and *Dlg4* (which encoded HOMER1, homer protein homolog 1; MAGL, monoacylglycerol lipase; and PSD-95, postsynaptic density protein 95) (Prini *et al.* 2018).

Gerra *et al.* (2018) also identified epigenetic changes related to cannabis use. These authors showed that cannabis users presented an hypermethylation of the exon 8 of *drd2*, as well as the CpG rich region of the *ncam1*, compared to controls, which led to transcriptional silencing of *ncam1* gene (neural cell adhesion molecule, also known as CD56).

Prenatal THC exposure studies demonstrated changes in gene expression. More recent research of the developmental effects of THC described epigenetic alterations germane to addiction disorders. Exposure to low-to-moderate THC dosing paradigms has generally induced significant alterations of the dopaminergic D_2 receptor (*drd2*) and the opioid neuropeptide proenkephalin (*penk*) genes. The studies revealed disturbances in the histone modification profile in the nucleus accumbens of adult rats with prenatal THC exposure, such as decreased levels of the H3K4me3 (trimethylation of lysine 4 on histone H3), and H3K9me2 (demethylation of lysine 9 on histone H3), as well as decreased RNA polymerase II association with the promoter and coding repair of the gene in the nucleus accumbens. The combined epigenetic alterations were consistent with the reduction of the *drd2* gene expression and also with the enduring consequences of THC exposure following prenatal development. Similarly, the same epigenetic alterations were identified in *penk* locus in the nucleus accumbens of adult rats following adolescent THC exposure with enduring upregulation of *penk* mRNA levels. In addition, THC treatment dose-dependently increased the expression of histone deacetylase 3 (HDAC3) in a human trophoblast cell line, suggesting the possibility for cannabinoid exposure to affect placental development (Szutorisz & Hurd 2016).

1.5.2. Synthetic cannabinoids

The recreational use of SCs, which represent a diverse group of designer psychoactive drugs, commonly marketed as more potent substitutes of cannabis and in comparison, to other substances, the SCs had a remarkable increase in consumption along this past decade ((EMCDDA) 2020; Alves *et al.* 2020). SCs were initially developed with the aim of studying the function of the endocannabinoid system and for therapeutic purposes to increase appetite and decrease the feeling of nausea in patients treated with chemotherapy but lacking the psychoactive side effects. Like endo- and phytocannabinoids, these synthetic molecules bind and activate CBRs. However, SCs are full agonists of CBRs, thus being more potent and producing stronger psychoactive effects, as well as adverse effects, compared to eCBs and phytocannabinoids ((EMCDDA) 2020; Cristino *et al.* 2020).

The use of these substances is typically motivated by curiosity, low cost, and desired effects, which include relaxation and feeling a pleasant high. The preferred route of SC administration is the inhalation by smoking, due to the rapid pharmacological effects. As these substances are usually found in packages mixed with plant material, users may be misled into believing that these are natural products. However, the toxicological mechanisms underlying SCs' effects remain poorly understood.

Adverse effects of severe SC-related poisoning include cardiovascular toxicity (likely leading to sudden death), respiratory depression, seizures, convulsions, hyperemesis, delirium, agitation, psychosis, aggressive and violent behaviour, coma or rapid loss of consciousness that could lead to choking on vomiting, hypothermia and self-inflicted injury. Fatal intoxications have also been reported following the use of these substances ((EMCDDA) 2020). In the US, the poison control centre's receive thousands of calls annually, related to SC exposure.

SCs have been increasingly used by adolescents in nightlife settings, but also by vulnerable groups, such as the homeless and prisoners ((EMCDDA) 2020). The focus on this group of users (adolescents, homeless and prisoners) is due to the easy access to these drugs. Of note, in 2010 a total of 11406 case involving SCs were registered, by Drug Abuse Warning Network, of which 75% were adolescents and young adult ages between 12 and 29 years (SAMHSA 2012).

Most important, the lack of understanding about the toxicity and abuse liability of these substances poses a threat to public health, since the risks related to its consumption are often unexpected and mostly unknown. Moreover, data on the neurobiology underlying the impact of SC use by pregnant women and women of childbearing age on their offspring remains scarce, with only a few studies correlating impaired cognition with prenatal SC exposure (Alexandre *et al.* 2019). Recently, our research group showed that two SCs, 5F-PB22 and THJ-2201, increased neurite outgrowth in a NG108-15 cell line in a CB_1 activation-dependent manner, at biologically relevant concentrations (Alexandre *et al.* 2020). Nevertheless, SCs' epigenetic imprint remains mostly unknown, although there are a few reports describing the potential of these substances to promote epigenetic changes. For example, Aguado *et al.* (2007) analyzed the regulation of glioma cells differentiation by the specific CBR agonists HU-210 (for CB_1) and JWH-133 (for CB_2), having observed an increased number of H3K9me3-positive cells, compared to control cells, following exposure to these agonists. The authors further concluded that such SC-mediated increase in H3K9me3-positive cells was regulated by CB_1 and CB_2 activation, as the presence of CBR antagonists (SR141716 and SR144528 for CB_1 and CB_2, respectively) prevented these outcomes.

Tomas-Roig and collaborators (2017) reported that administration of WIN55,212-2 to adolescent rats increased adult hippocampal AEA levels and induced DNA hypermethylation at the intragenic region of the intracellular signaling modulator Rgs7 (responsible for accelerating the GTP hydrolysis of G protein determining their fast inactivation and acting as intracellular antagonists of GPCR signaling), which was associated with a lower rate of mRNA transcription of the *rgs7* gene. Additionally, long-term administration of this SC during the rats' adolescence developmental phase led to an increase of eCBs levels, altering the *rgs7* expression in adulthood and establishing a potential link to epigenetic changes.

Hollins and collaborators (2014) studied the role of miRNA in the entorhinal cortex of adult rat. The exposure of rats to the synthetic cannabinoid HU-210 during adolescence was found to induce alterations in the expression of miRNA involved in the regulation of pathways important to neurodevelopmental processes. These alterations resulted in the malformation of entorhinal cortex region leading to functional disturbances as the ones observed in schizophrenia.

Andaloussi and coworkers (2019) studied the chronic exposure of adolescent male rats to WIN55,212-2 and observed that this SC increased the animals' vulnerability to stress-induced emotional and cognitive changes in the offspring, due to an alteration in global DNA methylation in the prefrontal cortex through the upregulation of DNA methyltransferase enzymes (namely DNMT1 and DNMT3a). In addition, this study evidenced a possible paternal transmittance of stress sensitivity to the offspring followed by a chronic cannabinoid exposure and emphasized a role for DNA methylation in stress-induced anxiety.

Objectives of the dissertation

Considering the recent changes in the legal status of cannabis and its derivatives worldwide, understanding the potential long-term impact of these substances on young adults (the main users of these substances), and their offspring, assumes extreme relevance. In particular, it becomes important to understand the mechanisms underlying this intricate interplay between the endocannabinoid system and epigenetic mechanisms, specifically during a stage at which the brain is most vulnerable (Cristino *et al.* 2020). In fact, the disruption of such interplay has been reported to lead to disturbances in normal neurodevelopment. To the best of our knowledge, the impact of SC use on epigenetic changes remains mostly unexplored. Here, we hypothesized that exposure of neuronal cells to SCs could impact their neurodifferentiation by inducing epigenetic modifications. To assess this hypothesis, the following aims were outlined:

1. To evaluate the effects of two SCs commonly detected in seizures in the European Union, THJ-2201 and ADB-FUBINACA, on the differentiation of NG108-15 neuroblastoma x glioma cells.

2. To assess epigenetic changes, namely global DNA methylation and histone H3 acetylation induced by those two SCs during neuronal differentiation.

These results will expectedly help to further understand the impact of SCs during neurodevelopment. This assumes utmost importance considering that there has been a large consumption of these compounds by young adults. Moreover, it will possibly contribute to raise awareness of the risks of using these substances.

Chapter II. Materials and Methods

2.1. Chemicals

Heat-inactivated fetal bovine serum (FBS), 0.25 % trypsin/EDTA, antibiotic with antimycotic (10 000 $U.ml^{-1}$ penicillin, 10 000 $\mu g.ml^{-1}$ streptomycin, 10 000 $\mu g.ml^{-1}$ amphotericin), Hank's balanced salt solution (HBSS), and phosphate buffered saline (PBS) were purchased from Gibco Laboratories (Lenexa, KS, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

2.1.1. Synthetic cannabinoids

The synthetic cannabinoid THJ-2201 ([1-(5-fluoropentyl)-1H-indazol-3-yl] (1-naphthyl) methanone) was kindly supplied by Dr. Ana Santos Carvalho (Centre for Neurosciences and Cell Biology, University of Coimbra, Portugal). This compound belongs to the naphtoylindoles cannabinoid class and it is a structural analogue of AM-2201, with an indazole group replacing the central indole ring. It is also a full agonist of CB_1 and CB_2, with binding affinities of 1.34 and 1.32 nM, respectively. In terms of potency, THJ-2201 has an EC_{50} of 0.45 and 1.68 nM at the CB_1 and CB_2, respectively. This synthetic cannabinoid has been linked to at least one hospitalization and death in Austin (Texas, USA), in 2014 (Trecki *et al.* 2015).

ADB-FUBINACA (N-1(-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-caboxamide) was kindly supplied by TicTac Communications, Ltd (UK). This synthetic compound belongs to the 1-alkyl-1H-indazole-3-carboxamide cannabinoid class, consisting of a benzene and pyrazole in its bicyclic structure. It is also a potent and full agonist of CB_1, with a binding affinity of 0.36 nM and an EC_{50} of 0.98 nM to this receptor. This SC has been linked to at least one hospitalization and death in Baton Rouge (LA, USA) in 2014 (Trecki *et al.* 2015; World Health Organization 2019).

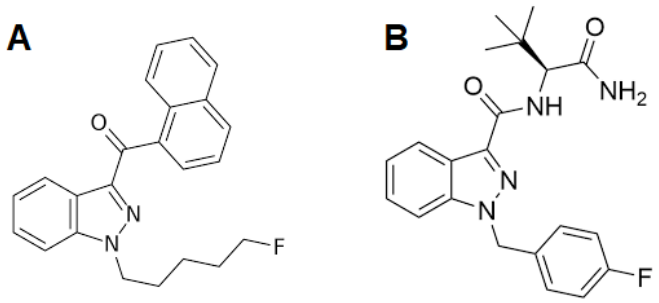


Figure 6. Structure of THJ-2201 (A) and ADB-FUBINACA (B).

SCs stock solutions were prepared in dimethyl sulfoxide (DMSO) at 5 mM and sequentially diluted in Hank's balanced salt solution (HBSS) prior to cell exposure to attain a final DMSO concentration below 0.1 % DMSO.

The selection of these SCs was based on their high prevalence in seizures in the European Union, in case reports related to hospitalizations related to their use, and to their structural differences, which make it possible to compare two different classes of cannabinoids.

2.2. Cell Culture

NG108-15 mouse neuroblastoma (N18TG-2) x rat glioma (C6Bu-1) hybrid cells were cultured in 75 cm^2 flasks using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and an antibiotic/antimycotic solution (containing 10 000 $U.ml^{-1}$ penicillin, 10 000 $\mu g.ml^{-1}$ streptomycin, 10 000 $\mu g.ml^{-1}$ amphotericin B), and maintained at 37 ºC in a humidified 5 % CO_2 atmosphere. The cells were sub-cultured once they reached 70-80 % confluence by trypsinization using a 0.25 % trypsin/EDTA solution.

Of note, NG108-15 cells represent an advantageous model for studying the mechanisms of neuronal development and differentiation due to their morphological properties, including neurite growth and synchronous differentiation in culture dishes. Under stress conditions, such as a state of limited nutrients, NG108-15 cells are representative of the neuritogenesis occurring in a blastoid state, thus allowing the examination of the onset of adhesive and morphological changes of neuronal differentiation (Campanha *et al.* 2014). These properties allow the examination of morphological changes triggered by SCs, especially due to the fact that this type of cell line express the CB_1 (Callén *et al.* 2012).

2.3. Neuronal differentiation

NG108-15 differentiation was performed according to a procedure previously described (Alexandre *et al.* 2020). Cells were seeded at $7.2x10^3$ cells/cm^2 in 96-well plates in complete cell culture medium (hereafter referred as maintenance medium, MM), and allowed to adhere overnight. On the following day, MM was replaced by DMEM supplemented with 1 % FBS, 30 µM forskolin and 10 µM retinoic acid (hereafter referred as differentiation medium, DM), and cells were incubated for 3 days at 37 ºC, 5 % CO_2.

THJ-2201 and ADB-FUBINACA were added to cultured cells at the time the differentiation was initiated, at concentrations of 1 pM, 1 nM, and 1 µM, which have been

previously determined to be non-cytotoxic (Alexandre *et al.* 2020). A vehicle control (0.1 % DMSO) was also tested.

After three days of incubation, neurite outgrowth in each condition was imaged using bright-field analysis in the Lionheart™ FX Automated Microscope (Bio-Tek, Winooski, VT, USA). Neurite outgrowth was determined by measuring the neurite length and total cell number using the Neurite Tracer plugin and the multi-point tool, respectively, available at the ImageJ open source image processing software (ImageJ 2.0.0 National Institutes of Health, Bethesda, MD, USA). The differentiation ratios were calculated dividing the number of neurites per total number of cells per well (Equation 1). The branching ratios were calculated in the same way, but using the number of branches instead of the number of neurite (Equation 2). Every outgrowth extending longer than 20 µm from the soma was considered to be a neurite, according to (Campanha *et al.* 2014).

Equation1: $$Differentiation\ ratio = \frac{Nr\ of\ Neurites\ (\geq 20\ \mu m)}{Total\ cell\ nr}\ x\ 100$$

Equation 2: $$Branching\ ratio = \frac{Nr\ of\ Branches\ (\geq 20\ \mu m)}{Total\ cell\ nr}\ x\ 100$$

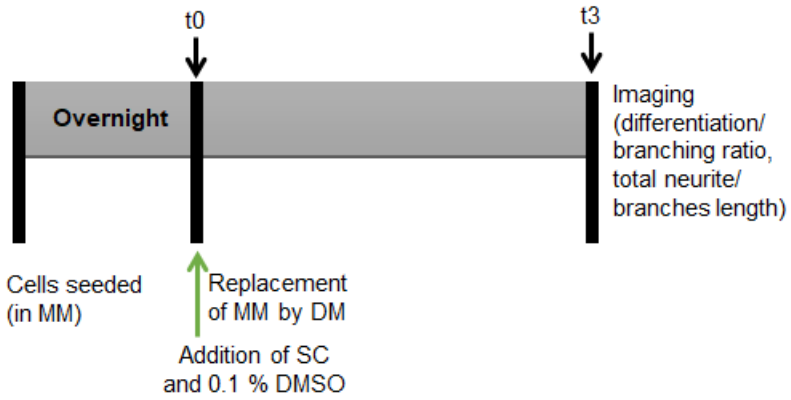


Figure 7. Chronological scheme of the protocol used to induce NG108-15 cell differentiation. NG108-15 cells were seeded and allowed to adhere overnight. On the following day (t0), neuronal differentiation was induced by replacing the Maintenance Medium by Differentiation Medium. Vehicle (0.1 % DMSO) or SCs were also added immediately after cell culture medium replacement. After 3 days in culture, differentiation/branching ratios and total neurite/branches length were assessed. t (days); MM: Maintenance Medium (DMEM supplemented with 10 % FBS); DM: Differentiation Medium (DMEM supplemented with 1 % FBS + 30 µM forskolin +10 µM retinoic acid).

2.4. Assessment of cell viability and phenotype maintenance

2.4.1. Hoechst – Propidium Iodide staining

Hoechst is a cell permeable, fluorescent dye that stains nucleic acids. It allows distinguishing apoptotic from non-apoptotic cells by assessing the presence of condensed chromatin, which is a typical feature of apoptotic cells. Propidium iodide (PI) is a red fluorescent dye that does not permeate the cell membrane unless the membrane is disrupted, thus allowing the identification of late apoptotic or necrotic cells (Silva *et al.* 2006).

Cells were seeded at $1.8x10^4$ cells/cm^2 in 24-well plates and neuronal differentiation performed as described in section 2.3. At days 3, 6, 9 and 12 after the initiation of differentiation, cells were incubated at 37 °C, 5 % CO_2 with 0.63 µM of propidium iodide for 5 min, followed by 10 µM of Hoechst 33342 for 15-20 min. Nuclear morphology (Hoechst 33342) and PI permeabilization were assessed by fluorescence microscopy (Lionheart™ FX Automated Microscope (Bio-Tek, Winooski, VT, USA) equipped with a UV filter (excitation at 377, emission at 447 nm) and another filter with excitation at 531, and emission at 593 nm. At least 400 cells were counted per condition and the number of nuclei showing condensed chromatin and/or intracellular PI accumulation was expressed as a percentage of the total number of cells.

2.4.2. Acetylcholinesterase activity

Acetylcholinesterase (AChE) is a crucial enzyme for nerve response and function, as it degrades the neurotransmitter acetylcholine into choline and acid acetic. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to terminate the synaptic transmission (Campanha *et al.* 2014). Upon differentiation, these cells characteristically display the expression of choline acetyltransferase activity, release of acetylcholine by bradykinin stimulation and the formation of cholinergic synapses with cultured myotubes (Tojima *et al.* 2000). As such, the assessment of AChE activity makes an appropriate biomarker for differentiated NG108-15 cells' phenotype.

NG108-15 cells were seeded at $1.8x10^4$ cells/cm^2 in 24-well plates and the cell phenotype was assessed in cells every 3 days from day 0 to day 15 after induction of differentiation, performed as described in section 2.3. At the referred time points, cells were collected by removing the cell culture medium and washing the wells with 500 µL phosphate buffer (composed by 75.4 mM $Na_2HPO_4 \cdot 7H_2O$ and 24.6 mM $Na_2HPO_4 \cdot H_2O$). Two hundred and fifty microliters of the same buffer were then added to scrape the cells with a cell scraper and these cells were transferred into clean 1.5 mL tubes. Then, the

samples were centrifuged at 1000 rpm for 10 min at 4 ºC (Biofuge Fresco, Heraeus, Germany) to homogenize the samples. Then, 40 µL of each sample and a negative control (cell lysates replaced by phosphate buffer) were added to a 96-well plate. A reaction mixture composed of 166 µL of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's Reagent), and 33 µL of acetylcholine (the substrate for acetylcholinesterase) in 5 mL of the phosphate buffer was prepared. The reaction was then initiated by adding the reaction mixture to the cell lysates, using a multichannel pipette. This reaction is based on the hydrolysis of acetylthiocholine into thiocholine, which complexes with DTNB to form a mixed disulphide and the yellow chromophore 5-thio-2-nitrobenzoic acid (TNB) (Frasco *et al.* 2005). The kinetics of color development was scanned over time at 412 nm in a Bio-Tek PowerWaveX microplate reader (Bio-Tek, Winooski, VT, USA). Total protein content in each sample was quantified using the DC Protein Assay (Bio-Rad, California, USA), according to the manufacturer's instructions, and the samples were stored at -20 ºC until use. Results were expressed as AChE activity (a.u./mg protein), which was calculated by dividing the slope of TNB formation by the amount of protein.

2.5. Epigenetic modifications

To evaluate whether SCs induce epigenetic modifications during neuronal differentiation, NG108-15 cells were seeded at $3.1x10^4$ cells/cm^2 in 6-well plates and differentiation was induced as described in section 2.3. THJ-2201 and ADB-FUBINACA were added to cultured cells at the concentrations of 1 pM, 1 nM, and 1 µM, in duplicate. Controls containing only Differentiation Medium (DMC, for Differentiation Medium Control), or 0.1 % DMSO (VC, vehicle control) were also included.

The epigenetic modifications promoted by SCs were evaluated following three different types of exposure settings: Treatment A consisted in one addition of SCs or vehicle (0.1 % DMSO) at the beginning of the differentiation with posterior collection of samples (i.e. genomic DNA or histone extracts) at the third day of differentiation (Fig. 8A). This treatment allowed assessing the epigenetic modifications triggered by the SCs at an early differentiation stage; In treatment B, the SCs were added once at the third day of the differentiation and the samples collected at the sixth day, to evaluate the impact of these substances at a later stage of differentiation (Fig. 8B); Treatment C consisted in two additions of the SCs, the first at the beginning of the differentiation, and the second one at the third day and, finally, sample collection occurred at the sixth day after the start of differentiation (Fig. 8C). The schemes of these treatments are shown in Figure 8.

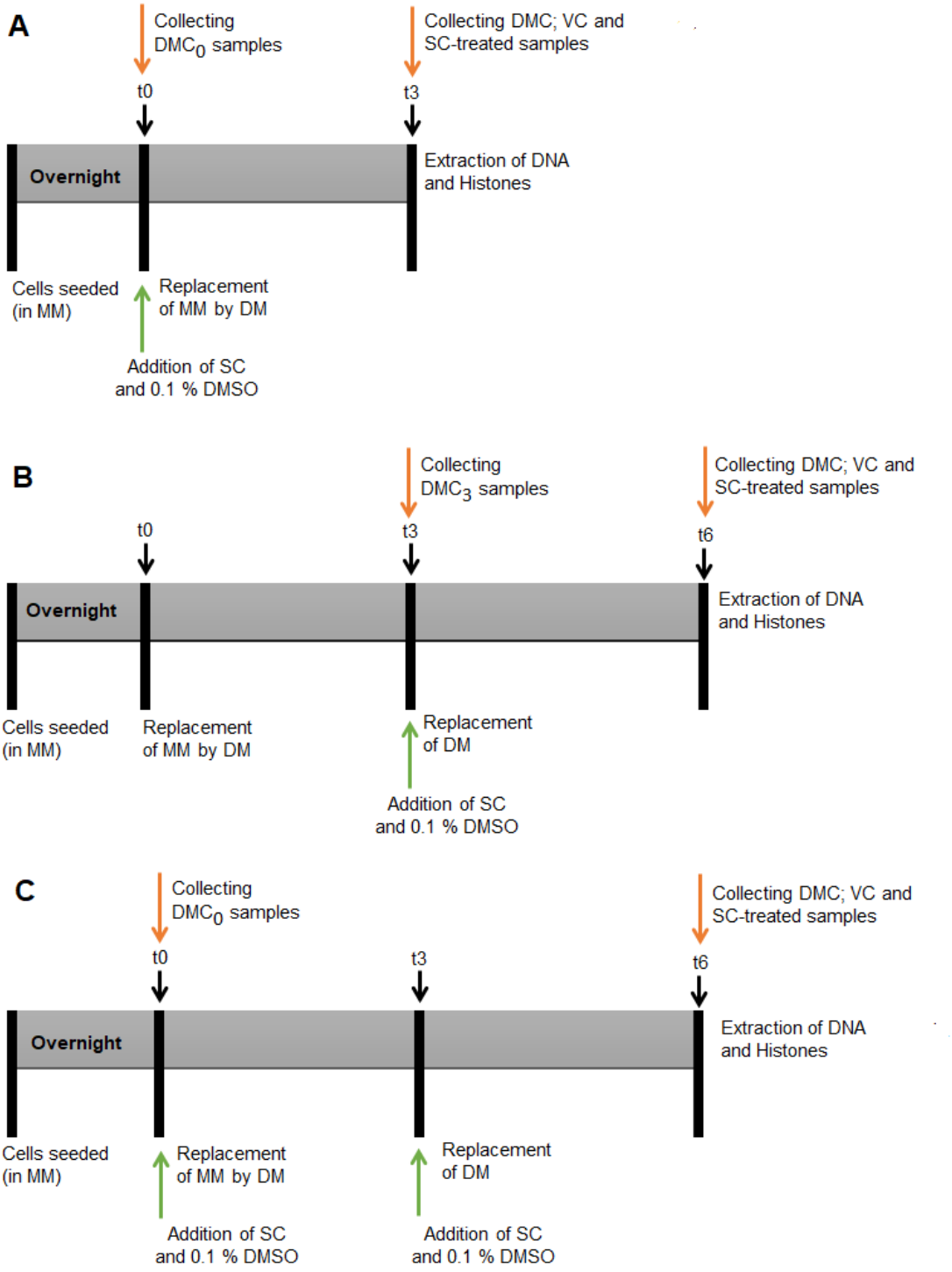


Figure 8. Schematic representation of the treatments used to evaluate epigenetic modifications. NG108-15 cell differentiation was induced as previously described in section 2.3. **A)** Samples were treated with one addition of SC or 0.1 % DMSO at the first day of differentiation and collected at the third day; **B)** Samples were treated once with SC or 0.1 % DMSO at the third day of differentiation and collected at the sixth day; **C)** Samples were treated twice with the SC or 0.1 % DMSO at the first and third day of the differentiation and collected at the sixth day. t (days); MM: Maintenance Medium (DMEM supplied with 10 % FBS); DM: Differentiation Medium (DMEM

supplied with 1 % FBS + 30 μM forskolin +10 μM retinoic acid): DMC_0/DMC_3: Differentiation Medium Control (without SC or 0.1 % DMSO) of the beginning of the differentiation; DMC: Differentiation Medium Control (without SC or 0.1 % DMSO) of the end of the differentiation; VC: Vehicle Control (0.1 % DMSO).

2.5.1. DNA extraction and purification

Extraction of genomic DNA was performed with the GRS Genomic DNA kit (Grisp, Porto, Portugal), according to the manufacturer's instructions. Briefly, at the indicated time points (Fig. 8, section 2.5), cells were collected by scrapping and centrifuged at 1000 rpm for 5 min (Eppendorf® centrifuge 5810 R). After discarding the supernatant, cells were resuspended in 200 μL of phosphate buffer saline (PBS), pH 7.4, and 20 μL pf proteinase K (10 mg/mL) were added and mixed by pipetting. The samples were incubated at 60 ºC for 5 min in a water bath. Cell lysis was promoted by adding 200 μL of Buffer BR2 to the samples, followed by vigorously shaking and further incubation at 60 ºC for 5 min in a water bath. During this incubation, the samples were regularly inverted. Two hundred microliters of absolute ethanol were added to the lysate and mixed by immediately shaking for 10 sec. The samples were transferred into a spin column placed in a 2 mL collection tube. Then, samples were centrifuged at 15 388 rpm (SIGMA, 1-14K, Germany) for 3 min and the collection tube discarded and replaced by a new one. Afterward, 400 μL oh wash buffer were added, the columns centrifuged for 1 min at 15 388 rpm, and the flow-through discard. The columns were again washed by adding 600 μL of wash buffer and centrifuged again for 1 min. The flow-through was again discarded and the matrix dried by centrifuging at 15 388 rpm for 3 min. Finally, the DNA was eluted in 100 μL of pre-heated elution buffer and centrifugation for 1 min at 15 388 rpm. The spin-column was discarded, and the samples stored at -20ºC or used immediately for DNA quantification.

The concentration of DNA in each sample was determined in a Qubit™ 4 fluorometer, using the Qubit® dsDNA Broad Range assay kit, according to the manufacturer's instructions.

2.5.2. Quantification of global DNA methylation

Global DNA methylation was assessed using the colorimetric Global DNA Methylation Assay Kit (5-Methyl Cytosine) (Abcam, Cambridge, UK), according to the manufacturer's instructions. This kit provides specific strip-wells treated to have a high affinity for DNA binding, and it is based on the detection of DNA methylation occurring by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNMT, forming 5-methylcytosine (5-mC). The methylated fraction of DNA (% 5-mC) is detected by colorimetry using capture and detection antibodies.

Briefly, 4 µL of sample containing 50 or 100 ng of DNA (from 2.5.1.) were added to 100 µL of Binding Solution in a 96-well plate, and mixed by gently shaking to ensure the homogeneous coating of the bottom of the wells. A standard curve prepared with known percentages of 5-mC, as well as a positive control, a diluted positive control, and a negative control, were also prepared. All samples, standards and controls were performed in duplicates. The plates were sealed with parafilm and incubated at 37 ºC, 5 % CO_2, for 60 min. After this incubation, the Binding Solution was removed from each well and the wells washed three times with 150 µL of the diluted 1X Wash Buffer. To detect methylated DNA, 50 µL of a 5-mC Detection Complex Solution (containing 5-mC Antibody, Signal Indicator, and Enhancer Solution) were added to each well, and the plates sealed and incubated at room temperature for 50 min. After incubation, the 5-mC Detection Complex Solution was removed and the wells washed five times with 150 µL of the diluted 1X Wash Buffer. Then, 100 µL of Developer Solution were added to the wells using a multichannel pipette. The plate was then manually shaken against the surface for 5-10 sec and incubated at room temperature for 3-4 min.

Color development in the samples, standards and control wells was monitored until the Developer Solution turned blue (except in the negative control wells, in which it remained unchanged). When the color in the 5 % Positive Control wells turned deep blue, the reaction was stopped by adding 100 µL of Stop Solution to each well using a multichannel pipette. The plates were manually gently shaken against the surface and allowed to rest for 1-2 min, to assure the reaction was completely stopped. The color changed to yellow after adding the Stop Solution and the absorbance was read at 450 nm in a Bio-Tek PowerWaveX (Bio-Tek,Winooski, VT, USA) microplate reader. Results were expressed as percentage of 5-mC, according to Equation 3:

Equation 3: $$5 - mC\ \% = \frac{Sample\ OD - Negative\ Control\ OD}{Slope\ x\ S}\ x\ 100$$

in which the slope was determined from the linear regression of the standard curve, and S corresponds to the total DNA amount in the samples.

2.5.3. Histone extraction

For histone extraction, cells were collected by scrapping and pelleted through centrifugation at 1000 rpm for 5 min at 4 ºC (Eppendorf centrifuge 5810 R), in 15 mL tubes. The cells were then resuspended in 500 µL of Triton Extraction Buffer (TEB, constituted by PBS supplemented with 0.5 % Triton X-100, 2 mM PMSF and 0.02 % NaN_3), transferred to 1.5 mL tubes, and incubated for 10 min on ice with regular gentle

stirring to promote cell lysis. The cell lysates were then centrifuged at 10000 rpm for 1 min at 4 ºC (Biofuge Fresco, Heraeus, Germany), and the supernatant was discarded. The cell pellets were resuspended in 200 µL of extraction buffer (composed by 0.5 N HCl supplemented with 10 % glycerol), and the suspension further incubated on ice for 30 min. The samples were centrifuged at 12000 rpm for 5 min at 4 ºC and the supernatant transferred into a new vial. Six hundred microliters of acetone were added to each sample and the tubes were stored overnight at -20 ºC. On the following day, the samples were centrifuged again at 12000 rpm for 5 min and the pellet was air-dried. Finally, the pellet was dissolved in 50 µL of distilled water. Total protein content in each sample was quantified using the DC Protein Assay (Bio-Rad, California, USA), according to the manufacturer's instructions, and the histone extracts were stored at -20 ºC until use.

2.5.4. Quantification of global Histone H3 acetylation

Global acetylation of histone H3 was assessed using the fluorometric Histone H3 Total Acetylation Detection Fast Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. This kit measures the total H3 acetylation by capturing the acetyl histone H3 to the strip wells coated with an anti-acetyl histone H3 antibody. Then acetyl histone H3 is detected with a labeled detection antibody followed by a fluorescent development reagent. The histone H3 was selected because, according to the literature, this histone is the most affected by this type of modification.

Briefly, 5 µL of sample containing 1 µg of histone extract (from 2.5.3.) were added to 50 µL of Antibody Buffer into the wells of a 96-well plate. A standard curve was also prepared by adding 1 µL of Standard Control at concentrations ranging from 1 to 100 ng/µL. A blank containing only Antibody Buffer was also prepared. All samples, standards and controls were performed in duplicates. The strip wells were then sealed with Parafilm and incubated at room temperature for 2 h. After this incubation, the acetylated histone H3 was fixed into the wells by binding to an anti-acetyl histone H3 antibody, the solution in the wells was aspirated and these were washed three times with 150 µL of 1x Wash Buffer. Fifty microliters of diluted Detection Antibody were then to each well and the strips incubated for 1 h at room temperature, on an orbital shaker, at 100 rpm. After this period, the solution was aspirated, and the wells washed six times with 150 µL of diluted 1x Wash Buffer. Fifty microliters of the Fluoro-Development Solution were added into the wells and the strips incubated at room temperature for 5 min, protected from light. Finally, fluorescence in the wells was measured in a Biotek Synergy HT (Bio-Tek,Winooski, VT, USA) microplate reader using a filter with excitation at 530 nm and emission at 590 nm. Results were expressed in percentage of acetylation calculated by dividing the subtraction

of the blank RFU in the treated samples RFU per the subtraction of the blank RFU in the untreated samples (control) RFU.

Equation 4: $$Acetylation\ \% = \frac{Treated\ (tested)\ sample\ RFU}{Untreated\ (control)\ sample\ RFU}\ x\ 100$$

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Normality of the distributions was determined prior to each analysis using Kolmogorov-Smirnov and Shapiro-Wilk normality tests, and considering the acceptability of skewness and kurtosis values. Based on the normality tests' results, one-way ANOVA, followed by a Dunnett's comparison post-test, or unpaired two-tailed t-tests were conducted whenever appropriate. The number of independent experiments, as well as the number of replicates performed, if any, are detailed in the figure legends.

Chapter III. Results

3.1. THJ-2201 and ADB-FUBINACA increase neuronal differentiation ratios

The impact of two different SCs, THJ-2201 and ADB-FUBINACA, on neuronal differentiation was determined by assessing the neurite outgrowth of differentiating NG108-15 cells exposed to both SCs in Differentiating Medium, at biologically relevant concentrations.

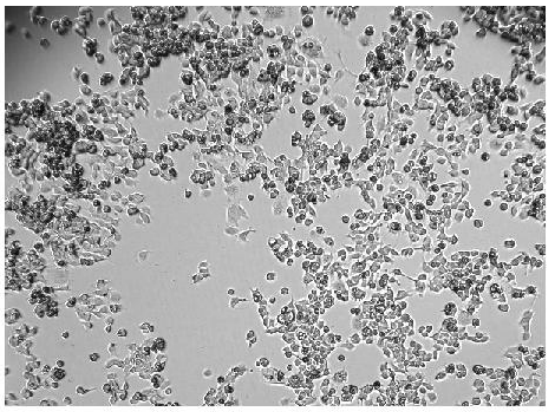
As observed in Fig. 9A, cells cultured in Differentiation Medium, in the presence of the vehicle (VC, 0.1 % DMSO) showed clear neurite outgrowth, in opposition to cells grown in Maintenance Medium (MM). Cells in MM continued to proliferate, attaining a high cell density after three days in culture, which prevented the analysis of neurite outgrowth. As such, neuronal differentiation assays were performed in the presence of Differentiation Medium to facilitate the analysis of the effects of SCs on neuronal differentiation without the interference from other neurogenesis-related processes like cell proliferation.

Figs. 9A and 9B (left panel) show that all the concentrations tested of THJ-2201 and ADB-FUBINACA (1 pM - 1 μM) increased the ratio of differentiation of primary neurites, compared to the VC (0.1 % DMSO). Concerning the number of branches per neurite, only 1 μM ADB-FUBINACA promoted a statistically significant increase when compared to the VC.

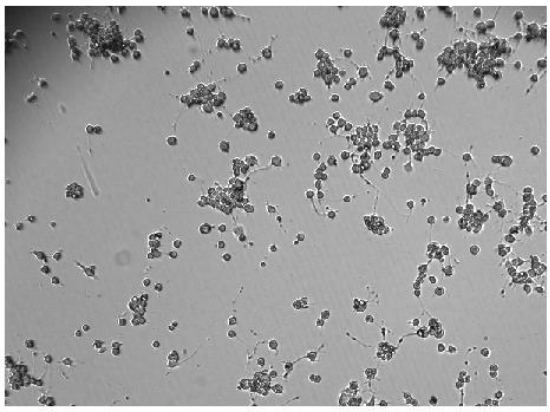
Fig. 9C shows the total length of the primary neurites (left panel), and their branches (right panel) after SC exposure. As noted, neither THJ-2201 or ADB-FUBINACA, at any of the concentrations tested, significantly altered the total length of primary neurites, compared to the vehicle control. However, at the highest concentration tested (1 μM), ADB-FUBINACA increased the total length of neurite branches, compared to the vehicle control.

A

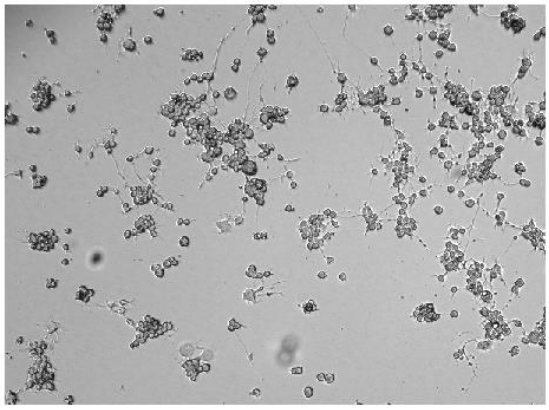
MM



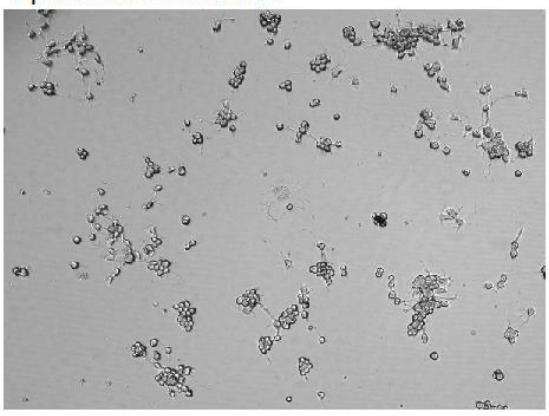
VC



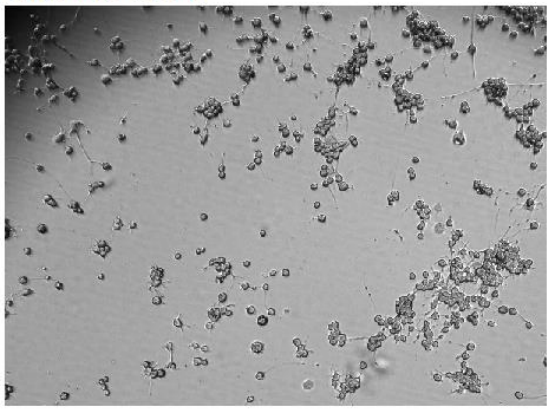
1 pM THJ-2201



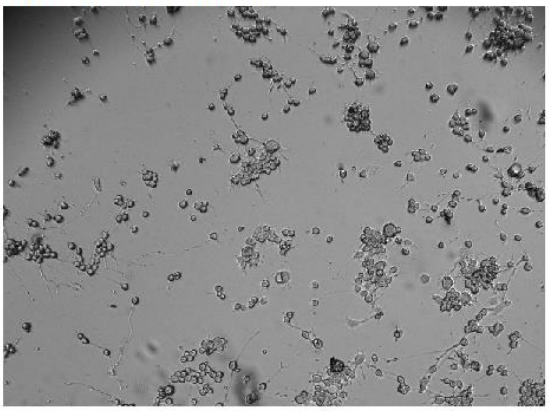
1 pM ADB-FUBINACA



1 µM THJ-2201



1 µM ADB-FUBINACA



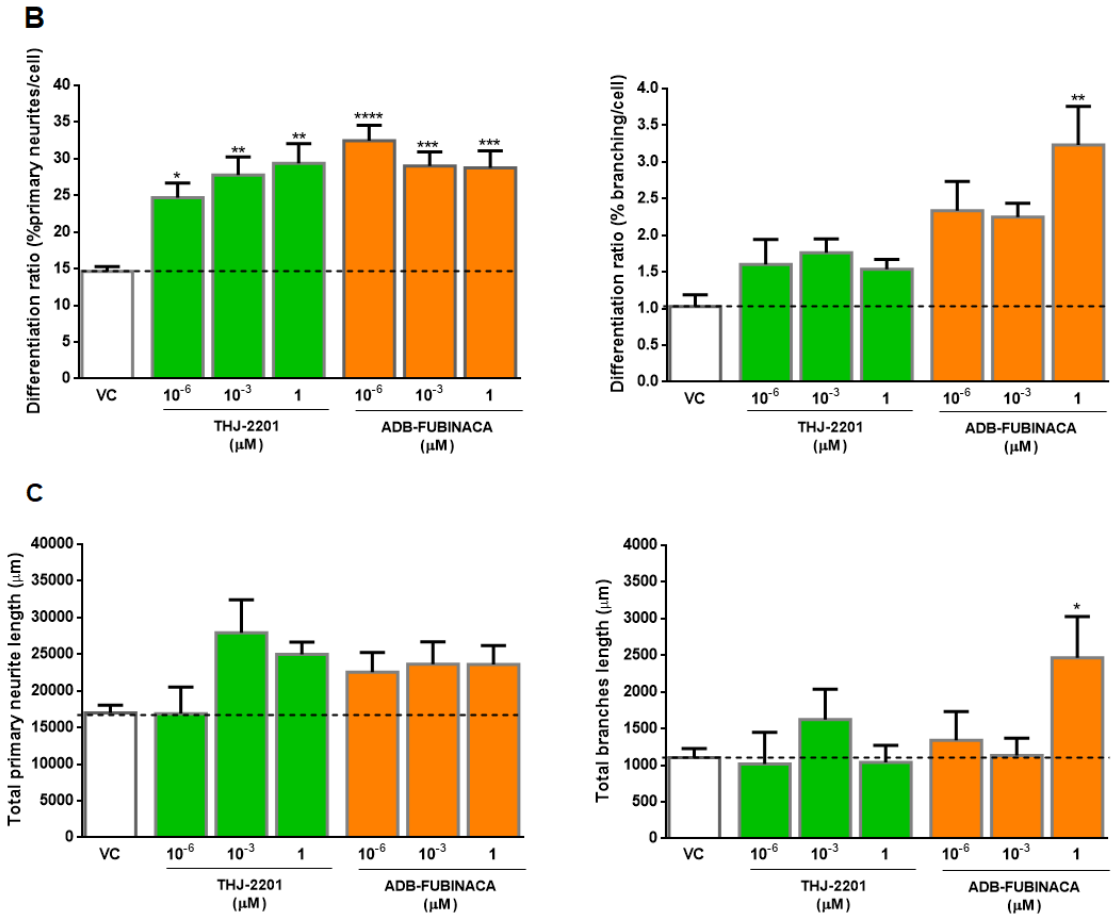


Figure 9.Effects of THJ-2201 and ADB-FUBINACA on neuronal differentiation. Neuronal differentiation was induced on NG108-15 cells by replacing maintenance medium (MM) by differentiation medium in the presence of the SCs or the vehicle (VC, 0.1 % DMSO). A) Representative images of NG108-15 cells 72 h following induction of differentiation. MM: Maintenance Medium; VC: Differentiation medium in the presence of the vehicle. Cells cultured in MM (in the absence of differentiation factors) continued to proliferate, preventing the analysis of neurite outgrowth. **B)** Differentiation ratios for primary neurites (left panel) and branches (right panel), calculated as the number of neurites longer than 20 μm divided by the total number of cells per well. **C)** Total neurite length of primary neurites (left panel) and branches (right panel). Bars show the mean ± SEM, from at least four independent experiments, performed in duplicate. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001, compared to vehicle control (one-way ANOVA, followed by Dunnett's post-test).

3.2. Assessment of NG108-15 phenotype over time

Differentiated NG108-15 cells show typical features of a cholinergic phenotype (Tojima *et al.* 2000). However, over time, they may lose this phenotype and/or initiate cell death mechanisms. As such, we assessed the viability and acetylcholinesterase activity of NG108-15 cells at different time points to determine the maximum period of time NG108-15 cells retained their cholinergic phenotype, without observing significant cell death.

Figure 10 shows the percentage of apoptotic and necrotic cells up to 12 days following the induction of NG108-15 cells differentiation. As observed, the percentage of apoptotic cells was very low over the 12 days that this parameter was assessed, increasing from 1.5 ± 0.3 % at day 0 to 4.5 ± 2.8 % at day 12. The percentage of necrotic cells was maintained below 7.1 % up to the sixth day. However, the presence of necrotic cells was significantly increased at day 9 and 12 to 53.0 ± 24.3 % and 74.1 ± 12.4 %, respectively. These results indicate that up to the sixth day of differentiation, the percentage of cell death is relatively low.

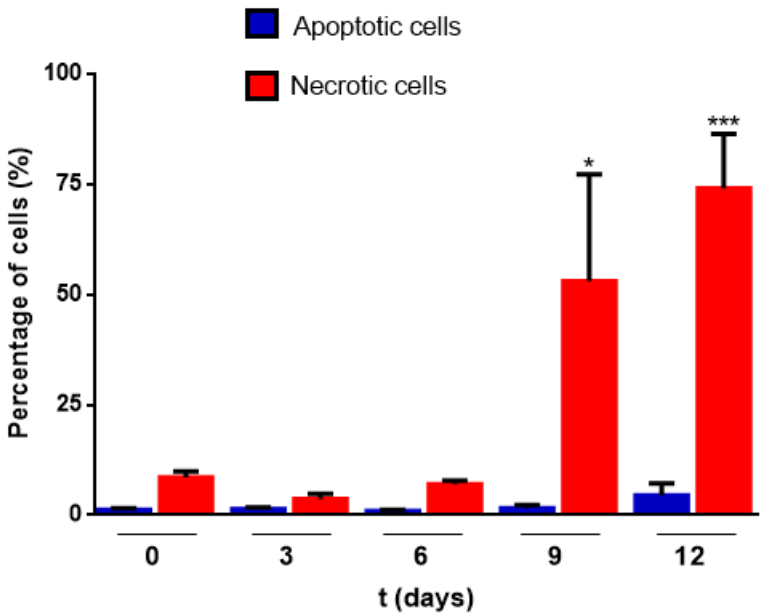


Figure 10. Evaluation of apoptosis and necrosis over 12 days using Hoechst and propidium iodide respectively. The evaluation of apoptotic and necrotic cells were based on a permeable blue and red fluorescent dye which identifies that type of cells, respectively. The evolution of this death mechanisms were quantified along 12 days and, assessing at every three days. Bars show the mean ± SEM, from at least six independent experiments, performed in duplicate. *** $p < 0.001$, compared to the first day after cultured the cells (t0) (one-way ANOVA, followed by Dunnett's post-test).

Figure 11 displays the changes in the activity of AChE monitored up to 15 days after neuronal differentiation was induced. As shown in this figure, AChE activity gradually increased until it reached a peak at the ninth day post-differentiation induction. Nevertheless, from the sixth day onward, activity values were statistically significant, compared to day 0 (beginning of differentiation). From day 9 and up to day 15, the activity of acetylcholinesterase decreased.

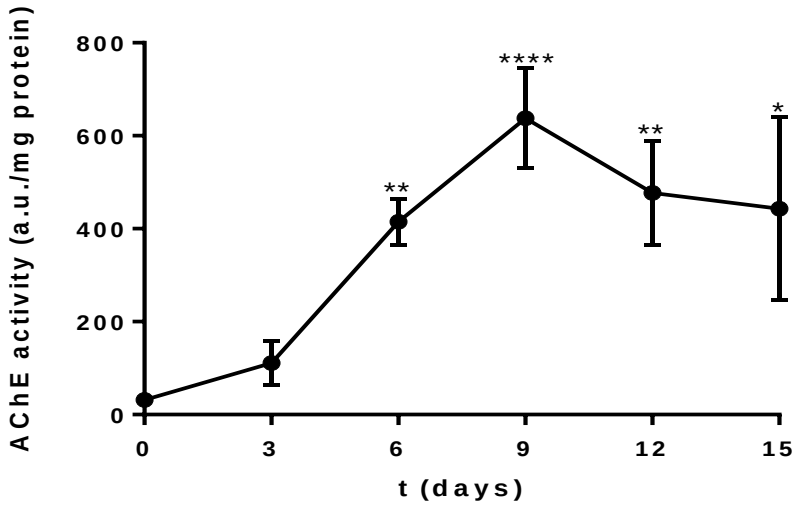


Figure 11. Evaluation of NG108-15 cells phenotype through the quantification of acetylcholinesterase (AChE) activity. The AChE activity was assessed every three days up to fifteen days, to evaluate the phenotype of differentiated cells during time. For each time point the mean ± SEM is shown, from at least eleven independent experiments, performed in duplicate. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, compared to day 0 (t0, the beginning of differentiation) (one-way ANOVA, followed by Dunnett's post-test).

Based on these results, we selected different exposure settings that allowed assessing epigenetic modifications in differentiating NG108-15 cells, which lasted no longer than 6 days after differentiation was induced, as at this time point a significant increase in AChE, without significant cell death, was noted.

3.3. Analysis of epigenetic modifications induced by THJ-2201 and ADB-FUBINACA

3.3.1. Global DNA methylation

The effects of THJ-2201 and ADB-FUBINACA on global DNA methylation were assessed in differentiating NG108-15 cells, according to three different exposure settings, schematized in Materials and Methods (section 2.5, Fig. 8). Treatment A, which allowed the assessment of the SCs' influence in global DNA methylation during neuronal differentiation, consisted in a single SC or vehicle addition at the beginning of differentiation (t0), with samples being collected at the third day of differentiation (t3); Treatment B evaluated the impact of the SCs in global DNA methylation in already differentiated cells, as SCs/vehicle were added once at the third day of differentiation (t3)

and the samples collected at the sixth day (t6). In treatment C, SCs/vehicle were added twice, once at the beginning of the differentiation (t0) and another at the third day (t3), with samples being collected at the sixth day at the differentiation (t6). DNA methylation baseline levels (Differentiation Medium Control, DMC) were assessed in the genomic DNA of cells cultured in differentiation medium, in the absence of vehicle or SCs, collected at the beginning of SC/vehicle exposure.

As shown in Fig. 12, there were no statistically significant differences between vehicle controls and baseline levels at the beginning of the SC/vehicle treatments for any of the exposure settings, indicating that the vehicle (0.1 % DMSO) alone did not affect global DNA methylation levels. Moreover, Fig. 12A shows that exposure of NG108-15 cells to 1 µM THJ-2201 (the highest concentration tested), or to 1 nM ADB-FUBINACA increased global DNA methylation in about 1.9- and 1.7-fold, respectively, compared to the vehicle control. When the SCs were added only at the third day of differentiation (Fig. 12B), only 1 nM ADB-FUBINACA affected DNA methylation levels, decreasing these values to about 0.3-fold compared to the vehicle. Of note, in this exposure setting, none of the THJ-2201concentrations tested presented any influence at the DNA methylation. Upon cell exposure to SCs at the start and at the third day of the differentiation (Fig. 12C), 1 pM THJ-2201 produced an increase of about 1.5-fold and 1 µM ADB-FUBINACA led to a decrease to about 0.1-fold compared to the vehicle.

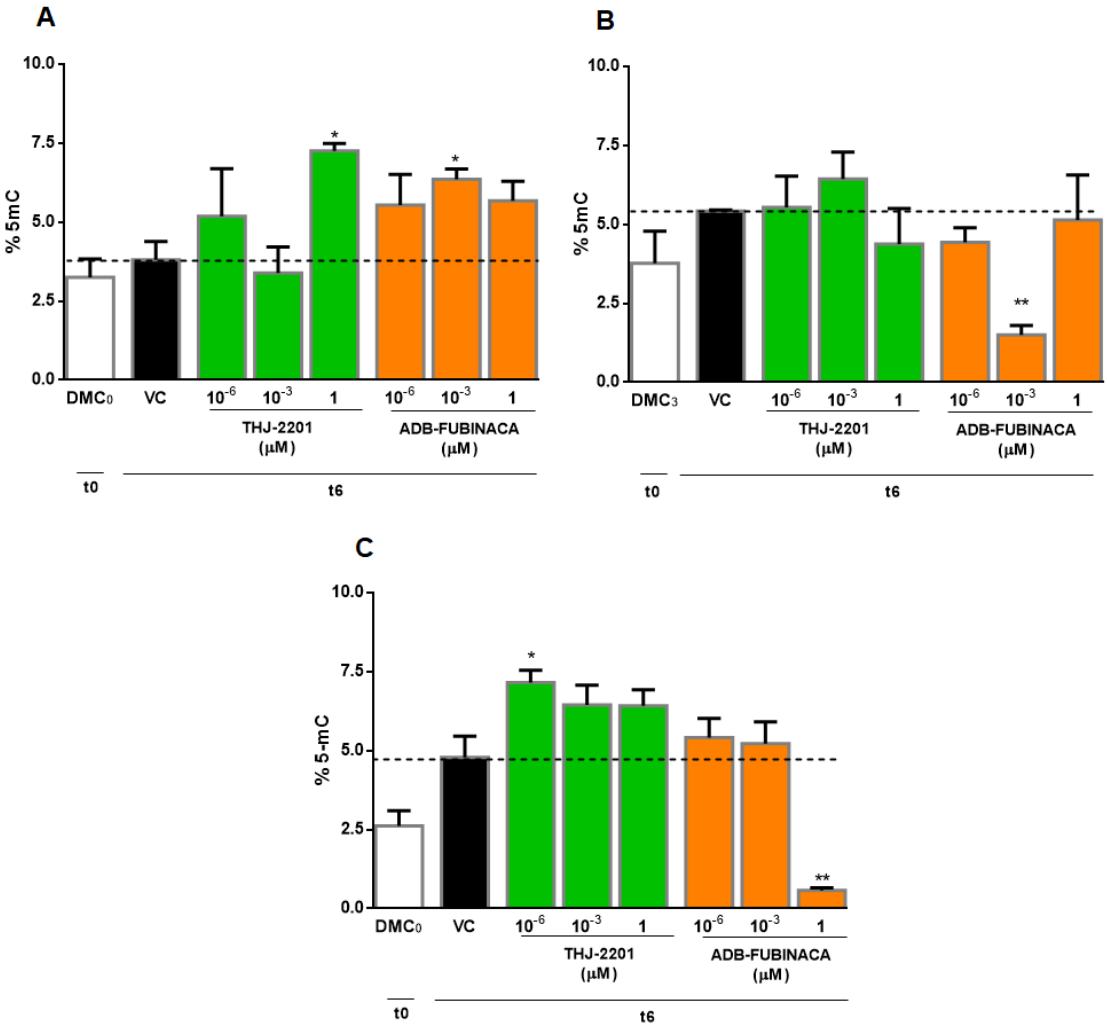


Figure 12. Effects of THJ-2201 and ADB-FUBINACA on global DNA methylation. Neuronal differentiation was induced in NG108-15 cells, as described in Materials and Methods. DNA methylation was evaluated after 3 or 6 days of exposure to 0.1 % DMSO (VC) or each SC at three concentrations (1 pM, 1 nM, and 1 μM). Differentiation Medium Control (DMC) represents the control containing only differentiation medium, without any vehicle or SCs at the beginning of the SC or vehicle treatments. At the end of SC exposure, the cells were collected, genomic DNA was extracted, and global DNA methylation were assessed using a commercially available kit. **A)** SCs or vehicle were added to NG108-15 once at the beginning of the differentiation (t0), and samples were collected at the third day of differentiation (t3). **B)** SCs or vehicle were added once at the third day of differentiation (t3) and collected at the sixth day (t6). **C)** SCs or vehicle were added twice, first at the beginning of the differentiation (t0), and once again at the third day of differentiation (t3). Samples were collected at the sixth day of differentiation (t6). Bars show the mean ± SEM, from at least three independent experiments, performed in duplicate. * $p < 0.05$; ** $p < 0.01$, compared to vehicle control (One-way ANOVA, followed by Dunnett's post-test).

3.3.2. Global Histone modifications

Similar to the assessment of the SCs' effects on DNA methylation, the changes possibly induced by THJ-2201 and ADB-FUBINACA on global histone H3 acetylation were assessed in differentiating NG108-15 cells, according to the same three different

exposure settings (Fig. 8). Histone H3 acetylation baseline levels (Differentiation Medium Control, DMC) were assessed in the histone extracts of cells cultured in differentiation medium, in the absence of vehicle or SCs, collected at the beginning of differentiation (DMC_0) and immediately prior to SC/vehicle exposure (DMC_3 or DMC_6).

As shown in Fig. 13, there were no statistically significant differences in histone H3 acetylation levels between vehicle and differentiation medium controls at the end of the SC/vehicle treatments (DMC_3 or DMC_6) for any of the exposure settings, indicating that the vehicle (0.1 % DMSO) alone did not affect histone H3 acetylation levels. There were also no significant changes in the percentage of histone H3 acetylation between the negative controls (with or without vehicle) at the day of histone extraction and baseline levels at the beginning of the differentiation (DMC_0) for exposure settings B (Fig. 13B) and C (Fig. 13C). However, the percentage of H3 acetylation at the third day of differentiation in the negative controls (VC and DMC_3) was significantly higher than that observed at the beginning of differentiation (DMC_0), as shown in Fig. 13A.

As also noted in Fig. 13A, none of the SCs, at the concentrations tested, presented any influence in global histone H3 acetylation. NG108-15 cells exposed to a single administration of 1 nM THJ-2201 at the third day of differentiation presented an increase of about 1.8-fold, compared to the VC (Fig. 13B). However, none of the ADB-FUBINACA concentrations tested altered the percentage of global histone H3 acetylation. In turn, cells exposed at days 0 and 3 of differentiation to 1 pM ADB-FUBINACA showed a significant decrease to about 0.4-fold in histone H3 acetylation levels compared to the VC. Of note, in this exposure setting, THJ-2201 did not affect histone H3 acetylation at any of the concentrations tested.

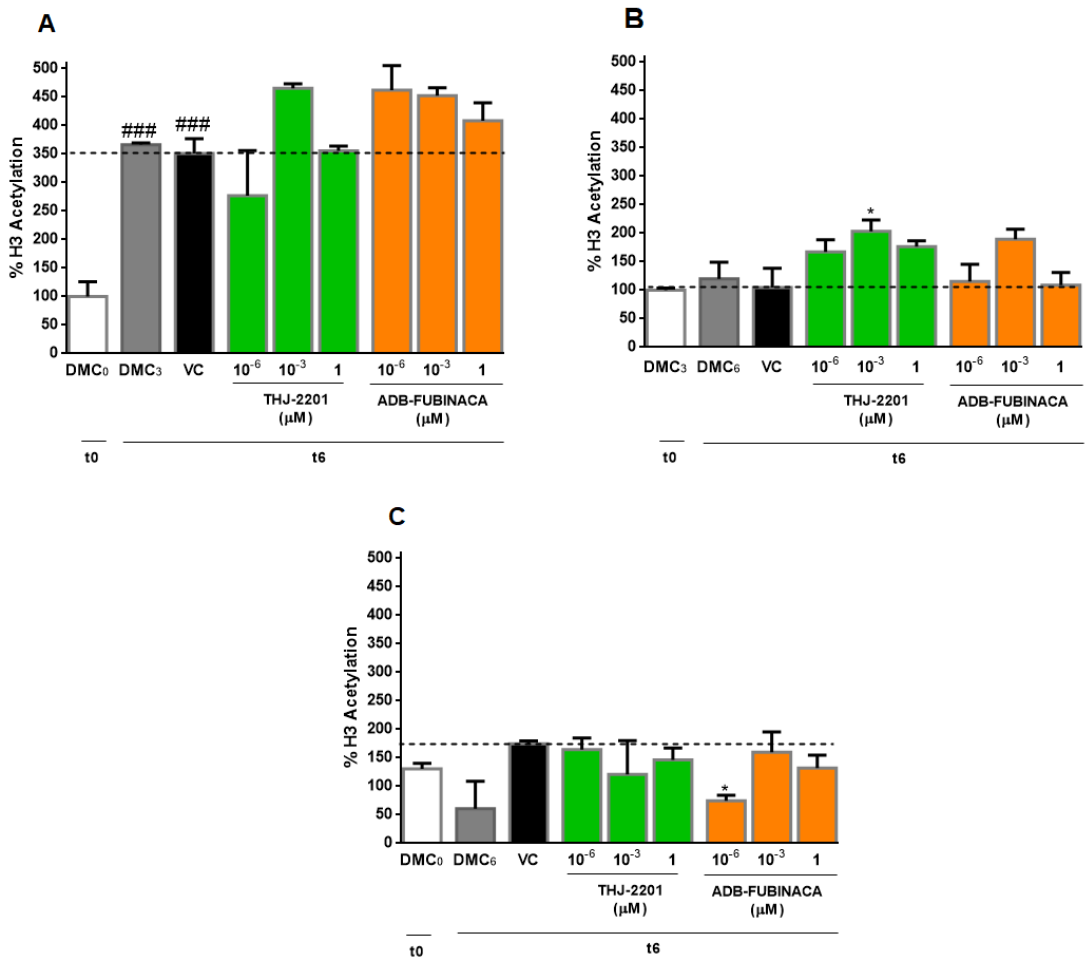


Figure 13. Effects of THJ-2201 and ADB-FUBINACA on histone H3 acetylation. Neuronal differentiation was induced in NG108-15 cells, as described in Materials and Methods. The percentage of H3 acetylation was evaluated after 3 or 6 days of exposure to 0.1% DMSO (VC, vehicle control) or each SC at three concentrations (1 pM, 1 nM, and 1 μM). Differentiation Medium Control (DMC) represents the control containing only differentiation medium, without any vehicle or SCs differentiation medium control at the beginning and at the end of the SC or vehicle treatment. At the end of SC exposure, the cells were collected, histones were extracted, and global histone H3 acetylation levels were assessed using a commercially available kit. **A)** SCs or vehicle were added to NG108-15 once at the beginning of the differentiation (t0), and samples were collected at the third day of differentiation (t3). **B)** SCs or vehicle were added once at the third day of differentiation (t3) and collected at the sixth day (t6). **C)** SCs or vehicle were added twice, first at the beginning of the differentiation (t0), and once again at the third day of differentiation (t3). Samples were collected at the sixth day of differentiation (t6). Bars show the mean ± SEM, from at least three independent experiments, performed in duplicate. * $p < 0.05$; ** $p < 0.01$, compared to vehicle control (One-way ANOVA, followed by Dunnett's post-test). ### $p < 0.001$ compared to DMC_0 [at the beginning of the differentiation (t0)] (two-tailed, unpaired t-test).

Chapter IV. Discussion

The recreational use of synthetic cannabinoids has increased in the past two decades, especially in young people. Since the brain is the major target organ for SCs, and considering that the brain is most vulnerable during its developmental stage, it becomes important to assess the impact of these substances in neurogenic processes. Here, we analyzed the impact of two SCs, THJ-2201 and ADB-FUBINACA, on neuronal differentiation, particularly ascertaining if such process could be related by the SC-mediated induction of epigenetic modifications.

We observed a clear modulation of in vitro neurodifferentiation by THJ-2201 and ADB-FUBINACA, indicated by an increase in differentiation ratios at biologically relevant concentrations (1 pM – 1 µM). This range of concentrations has been previously shown to be non-toxic to NG108-15 cells (Alexandre *et al.* 2020) (data for ADB-FUBINACA has not been published yet). Of note, differentiation assays were performed in serum-deprived (1 % FBS) cell culture medium containing forskolin and retinoic acid as differentiation factors to promote differentiation without the interference of other processes involved in neurogenesis, like proliferation. Retinoic acid is a metabolite of vitamin A (retinol) whose primary function is the regulation of cell cycle, by stopping proliferation, and then redirecting the cellular machinery to differentiation. Retinoic acid enters the cell and binds to cellular retinoic acid binding protein (CRABP), resulting in the formation of the complex RA-CRABP. In turn, this complex enters the nucleus to bind retinoid specific receptors (RARs) and the retinoid X receptors (RXRs). Afterward, the new complex binds to DNA sequences and induces the transcription of target genes such as *oct4* or *hox*, which are required to differentiate embryonic stem cells into neurons (George *et al.* 2019; Janesick *et al.* 2015; Salehi *et al.* 2016). Forskolin is an extract of the plant *plectranthus barbatus*, which activates the enzyme adenylyl cyclase and accelerates the coupling between stimulatory GTP-binding protein and adenylate cyclase, and enhances the K^+/Ca^{2+} influx leading to cell differentiation. Also, the activation of adenylyl cyclase increases intracellular cAMP levels, which stimulate neurite outgrowth and axonal elongation (Ammer & Schulz 1997; Tohda & Nomura 1988; Zhang *et al.* 2006). These two compounds combined (retinoic acid and forskolin) increase the formation of neurites (Zhang *et al.* 2006).

Results concerning THJ-2201 are in line with (Alexandre *et al.* 2020), who have previously demonstrated that this SC increased neurodifferentiation at concentrations between 1 pM and 1 µM. Here, we further showed that this SC is able to promote neurite outgrowth at a concentration as high as 1 µM. Moreover, this work shows first-hand the ability of ADB-FUBINACA to also increase neurite outgrowth in NG108-15 cells. Other SCs have already been reported to interfere with neurogenic processes. For example,

Bromberg and coworkers (2008) observed that SCs play a vital role in neurodifferentiation through the regulation of Pax6, a modulator of neuronal proliferation and differentiation during neurogenesis. These authors found that the SC HU-210-mediated modulation of the PI3k/Akt pathway promoted the phosphorylation of Pax6 in differentiating Neuro2A cells, which in turn led to the activation of kinase signaling and increased the growth of neurites as well as neuronal differentiation. Additionally, Ferreira and collaborators (2018) described the impact of the SCs HU-308 (a selective agonist of CB_2) and WIN55,212-22 (a selective agonist of CB_1) in the neurogenesis at the subventricular zone and dentate gyrus, promoting cell differentiation due to a dependence on an interaction between BDNF and endocannabinoid signaling.

Moreover, only 1 μM ADB-FUBINACA (the highest concentration tested) increased the branching ratio and total branches length. As described in section 1.1.4, neuronal differentiation may occur as a result of different signaling pathways, such as the activation of the MAPK family cascade, involving the activation of ERK1/2 through the inhibition of Rap-1 and B-Raf, and also by the Akt signaling pathway (Galve-Roperh *et al.* 2013; Harkany *et al.* 2007). Similar to endocannabinoids, acute SC exposure may be related to the stimulation of presynaptic CB_1-mediated signaling and the inhibition of neurotransmitter release affecting different types of synapses (Mackie 2013). Additionally, the G protein has a particularity called biased signaling, which suggests that the GPCR could be modelled allosterically and acquire different conformations in response to different ligands (Smith *et al.* 2018). For example, Laprairie and coworkers (2016) showed that THC has more affinity to $G\alpha_s$ in comparison to $G\alpha_{i/o}$, and in turn, a preference to $G\alpha_{i/o}$ in comparison to β-arrestin 1-, $G\alpha_q$-, or Gβγ (Laprairie *et al.* 2016). Therefore, it is possible that THJ-2201 and ADB-FUBINACA may have different mechanisms in the modulation of neuronal differentiation due to the interaction with each of the conformations of the G protein mentioned above, activating different signaling pathways. For example, it is plausible that 1 μM ADB-FUBINACA may have changed the expression of some molecules involved in these processes related to neurite ramifications, resulting in higher branching ratio and length.

Ly and collaborators have shown that other psychoactive substances [e.g. lysergic acid diethylamide (LSD) or N-dimethyltryptamine (DMT)] increased neurogenesis and synaptogenesis, resulting in a subsequent increase of neuroplasticity, which has been associated with the reduction of anxiety and depression-like behaviors (Hill *et al.* 2015). However, these effects may not be necessarily beneficial. Every process during neurodevelopment (e.g. cell division, protein synthesis or DNA replication) is tightly regulated and has a determined and specific time frame to occur. An untimely activation of

those processes, such as an increase in differentiation ratio like the one occurring with SCs and other psychoactive substances, may result in central nervous system abnormalities (e.g. exencephaly, holoprosencephaly, or cortical dysplasia) (Gilbert *et al.* 2016).

Considering that epigenetic modifications induced by a given agent may take some time to be noticed, the NG108-15 cells' phenotype (acetylcholinesterase activity) and cell viability (Hoechst – PI staining) was assessed over time to determine the maximum period that NG108-15 cells retained their cholinergic phenotype, without a significant loss in cell viability. Our data showed that acetylcholinesterase activity gradually increased over time, reaching a peak at day 9 after differentiation was induced. However, at day 9, we also observed around 53 % of necrotic cells, a number that doubled at day 12. Of note, no major changes in the percentage of apoptotic cells was noted over the days, compared to the first day of differentiation (t0). Based on these results, we designed three types of experimental settings, none lasting more than 6 days of cell exposure to SCs to assure that NG108-15 kept their cholinergic phenotype without showing any significant cell death.

Our results showed that depending on the exposure setting, the outcome of SC exposure on epigenetic modifications may differ. For example, a single addition of the 1 µM of THJ-2201 and 1 nM of ADB-FUBINACA at the beginning of neurodifferentiation (treatment A), or two additions of 1 pM THJ-2201 (treatment C), increased global DNA methylation. However, DNA methylation levels decreased in treatment B and C, after exposure to 1 nM and 1 µM ADB-FUBINACA, respectively. Histone H3 acetylation increased in response to a single addition of 1 nM THJ-2201 at the third day after differentiation induction (treatment B) and decreased with 1 pM ADB-FUBINACA when cells were treated twice with the SC (treatment C). DNA methylation is one of the most important epigenetic modifications involved in the regulation of neuronal cell fate. Actually, neurogenesis seems to be favored over gliogenesis during early stages of brain development as a result of the repression of glia cell-related genes by DNA methylation at such stages. Of note, changes in DNA methylation frequently overlap with key signaling pathways regulating cell fate (e.g. Pax6). Moreover, most of DNA methylation has been reported to occur during fetal development and childhood, being less plastic in adulthood. Changes in DNA methylation patterns may persist throughout childhood and influence the child's cognitive performance, learning, and memory behaviors. Additionally, alterations in global DNA methylation during neurodifferentiation often result in cell death (Cardenas *et al.* 2017; Stricker & Götz 2018).

We observed that in treatment A, comprising the initial stage of neuronal differentiation, DNA methylation levels were increased following some of the SC exposure

conditions. These data are in accordance with the increased differentiation ratios observed at the same conditions, which suggests that the SC-induced increase in DNA methylation may relate to the higher differentiation ratios also elicited by those SCs. Similarly, the decreased DNA methylation observed at day 6 of differentiation following a single or two SC additions of ADB-FUBINACA (treatment B and C) may relate to a later stage of neurogenesis, possibly corresponding to a neuronal maturation stage.

Histone acetylation is required for the induction of axonal regeneration and neuronal development including neurodifferentiation. Similar to DNA methylation, increased histone acetylation, as the one observed for 1 nM THJ-2201, is also associated with increased neurodifferentiation. In contrast, a reduction in histone acetylation, which is the case of exposure to 1 pM ADB-FUBINACA in treatment C, may promote neural proliferation, and blocking of premature differentiation of neuronal stem cells, which is required to differentiate oligodendrocytes, and regulate dendritic growth (Lilja *et al.* 2013). Along with the decreased DNA methylation observed in treatments B and C, this SC-mediated reduced ADB-FUBINACA histone H3 acetylation further supports the notion that at day 6 cells may be at a later stage of differentiation and it is possible that part of the cell population may be starting to be targeted for proliferation. Nevertheless, as the ADB-FUBINACA concentration that affected histone H3 acetylation was different from the ones reducing DNA methylation, it would be important to perform additional research to better understand these disparities in terms of the response to the different ADB-FUBINACA concentrations tested. Of note, the percentage of histone H3 acetylation in our study was much higher in cells collected three days after differentiation was induced, underlining the importance of this modification for neuronal differentiation (Lilja *et al.* 2013).

All these epigenetic data assume great importance considering that the occurrence of epigenetic modifications during neurodevelopment may lead to neurodevelopmental-related disorders. For example, Dall'Aglio (2018) identified a decrease in DNA methylation in *vipr2* gene resulting in hyperactivity in patients with ADHD. Liu and coworkers (2008) evaluated the DNA methylation at CpG sites across the genome of 98 schizophrenia patients and 108 healthy controls, and correlated eleven CpG sites with reality distortion symptoms. Of those, seven were hypermethylated and the other four were hypomethylated, which may neutralize symptoms such as hallucination and delusion. These data further suggest that in addition to the analysis of global DNA methylation, the assessment of methylation levels in specific genes may also be important. Hyperacetylation of nucleosome core histones appear in *mecp2*-deficient brains, resulting in Rett Syndrome (Jakovcevski & Akbarian 2013). In patients with Beckwith-Wiedemann

syndrome, histone modifications like trimethylation and H3K27ac were frequently found (Dall'Aglio *et al.* 2018).

The impact of exogenous cannabinoids on epigenetic modifications has already been reported. For example, Prini and coworkers (2018) showed that adolescent female rats exposed to THC presented a 30% increase in H3K9ac after 48 h exposure. Other have described a decrease in levels of H3K4me3 and H3K9me2 in the nucleus accumbens of adult rats prenatally exposed to THC (Szutorisz & Hurd 2016). However, the impact of SCs on epigenetic modifications is not yet fully understood. Tomas-Roig *et al.* (2017) reported that the administration of WIN55,212-2 to C57B16/J male mice induced DNA hypermethylation at the intragenic region of the intracellular signaling modulator Rgs7, which was associated with a lower rate of mRNA transcription of this gene, contributing to the learning and memory deficits. The same SC was reported to increase global DNA methylation in the prefrontal cortex in male wistar rats, through upregulation of DNMT enzymes in stress-induced anxiety behavior (Ibn Lahmar Andaloussi *et al.* 2019).

Of note, epigenetic changes are the result of the action of a wide range of enzymes, factors, and regulation of signaling pathways. As noted in the literature, depending on the affected genes, epigenetic changes can result in different consequences, such as different neurodevelopmental disorders as those mentioned in **Table I**. This suggests that SC can interfere in different stages of these processes, including the expression of enzymes that promote or decrease the epigenetic modifications (e.g. histone methyltransferases, histone deacetylases, histone acetyltransferases, and DNA methyltransferases), as well as in the regulation of different key signaling pathways involved in neurodifferentiation, such as the PI3K/Akt, through the inhibition/activation of target genes expression.

Further studies are thus required to understand how the SC-induced epigenetic changes may affect neuronal differentiation, as well as the mechanisms involved in the onset of such SC-elicited epigenetic modifications.

Chapter V. Conclusions and Future Perspectives

Overall, we showed, for the first time, the ability of ADB-FUBINACA to promote neurite outgrowth in NG108-15 neuroblastoma x glioma cells, while confirming the induction of such effect by THJ-2201. Most important, our data suggested that such SC-induced neuronal differentiation could be, at least partly, influenced by the changes in global DNA methylation and histone H3 acetylation levels promoted by THJ-2201 and ADB-FUBINACA. However, no clear pattern in terms of concentration-response was observed for any of the SCs tested. For this reason, further research is required to clarify the influence of SCs on epigenetic modifications during neuronal differentiation. For example, it would be important to analyze the effects of SCs on the enzymes involved in DNA methylation and histone acetylation/deacetylation, such as DNMTs, HAT and HDAC. Also, it would be important to address the effects of SCs on the acetylation of histone H4 since this type of modification in specific genes seems to play a role in neurodevelopment regulation. It would be also interesting to analyze other proteins and molecules involved in the regulation of epigenetic modifications, as DNA/histone-binding proteins or effector molecules [e.g. methyl-CpG-binding domain (MBD), kaiso protein family, chromodomain, Tudor, malignant brain tumor (MBT) proteins)] to verify the impact of the alterations of such molecules. Moreover, analyzing the epigenetic changes in specific genes could provide useful insights into these mechanisms of action.

As observed in the literature, in addition to histone acetylation, histone methylation also seems to play an important role during neurodevelopment. As such, it could be important to study the impact of SCs in this type of histone modifications. Additionally, studies with an antagonist of CB_1 are required to better understand the involvement of this receptor in the modulation of these epigenetic mechanisms, as well as in the differentiation assays with ADB-FUBINACA. In addition, it would be interesting to ascertain how these data compare with the effects of THC.

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