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# AVALIAÇÃO DE POSSÍVEL EFEITO ADVERSO DO CANABIDIOL E DERIVADOS SINTÉTICOS DURANTE E APÓS O DESENVOLVIMENTO NEURONAL: ENVOLVIMENTO DO SISTEMA ENDOCANABINOIDE

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Orientador: Fabio Klamt (Prof. Dr.)

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## Apresentação

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**Índice**

# Parte I

**Resumo**

A via de sinalização retrógrada endocanabinoide é ubíqua no sistema nervoso central (SNC), onde modula as sinapses, entre outros processos. Os componentes do sistema endocanabinoide (SEC) - receptores canabinoides, endocanabinoides e enzimas de síntese/degradação - são expressos e funcionais desde os estágios iniciais de desenvolvimento embrionário até o final do desenvolvimento cortical na adolescência, regulando o destino das células progenitoras, a diferenciação neural, sua migração e sobrevivência. Esses fatores podem conferir maior vulnerabilidade aos efeitos adversos da exposição precoce aos canabinoides. O canabidiol (CBD) é um dos fitocanabinoides exógenos mais estudados, e tem sido usado no tratamento de crianças com epilepsia refratária. No entanto, não há evidências suficientes sobre potenciais consequências prejudiciais dos canabinoides a longo prazo no desenvolvimento do SNC. O CBD é capaz de atuar direta e indiretamente no SEC, podendo perturbar processos regulatórios mediados por este sistema. Nos últimos anos, análogos sintéticos do CBD foram desenvolvidos visando aumentar seus efeitos benéficos, mas reduzindo os efeitos colaterais. Neste estudo avaliamos os efeitos neuroprotetores/neurotóxicos e anticonvulsivantes do CBD e de alguns de seus derivados sintéticos durante e após fases do desenvolvimento neuronal, verificando o papel do SEC nesses efeitos. Para isso, foi utilizado um modelo animal com maior susceptibilidade a crises epilépticas, tratados com 10 mg/kg de CBD com 15 e 44 dias de vida e submetidos a crises epilépticas induzidas por ácido kaínico (KA). Neste modelo, o CBD foi capaz de aumentar os níveis hipocampais de endocanabinoides, associado a um aumento da susceptibilidade a crises epilépticas em animais de 15 dias. Animais com 44 dias não tiveram alterações nos níveis de endocanabinoides e apresentaram uma discreta redução na susceptibilidade a crises epilépticas após a administração de CBD. Esses resultados reforçam a hipótese de que o aumento da ativação endocanabinoide seria um dos mecanismos de ação do CBD. Para as análises *in vitro*, utilizamos células da linhagem de neuroblastoma humano SH-SY5Y diferenciadas em neurônio maduro – como um modelo de toxicidade em neurônios terminalmente diferenciados –, e durante a diferenciação – como um modelo de toxicidade durante o desenvolvimento neuronal. Nestes modelos, foi possível verificar efeitos neurotóxicos do CBD e de 3 de seus derivados sintéticos com ou sem o desafio com a neurotoxina redox-ativa 6-OHDA durante a diferenciação neuronal. Além disto, o co-tratamento destas células com cada canabinoide e com um agonista e um antagonista de CB1, aumentou e reduziu, respectivamente, a viabilidade celular observada para cada canabinoide, evidenciando uma participação do sistema endocanabinoide nos efeitos observados. *In vitro*, todos os derivados sintéticos apresentaram neurotoxicidade em 2,5 μM. Os efeitos neurotóxicos observados podem ser atribuídos a um possível desequilíbrio redox causado em células tratadas durante a diferenciação com canabinoides tanto isoladamente quanto em desafios com a 6-OHDA. No entanto, como os co-tratamentos com agonista e antagonista CB1 interferiram nestes resultados, é provável que o SEC esteja envolvido nos mecanismos de neuroproteção/neurotoxicidade observados. Além disso, a afinidade destes canabinoides por CB1 predita *in silico* sugere que pode haver interação direta nas concentrações testadas. Os resultados obtidos nos modelos *in vivo* e *in vitro* corroboram entre si, pois animais neonatos e neurônios em diferenciação foram mais sensíveis aos canabinoides e reforçam a hipótese d o uso de canabinoides em indivíduos muito jovens, com cérebros imaturos, deve ser melhor avaliado.

**Abstract**

The endocannabinoid retrograde signaling pathway is ubiquitous in the central nervous system (CNS), where it modulates synapses, among other processes. The components of the endocannabinoid system (ECS) - cannabinoid receptors, endocannabinoids and its synthesis / degradation enzymes - are expressed and functional from the early stages of embryonic development until the end of cortical development in adolescence, regulating progenitor cells' fate, neural differentiation, their migration and survival. These factors may confer greater vulnerability to the adverse effects of early exposure to cannabinoids. Cannabidiol (CBD) is one of the most widely studied exogenous phytocannabinoid and has been used in the treatment of children with refractory epilepsy. However, there is insufficient evidence on the potential long-term harmful effects of cannabinoids on CNS development. CBD is able to act directly and indirectly over the ECS and may disturb regulatory processes mediated by this system. In recent years, synthetic analogues of CBD have been developed to increase their beneficial outcomes but reduce side effects. In this study we evaluated the neuroprotective / neurotoxic and anticonvulsive effects of CBD and some of its synthetic derivatives during and after phases of neuronal development, verifying the role of the ECS in these effects. For this, an animal model with increased susceptibility to seizures, treated with 10 mg / kg of CBD at 15 and 44 days of life and submitted to kainic acid (KA)-induced seizures, was used. In this model, CBD was able to increase hippocampal levels of endocannabinoids, associated with increased susceptibility to epileptic seizures in animals of 15 days. Animals with 44 days had no alterations in endocannabinoid levels and presented a slight reduction in susceptibility to epileptic seizures after CBD administration. These results reinforce the hypothesis that increased endocannabinoid activation would be one of the mechanisms of action of CBD. For the *in vitro* analyzes, the human neuroblastoma SH-SY5Y cell line was used after differentiation into mature neurons - as a terminally differentiated neuronal toxicity model - and during differentiation - as a neuronal developmental toxicity model. In these models, it was possible to verify neurotoxic effects of CBD and of 3 of its synthetic derivatives with or without the challenge with the redox-active 6-OHDA neurotoxin during neuronal differentiation. In addition, the co-treatment of these cells with each cannabinoid and with an agonist and a CB1 antagonist, respectively, increased and reduced the cellular viability observed for each cannabinoid, evidencing a participation of the endocannabinoid system in the observed effects. *In vitro*, all synthetic derivatives showed neurotoxicity in 2.5 μM. The observed neurotoxic effects can be attributed to a possible redox imbalance caused in cells treated during differentiation with cannabinoids both alone and in challenges with 6-OHDA. However, as co-treatments with the CB1 agonist and antagonist alter these results, it is likely that ECS is involved in the neuroprotection / neurotoxicity mechanisms observed. In addition, both the effects of cannabinoids and the participation of ECS in these effects appear to be dose dependent and age related. Furthermore, the affinity of cannabinoids for CB1 predicted in silico suggests that there may be direct interaction between them at the concentrations tested. The results obtained in the *in vivo* and *in vitro* models corroborate to each other, since neonates and neurons treated during differentiation were more sensitive to cannabinoids and reinforce the hypothesis that the use of cannabinoids in very young individuals with immature brains should be better evaluated.

**Lista de Abreviaturas**

(−)-5′-DMH-CBD – (-)-5'-Dimethylheptyl-cannabidiol

6-OHDA – 6-hidroxidopamina

AA – Ácido araquidônico

AC – Adenilil ciclase

AEA – Anandamida, N-aracdonoiletanolamida

AR – Ácido Retinoico

Aβ – Peptídeo β-amiloide

cAMP – AMP cíclico

CB1 – Receptor Canabinoide tipo I

CB2 – Receptor Canabinoide tipo II

CBD – Canabidiol

$CL_{50}$ – Concentração letal 50

DA – Doença de Alzheimer

DAT – Transportador de Dopamina

DMSO – Dimetilsulfóxido

DP – Doença de Parkinson

ET – Etanolamina

FAAH – Amida hidrolase de ácidos graxos

FABPs – Proteínas ligadoras de ácidos graxos, do inglês *fatty acid binding proteins*

FDA – *Food and Drug Administration*

GABA - Ácido gama-aminobutírico

$H_2O_2$ – Peróxido de Hidrogênio

HUF-101 – 4'-fluoro-cannabidiol

KA – Ácido kaínico

$k_i$ – Constante de inibição

LCMS – Cromatografia Líquida Com Espectometria de Massas

LTD – Depressão de longo prazo, do inglês *Long-term Depression*

LTP – Potenciação de longo prazo, do inglês *Long-term Potentiation*

MAGL – Monoacilglicerol lipase

MAPK – Proteína cinase ativada por mitógeno

MPP+ – 1-metil-4-fenil-piridina

PIP2 – Bifosfato de fosfatidilinositol

PKA – Proteína cinase A

PND – Dia pós-natal, do inglês *Post Natal Day*

PPARγ – Receptor-γ ativado por proliferadores de peroxissoma

SNC – Sistema Nervoso Central

SOD – Superóxido Dismutase

STD – Depressão de curto prazo, do inglês *Short-term Depression*

TAR – Reatividade antioxidante total, do inglês *Total Antioxidant Reactivity*

TNF-α – Fator de necrose tumoral α

TRAP – Potencial antioxidante total, do inglês *Total Reactivity Antioxidant Potential*

TRP – Receptor Catiônico de Potencial Transiente

TRPV-1 – Receptor Catiônico de Potencial Transiente Tipo 1 / Receptor Vanilóide

Δ9-THC – Δ9-tetrahidrocanabinol

## 1. Introdução

A planta *Cannabis sativa* é conhecida mundialmente como uma droga recreativa, mas tem sido usada para fins medicinais por milhares de anos por diferentes culturas (Cassol-jr et al., 2010). Nos últimos anos, o aumento na legalização da *Cannabis* favoreceu positivamente a pesquisa e uso de seu extrato e de seus compostos isolados. A primeira evidência do uso desta planta data de 4000 AC na China (Li, 1973), onde provavelmente era cultivada para obtenção de suas fibras para produção de têxteis, para alimentação (frutas) e como medicamento (Touw, 1981).

A *Cannabis* foi introduzida na Medicina Ocidental no século XIX, sendo bastante utilizada até o final deste século. Porém, no início do século XX, esse uso diminuiu devido aos efeitos psicotrópicos apresentados (Zuardi, 2006), e por ter se tornado uma droga ilegal.

Na resina de plantas fêmeas da *Cannabis* são encontrados cerca de 100 compostos lipossolúveis, os chamados fitocanabinoides, (Elsohly and Slade, 2005; Izzo et al., 2009). Todos fitocanabinoides são encontrados exclusivamente na *Cannabis* e seus principais componentes, o $\Delta^9$-tetrahidrocanabinol ($\Delta^{9-}$THC, principal composto psicoativo) e o canabidiol (CBD) são produtos do metabolismo secundário de plantas a partir de um mesmo precursor, o canabigerol (Galve-Roperh et al., 2013).

### 1.1. Fitocanabinoides e o Sistema Endocanabinoide

Os fitocanabinoides foram isolados, identificados e sintetizados pela primeira vez décadas atrás (Gaoni e Mechoulam 1971), mas apenas quando os receptores canabinoides foram descritos no cérebro, o modo de ação da *Cannabis* e dos fitocanabinoides começou a ser esclarecido, levando também à identificação e isolamento de seus homólogos endógenos (Howlett et al., 2010), os endocanabinoides. Os primeiros a serem descritos e

mais importantes são a N-araquidonoiletanolamida (anandamida, ou AEA), o 2-araquidonoilglicerol (2-AG) e o 2-araquidonilgliceril éter (noladina) (Luchicchi and Pistis, 2012; Pertwee and Ross, 2002). Os endocanabinoides são sintetizados e liberados em resposta a estímulos fisiológicos ou patológicos, ligam e ativam seus receptores, causando diversos efeitos biológicos em diferentes tecidos (Pertwee et al., 2010). Seus alvos clássicos são os receptores canabinoides tipo 1 (CB1) e tipo 2 (CB2) - CB1 é amplamente expresso no sistema nervoso central (SNC), enquanto o CB2 é expresso principalmente em células do sistema imune, mas também em algumas células do SNC (Skaper and Marzo, 2012) como células piramidais em CA3 e CA2 do hipocampo, onde este receptor participa dos mecanismos de plasticidade sináptica (Stempel et al., 2016). Esses receptores constituem o sistema endocanabinoide (SEC), juntamente com enzimas que catalisam a biossíntese e a degradação dos endocanabinoides (Pertwee et al., 2010).

Assim como os endocanabinoides, a maioria dos fitocanabinoides exerce suas propriedades terapêuticas sobre o SNC principalmente através do SEC, embora existam outros alvos conhecidos (Morales et al., 2017a). A sinalização endocanabinoide desempenha papéis cruciais em vários aspectos do cérebro maduro e durante o desenvolvimento (Meyer et al., 2017). Portanto, distúrbios neste sistema podem interferir no desenvolvimento neural e neuronal.

A clássica via de sinalização da SEC é mostrada na Figura 1 (Schonhofen et al., 2018) (revisado por (Lu and MacKie, 2016)). No cérebro maduro, o SEC modula as sinapses (excitatórias e inibitórias) através da liberação de endocanabinoides AEA e 2-AG. Estes agem como mensageiros retrógrados, com sua liberação pelo neurônio pós-sináptico ativando os receptores CB1 no neurônio pré-sináptico, levando à diminuição da liberação de neurotransmissores na fenda sináptica (Alger, 2002; Lu and MacKie, 2016; Velasco et al., 2012). Este processo é iniciado pelo aumento do influxo de $Ca^{2+}$ causado pela

neurotransmissão no neurônio pós-sináptico que ativa a síntese de endocanabinoides a partir de seus precursores na membrana plasmática. AEA é gerada a partir da hidrólise mediada pela fosfolipase D do N-araquidonoilfosfatidiletanolamina (NAPE), enquanto o 2-AG se origina da hidrólise mediada pela diacilglicerol lipase do diacilglicerol (DAG), derivada principalmente do bifosfato de fosfatidilinositol (PIP2) localizado na membrana. AEA e 2-AG se difundem em direção aos terminais pré-sinápticos e, assim como os canabinoides exógenos, como o $\Delta^9$-THC, se ligam e ativam os receptores CB1 pré-sinápticos acoplados à proteína G. Esta ligação desencadeia a ativação e liberação de proteínas Gi/Go do CB1, inibindo a adenilil ciclase (AC) e, assim, diminuindo a formação de AMP cíclico (cAMP) e subsequente atividade da proteína cinase A (PKA). Esses eventos levam à abertura de canais de $K^+$, causando hiperpolarização do terminal pré-sináptico e fechamento dos canais de $Ca^{2+}$, impedindo a liberação de neurotransmissores armazenados. Finalmente, AEA e 2-AG são recaptados nos terminais pré ou pós-sinápticos, onde são catabolizados, respectivamente, pela amida hidrolase de ácidos graxos (FAAH) e pela monoacilglicerol lipase (MAGL), para produzir ácido araquidônico (AA) e etanolamina (ET) no caso de AEA, ou AA e glicerol para 2-AG. O transporte de endocanabinoides através da membrana plasmática ainda não é completamente compreendido. Embora alguns estudos tenham proposto a existência de um transportador endocanabinoide, o tráfego de AEA, que tem sido mais extensivamente estudado, ocorre através de transporte facilitado de membrana (Fowler, 2013). Recentemente, um novo mecanismo de transporte endógeno de endocanabinoides foi identificado (Deutsch, 2016), no qual proteínas ligadoras de ácidos graxos (*fatty acid binding proteins* - FABPs) seriam transportadores intracelulares que auxiliam na solubilidade de a AEA, transportando AEA para a FAAH, facilitando sua degradação.

Além disso, a ativação do receptor CB1 leva à estimulação da atividade da proteína cinase ativada por mitógeno (MAPK), um mecanismo pelo qual os canabinoides afetam a plasticidade sináptica, a migração celular e, possivelmente, o crescimento neuronal (Mechoulam and Parker, 2013).

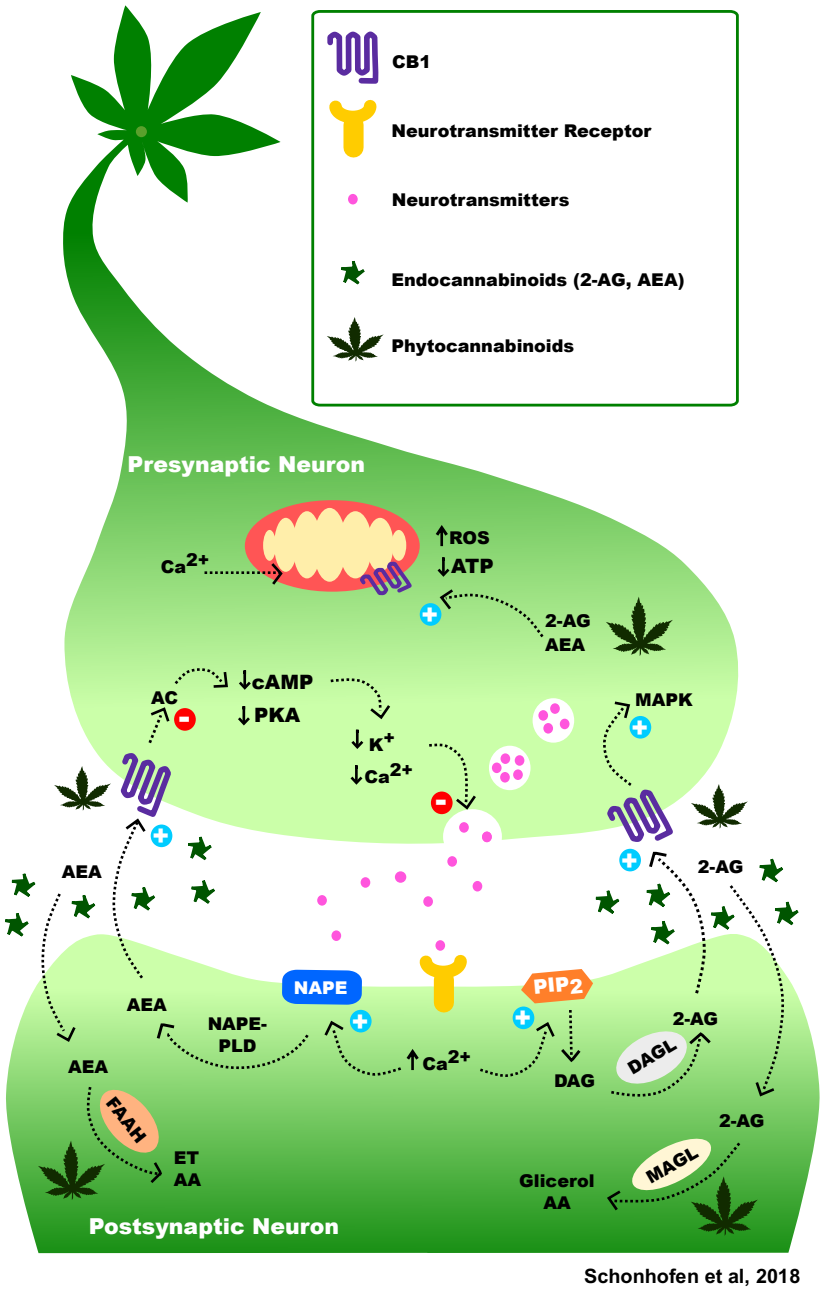


**Fig. 1**. Sistema endocanabinoide. No SNC, endocanabinoides são sintetizados sob demanda no neurônio pós-sináptico, em resposta ao aumento no influxo de $Ca^{2+}$ intracelular, e liberados na fenda

sináptica, onde ativam CB1. Como consequência, no neurônio pré-sináptico ocorre redução do influxo de $Ca^{2+}$, resultando em uma menor liberação de neurotransmissores (Schonhofen et al., 2018).

Assim, o componente central do SEC nos neurônios é o receptor CB1 (Fig. 1). No SNC, o CB1 é particularmente enriquecido no córtex, no hipocampo, na amígdala, nas vias de saída dos núcleos da base e no cerebelo. Esta distribuição corresponde aos efeitos comportamentais mais proeminentes da *Cannabis* e ajuda a prever os efeitos neurológicos e psicológicos da manipulação da SEC (Mackie, 2005). Os receptores CB1 também são observados em compartimentos intracelulares como a superfície mitocondrial, onde são capazes de ativar a sinalização dependente de proteína G e modificar os níveis intracelulares de ATP, $Ca^{2+}$, e espécies reativas de oxigênio, todos com reconhecido impacto na transmissão sináptica (Djeungoue-Petga and Hebert-Chatelain, 2017).

No cérebro maduro, a sinalização retrógrada do SEC medeia a plasticidade sináptica através de depressão de curto prazo ou depressão a longo prazo (STD / LTD) (Lu and MacKie, 2016) e potenciação de longo prazo (LTP) (Silva-Cruz et al., 2017). Tanto LTP e LTD têm papéis na aprendizagem e desenvolvimento neural (Dow-Edwards and Silva, 2017).

O sistema endocanabinoide é expresso e ativo desde as fases iniciais do período embrionário. No SNC em desenvolvimento e nas áreas neurogênicas remanescentes no cérebro adulto (zona subgranular do hipocampo e zona subventricular), o SEC exerce um papel regulador na sobrevivência, proliferação, diferenciação e migração de células progenitoras neurais via CB1 (Díaz-Alonso et al., 2012; Harkany et al., 2007), afetando assim possivelmente a formação de tecidos especializados adultos (Habayeb et al., 2008). Recentemente, o SEC também foi descrito como regulador da proliferação e diferenciação de células-tronco hematopoieticas e mesenquimais derivadas de mesoderma, com um

papel-chave na determinação da formação de vários tipos de células nos tecidos periféricos (Galve-Roperh et al., 2013).

Para fins terapêuticos, em relação ao SNC maduro, demonstrou-se que o SEC modula a ansiedade, a depressão, a neurogênese, a recompensa, a cognição, a aprendizagem e a memória (Mechoulam and Parker, 2013). Além disso, sua sinalização retrógrada age para regular a atividade epileptógena e a hiperexcitabilidade neuronal - canabinoides demonstraram atividade em CB1 em modelos experimentais de crises epilépticas e epilepsia (Blair et al., 2015; Gaston and Friedman, 2017). Assim, direcionar o SEC pode ser de interesse terapêutico. Entretanto, o uso de agonistas do CB1, como o $\Delta^9$-THC, ou mesmo o extrato de *Cannabis*, como estratégia terapêutica, é inviável devido a seus efeitos psicoativos, potencial de abuso e desenvolvimento de tolerância (Blair et al., 2015). Por outro lado, o antagonismo do CB1 também pode exacerbar a atividade epileptógena (Braakman et al., 2009).

Assim, a modulação do SEC como uma abordagem terapêutica é relevante, mas também desafiadora, já que seu bloqueio ou sua exacerbação podem levar a desfechos indesejáveis, especialmente durante o desenvolvimento neuronal, evidenciando a necessidade de estudos mais aprofundados.

Fitocanabinoides também interagem com enzimas do sistema endocanabinoide. Por exemplo, CBD inibe a FAAH, regulando a disponibilidade deste endocanabinoide pela redução da degradação de AEA (De Petrocellis et al., 2011). Ou seja, CBD aumenta os níveis teciduais de AEA, o que pode mediar alguns efeitos farmacológicos do CBD e seus análogos (Bisogno et al., 2001). Os canabinoides – fitocanabinoides, endocanabinoides e derivados sintéticos – também possuem uma variedade de mecanismos não associados ao SEC, incluindo vários canais iônicos clássicos, receptores, transportadores e enzimas, conforme revisado recentemente (Ibeas Bih et al., 2015).

### 1.2. Propriedades neuroprotetoras e mecanismos de ação do Canabidiol

O uso da *Cannabis* e de seus extratos leva a muitos efeitos psicotrópicos, mediados principalmente pela ação agonista do $\Delta^9$-THC no receptor CB1 (Silveira et al., 2017), o que dificulta a sua utilização *in natura*. Por outro lado, estudos experimentais demonstraram várias propriedades terapêuticas de canabinoides isolados em diversos modelos *in vitro* e *in vivo* (Kaur et al., 2016).

O CBD é um dos fitocanabinoides mais relevantes, representando mais de 40% do extrato total da *Cannabis*, sem apresentar os típicos efeitos psicoativos do $\Delta^9$-THC (Grlic, 1976; Karniol et al., 1974). Este canabinoide tem sido associado a propriedades neuroprotetoras em um grande número de estudos, como anti-inflamatório e antioxidante e como atenuante dos efeitos prejudiciais à memória produzidos pelo $\Delta^9$-THC, entre outros efeitos (Mechoulam and Parker, 2013). Isso abre uma ampla gama de usos terapêuticos possíveis em distúrbios neurodegenerativos, incluindo doença de Parkinson (DP), doença de Alzheimer (DA) e isquemia cerebral (Fernández-Ruiz et al., 2013). Além disto, CBD é anti-emético, como a maioria dos canabinoides, e esta capacidade pode estar relacionada à modulação de receptores de serotonina (Parker et al., 2011). Possui propriedades antitumorais contra muitos tipos de câncer (Massi et al., 2013) e também é sugerido que tenha efeitos antipsicóticos, ansiolíticos e antidepressivos (Crippa et al., 2010). Finalmente, numerosos estudos mostraram que o CBD tem propriedades anticonvulsivantes (Campbell et al., 2017), talvez o uso mais proeminente do CBD atualmente.

O CBD possui um anel fenólico em sua estrutura química que lhe confere potencial antioxidante (Borges et al., 2013) (Figura 2), o que já foi verificado em diversos estudos. Em modelos de DP, o CBD reverteu a redução da atividade da tirosina hidroxilase e a depleção de dopamina na *substantia nigra* e estriado após a micro injeção de 6-OHDA

(García-Arencibia et al., 2007; Lastres-Becker et al., 2005) e aumentou os níveis RNAm da enzima superóxido dismutase (SOD) na *substantia nigra* (García-Arencibia et al., 2007). Além disso, em animais hipóxico-isquêmicos, o CBD preveniu a diminuição do número de neurônios viáveis e o aumento da excitotoxicidade, estresse oxidativo e inflamação através de mecanismos envolvendo os receptores CB2 e $5HT_{1A}$ (Pazos et al., 2013). Assim, mecanismos antioxidantes e / ou ativação de diferentes receptores parecem ser responsáveis pelo perfil neuroprotetor atribuído ao CBD.

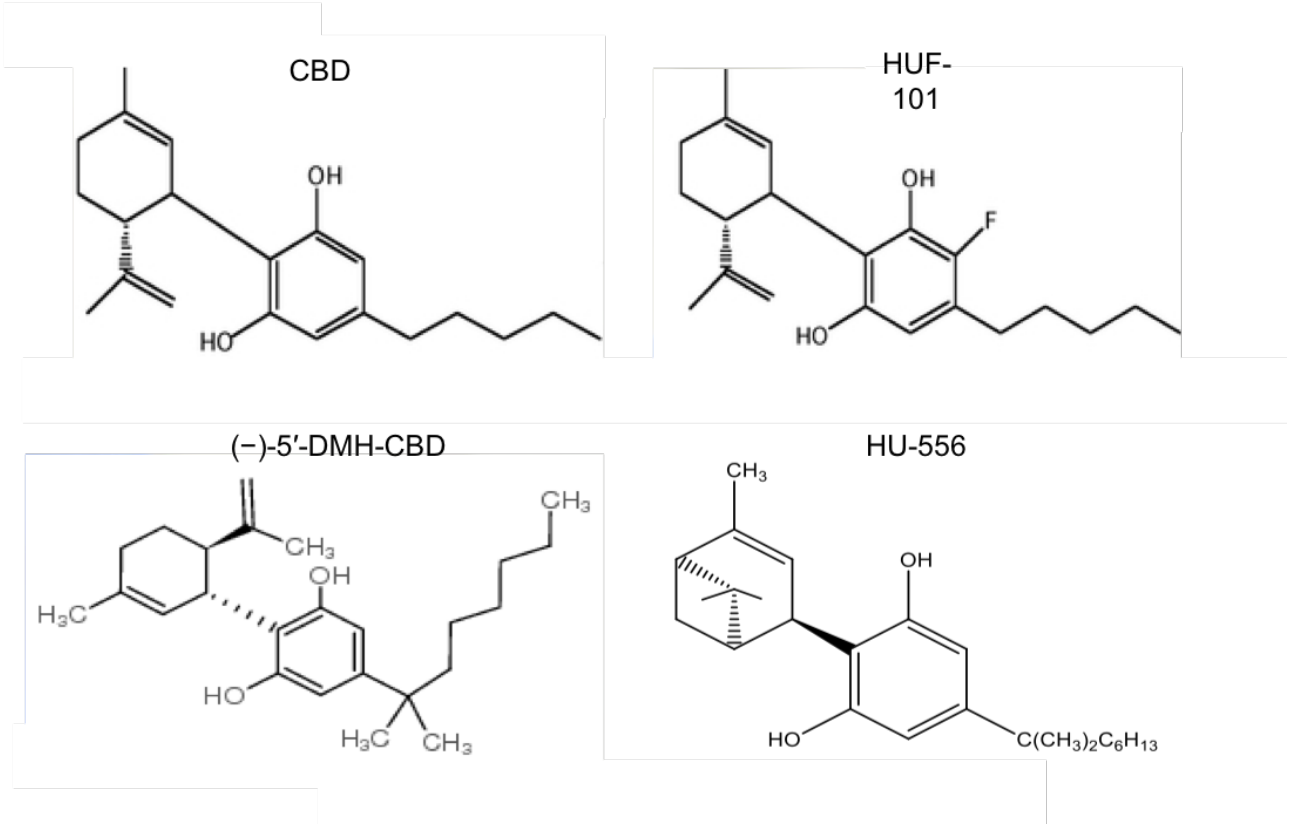


Fig 2. Estruturas químicas dos canabinoides Canabidiol (CBD) e seus derivados sintéticos – HUF-101, (−)-5′-DMH-CBD e HU-556.

Embora diferentes doenças que acometem o SNC tenham suas causas específicas, a neurodegeneração é um fator em comum na fisiopatologia da maioria destas doenças,

como causa ou consequência (Halliwell, 2006, 2001). Em contrapartida, a neuroproteção constitui um mecanismo importante para a preservação da estrutura e função de células neurais, pois promove a proteção contra estresse oxidativo, íons de ferro, excitotoxicidade, agregação proteica, danos a organelas e inflamação (Filipović et al., 2017; Kaur and Ling, 2008).

Em condições normais, defesas antioxidantes e espécies reativas estão em equilíbrio, embora alguns oxidantes não sejam removidos por participarem de funções biológicas importantes como o combate a infecções e resposta inflamatória (Halliwell, 2006). Quando este equilíbrio é desfeito, temos o estresse oxidativo, que por definição, leva ao acúmulo de danos celulares (Halliwell, 2001). O estresse oxidativo está implicado na patogênese de uma série de distúrbios neurológicos, como a DA, DP, esclerose múltipla e acidente vascular cerebral no adulto, bem como em condições como lesão da substância branca periventricular no cérebro neonatal (Filipović et al., 2017; Kaur and Ling, 2008).

O tecido cerebral é especialmente sensível ao dano oxidativo devido ao alto consumo de oxigênio, à presença de aminoácidos citotóxicos como o glutamato, de neurotransmissores auto-oxidáveis, de altos níveis de ferro entre outras especificidades (Halliwell, 2006, 1992). O cérebro em desenvolvimento tem sido descrito como mais suscetível do que o cérebro adulto ao estresse oxidativo que ocorre em muitas condições tais como hipóxia/isquemia (Lee et al., 2005).

As espécies reativas de oxigênio têm a capacidade de danificar as proteínas e as membranas das células, com consequente disfunção mitocondrial e comprometimento da comunicação celular, podendo resultar, por exemplo, em crises epilépticas (Shin et al., 2011). Além disso, modelos experimentais indicaram que os animais geneticamente propensos a uma baixa capacidade de reduzir os radicais livres mitocondriais são mais propensos a ter crises epilépticas do que os animais normais (Liang and Patel, 2004). O

hipocampo é uma das regiões mais suscetíveis do cérebro a sofrer com a lesão induzida pelo *status epilepticus*, que pode ocorrer por inúmeras causas e é mais frequente em crianças e idosos. O CBD já foi descrito como atenuante a neurodegeneração induzida por *status epilepticus*, neuroinflamação, deficiências cognitivas e do humor, e as crises epilépticas recorrentes espontâneas (Upadhya et al., 2018).

Uma vez que, como dito acima, a produção de espécies reativas é importante na neurodegeneração, que está presente em diversas disfunções do SNC, a busca e descrição de novas moléculas antioxidantes com potencial neuroprotetor são constantes na pesquisa científica e servem de alvos potenciais para desenvolvimento de novas terapias (Posser et al., 2008), aspecto no qual o CBD novamente se destaca por suas propriedades antioxidantes.

CBD tem um grande número de alvos moleculares possíveis além do SEC em uma ampla gama de condições médicas, aumentando a possibilidade de efeitos significativos fora do alvo (do inglês *off-target effects*) (Morales et al., 2017a). Por exemplo, o CBD é descrito como um agonista completo do receptor 5-$HT_{1A}$, um agonista parcial fraco do receptor 5-$HT_{2A}$ e um antagonista não competitivo do receptor 5-$HT_{3A}$ (Rock et al., 2012). O CBD pode desempenhar um papel na regulação dos canais de cálcio tipo-T e a atividade do receptor-γ ativado por proliferadores de peroxissoma (PPARγ) nuclear, os quais têm sido implicados na atividade epileptógena (Cilio et al., 2014). Outros alvos moleculares também foram estudados, dentre eles os receptores de glicina (Xiong et al., 2012), GABAA (Bakas et al., 2017), e receptores de potencial transiente (TRP) (De Petrocellis et al., 2011). Estudos focados na possível regulação epigenética de genes de diferenciação cutânea pelo CBD revelaram que ele pode atuar como um repressor transcricional, controlando a proliferação e diferenciação celular através da metilação do DNA (Pucci et al., 2013).

Embora evidências atuais sugiram que o CBD teria afinidade pelo receptor CB1 apenas *in vitro* e em concentrações suprafisiológicas (acima da quantidade de CBD absorvida em doses toleradas *in vivo*) (Ibeas Bih et al., 2015), ele também já foi descrito como agonista ou antagonista do receptor CB1. O CBD pode exercer agonismo indireto, compreendendo o aumento do efeito de um agonista do receptor sem ter qualquer efeito agonista direto em si, nos receptores CB1 - seja aumentando a atividade constitutiva do CB1 (Sagredo et al., 2011), ou aumento dos níveis de ativação do SEC através da inibição da hidrólise de AEA, inibição do transportador putativo de AEA e aumento dos níveis de 2-AG (McPartland et al., 2015), ou mesmo pela competição com proteínas transportadoras (Deutsch, 2016). O CBD também pode antagonizar os efeitos farmacológicos dos agonistas do CB1 através da ligação a um sítio alostérico nos receptores CB1 que é funcionalmente distinto do sítio ortostérico para seus agonistas, atuando como modulador alostérico negativo deste receptor (Laprairie et al., 2015; McPartland et al., 2015; Thomas et al., 2007).

Os efeitos neuroprotetores do CBD também podem envolver mecanismos neuro-inflamatórios. A administração de CBD neutraliza as consequências deletérias da neuro-inflamação e a imunidade inata microglial / macrofágica (Kozela et al., 2010).

Como outros canabinoides, o CBD produz curvas de dose-resposta em forma de sino invertido e pode agir por diferentes mecanismos de acordo com sua concentração ou a presença simultânea de outros ligantes canabinoides (Campos et al., 2017, 2012; Ligresti et al., 2006).

### 1.3. Modelos experimentais para o desenvolvimento neuronal

Apesar do conhecimento atual sobre os alvos moleculares do CBD, não há consenso sobre mecanismos de ação em doenças (Ibeas Bih et al., 2015) e principalmente sobre o desenvolvimento cerebral (Schonhofen et al., 2018). Ainda que já exista um uso crescente

de CBD em crianças e adolescentes cujos cérebros ainda estão em desenvolvimento, a maioria dos estudos *in vitro* e *in vivo* usa células maduras ou modelos animais adultos e, portanto, não mimetizam peculiaridades do SNC juvenil. Além disso, a avaliação da neurotoxicidade / neuroproteção *in vivo* é dispendiosa, demorada, eticamente questionável e inadequada para triagem de grande número de compostos. Por esta razão, as linhagens celulares têm sido amplamente utilizadas para a rápida avaliação toxicológica de um grande número de produtos químicos (Radio and Mundy, 2008). Uma dessas linhagens, a do neuroblastoma humano SH-SY5Y, apresenta várias vantagens para estudos em neurociências, como sua origem humana, a facilidade de cultivo, e ainda é um modelo adequado para estudar mecanismos moleculares de ação e de triagem da neuroproteção / neurotoxicidade de compostos (Bal-Price et al., 2008; Lopes et al., 2012). Nessas células, independentemente da sua origem tumoral, a morfologia neuronal pode ser acessada por um processo de diferenciação em fenótipo dopaminérgico através do tratamento com ácido retinoico (AR) (Figura 3) (Lopes et al., 2017, 2010). Também pode ser um modelo para triagem de drogas durante o desenvolvimento neuronal quando as mesmas são administradas durante o processo de diferenciação (Schönhofen et al., 2015). Além disso, a expressão do gene do receptor CB1 (gene *CNR1*) está aumentada em células diferenciadas por AR em comparação com células indiferenciadas (proliferativas) (Schönhofen et al., 2015).

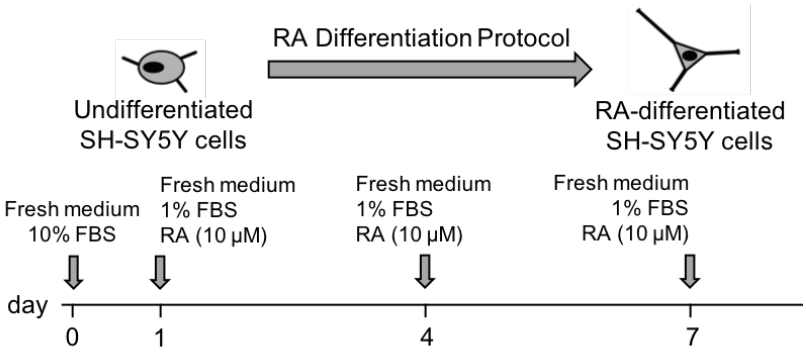


Fig. 3. Desenho experimental do protocolo de diferenciação neuronal de células da linhagem de neuroblastoma humano SH-SY5Y (Adaptado de Lopes et al, 2017).

Nosso grupo de pesquisa mostrou, em um estudo usando células SH-SY5Y diferenciadas por AR, que uma concentração subletal de CBD com atividade antioxidante não exibiu neuroproteção em desafios com neurotoxinas redox-ativas no modelo de neurônios diferenciados. Quando células SH-SY5Y foram expostas à mesma concentração de CBD durante a diferenciação neuronal, o CBD potencializou a neurotoxicidade induzida por todas as toxinas testadas (Schönhofen et al., 2015). Estes resultados sugerem um possível efeito adverso oculto do CBD durante o desenvolvimento neuronal, reforçando que os resultados observados para uma dada molécula podem variar amplamente de acordo com o modelo experimental usado. Já em um modelo genético da Síndrome de Dravet em camundongo, o tratamento com CBD do 21º ao 27º dia de vida diminuiu a duração e a severidade das crises epilépticas induzidas termicamente e a frequência de crises epilépticas espontâneas e, em doses mais baixas, também melhorou os déficits de interação social do tipo autista nestes animais (Kaplan et al., 2017).

Em um modelo alternativo de desenvolvimento, peixe-zebra exposto da blástula ao estágio larval com concentrações micromolares de $\Delta^9$-THC (1 - 16 µM) ou CBD (0,25 - 4 µM), apresentou alterações morfológicas para ambos os compostos, enquanto o $CL_{50}$

(concentração letal para 50% da população) para CBD foi quase sete vezes menor do que $\Delta^9$-THC. Os autores também relataram efeitos teratogênicos de baixas concentrações de CBD (Carty et al., 2017). Em contraste, outra pesquisa não encontrou malformação no desenvolvimento de embriões de peixe-zebra expostos ao CBD 20-300µg / L, embora a dosagem máxima tenha causado atraso na eclosão dos embriões. Além disso, eles eram temporariamente mais ativos que o controle. Os autores discutiram que os efeitos observados estão intimamente relacionados ao receptor CB1 (Valim Brigante et al., 2018). Assim, o CBD apresenta efeitos positivos e deletérios em modelos animais e celulares dos estágios iniciais de desenvolvimento, o que evidencia a necessidades de mais estudos nestas fases de desenvolvimento.

Como alternativa, recentemente, derivados naturais e sintéticos do CBD estão sendo estudados visando melhorar a potência, eficácia ou propriedades farmacocinéticas do CBD (Morales et al., 2017b). Estes derivados são construídos a partir de modificações da molécula do CBD, como demonstrado na figura 2. Por exemplo, a conversão de CBD oral em $\Delta^9$-THC em um ambiente ácido (por exemplo, o estômago) é uma preocupação, embora não tenha sido observada *in vivo* até agora (Nahler et al., 2017). Um novo derivado do CBD, HU-444, tem apresentado efeitos positivos em uma grande variedade de estudos e não pode ser convertido por ciclização ácida em $\Delta^9$-THC (Haj et al., 2015).

Entretanto, assim como os canabinoides naturais, diferentes derivados do CDB variam em suas propriedades farmacológicas e terapêuticas, evidenciando a necessidade de um melhor entendimento de seus efeitos e mecanismos de ação (Schonhofen et al., 2018). Abordagens *in silico* podem ser úteis em tais estudos mecanísticos, através de simulações das interações de um receptor com um potencial ligante, que tem demonstrado ser eficaz e confiável no cálculo de alguns parâmetros cinéticos e termodinâmicos (Perricone et al., 2018).

## 1.4. **Uso de produtos à base de CBD em pacientes pediátricos**

As semanas iniciais do período pós-natal envolvem o crescimento e desenvolvimento rápidos do cérebro, com pico de sinaptogênese, gliogênese e maturação de sistemas de neurotransmissores (Dobbing and Sands, 1979). Assim, alterações nas redes neurais favorecem a emergência de várias desordens neurológicas e psiquiátricas, incluindo comportamento emocional, humor e cognição alterados (Dawson et al., 2014), bem como distúrbios na consolidação da memória e aprendizado (Lee et al., 2004). Nestas fases de desenvolvimento, estudos epidemiológicos revelaram efeitos cognitivos deletérios e/ou falta de significância dos efeitos positivos associados à *Cannabis* e a canabinoides isolados principalmente em adolescentes e em filhos de mulheres usuárias de *Cannabis* (A. et al., 2015; Whiting et al., 2015). Em um estudo com $\Delta^9$-THC, foi observada a perda de capacidade motora e cognitiva e alterações neurodesenvolvimentais em camundongos expostos no período pré-natal; estes efeitos foram associados diretamente ao sistema endocanabinoide e observados mesmo a longo prazo (de Salas-Quiroga et al., 2015).

Atualmente, o CBD é usado clinicamente em associação com o $\Delta^9$-THC em um preparado à base de *Cannabis* (Sativex®) que contém conteúdo equimolar de ambos, para o manejo dos sintomas neuropáticos associados à esclerose múltipla (Fernández, 2016). Alívio da espasticidade e da dor foram relatados para pacientes com esclerose múltipla que fumam *Cannabis*, mas, para esses pacientes, exames de ressonância magnética estrutural sugeriram que uma redução no volume cerebral, podendo estar associado com comprometimentos cognitivos mais acentuados em comparação com pacientes não usuários de *Cannabis* – por exemplo, déficits na memória verbal e espacial, redução da velocidade de processamento de informações (Romero et al., 2015). Da mesma forma, em usuários recreativos, o uso da *Cannabis* resulta em alterações volumétricas, na massa

cinzenta e na substância branca no cérebro, em particular no hipocampo e na amígdala (Weinstein et al., 2017), uma evidência de que a *Cannabis* (consumida em cigarros e possivelmente em extratos) pode ser prejudicial no cérebro adulto. Em contrapartida, em baixas doses e administrado de forma crônica, o $\Delta^9$-THC isolado se mostrou capaz de restaurar parcialmente déficits cognitivos em camundongos idosos, através do aumento da expressão de proteínas sinápticas e aumento da densidade de espinhos sinápticos hipocampais de forma dependente de CB1 (Bilkei-Gorzo et al., 2017). O processo de envelhecimento é acompanhado pela redução dos níveis dos constituintes dos SEC e, neste caso, o $\Delta^9$-THC age de forma a restabelecer estes níveis através de seu agonismo por CB1 (Bilkei-Gorzo, 2012).

Em 2016, a *GW Pharmaceuticals* relatou os primeiros resultados positivos com CBD puro (Epidiolex®) em ensaios clínicos fase III para uso em crises epilépticas resistentes ao tratamento medicamentoso, incluindo as síndromes de Lennox-Gastaut e de Dravet (Devinsky et al., 2017, 2015). Mais recentemente, os mesmos autores publicaram mais resultados de um estudo randomizado, duplo-cego, controlado por placebo, utilizando o CBD puro (Devinsky et al., 2018a; Thiele et al., 2018). Nestes estudos, nos quais a maioria dos pacientes são crianças e adolescentes refratários, apesar de alguns efeitos colaterais e da falta de acompanhamento de longo prazo, o CBD levou à melhora significativa das crises epilépticas e das funções cognitivas dos pacientes. Com base nesses resultados, recentemente, um produto comercial à base de CBD purificado (GW-Epidiolex®) foi aprovado pelo FDA (*Food and Drug Administration*), órgão norte americano que regula alimentos e produtos farmacêuticos, para tratamento de epilepsias refratárias infantis. Esta aprovação foi possível depois de recentes testes clínicos com pacientes pediátricos e jovens adultos que comprovaram uma boa eficácia e segurança do CBD (Devinsky et al., 2018b; Schonhofen et al., 2018; Thiele et al., 2018). Apesar dos resultados positivos

obtidos, ainda não há dados sobre a segurança no longo prazo, bem como possíveis riscos ao desenvolvimento normal.

É importante ressaltar que as epilepsias infantis geralmente se manifestam já durante o primeiro ano de vida, como no caso da síndrome de Dravet (Higurashi et al., 2013). Nesta idade, o SNC está ainda em intensa maturação e apresenta grande plasticidade sináptica, processos que envolvem a ação do sistema endocanabinoide. Portanto, a utilização de canabinoides em pacientes em fases de desenvolvimento neuronal pode representar riscos ainda não conhecidos já que os poucos estudos clínicos realizados até o momento carecem de acompanhamento a médio e longo prazo. De fato, deve haver uma cuidadosa avaliação caso-a-caso sobre o equilíbrio entre riscos e benefícios do uso de CBD, já que nos casos mais graves as crises epilépticas repetitivas durante a infância podem causar grave comprometimento do desenvolvimento, cognitivo e motor. Estas comorbidades são obviamente mais prejudiciais do que os efeitos adversos e possíveis implicações no desenvolvimento neurológico causados pelo CBD. Portanto, o CBD pode ser uma opção terapêutica atraente nesses casos.

Extratos de *Cannabis* não são recomendados em crianças e adolescentes devido ao potencial de efeitos deletérios, principalmente pela presença de $\Delta^9$-THC. O desenvolvimento fetal é largamente afetado pelo consumo recreativo de *Cannabis* materno durante o período pré-natal, enquanto durante a infância há um impacto negativo sobre os resultados cognitivos e comportamentais (Huizink, 2014). A exposição precoce a canabinoides, principalmente $\Delta^9$-THC, pode prejudicar todos os estágios da memória, da codificação à consolidação e recuperação (Ranganathan and D'Souza, 2006). Além disso, o uso de *Cannabis* durante a adolescência aumenta significativamente o risco de desenvolver transtornos psicóticos, como esquizofrenia (Bossong and Niesink, 2010; Bourque et al., 2018). No entanto, estes efeitos estão principalmente associados ao $\Delta^9$-THC

e o CBD já foi associado à neutralização de tais efeitos (Niesink and van Laar, 2013). Ainda assim, em um relato de caso recente, por exemplo, duas crianças apresentaram sintomas típicos de intoxicação por $\Delta^9$-THC (riso inadequado, ataxia, atenção reduzida e vermelhidão ocular) após o uso de um extrato de *Cannabis* enriquecido com CBD. O extrato foi substituído pela mesma dose de CBD purificado, resultando em diminuição dos sintomas de intoxicação e remissão das crises epilépticas (Crippa et al., 2016). Isso indica que o CBD puro seria uma opção terapêutica melhor em vez de extratos de *Cannabis* comuns ou enriquecidos com CBD.

Devido às evidências clínicas de efeitos anticonvulsivantes, bem como vários casos relatados pela mídia, muitos países aprovaram o CBD para o tratamento da epilepsia, particularmente em casos refratários aos tratamentos regularmente empregados. Assim, o CBD já se mostrou promissor no controle das crises epilépticas em epilepsia, o que faz dele um bom candidato para o tratamento de epilepsias resistentes a medicamentos. No entanto, apresar do grande número de estudos clínicos já finalizados e em andamento (clinicaltrials.gov), os efeitos colaterais do CBD com o uso a longo prazo para o controle das crises epilépticas em epilepsia são desconhecidos, especialmente seus efeitos sobre as comorbidades da epilepsia, como deficiências cognitivas e depressão – os estudos clínicos mais recentes relatam melhoras nestes aspectos, mas avaliaram os pacientes no curto ou médio prazo apenas. Neste contexto, estudar os vários efeitos da administração a longo prazo de CBD em modelos animais de epilepsia é criticamente necessário (Upadhya et al., 2018).

Mesmo com questões não respondidas, entretanto, a capacidade do CBD de reduzir a neurodegeneração associada à inflamação e suas propriedades antioxidantes, a falta de atividade psicotomimética e uma ampla gama de efeitos potencialmente benéficos indicam

que essa droga pode ser uma nova abordagem útil para tratar vários distúrbios neuropsiquiátricos. Além disto, novas moléculas derivadas do CBD destinadas a melhorar a eficácia e / ou a potência dos fitocanabinoides naturais foram recentemente desenvolvidas (Breuer et al., 2016; Campos et al., 2017; Haj et al., 2015), cujos efeitos e mecanismos ainda estão sendo avaliados.

Assim, é crescente a necessidade de mais estudos acerca da efetividade e segurança do CBD e de seus derivados sintéticos bem como para entender seu mecanismo de ação, principalmente sobre o SEC durante o desenvolvimento neuronal. Para isso, são necessários mais estudos em modelos *in vivo* e *in vitro* adequados.

## 2. Objetivos

### 2.1. Objetivo geral

Avaliar *in vivo* e *in vitro* os efeitos neuroprotetores/neurotóxicos e anticonvulsivos do CBD e de seus derivados sintéticos durante e após o desenvolvimento neuronal, bem como determinar o papel do sistema endocanabinoide nesses efeitos.

### 2.2. Objetivos específicos

- Realizar uma revisão bibliográfica sobre os efeitos conhecidos do CBD, seus mecanismos de ação e estudos clínicos;
- Avaliar o efeito da administração de CBD no período pós-natal e adolescência em camundongos com maior susceptibilidade a crises epilépticas quimicamente induzidas.
    - Utilizar um modelo para maior susceptibilidade a crises epilépticas quimicamente induzidas em camundongos através de um evento de hipóxia no sétimo dia pós-natal (PND7);
    - Avaliar a susceptibilidade a crises epilépticas quimicamente induzidas após o tratamento com CBD neste modelo animal na infância (PND15) e adolescência (PND44);
    - Avaliar os efeitos do CBD sobre o conteúdo de endocanabinoides em amostras de hipocampo;
- Avaliar os efeitos neuroprotetores/neurotóxicos do CBD e de 3 de seus derivados sintéticos, bem como o papel do receptor CB1 em seus efeitos, em modelo celular de neurônios humanos maduros e durante seu desenvolvimento.

- Avaliar a neurotoxicidade de diferentes concentrações de cada canabinoide, sozinhos ou em co-tratamento com um agonista/antagonista de CB1, em neurônios diferenciados e durante a diferenciação.
- Avaliar a neuroproteção de cada tratamento em neurônios diferenciados e durante a diferenciação, posteriormente desafiados com a neurotoxina 6-OHDA, simulando os mecanismos neurodegenerativos.

# Parte II

## 3. Resultados

Os resultados desta tese estão apresentados na forma de artigos científicos (Capítulo I, II e III).

No capítulo I, temos um artigo de opinião, publicado em 2018 na revista CNS Drugs, no qual revisamos o SEC e seu papel no desenvolvimento cerebral, os estudos *in vivo* e *in vitro* realizados durante fases do desenvolvimento, bem como os resultados de testes clínicos em crianças, ressaltando possíveis riscos da utilização do CBD em indivíduos muito jovens.

No capítulo II, estão apresentados os resultados obtidos durante meu doutorado sanduíche na Alemanha, um estudo *in vivo* sobre efetividade do CBD em animais neonatos em comparação com adolescentes, focando na modulação dos níveis de endocanabinoides como mecanismo de ação. Os dados serão submetidos à revista JAMA Psychiatry sob a forma de *Research Letter.*

Já o capítulo III apresenta os dados *in vitro* produzidos no Brasil avaliando os efeitos neuroprotetores e neurotóxicos do CBD e de 3 de seus derivados sintéticos em neurônios maduros e durante a diferenciação, além de avaliar os efeitos *in vitro* da modulação do SEC. Este artigo será submetido à revista Molecular Neurobiology.

**Capítulo I**

Artigo do tipo *Opinion* publicado no periódico CNS Drugs

Título: Cannabinoid-Based Therapies and Brain Development: Potential Harmful Effect of Early Modulation of the Endocannabinoid System

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CURRENT OPINION

# Cannabinoid-Based Therapies and Brain Development: Potential Harmful Effect of Early Modulation of the Endocannabinoid System

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## Abstract

The endocannabinoid retrograde signaling pathway is widely expressed in the central nervous system, where it plays major roles in regulating synaptic plasticity (excitatory and inhibitory) through long-term potentiation and long-term depression. The endocannabinoid system (ECS) components—cannabinoid receptors, endocannabinoids and synthesis/degradation enzymes—are expressed and are functional from early developmental stages and throughout adolescent cortical development, regulating progenitor cell fate, neural differentiation, migration and survival. This may potentially confer increased vulnerability to adverse outcomes from early cannabinoid exposure. Cannabidiol (CBD) is one of the most studied exogenous cannabinoids, and CBD-enriched *Cannabis* extracts have been widely (and successfully) used as adjuvants to treat children with refractory epilepsy, and there is even a Food and Drug Administration (FDA)-approved drug with purified CBD derived from *Cannabis*. However, there is insufficient information on possible long-term changes in the central nervous system caused by cannabinoid treatments during early childhood. Like the majority of cannabinoids, CBD is able to exert its effects directly and indirectly through the ECS, which can perturb the regulatory processes mediated by this system. In addition, CBD has a large number of non-endocannabinoid targets, which can explain CBD's effects. Here, we review the current knowledge about CBD-based therapies—pure and CBD-enriched *Cannabis* extracts—in studies with pediatric patients, their side effects, and their mechanisms of action regarding the central nervous system and neurodevelopment aspects. Since *Cannabis* extracts contain $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), we consider that pure CBD is possibly safer for young patients. Nevertheless, CBD, as well as other natural and/or synthetic cannabinoids, should be studied in more detail as a therapeutic alternative to CBD-enriched *Cannabis* extracts for young patients.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s40263-018-0550-4) contains supplementary material, which is available to authorized users.

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**Key Points**

Cannabidiol (CBD) targets the endocannabinoid system directly via cannabinoid receptor type 1 ($CB_1$) receptors or indirectly by regulating endocannabinoid levels, in both developing and mature brains.

$\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THC) is believed to be responsible for the majority of the potential harmful effects of CBD-enriched *Cannabis* extracts, although further direct evaluation of the effects of CBD upon brain development is necessary.

For young patients, pure CBD, both synthetic or plant derived, produced in accordance with good manufacturing practices (GMP-grade), is recommended as a therapeutic option instead of CBD-enriched *Cannabis* extracts, and a recently CBD-based product (Epidiolex®) was approved by the Food and Drug Administration (FDA) for the treatment of Dravet and Lennox-Gastaut syndromes.

There is a lack of trials of chronic administration of CBD-based therapies with long-term follow-up periods; conducting such trials would allow a more realistic comparison of the effects of these therapies with those of current treatment options.

## 1 Introduction

The plant *Cannabis sativa* has been used for medicinal purposes for thousands of years by different cultures [1]. *Cannabis* extract contains more than 80 components, of which $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (the main psychoactive ingredient) and cannabidiol (CBD) are the most abundant [2, 3]. These compounds were first identified several decades ago [4], but it is only more recently that the discovery of cannabinoid receptors and their endogenous homologues, the endocannabinoids [5] such as *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) [6], has occurred. Together with their related enzymes, endocannabinoids and their receptors form the endocannabinoid system (ECS) (Fig. 1) [7]. Cannabinoids—both endogenous and plant derived—target the G protein-coupled cannabinoid receptor type 1 ($CB_1$), which is widely expressed in the nervous system, and cannabinoid receptor type 2 ($CB_2$), which is mainly expressed in immune cells [8, 9]. Presently, it is proposed that the ECS has roles in the pathological mechanisms of several psychiatric disorders, including schizophrenia [10]. Besides, cannabinoids such as CBD also interact with a variety of non-endocannabinoid mechanisms, including numerous classical ion channels, receptors, transporters, and enzymes, as reviewed recently [11].

The effects of isolated cannabinoids and *Cannabis* extracts in different diseases have been studied for many years [12]. In the United States, recent medical and recreational marijuana legalization increased *Cannabis* accessibility and use [13]. Additionally, despite widely known deleterious effects during central nervous system development, medical marijuana usage by minors, with the consent from a legal guardian and certification from a physician, is approved [14]. Marijuana-derived products have their main effects against childhood severe epilepsies, including Dravet and Lennox-Gastaut syndromes. These early onset disorders are characterized by frequent, refractory seizures and neurodevelopmental delays, which lead to impaired quality of life in these individuals. This scenario compels families to seek alternative treatment methods, such as CBD-based therapies, which include pure synthetic or plant-derived CBD and CBD-enriched *Cannabis* extracts. In children, plant-derived, pharmaceutical-grade isolated CBD has been tested in clinical trials in patients with such syndromes [15–17], and this drug (Epidiolex®) has recently been approved in the USA as an orphan drug for those syndromes. Clinical trials with synthetic isolated CBD are ongoing (clinicaltrials.gov website). In addition, reports on the use of different forms of *Cannabis* extracts in children with epilepsy have also been published [18–20]. However, only few adequately powered, placebo-controlled, randomized studies have evaluated the safety and efficacy of CBD-based therapies in children [21]. Nevertheless, most of these therapies have been reported to have a greater reduction in convulsive seizure frequency than placebo, being associated, however, with higher rates of adverse events [22].

The constituents of the ECS, receptors and endocannabinoids, are expressed and are functional from very early developmental stages, whereby they regulate inhibitory and excitatory synapses. Even during adolescence, the brain and the ECS undergo active development, which may confer increased vulnerability to adverse long-term outcomes from early cannabinoid exposure [23]. Endocannabinoids have been shown to regulate cortical development throughout life in humans, and exogenous cannabinoids can alter cortical development of both the somatosensory and the prefrontal cortex [24].

Nevertheless, the current widespread use of CBD-based therapies in children and young adults, without sufficient studies on the potential consequences regarding neuronal and other systems' development, is of concern to the scientific and medical communities. One area of particular concern is the uncontrolled amount of $\Delta^9$-THC present in such extracts. Moreover, in 2017, an ad hoc committee of the National Academies of Sciences, Engineering, and Medicine presented a report regarding the health effects of *Cannabis*

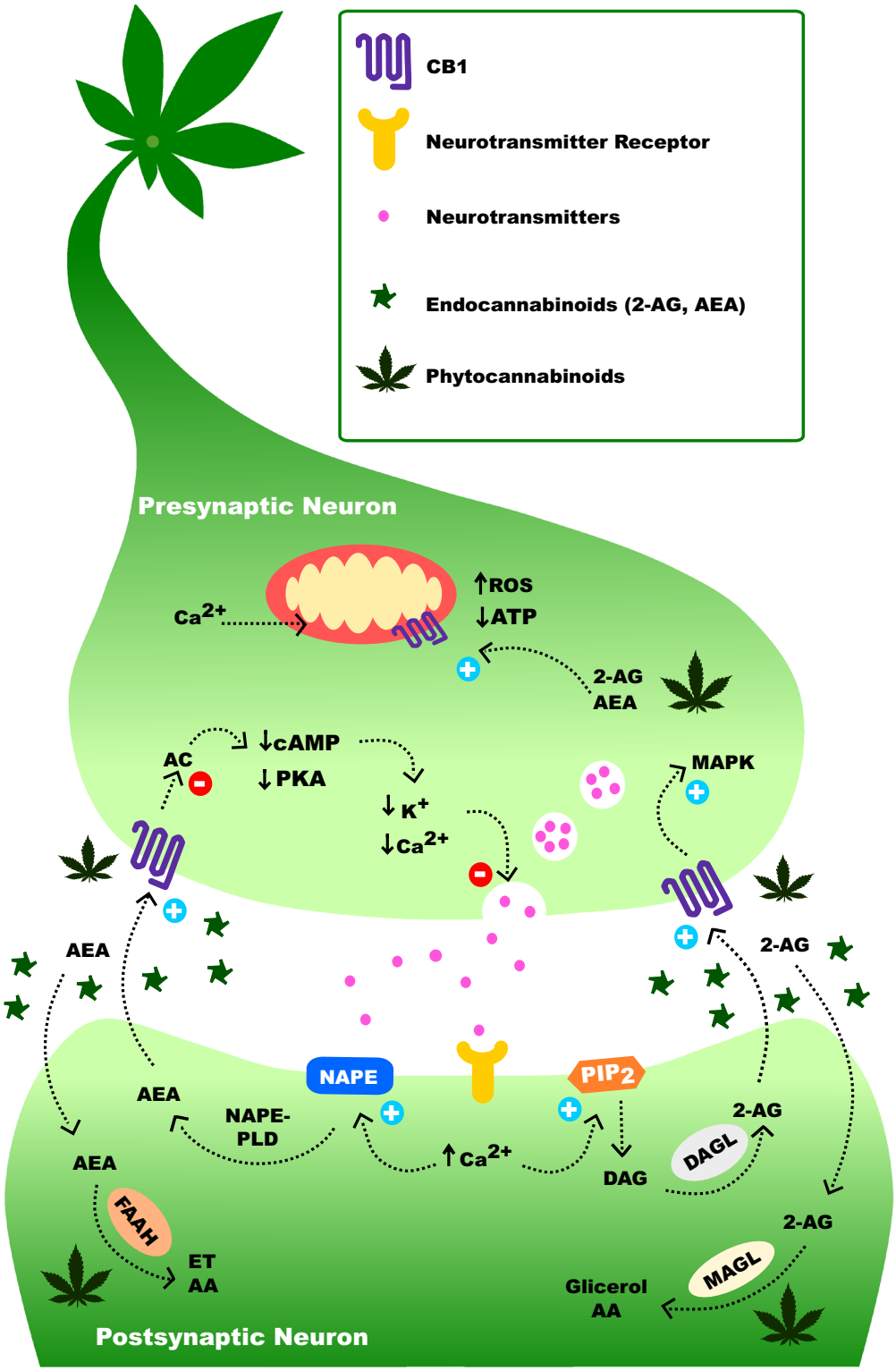


**Fig. 1** Retrograde endocannabinoid signaling. Endocannabinoids are produced on demand in the post-synaptic neuron, released in the synaptic cleft and activate $CB_1$ receptors in the pre-synaptic neuron. *2-AG* 2-arachidonoylglycerol, *AA* arachidonic acid, *AC* adenylyl cyclase, *AEA* anandamide, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *$CB_1$* cannabinoid receptor type 1, *DAG* diacylglycerol, *DAGL* diacylglycerol lipase, *ET* ethanolamine, *FAAH* fatty acid amide hydrolase, *MAGL* monoacylglycerol lipase, *MAPK* mitogen-activated protein kinase, *NAPE* *N*-arachidonoylphosphatidylethanolamine, *NAPE-PLD* *N*-arachidonoylphosphatidylethanolamine phospholipase-D, *$PIP_2$* phosphatidylinositol biphosphate, *PKA* protein kinase A, *ROS* reactive oxygen species

and CBD use, which revealed no or insufficient evidence to either support or refute the use of such compounds as an effective treatment for epilepsy [25]. Hence, this article reviews the current knowledge about the use of CBD-based therapies in pediatric patients, the alleged side effects, and the mechanisms of action regarding the central nervous system and neurodevelopmental aspects. We highlight that CBD administration before adulthood must be carefully evaluated, and the use of pure CBD and/or synthetic cannabinoids as a preferential alternative to *Cannabis* extracts for children and young adults needs to be studied further.

## 2 The Endocannabinoid System

Most cannabinoids exert their therapeutic properties upon the central nervous system primarily via the ECS, although there are other known targets [26]. Here, we discuss their effects upon the ECS. Endocannabinoid signaling plays crucial roles in various aspects of both the underdeveloped and the mature brain [27]. Therefore, disturbances in this system may disrupt neural development.

The classical ECS signaling pathway is shown in Fig. 1 (for review see [10]). In the mature brain, the ECS modulates synapses (excitatory and inhibitory) through the release of endocannabinoids AEA and 2-AG. These act as retrograde messengers, their release by the postsynaptic neuron activating $CB_1$ receptors in the pre-synaptic neuron, leading to decreased release of neurotransmitters into the synaptic cleft [10, 28, 29]. This process is initiated by increased $Ca^{2+}$ influx caused by neurotransmission in the postsynaptic neuron, which activates endocannabinoid synthesis from its precursors in the plasma membrane. AEA is generated from phospholipase D-mediated hydrolysis of the membrane lipid *N*-arachidonoylphosphatidylethanolamine (NAPE), while 2-AG originates from the diacylglycerol lipase-mediated hydrolysis of diacylglycerol (DAG), derived mainly from membrane-localized phosphatidylinositol biphosphate ($PIP_2$). AEA and 2-AG diffuse towards the pre-synaptic terminals and, like exogenous cannabinoids such as $\Delta^9$-THC, bind to and activate the pre-synaptic, G protein-coupled $CB_1$ receptors. This binding triggers the activation and release of Gi/Go proteins from the $CB_1$, inhibiting adenylyl cyclase (AC) and thus decreasing cyclic adenosine monophosphate (cAMP) formation and subsequent protein kinase A (PKA) activity. These events lead to opening of inwardly rectifying $K^+$ channels, causing a hyperpolarization of the pre-synaptic terminal, and closing of $Ca^{2+}$ channels, arresting the release of stored neurotransmitters. Finally, AEA and 2-AG re-enter the post- or pre-synaptic terminals, where they are catabolized respectively by fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL), to yield either arachidonic acid (AA) and ethanolamine (ET) in the case of AEA, or AA and glycerol for 2-AG. The transport of endocannabinoids through the plasma membrane is still not completely understood. Although some studies have proposed the existence of an endocannabinoid transporter, the trafficking of AEA, which has been most extensively studied, is proposed to occur through facilitated membrane transport followed by intracellular shuttling and sequestration [30].

Additionally, $CB_1$ receptor activation leads to stimulation of mitogen-activated protein kinase (MAPK) activity, a mechanism by which cannabinoids affect synaptic plasticity, cell migration, and possibly neuronal growth [23]. In mature neurons, the MAPK cascade, which leads to the activation of extracellular signal-regulated kinases (ERK), is stimulated by excitatory glutamatergic signaling. Subsequently, ERK activity regulates two processes that underlie changes in synaptic transmission—the activity of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and structural plasticity [31]. ECS retrograde signaling mediates synaptic plasticity through three classical mechanisms: depolarization-induced suppression of inhibition or excitation, metabotropic-induced suppression of inhibition or excitation, and endocannabinoid-mediated, short-term depression or long-term depression (STD/LTD) [10]. Also, $CB_1$ agonists can prevent long-term potentiation (LTP) of synaptic transmission, but the influence of endogenously formed cannabinoids on hippocampal LTP remains ambiguous [32]. Both LTP and LTD have roles in learning and neural development [24].

Thus, the central component of the ECS in neurons is the $CB_1$ receptor (Fig. 1). In the central nervous system, $CB_1$ is particularly enriched in the cortex, hippocampus, amygdala, basal ganglia outflow tracts, and cerebellum. This distribution corresponds to the most prominent behavioral effects of *Cannabis* and helps to predict neurological and psychological effects of ECS manipulation [33]. $CB_1$ receptors are also observed in intracellular compartments such as the mitochondrial surface, where they are able to activate G protein-dependent signaling and modify intracellular levels of adenosine triphosphate (ATP), $Ca^{2+}$, and reactive oxygen species, all of which impact upon synaptic transmission [34].

In the developing nervous system and the remaining neurogenic areas in the adult brain (the hippocampal subgranular zone and subventricular zone), the ECS exerts a regulatory role on neural progenitor cell survival, proliferation, differentiation and migration via $CB_1$ [35, 36], thus possibly affecting the formation of adult specialized tissues [37]. Recently, the ECS has also been shown to regulate proliferation and differentiation of mesoderm-derived hematopoietic and mesenchymal stem cells, with a key role in determining the formation of several cell types in peripheral tissues [38].

The importance of the ECS during embryonic development has been investigated through many experimental

models and approaches, mainly focusing upon the deleterious effect of early $\Delta^9$-THC administration. For example, $\Delta^9$-THC administration to pregnant mice interfered with sub-cerebral projection neuron generation, thereby altering corticospinal connectivity, and produced long-lasting alterations in the fine motor performance and seizure susceptibility of the adult offspring. These deleterious consequences were solely attributed to $\Delta^9$-THC's ability to disrupt the neurodevelopmental role of $CB_1$ signaling [39].

During adolescence, the ECS has a role in the development of the cortex, amygdala, hippocampus and hypothalamus, and exogenous cannabinoids have long-term effects on cognition, anxiety and stress-related behaviors, leading to mood disorders and substance abuse [24]. At this age, cannabinoids may produce abnormal LTD in prefrontal cortex by disrupting LTD mediated by metabotropic glutamate receptors and $CB_1$ [40]. The ECS maintain the homeostasis of prefrontal cortex interactions with the amygdala and hippocampus, which are responsible for behaviors such as emotional memory and anxiety-related behaviors. Endocannabinoids are required for the normal stress response, a process which matures during adolescence [24]. Besides, as the prefrontal cortex is the last brain region to finish development after adolescence, the abundance of $CB_1$ receptors may explain the negative effects of *Cannabis* use in this age range [27]. Finally, endocannabinoids are necessary for the normal regulation of neuronal excitation and inhibition; hence, disturbances in this delicate equilibrium likely result in changes in the balance of excitation/inhibition in individual neurons and networks, processes which are necessary for normal cortical development [24].

For therapeutic purposes, regarding the mature central nervous system, the ECS has been shown to modulate anxiety, depression, neurogenesis, reward, cognition, learning, and memory [23]. Moreover, its retrograde signaling acts to regulate seizure activity and neuronal hyper-excitability—cannabinoids have shown $CB_1$ activity in experimental models of seizure and epilepsy [41, 42]. However, the use of $CB_1$ agonists such as $\Delta^9$-THC, or even *Cannabis* extract, as a therapeutic strategy is unfeasible because of their psychoactive effects, abuse potential and development of tolerance [42]. On the other hand, antagonism of $CB_1$ can also exacerbate seizure activity in the epileptic phenotype [43].

Thus, the modulation of the ECS as a therapeutic approach is challenging because its blockage or its exacerbation could lead to undesired outcomes, especially during neuronal development. More studies are required to clarify its physiological functions and to predict the effect of $CB_1$ agonists and antagonists, both in adult and pediatric patients, to support its targeting for therapeutic purposes.

## 3 Therapeutic Uses and Mechanisms of Action of Cannabidiol (CBD)

*Cannabis* causes many psychotropic effects, mainly mediated by $\Delta^9$-THC agonism of $CB_1$ [44], which makes it unlikely to be used *in natura*. On the other hand, experimental studies have demonstrated several therapeutic properties of isolated cannabinoids in a number of in vitro and in vivo models [45]. Here, we discuss the therapeutic uses of the most prominent of these cannabinoids, CBD, and its mechanisms of action, highlighting its activity towards the $CB_1$ receptor.

Although only a limited number of studies have focused upon CBD, recently, it has been shown to be a potent anti-inflammatory and antioxidant agent and to attenuate the memory-impairing effects produced by $\Delta^9$-THC, amongst other effects [23]. This opens a wide range of possible therapeutic uses in neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, and cerebral ischemia [46]. Moreover, CBD is anti-emetic [47], has antitumoral properties against many types of cancer [48], and is also suggested to have antipsychotic, anxiolytic and antidepressant effects [49]. Finally, as already mentioned above, numerous studies have shown CBD to have anticonvulsive properties [50].

CBD has been reported to have a large number of possible molecular targets other than the ECS in a wide range of medical conditions, raising the possibility of significant off-target effects [26]. For instance, CBD is described as a full serotonin 1A ($5\text{-}HT_{1A}$) receptor agonist, a weak partial $5\text{-}HT_{2A}$ agonist and a non-competitive $5\text{-}HT_{3A}$ antagonist [51]. The ability of CBD to activate the $A_{1A}$ adenosine receptor has also been reported [52]. CBD may play a role in the regulation of T-type calcium channels and the activity of nuclear peroxisome proliferator-activated receptor-γ (PPARγ), both of which have been implicated in seizure activity [53]. Other molecular targets have also been studied, among them the PPARγ nuclear receptors [54], glycine receptors [55], $GABA_A$ receptors [56], and transient receptor potential (TRP) channels [57]. Studies focused on the possible epigenetic regulation of skin differentiation genes by CBD revealed that it can act as a transcriptional repressor, controlling cell proliferation and differentiation through DNA methylation [58]. Hence, the molecular mechanistic basis for the effects of CBD appears to be complex and thus remains to be fully elucidated.

Although current evidence suggests that CBD does not directly interact with the ECS except in vitro at supraphysiological concentrations [11], it can also indirectly act as agonist or antagonist of the $CB_1$ receptor. In the nanomolar range [below the reported affinity ($K_i$) for CBD to these receptors], CBD can antagonize the pharmacological effects

of $CB_1$ agonists such as $\Delta^9$-THC and AEA, despite having low direct affinity in the micromolar range for $CB_1$ in vitro [59, 60]. McPartland et al. reviewed in vitro and ex vivo mechanistic studies of CBD and found one study that reported slight agonism and one study that reported slightly inverse agonism comprising binding to the inactive form of the receptor, blocking agonist effects, both of which occurred at high concentrations of CBD (≥ 10 μM) [59]. Surprisingly, in some mechanistic studies, the effects of CBD could be reversed by $CB_1$ receptor inverse agonists, or were absent in $CB_1$ receptor knockout mice [59]. This suggests that CBD may exert indirect agonism, comprising enhancement of the effect of a receptor's agonist without having any direct agonist effect itself, at $CB_1$ receptors—either augmenting $CB_1$ constitutional activity [61] or augmenting endocannabinoid tone through inhibition of AEA hydrolysis, inhibition of the putative AEA transporter and increase of 2-AG levels [59].

Recent evidence supports the hypothesis that CBD also binds to an allosteric site on $CB_1$ receptors that is functionally distinct from the orthosteric site for its agonists. CBD reduced the potency and efficacy of $CB_1$ agonists at concentrations lower than the predicted affinity of CBD for the orthosteric site of $CB_1$ receptors [62]. The presence of this allosteric site is still to be directly demonstrated due to difficulties in the resolution of the crystallographic structure of this receptor [63]. Despite such methodological issues, in vitro pharmacological experiments have demonstrated that, at very low concentrations, CBD is a negative allosteric modulator of $CB_1$ [62].

Therefore, depending on the conditions, CBD seems to be able to interact both directly and indirectly with the $CB_1$ receptor via the regulation of endocannabinoid levels. Thus, since the ECS has a broad spectrum of physiological functions during neural development, it is reasonable to assume that CBD is potentially able to interfere with processes regulated by $CB_1$ when administered in infants. In fact, depending on the dosage and the clinical condition, potential CBD activity over $CB_1$ (agonism or antagonism) results in different outcomes—either therapeutic or harmful [27]; therefore its use must be very carefully considered in such ages. Besides, as CBD has effects on other targets at lower concentrations, the mechanisms underlying its therapeutic properties are not yet clearly understood [42].

## 4 Studies with CBD-Enriched *Cannabis* Extracts and Pure CDB in Pediatric Patients

Currently, CBD is clinically used in association with $\Delta^9$-THC in a *Cannabis*-based preparation (Sativex®) that contains equimolar content of both, for the management of neuropathic symptoms associated with multiple sclerosis [64]. Relieve of spasticity and pain have been reported for multiple sclerosis patients that smoke *Cannabis*, but for these patients, structural magnetic resonance imaging (MRI) scans have suggested reduced brain volume is associated with cognitive impairment [65]. Likewise, in recreational users, *Cannabis* has been shown to result in volumetric gray matter and white matter structural changes in the brain, in particular, in the hippocampus and the amygdala [66], further evidence that *Cannabis* (smoked and possibly in extracts) can be harmful in adult brain.

In 2016, GW Pharmaceuticals reported the first results of pure CBD (Epidiolex®) in phase III clinical trials for use in treatment-resistant seizure disorders, including Lennox-Gastaut and Dravet syndromes [17, 22]. More recently, the same authors have released further results from a randomized, double-blind, placebo-controlled trial using pure CBD [67, 68]. Moreover, CBD-enriched *Cannabis* extract is still widely used as a therapeutic option. In this section, we review the available data on clinical trials, case reports and parental surveys available from January 2000 to May 2018. We focused on literature containing data about isolated CDB administration and relevant oral *Cannabis* extracts with high CBD content in pediatric and young patients, as well as relevant studies in adult volunteers.

The use of common *Cannabis* extracts is not recommended in children and adolescent patients because of the potential for deleterious effects. Fetal development is affected by prenatal maternal *Cannabis* use, while during infancy there is a negative impact upon cognitive and behavioral outcomes [69]. Early exposure to cannabinoids, mainly $\Delta^9$-THC, can impair all stages of memory, from encoding to consolidation and retrieval [70]. Additionally, *Cannabis* usage during adolescence increases the risk of developing psychotic disorders such as schizophrenia later in life [71, 72]. Nevertheless, these effects are mainly associated with $\Delta^9$-THC, and CBD is able to counteract such effects [73]. This indicates that pure CBD would be a better therapeutic option instead of CBD-enriched or common *Cannabis* extracts. Careful consideration and attention should be taken when using CBD-enriched *Cannabis* extracts, in particular, within pediatric contexts. In a recent case report, for example, two children presented typical symptoms of $\Delta^9$-THC intoxication (inappropriate laughter, ataxia, reduced attention, and eye redness) after using a CBD-enriched *Cannabis* extract. The extract was replaced by the same dose of purified CBD, resulting in decreased intoxication symptoms and seizure remission [74].

Table 1 summarizes the main findings in children and young adult patients treated with pure CBD and CBD-enriched *Cannabis* extract. As most studies that established safety and dose tolerance were performed in adults, they were also reviewed (see the electronic supplementary

**Table 1** Clinical trials using pure CBD published until May 2018 with children and young patients

| Author/year | Design | Target/aim | Age (years) | Number of subjects included | Dose and administration route | Duration of treatment | Main results | Adverse/undesired effects |
|---|---|---|---|---|---|---|---|---|
| Thiele et al., 2018 [67] | Randomized, double-blind, placebo-controlled trial | Lennox-Gastaut syndrome | 2–55 | 171 | 20 mg/kg/day orally | 14 weeks | Reduction in seizure frequency of 43.9% in the CBD group and 21.8% in the placebo group | 86% of CBD group: diarrhea, somnolence, pyrexia, decreased appetite, and vomiting; 14% withdrew from the study; 1 patient died in the CBD group, but unrelated to treatment |
| Devinsky et al., 2018 [68] | Double-blind, placebo-controlled trial | Lennox-Gastaut syndrome | 2–55 | 225 | 10 or 20 mg/kg/day orally, in 2 equally divided doses daily | 14 weeks | Reduction in seizure frequency was 41.9% in the 20-mg group, 37.2% in the 10-mg group, and 17.2% in the placebo group | Somnolence, decreased appetite, and diarrhea; 6 patients in the 20-mg CBD group and 1 patient in the 10-mg CBD group withdrew from the study; 14 patients had elevated liver aminotransferase concentrations |
| Devinsky et al., 2018 [16] | Randomized, dose-ranging safety trial | Safety and pharmacokinetics of CBD in Dravet syndrome | 4–10 | 34 | 5, 10, or 20 mg/kg/day orally in 2 equally divided doses daily | 3-week treatment, 10-day taper, and 4-week follow-up periods | Content of CBD and its metabolites increased proportionally with dose; CBD did not affect concomitant antiepileptic drugs | Pyrexia, somnolence, decreased appetite, sedation, vomiting, ataxia, and abnormal behavior; 6 patients taking CBD and valproate developed elevated transaminase levels |
| Devinsky et al., 2017 [22] | Randomized controlled trial | Dravet syndrome | 2–18 | 120 | Titrated up to 20 mg/kg/day twice a day orally | 14 weeks | Reduction in convulsive seizure frequency; patients' overall condition improved 62% | Diarrhea, vomiting, fatigue, pyrexia, somnolence, and abnormal results on liver-function tests; 15% discontinued treatment; no significant reduction in non-convulsive seizures |

**Table 1** (continued)

| Author/year | Design | Target/aim | Age (years) | Number of subjects included | Dose and administration route | Duration of treatment | Main results | Adverse/undesired effects |
|---|---|---|---|---|---|---|---|---|
| Gofshteyn et al., 2017 [101] | Open-label trial | Febrile infection-related epilepsy syndrome | 3–8 | 7 | Titrated up to 25 mg/kg/day orally | Acute and chronic treatment after status epilepticus for 4 weeks and 48 months | Cessation of the status epilepticus, with 100% reduction in all seizures in an acute patient; 90.9% decrease in frequency at 4 weeks and a 65.3% decrease at 48 weeks in chronic patients; reduction of other antiepileptic drugs | Dizziness, decreased appetite and weight loss, and nausea/vomiting; 4 of 7 developed a persistent tremor, but this was believed to be secondary to underlying central nervous system pathology; all living subjects continued to have cognitive impairment |
| Kaplan et al., 2017 [102] | Open-label trial | Sturge-Weber syndrome | 2–19 | 5 | Titrated up to intolerance or to 25 mg/kg/day orally | 48 weeks | Seizure frequency decreased in 4 of 5 subjects; motor and cognitive improvements | Temporary increased seizures in 3 subjects, and behavioral issues in 2 subjects |
| Devinsky et al., 2016 [17] | Open-label trial | Treatment-resistant epilepsy | 1–30 | 214 | Titrated until intolerance or to 25 or 50 mg/kg/day orally | 12 weeks | Motor seizures reduced in 36.5%; 4% were free of all motor seizures at the end of the treatment; 4 weeks after the end of treatment, 11% of patients were free of all motor seizures and 7% were free of all seizures; 37% had a reduction of 50% or more; 22% had a response of 70% or more; 8% had a response of 90% or more | 79% of the safety group: somnolence, decreased appetite, diarrhea, fatigue, convulsion, increased appetite, status epilepticus, lethargy, weight increased, weight decreased, drug concentration increased; 11 patients withdrew the study; 1 death not related to CBD occurred |

**Table 1** (continued)

| Author/year | Design | Target/aim | Age (years) | Number of subjects included | Dose and administration route | Duration of treatment | Main results | Adverse/undesired effects |
|---|---|---|---|---|---|---|---|---|
| Geffrey et al., 2015 [78] | Clinical trial | Drug interactions in pediatric epilepsy | 4–19 | 13 | 5–25 mg/kg/day orally with clobazam | 36 weeks | 70% had a 50% or more decrease in seizures; enhanced blood levels of clobazam; reduction of clobazam doses for 77% of subjects | Side effects were reported in 77% of subjects: drowsiness, ataxia, irritability, restless sleep, urinary retention, tremor, and loss of appetite |

Clinical trials performed until May 2018 with pure, synthetic or plant-derived CBD, presenting dosages and duration of treatments, main results and adverse effects. References and their corresponding results are presented according to date of publication in descending order

*CBD* cannabidiol

material, Supplementary Table 1). The majority of published articles focused on neurological and neuropsychiatric conditions. In adult volunteers, CBD presented few adverse events and appeared to be safe, although its effectiveness was not always confirmed. In most of these studies, CBD was administered in a single dose. A recent article on the safety and tolerability of pure CBD in 34 children between 4 and 10 years old with Dravet syndrome showed that CBD did not alter plasma antileptic drug levels, when randomized into different dosages or placebo for 3 weeks of treatment followed by a 4-week follow-up period [16]. The main adverse effects were pyrexia, somnolence, decreased appetite, sedation, vomiting, ataxia, and abnormal behavior. As observed in the abovementioned studies and reviewed by Wong and Wilens (2017) [13], the methodological quality of those clinical studies varied significantly (e.g., studies lacking control groups; limited by small sample size). Studies are also heterogeneous in the dosage and duration of treatment, and many lacked any long-term follow-up reviews to identify potential adverse effects [13]. This variability in employed protocols makes it difficult to evaluate the real benefits and risks of CBD-based therapies.

Until a few years ago, the suggested beneficial outcomes of CBD-based therapies for pediatric patients were based mainly on case reports and surveys of parents with epileptic children (see the electronic supplementary material, Supplementary Table 2). Such anecdotal studies were the first to report improvement in the general condition of children with refractory epilepsies with *Cannabis* extracts, and so they attracted the interest of the scientific community for cannabinoid-based treatments. Many surveys of parents of children with refractory seizures who self-administered CBD-enriched *Cannabis* extracts have been published in the last few decades. One such survey, involving a small cohort of patients, showed that 42% of children had a greater than 80% reduction in seizure frequency [75]. Another survey, using a larger cohort of 75 pediatric patients, reported that 38% of children achieved a greater than 50% reduction in seizures [20]. An online survey of 117 parents of children with epilepsy reported that 85% of children had a reduction in seizure frequency, whilst 14% reported complete freedom from seizures after CBD-enriched *Cannabis* treatment [19]. These surveys, even though not controlled, reported general improvements in cognitive and motor function in patients undergoing CBD-based therapies, along with some mild side effects.

On the other hand, not all studies have reported favorable results (e.g., CBD-enriched *Cannabis* extract resulted in no improvement in the general condition or seizure relief of an 18-year-old male with severe refractory epilepsy) [76]. Moreover, case reports and parent surveys rarely describe side effects or even drug administration issues. For this reason, clinical trials are indispensable for investigating both

the therapeutic and toxicological aspects of CBD-based therapies, as well as standardizing drug administration protocols to allow direct study comparisons.

However, as anecdotal studies have stimulated a growing interest in the anticonvulsive properties of CBD, pure CBD or CBD-enriched *Cannabis* extracts are now being tested in controlled clinical trials, with relevant positive outcomes thus far reported (Table 1). Such studies are still somewhat limited in number; however, a brief survey on clinicaltrials.gov website reported at least 20 clinical trials that are currently recruiting young patients or already in progress [77]. An open-label clinical trial of 214 patients (aged 1–30 years) with severe, intractable, childhood-onset, treatment-resistant epilepsy investigated the efficacy and safety of pure CBD. Patients in the efficacy analysis group reported a median reduction in monthly motor seizures of 36.5% compared to the placebo group. Adverse events were reported in 79% of the safety analysis group, and serious adverse events were reported in 30% of patients, including one death—a sudden, unexpected death due to the patient's epilepsy which was determined as unrelated to CBD. Twelve percent of patients had severe adverse events possibly related to CBD use, the most common of which was status epilepticus (6%). Three percent of patients discontinued treatment because of an adverse event [17].

A randomized, placebo-controlled, clinical trial of pure CBD reported a significant reduction in total seizures of all types. Although there was no significant reduction in non-convulsive seizures, the trial did demonstrate a greater reduction in convulsive seizure frequency, with 62% of patients reporting an improvement in overall condition, with 5% of patients becoming seizure-free. Adverse events included diarrhea, vomiting, fatigue, pyrexia, somnolence, and abnormal liver function tests [22]. This report, however, did not evaluate possible drug–drug interactions between CBD and clobazam, of which 65% of patients enrolled on the study were prescribed. CBD can increase plasma clobazam concentrations [78]; hence, the beneficial effects of CBD may have arisen indirectly due to the increased pharmacological effects of clobazam and not as a direct pharmacological effect of CBD itself.

In 2018, a randomized, double-blind, placebo-controlled trial encompassing 24 clinical sites in the USA, the Netherlands, and Poland was published. In this study, pure CBD (20 mg/kg/day) or placebo was administered to patients with treatment-resistant Lennox-Gastaut syndrome (aged 2–55 years) for 14 weeks. Of the 171 randomly assigned patients who received CBD ($n=86$) or placebo ($n=85$), 14 patients in the CBD group and one in the placebo group discontinued study treatment. The monthly drop in seizure frequency was reduced by 43.9% in the CBD group and 21.8% in the placebo group. Adverse events, which were mostly mild or moderate, occurred in 86% of patients in the CBD group and in 69% of patients in the placebo group [67].

Another recent double-blind, placebo-controlled trial, in which 225 patients with the Lennox-Gastaut syndrome (age range of 2–55 years) were randomly assigned to receive CBD at 10, 20 mg/kg/day, or placebo administered in two equally divided doses daily for 14 weeks, showed significant decreases in seizure frequency [68]. Seizure frequency decreased by 41.9% in the 20-mg CBD group, 37.2% in the 10-mg CBD group, and 17.2% in the placebo group. Six patients in the 20-mg CBD group and one patient in the 10-mg CBD group were withdrawn from the trial because of adverse events. Fourteen patients who received CBD (9%) had elevated plasma liver aminotransferase levels. The most common adverse events among the patients in the CBD groups were somnolence, decreased appetite, and diarrhea; these events occurred more frequently in the higher-dose group. Yet, even in these two recent clinical trials, although they are scientifically relevant and reliable, a longer treatment and follow-up period was missing.

In general, in pediatric patient clinical trials, the most common side effects reported were either mild (somnolence, fatigue, altered appetite, weight gain/loss, diarrhea and other gastrointestinal disturbances, irritability) or serious (drowsiness/dizziness, ataxia, tremor, mental sedation), with severe adverse effects such as increased seizure frequency and worsening seizure phenotype also being observed. Alimentary effects can be explained by the presence of the ECS in the gastrointestinal tract, where it has effects on motility, inflammation and immunity, intestinal and gastric acid secretion, nociception and emesis pathways, and appetite control [79]. In the brain, ECS modulates several brain functions, such as memory, mood, food intake, pain perception and the sleep–wake cycle [80], which may explain, at least partially, the central nervous system-mediated adverse effects observed in clinical trials. Besides, as discussed above, other cannabinoids present in *Cannabis* extracts as well as CBD are able to interact and possibly disturb the important roles played by the ECS during neurodevelopmental stages.

It is likely that non-endocannabinoid targets of CBD may explain some of the positive and adverse effects observed [11]. For example, in a mouse model of Dravet syndrome, the beneficial effects of CBD on inhibitory neurotransmission were mimicked and blocked by an antagonist of the orphan G protein-coupled receptor 55 (GPR55), suggesting that the therapeutic effects of CBD are mediated through this lipid-activated, G protein-coupled receptor and thus identify it as a third cannabinoid receptor [81].

A careful case-to-case evaluation on the risk/benefit balance of CBD usage must be taken, as in the most serious cases, repetitive infantile seizures can cause severe developmental, cognitive and motor impairment. These are obviously more detrimental than the adverse effects and possible

neurodevelopmental implications of CBD; hence, CBD may be an attractive therapeutic option in these cases.

Finally, CBD therapy does not always work for all patients. Also, some of the studies used CBD-enriched *Cannabis* extracts, which contain $\Delta^9$-THC. Even controlled clinical trials investigating pure CBD used mostly short treatment periods and short follow-up periods, which will not reveal the possible long-term effects of CBD and possible developmental adverse effects. Hence, more clinical trials, with larger population sizes and longer chronic pure CBD administration, are warranted in order to clarify under which conditions it is worthwhile and safe to use. In addition, it is still unknown how CBD acts on hormones, hepatic enzymes, and drug transporters, along with its interactions with other drugs [12].

## 5 CBD During Development: Effects in Cell Culture and Animal Models of the Developing Brain

Despite the increasing use of CBD-based therapies in children and adolescents whose brains are still developing, most in vitro and in vivo studies use mature cells or adult animal models and are thus not faithful mimics of the juvenile central nervous system. Experiments with immature animals or cells have greater potential for identifying CBD's effects and the molecular mechanisms by which such effects are mediated with greater relevance to juveniles. However, few studies have evaluated the developmental phases which are equivalent to human central nervous system development. Here, we present some of the recent studies using pure CBD in relevant cellular and animal models of the developing brain.

In a genetic mouse model of Dravet syndrome, caused by loss-of-function mutations in the voltage-gated sodium channel NaV1.1, CBD treatment from postnatal day 21 to 27 decreased the duration and severity of thermally induced seizures and the frequency of spontaneous seizures. Lower doses of CBD also improved autistic-like social interaction deficits [81]. This mouse model represents a very specific cause of children refractory epilepsy, a single mutation in a sodium channel subunit, and its positive outcomes must be considered carefully when extrapolated to other pathologies.

Single-dose administration of CBD to newborn piglets shortly after hypoxia ischemia had a protective effect upon neurons and astrocytes, preserved brain activity, prevented seizures and improved neurobehavioral performance [82, 83]. In newborn rat brains, CBD administration also prevented necrotic and apoptotic cell death in an in vivo model of hypoxia ischemia damage [84], and rescued neuron function after sciatic nerve transection [85]. However, both studies used a single dose of CBD at a very specific moment, namely immediately after an intensive brain injury, to evaluate its acute effects. Thus, these results may not be representative of long-term treatments with CBD.

Although recent literature has primarily searched for potential protective and therapeutic effects of CBD, a recent research paper has reported negative effects. Zebrafish, exposed from blastula through to larval stage to micromolar concentrations of $\Delta^9$-THC (1–16 μM) or CBD (0.25–4 μM), presented similarity in dysmorphologies to both compounds (i.e., edemas, curved axis, eye/snout/jaw/trunk/fin deformities, swim bladder distention, and behavioral abnormalities), whilst the $LC_{50}$ (lethal concentration 50—concentration to kill 50% of the population) for CBD was nearly seven times lower than that for $\Delta^9$-THC. The authors also reported teratogenic effects of low concentrations of CBD [86]. In contrast, other research found no malformation in development of zebrafish embryos exposed to CBD 20–300 μg/L, although the maximal dosage caused delay in embryo hatching. Besides, they were temporarily more active than control. The authors discussed that the effects observed are intimately related to the $CB_1$ receptor [87]. Again, the chosen doses may be responsible for the difference in results observed in these two studies. Additionally, 10 μM of $\Delta^9$-THC, but not 10 μM of CBD, arrested the development of pre-implantation mouse embryos [88].

Notwithstanding that very few studies offer insight into CBD toxicity, some deleterious effects have been reported for CBD in vitro and in vivo. These include alterations in cell viability, reduced fertilization capacity, and inhibition of hepatic drug metabolism and drug transporters [89]. Our research group showed in a study using an in vitro model of human neurons (human neuroblastoma SH-SY5Y cells differentiated with retinoid acid) that a sublethal dose of CBD with antioxidant activity did not exhibit neuroprotection against the neurotoxic effect of glycolaldehyde, methylglyoxal, 6-hydroxydopamine, and hydrogen peroxide in terminally differentiated neurons. When SH-SY5Y cells undergoing neuronal differentiation were exposed to the same dose of CBD, besides the lack of neuroprotection and antioxidant activity, CBD potentiated the neurotoxicity induced by all redox-active drugs tested [90]. These results suggest a possible hidden negative effect of CBD during neuronal development, reinforcing the observation that effective dosages for CBD and the resulting pathologies observed can vary widely according to the experimental model used.

Thus, pure CBD presents both positive and deleterious effects in animal and cellular models of early stages of development. We recommend that the therapeutic use of CBD and other cannabinoids during brain developmental stages must be always supported by experimental studies in appropriate cellular and animal models, with special attention to the therapeutic window of CBD. It is particularly important

to consider that the effect of CBD in humans follows an inverted U-shaped dose–effect curve pattern of effectiveness as observed in many animal studies [91, 92].

## 6 Therapeutic Perspectives

Although a number of physiological effects of CBD in the brain have been identified, the mechanism(s) underlying its therapeutic properties in neurological diseases and during neurodevelopment are not yet clearly understood. Depending on the experimental model, the dosage used and the protocol, CBD can act upon $CB_1$ as an agonist, as an antagonist of endogenous ligands, or as an allosteric modulator, as well as acting upon non-endocannabinoid targets. Nevertheless, $\Delta^9$-THC, which is able to interact with the ECS, is present in CBD-enriched *Cannabis* extracts used in some studies. Since the ECS performs primordial functions during embryonic development and neurodevelopment, in addition to neurogenesis in adults, it makes sense to hypothesize that any molecule that disturbs ECS activity, such as $\Delta^9$-THC (and potentially CBD), might disrupt the processes regulated by this cellular signaling system.

Regarding CBD therapeutic use for the treatment of children, there are several positive results in clinical trials and case reports in children with refractory epilepsy. However, for CBD-enriched *Cannabis* extracts the controversial effects of $\Delta^9$-THC points to a possible risk of adverse effects for its use in young patients. *Cannabis* has been associated with development of psychotic symptoms later in life, and a recent publication was able to establish a causal role of *Cannabis* use during adolescence and the emergence of such symptoms in the subsequent year [72]. Such effects are attributed to $\Delta^9$-THC activity on $CB_1$. As CBD has low affinity for $CB_1$, although it interferes in other steps of ECS signaling, this cannabinoid may be preferable and safer. Thus, formulations containing $\Delta^9$-THC should be avoided. Moreover, adverse effects of CBD and its extracts—even though they are mainly not severe—as well as absence of therapeutic effects were also reported. Seizure reduction has a significant effect on the patient's quality of life, but the need to take into account other changes that CBD could cause in social behavior, cognitive function, or motor skills is also important. Another concern is that the use of CBD-based therapies for pediatric epilepsy and anxiety (see Table 1 and Supplementary Table 2), together with the common belief that natural products are always harmless, could represent a precedent for its use to treat other neurological diseases. It is not completely clear how CBD affects children's brain development and how it could represent a risk of developing diseases later in adulthood. Thus, despite evidence for potential benefits in pediatric patients, pediatricians and families must balance the decision to use CBD with the associated risks [13]. An evaluation must occur on a case-to-case basis, with, at each instance, consideration of the damage to the patient that may arise from uncontrolled epileptic seizures, the adverse effects of the established antiepileptic drugs and the uncertainties in the effects of CBD during brain development.

Recently, natural and synthetic derivatives of CBD have attracted the attention of both industry and academia. Indeed, some of these molecules are being studied for a variety of purposes, most of them aiming to improve the potency, efficacy, or pharmacokinetic properties of CBD [93]. For instance, a natural CBD derivative, cannabidiolic acid (CBDA), does not have an effect on inhibition of AEA uptake, while keeping the low $CB_1$ affinity [93]. Thus, CBDA probably does not interfere in ECS signaling, which lowers the risk for adverse effects during brain development. The conversion of oral CBD into $\Delta^9$-THC in an acidic environment (e.g., the stomach) is another concern, although it has not been observed in vivo thus far [94]. A novel CBD derivative, HU-444, is a potential novel drug which cannot be converted by acid cyclization into a $\Delta^9$-THC–like compound. In vitro, HU-444 has an anti-inflammatory activity, leading to the suppression of tumor necrosis factor-α production and amelioration of liver damage, whilst not causing $\Delta^9$-THC–like effects in mice [95]. Another synthetic cannabinoid, HU-320, produced strong anti-inflammatory and immunosuppressive effects in an in vivo model of collagen-induced arthritis [95].

For the generation of another class of CBD derivatives, the introduction of the dimethylheptyl (DMH) alkyl chain in the (−)-DMH-CBD series did not alter the lack of $CB_1$ and $CB_2$ receptor affinity [96]. (−)-DMH-CBD analogs have displayed anxiolytic, analgesic, anti-inflammatory, and antiproliferative effects in diverse assays [93]. (−)-DMH-CBD has been shown to have anti-inflammatory and antiproliferative properties in human acute myeloid leukemia [97]. Interestingly, (−)-7-OH-DMH-CBD exhibited potent inhibition of electrically evoked contractions of the mouse vas deferens that was not mediated through $CB_1$, $CB_2$, TRPV1, opioid, or α2-adrenergic receptors [98, 99].

Measurements of the binding affinities for the $CB_1$ and $CB_2$ cannabinoid receptors yielded unexpected outcomes of some CBD enantiomers. Contrary to naturally occurring (−)-CBD analogs, some synthetic derivatives, such as (+)-CBD, H2-CBD, H4-CBD, and HU-465, bind to $CB_1$, and several of them have shown interesting pharmacological properties for various pathologies [93]. However, as $CB_1$ activity is not desirable for an antiepileptic drug, because of all the ECS roles at developmental stages, such derivatives might not be an alternative in these cases. Thus, different CBD derivatives vary in their pharmacological and therapeutic properties, as well as naturally occurring

cannabinoids, evidencing the need for a better understanding of their mechanism of action.

## 7 Conclusion

As *Cannabis* extracts contain $\Delta^9$-THC, which has psychoactive effects and is a $CB_1$ agonist and may potentially disturb the ECS processes during brain development, pure GMP-grade CBD, synthetic or plant derived, is probably a safer option for use in pediatric and juvenile patients. Recently, a CBD oral solution purified from a *Cannabis* extract and developed and tested by GW Research has been approved by the Food and Drug Administration (FDA) of the United States as an adjuvant in the treatment of seizures associated with Lennox-Gastaut syndrome and Dravet syndrome in patients 2 years of age and older. According to the released document, the approval was based on CBD's effectiveness in preclinical and clinical trials and due to its mechanisms of action (low $CB_1$ affinity, reduction of neuronal hyperexcitability and inflammation) [100].

However, since CBD can potentially affect the ECS also, further studies are recommended in order to clarify its mechanisms of action and developmental implications. Besides, longer chronic treatment and follow-up periods are recommended in clinical trials and animal studies in order to evaluate CBD's long-term effects, as well as the most effective dosage and the age which the therapeutic use of pure CBD is not only effective but safe.

At the moment, we consider that CBD is recommended as the last option for the treatment of non-responsive epileptic children. For other neurological or psychiatric diseases, such as childhood anxiety, there is insufficient evidence to support the effectiveness of CBD. Besides, we suggest that more studies should use adequate experimental models to focus on pure CBD, in order to establish its safe and effective dosage and therapeutic targets, as well as synthetic CBD derivatives, aiming to identify a CBD analog with therapeutic properties, but with fewer risks to the developing brain.

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### Compliance with Ethical Standards

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**Conflict of interest** AWZ, JECH and JAC are co-inventors of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to *Phytecs Pharm* (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with *Prati-Donaduzzi* (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders." JECH and JAC have received travel support from and are medical advisors of BSPG-Pharm. AWZ is medical advisor of BSPG-Pharm. PS, FK, IJB, RBP declare no conflics of interest.

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Title: Cannabinoid-based Therapies and Brain Development: Potential Harmful Effect of Early Modulation of the Endocannabinoid System

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**Supplementary table 1.** Adult volunteer studies on adverse effects of pure CBD and relevant CBD-enriched extract (CBD-based therapies).

| Author / year | Design | Target / aim | Age (years) | Number of subjects included | Dose and administration route | Duration of treatment | Main results | Adverse / undesired effects |
|---|---|---|---|---|---|---|---|---|
| Hindocha et al., 2015 [1] | Randomized, double-blind, placebo controlled study | Emotional affect recognition | 19-23 | 48 *Cannabis* users volunteers | $\Delta^9$-THC (8mg), CBD (16mg), $\Delta^9$-THC+CBD (8mg+16mg) and placebo, by inhalation. | 4 testing sessions separated by a one week. Evaluations occurred 2 min after drug administration and every 30 min. | CBD improved emotional facial affect recognition at 60%; $\Delta^9$-THC was detrimental to the recognition of ambiguous faces of 40% intensity; $\Delta^9$-THC+CBD produced no impairment. | Not mentioned. |
| Morgan, Das, Joye, Curran, & Kamboj, 2013 [2] | Double-blind placebo controlled study | Cigarette consumption | 18-35 | 24 smoker volunteers | CBD (400 µg) or placebo by inhalation, whenever subjects felt like smoking. | 1 week | 40% reduction in number of cigarettes smoked; maintenance of this effect during follow-up. | CBD did not produce changes in self-rated anxiety or increase sedation and depression. |

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| Das et al., 2013 [3] | Double-blind, placebo-controlled trial | Consolidation of fear extinction | 18-35 | 48 healthy volunteers | CBD (32 mg) or placebo orally. | Single dose. | CBD administered post-extinction enhanced consolidation of extinction learning; CBD administered at either time produced trend level reduction in reinstatement of autonomic contextual responding. | No adverse effects were mentioned.<br>No acute effects of CBD were found on extinction. |
| Englund et al., 2013 [4] | Double-blind, randomized placebo-controlled trial. | $\Delta^9$-THC paranoid effects and memory impairment. | 21-50 | 48 healthy volunteers | CBD (600 mg) or placebo orally after intravenous $\Delta^9$-THC (1.5 mg). | Single dose | Positive psychotic symptoms and post $\Delta^9$-THC paranoia were less likely; Episodic memory was improved. | Not mentioned. |
| Martin-Santos et al., 2012 [5] | Randomized, double-blind, cross-over, placebo controlled trial | Acute effects of $\Delta^9$-THC and CBD in healthy people. | 20-42 | 16 healthy volunteers | 10 mg $\Delta^9$-THC, 600 mg CBD or placebo orally. | 3 hours observation | CBD was less associated with anxiety, dysphoria, positive psychotic symptoms, physical and mental sedation, subjective intoxication and increase in heart rate compared to $\Delta^9$-THC. | There were no differences between CBD and placebo on any symptomatic, physiological variable. |
| Winton-Brown et al., 2011 [6] | Balanced double-blinded pseudo-randomized crossover study. | Modulation of auditory and visual processing during fMRI scanning | 20-42 | 14 healthy volunteers | 10 mg $\Delta^9$-THC, 600 mg CBD, or placebo orally. | Each participant was scanned three times (placebo, $\Delta^9$-THC, CBD), with a 1-month interval between sessions. | CBD was associated with activation in right temporal cortex during auditory processing; $\Delta^9$-THC and CBD had opposite effects in the right posterior superior temporal gyrus. | CBD caused no significant changes in the evaluated ratings for side effects. |
| Crippa et al., 2004 [7] | Double-blind placebo-controlled, cross-over design | Anxiety, Regional cereb ral blood flow | 25-42 | 10 healthy male volunteers | CBD (400 mg) or placebo orally. | Each subject was studied on two occasions, 1 week apart. | Decreased subjective anxiety; Enhanced regional blood flow in the medial temporal cortex; Decreased regional blood flow: in portions of the amygdala, cingulate gyrus, paracentral lobule, cerebellum, cortex (occipital, temporal, posterior lateral frontal). | Increased mental sedation. |

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| Hallak et al., 2011 [8] | Double-blind placebo-controlled | Attenuation of behavioral effects of ketamine | 20-36 | 10 male healthy volunteers | CBD (600 mg) or placebo orally. | Two sessions when subjects received ketamine preceded by either CBD (600 mg) or placebo. | CBD significantly augmented the psychomotor activation produced by ketamine. | CBD showed a trend to reduce ketamine-induced depersonalization. |
| Bhattacharyya et al., 2010 [9] | Double-blind placebo-controlled | Acute psychotic symptoms induced by $\Delta^9$-THC. | 20-42 | 15 healthy men with minimal earlier exposure to *Cannabis* | $\Delta^9$-THC (10mg), CBD (600mg) or placebo orally. | Three fMRI scans, each preceded by $\Delta^9$-THC, CBD, or placebo. | Some of the effects of CBD on brain function and psychiatric symptoms are in the opposite direction to those of $\Delta^9$-THC. | Not mentioned. |

Relevant trials published from 2010 to May 2018 with pure CBD and relevant CBD-enriched *Cannabis* extract (CBD-based therapies) with healthy adult volunteers, presenting dosages and duration of treatments, main results and adverse effects. References and their corresponding results are presented according to date of publication in descending order. CBD (Cannabidiol); Tetrahydrocannabinol ($\Delta^9$-THC); Functional Magnetic Ressonance Imaging (fMRI).

**Supplementary table 2.** Pure CBD and relevant CBD-enriched extract (CBD-based therapies) case reports, parent surveys and retrospective chart reviews published until May 2018 with children and young adults.

| **Author / year** | **Design** | **Target / aim** | **Age** | **Number of subjects included** | **Dose and administration** route | **Duration of treatment** | **Main results** | **Adverse / undesired effects** |
|---|---|---|---|---|---|---|---|---|
| Treat, Chapman, Colborn, & Knupp, 2017 [10] | Retrospective chart review | Epilepsy | 1 month to 18 years | 119 patients | *Cannabis* extract orally. | 0.3 to 57 months | Seizures improved in 49% of patients; 24% report 50% reduction in seizures. | Adverse events were reported in 19% of patients: worsening seizures, somnolence, and gastrointestinal symptoms; 71% withdrew the study. |
| Rosemergy, Adler, & Psirides, 2016 [11] | Case report | New-onset refractory status epilepticus syndrome | 18-year-old male | 01 patient | *Cannabis* extract (containing 18% CBD) up titrated to a maximum of 24 mg/kg/day orally. | 2 weeks | CBD was not effective. | EEG confirmed unchanged, sustained, bilateral electrographic seizures; Clinical focal and generalized seizures continued occurring; Patient died (expected outcome for the disease). |
| Crippa et al, 2016 [12] | Case report | refractory epilepsy | 7 and 10 years | 02 patients | 208 mg/day CBD divided in three doses of 70 mg or | at least 1 year | Complete seizure remission; Progressive improvement of the remaining general | No side-effects were reported. |

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| | | | | | 300 mg/day CBD divided in two doses of 150 mg orally. | | symptoms; Improvement of previous $\Delta^9$-THC intoxication. | |
| Carlos G. Aguirre-Velázquez, 2016 [13] | Parent survey | Refractory epilepsy | 9 months 18 years | 43 patients | 76,6 % of patients used a CBD-based with 0.1% $\Delta^9$-THC; 11.5% used a combination of CBD + $\Delta^9$-THC; 11.6% used different home-made *Cannabis* extract products orally. | 1 to 12 months | 81.3% reduced frequency, duration and intensity of seizures; 16% were free from seizure; Reduced number of antiepileptic drugs in 20.9%; Improvements in emotional state, attention, communication, sleep patterns, and diet. | Adverse effects were reported in 42% of cases, such as increased appetite or changes in sleep patterns; No change 11.6%; 2 cases (4.6%) showed an exacerbation of seizures. |
| Shannon & Opila-Lehman, 2016 [14] | Case report | Anxiety related to posttraumatic stress disorder | 10-year-old girl | 01 patient | CBD oil (25 mg/day) in a sublingual spray as needed for anxiety. | 5 months (CBD was not withdrawn after the follow-up period) | A gradual increase in sleep quality and quantity; Decrease in anxiety. | No side effects observed. |
| Tzadok et al., 2016 [15] | Retrospective chart review | Treatment-refractory epilepsy | 1 to 18 years | 74 patients | CBD-enriched *Cannabis* extract with 1–20 mg/kg/day of CBD orally, titrated by seizure response and side effects | 3 to12 months, with median 10 months follow-up | 89% reported reduction in seizure frequency: 18% reported 75 to 100% reduction; 34% reported 50 to 75% reduction; 12% reported 25 to 50% reduction; 26% reported less than 25% reduction; Improvement in behavior and alertness, language, communication, motor skills, and sleep. | Adverse reactions included somnolence, fatigue, gastrointestinal disturbances and irritability; 7% reported aggravation of seizures which led to CBD withdrawal. |
| Press, Knupp, & | Parental retrospective chart | pediatric treatment- | 30 days to 18 years | 75 patients | Any oral *Cannabis* | Not informed (1 to 24 months follow up) | 57% had improvement in seizures; 33% had more than 50% reduction in | Side effects were observed in 44% of all patients: worsening |

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| Chapman, 2015 [16] | | resistant epilepsy | | | extracts as a daily medication (mostly high CBD extract) orally. | | seizures; Two patients (0.3%) were free from seizure; Improved behavior/alertness, Language, and motor skills. | of seizures, somnolence, and gastrointestinal symptoms. |
| Saade & Joshi, 2015 [17] | Case report | Malignant migrating partial seizures in infancy | 10-month-old boy | 01 patient | CBD titrated from 10 to 25 mg/kg/day twice daily orally | 6 months | Developmental gains; Seizure frequency decreased. | Not observed. |
| Hussain et al., 2015 [18] | Parent survey | Treatment-refractory epilepsy | 3 to 10 years old | 117 patients | CBD-enriched *Cannabis* extract with at least 15:1 CBD:$\Delta^9$-THC ratio orally; Median reported CBD dose of 4.3 mg/kg/day. | Median 6.8 months | Reduced seizures in 85% of patients. | Increased appetite (30%); Weight gain (29%). |
| Maa & Figi, 2014 [19] | Case report | Dravet syndrome | 5 years old girl | 01 patient | CBD-enriched *Cannabis* extract *Aproximatly 4 mg CBD/lb/day orally. | Continuous | 90% reduction in generalized tonic–clonic seizures; Weaned from other antiepileptic drugs; Improved autistic behaviors. | Reduction of dose caused recurrence of seizures. |
| Porter & Jacobson, 2013 [20] | Parent survey | pediatric treatment-resistant epilepsy | 2 to 16 years | 19 patients | CBD-enriched *Cannabis* extract. CBD dosages ranged from 0.5 to 28.6 mg/kg/day orally. | 2 weeks to over one year | 84% presented reduction in seizure frequency; Two became seizure-free; 8 had more than 80% reduction in seizure frequency; 12 were weaned from other drugs; Improvements in mood, alertness, sleep. | Drowsiness, fatigue and decreased appetite; 3 parents reported no change. |

Case reports, parent surveys and retrospective chart reviews performed until May 2018 with pure CBD and relevant CBD-enriched *Cannabis* extract (CBD-based therapies), presenting dosages and duration of treatments, main results and adverse effects. References and their corresponding results are presented according to date of publication in descending order. CBD (Cannabidiol); Tetrahydrocannabinol ($\Delta^9$-THC); Electroencephalography (EEG).

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**Capítulo II**

Manuscrito do tipo *Research letter* preparado para submissão à revista JAMA Psychiatry

Título: Cannabidiol enhances anandamide hippocampal levels in young animals in an infantile refractory epilepsy model with no alteration in adults

Instruções para os autores:

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- Máximo de 600 palavras;
- Até 7 autores;
- Máximo de 6 referências;
- Até 2 tabelas ou figuras;
- Sem material suplementar;
- Sem abstract ou pontos chave.

Title: Cannabidiol enhances anandamide hippocampal levels in young animals in an infantile refractory epilepsy model with no alteration in adults

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**Introduction**

Worldwide, the growing legalization of Cannabis promises to increase access to the therapeutic effects of Cannabis itself and its isolated components, such as Cannabidiol (CBD), which has been successfully used in the treatment of infantile refractory epilepsy. Based on recent outcomes with Dravet and Lennox-Gastaut Syndromes, the US Food and Drug Administration (FDA) has approved Epidiolex®, a drug with pure plant-derived CBD.

Despite the increasing number of concluded and ongoing controlled clinical trials, there are still poor data over CBD's effects in the underdeveloped brain and its mechanisms of action (Schonhofen et al., 2018). In addition, CBD modulates the levels of endocannabinoid system's components (Toczek and Malinowska, 2018), a retrograde signaling that participates in neural development and regulates synapses. Here we present data which will contribute to the clarification of CBD's mechanisms of action in epileptic children.

Methods

C57BL / 6J mice were submitted to global hypoxia (5% O2/95% N2) in the postnatal day (PND) 7 (Rodriguez-Alvarez et al., 2015) for 15 minutes, to induce increased susceptibility to pharmacologically-induced seizures and reduced response to anticonvulsive drugs – symptoms of refractory epilepsy. Normoxic animals were exposed to 21% O2. CBD (10 mg/kg) was subcutaneously administered in PND15 and PND44 mice. One hour later, susceptibility to seizures was accessed through the intraperitoneal injection of kainic acid (KA) (3 mg/kg for PND15 and 15 mg/kg for PND44). Behavioral scores were ranked according to the Racine scale (Racine, 1972) during 90 min, after which, brains were removed and processed to measure N-arachidonylethanolamine (AEA or Anandamide), 2-arachidonoyl glycerol (2-AG), and CBD hippocampal levels through liquid chromatography with mass spectrometer (LC-MS).

Results

Figure 1.A compares behavioral scores and endocannabinoids' levels in normoxic and hypoxic animals. PND15 animals were more susceptible to KA-induced seizures and presented no alterations in endocannabinoids levels. PND44 presented no behavioral alterations, but hypoxia increased AEA hippocampal levels.

PND15 normoxic animals treated with CBD had increased susceptibility to seizures in the first 50 minutes after KA injection and decreased after 60 min (Fig 1.B) and increased AEA (Fig 1.D). Normoxic PND44 animals treated with CBD presented unaltered behavioral scores (Fig 1.C) and AEA levels (Fig 1.E).

In hypoxic PND15, CBD did not alter seizure susceptibility (Fig 2. A), but enhanced AEA levels (Fig 2.C). PND44 hypoxic animals were less susceptible to seizures only at some

points (Fig 2.B) and no alterations in endocanabinnoids levels (Fig 2.D). Besides, PND44 animals CBD presented higher CBD hippocampal levels.

## Discussion

Our mouse model presented enhanced seizure susceptibility only in PND15, as expected, since epileptic seizures tend to become less frequent and severe after childhood (Genton et al., 2011). Also, the increased AEA in PND44 hypoxic animals is in accordance with previous findings for AEA and/or 2-AG, which usually increase in human disorders and animal disease models, including KA-induced seizures (Toczek and Malinowska, 2018).

Importantly, CBD was able to enhance AEA levels only in PND15 animals, worsening the convulsive effects of KA in normoxic and with no effects in hypoxic animals. It is know that CBD can enhance endocannabinoid tone by competition with AEA for its intracellular carriers, the fatty acid binding proteins (FABPs), raising AEA levels (Toczek and Malinowska, 2018). It promotes an optimal endocannabinoid tone, a more selective strategy since it mainly targets sites where the endocannabinoid signaling is already activated, reducing side effects (Vilela et al., 2013).

CBD was present in a greater quantity in normoxic and hypoxic animals with 44 days. Possibly because although neonates are lighter, their hippocampi have weights similar to adult hippocampi - so that they receive proportionally less CBD in the hippocampus. In other words, younger animals had less CBD in the hippocampus and nevertheless experienced significant changes in susceptibility to epileptic seizures and AEA levels. Such differences in CBD uptake can be related to drug metabolism which changes with age (A. et al., 2015). In addition, the absorption of drugs administered subcutaneously or intramuscularly is not good in neonates due to low regional blood flow and low reserve of muscle mass (Juárez-Olguín et al., 2014).

Our results reinforced this hypothesis as the mechanism of action for CBD only for young animals, in a low dosage associated with no anticonvulsive activity and enhanced seizure susceptibility. In conclusion, as a low dose of CBD leads to the modulation of AEA levels in underdeveloped brains and synaptic plasticity and brain development are dependent of the endocannabinoid signaling, this age-related selectivity of CBD may represent a risk for its administration in very young children.

Fig.1) Behavioral scores and endocannabiniod levels in normoxic and hypoxic infant and adolescent mice.

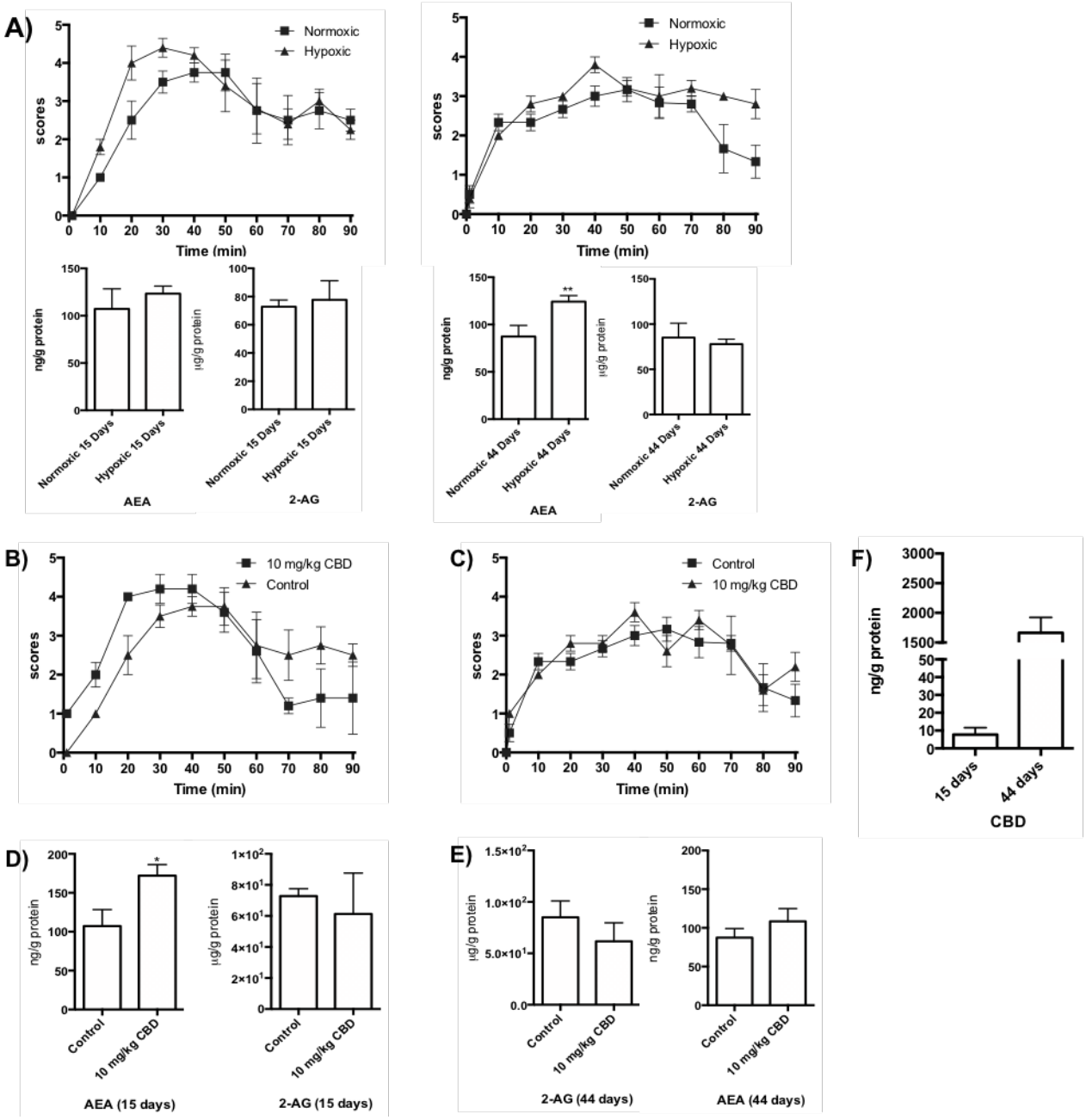
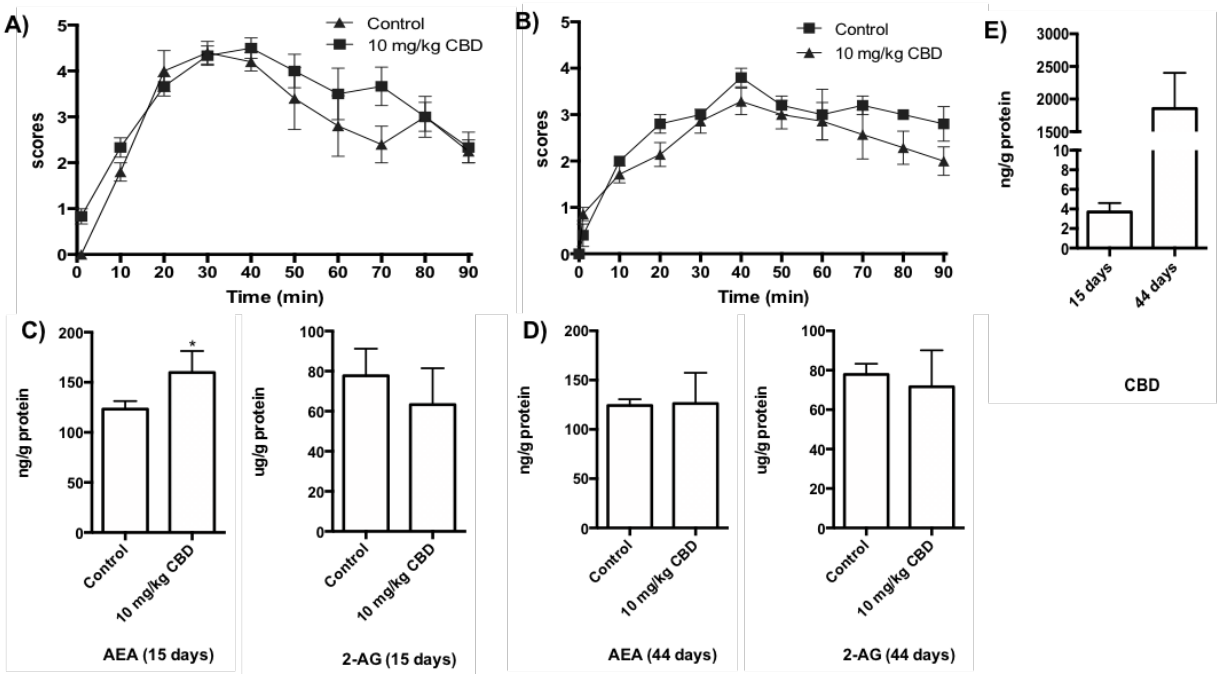


Fig.2) Behavioral scores and endocannabiniod levels in hypoxic animals.




## Figure legends

Figure 1. Behavioral scores and hipocampal levels of endocannabinoids in Normoxic and Hipoxic control animals, and CBD-treated Normoxic animals A) Behavioral scores and endocannabinoids' levels in Hypoxic and Normoxic control PND15 (left) and PND44 (right). B) Behavioral scores of KA-injected hypoxic PND15 and C) PND44 mice treated with CBD in comparison to control. D) Endocannabinoids hippocampal levels in hypoxic PND15 and E) PND44. F) CBD hippocampal level. Statistical analyses of the difference between group means were carried out by two-tailed unpaired Student's t-test and were considered significant at a p-value < 0.05 (n = at least 3 per group for endocannabinoids quantification and at least 5 per group for behavioral scores).

Figure 2. Behavioral scores and hipocampal levels of endocannabinoids in CBD-treated Hypoxic animals A) Behavioral scores of KA-injected Hypoxic PND15 and B) PND44 mice treated with CBD in comparison to control. C) Endocannabinoids hippocampal levels in Hypoxic PND15 and D) PND44. E) CBD hippocampal level. Statistical analyses of the

difference between group means were carried out by two-tailed unpaired Student's t-test and were considered significant at a p-value < 0.05 (n = at least 3 per group for endocannabinoids quantification and at least 5 per group for behavioral scores).

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**Capítulo III**

Manuscrito do tipo *Research article* preparado para submissão à revista Molecular Neurobiology

Título: Neuroprotective and neurotoxic effects of Cannabidiol and its synthetic analogs in mature and underdevelopment neurons: role of the endocannabinoid system modulation

Instruções para os autores:

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Title:

Neuroprotective and neurotoxic evaluations of Cannabidiol and its synthetic analogs using mature and underdevelopment neurons: role of the endocannabinoid system modulation

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Abstract

Cannabidiol (CBD) is one of the most studied phytocannabinoid and has been used in the treatment of children with refractory epilepsy. Although there are many lines of positive evidences regarding the clinical use of CBD, there is insufficient evidence on the potential long-term harmful effects of cannabinoids on central nervous system development. CBD is able to act directly and indirectly over the endocannabinoid system (ECS) and may disturb regulatory processes mediated by this system. In recent years, synthetic analogues of CBD have been developed to improve their beneficial outcomes while reducing potentials side effects. In this study, we evaluated the neuroprotective/neurotoxic effects of CBD and 3 synthetic derivatives in mature neurons and under neuronal differentiation, investigating the role of the ECS in these effects. Human neuroblastoma SH-SY5Y cells was used after differentiation into mature neurons - as a terminally-differentiated (mature) neuronal toxicity model - and during the neuronal differentiation - as a neuronal developmental toxicity model. Neuronal toxicity of CBD and 3 synthetic derivatives was observed when administered during neuronal differentiation and can be attributed to redox imbalances both alone and in combination with the neurotoxin 6-hydroxydopamine (6-OHDA). Modulation in ECS is involved in the neuroprotection/neurotoxicity mechanisms observed for CBD and 3 synthetic derivatives since co-treatment with CB1 agonist and antagonist showed interactions with the cannabinoids tested. Furthermore, cannabinoids affinities for CB1 were predicted *in silico* using public available crystallographic data and demonstrated a potential physiological direct interaction between the drugs and CB1 receptor in the concentration tested. In conclusion, neurons treated during differentiation were more sensitive to CBD and 3 synthetic derivatives, what reinforce the hypothesis that the use of cannabinoids in very

young individuals with immature brains should be better evaluated.

*Keywords:* Cannabidiol, Synthetic Derivatives, Cannabinoid Receptor 1, Endocannabinoid System, Neuroprotection, Neurotoxicity, Neurodevelopmental Toxicity.

## Introduction

The plant *Cannabis sativa* is known worldwide as a recreational drug, but it has been used for medicinal purposes for thousands of years by different cultures [1]. In recent years, the increasing in legalization of *Cannabis* for recreational use has positively favored the research of its extract and isolated lipid-soluble phytocannabinoids, or plant-derived cannabinoids [2]. Their endogenous homologous, the endocannabinoids [4], of which the best characterized are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) [5] targets the cannabinoid receptors type 1 (CB1) and type 2 (CB2). CB1 is widely expressed in the nervous system, whereas CB2 is expressed mainly in cells of the immune system, but also in some cells of the nervous system [6] as hippocampal CA3 and CA2 pyramidal cells, where this receptor participates in the mechanisms of synaptic plasticity [7]. These receptors constitute the endocannabinoid system (ECS), together with enzymes that catalyze endocannabinoids' biosynthesis and degradation [8].

In the mature brain, the ECS modulates synapses (excitatory and inhibitory) through the release of endocannabinoids AEA and 2-AG. They act as retrograde messengers, released by the postsynaptic neuron and acting on CB1 receptors in the pre-synaptic neuron, leading to decreased release of neurotransmitters into the synaptic cleft [9–11]. ECS retrograde signaling mediates synaptic plasticity through short-term or long-term

depression (STD/LTD) [11] and long-term potentiation (LTP) [12]. Both have roles in learning and neural development [13].

In the developing nervous system and in remaining neurogenic areas of the adult brain (the hippocampal subgranular zone and subventricular zone), the ECS exerts a regulatory role on neural progenitor cell survival, proliferation, differentiation and migration via CB1 [14, 15], thus possibly affecting the formation of specialized tissues in adult [16]. Recently, the ECS has also been shown to regulate proliferation and differentiation of mesoderm-derived hematopoietic and mesenchymal stem cells, with a key role in determining the formation of several cell types in peripheral tissues [17].

For therapeutic purposes, the ECS has shown to modulate anxiety, depression, neurogenesis, reward, cognition, learning, and memory [18]. Moreover, its retrograde signaling acts to regulate seizure activity and neuronal hyper-excitability – cannabinoids have shown CB1 activity in experimental models of seizure and epilepsy [19, 20]. Thus, targeting the ECS might be of therapeutic interest.

The ECS' cannabinoid receptors are the main target of *Cannabis*. However, it causes many undesired psychotropic effects, which makes it unlikely to be used clinically *in natura*. On the other hand, experimental studies have demonstrated several therapeutic properties of isolated cannabinoids in a number of *in vitro* and *in vivo* models [21]. Among the products derived from *Cannabis*, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, the main psychoactive ingredient), and cannabidiol (CBD) are the two major components. CBD represents up to 40% of its extract and is devoid of the typical psychoactive effects of $\Delta^9$-THC [22, 23], and has shown positive results in several diseases models.

CBD is predicted to have antioxidant properties due to the presence of a phenolic ring

in its structure [24]. There are some studies evaluating the neuroprotective role of CBD *in vivo* and *in vitro* against redox-active neurotoxins such as 6-hydroxydopamine (6-OHDA) [25], β-amyloid (Aβ) peptide, hydrogen peroxide ($H_2O_2$) among others [26–28]. CBD was able to reverse iron-induced reductions in synaptophysin and increases in caspase-3 levels [29], and it has either improved memory impairments associated to iron toxicity in a rat model [30]. CBD administration, after hypoxia-ischemia in newborn rats, can also reduce brain injury and restore neurobehavioral function [31]. Still in newborn rats, treatment with CBD after transection of the sciatic nerve has resulted in both motor and sensory neuron rescue [32].

Finally, numerous studies have shown CBD to have anticonvulsive properties [33] as it has been widely (and successfully) used as adjuvant to treat children with refractory epilepsy [34]. It is important to emphasize that infantile epilepsies usually present during the first year of life, as in the case of Dravet Syndrome [35]. At this age until the end of adolescence, the nervous system is still in intense maturation and presents great synaptic plasticity, processes directly involved with the ECS [13]. CBD acts on several components of this system, although it exerts its effects primarily through mechanisms not mediated by CB1 [36]. For example, it is known that CBD is able to enhance endocannabinoid tone by inhibiting AEA degradation [37], and it can act as a negative allosteric modulator of CB1 [38], among other targets [39]. Since the ECS is very important for the normal functioning of synapses, for the regulation of cognitive and behavioral processes, besides participating in the regulation of all phases of neuronal development, any perturbations in endocannabinoid signaling may represent a risk [36].

Notwithstanding the current knowledge about CBD's molecular targets, there is not a consensus about the mechanisms of action in diseases [39] and in brain development [36].

Despite the increasing use of CBD in children and adolescents whose brains are still developing, most *in vitro* and *in vivo* studies use mature cells or adult animal models and are thus not faithful mimics of the juvenile CNS. Experiments with immature animals or cells under differentiation have greater potential for identifying CBD's effects and the molecular mechanisms by which such effects are mediated with greater relevance to juveniles.

Our research group showed, using RA-differentiated SH-SY5Y cells, that a sublethal concentration of CBD with antioxidant activity did not exhibit neuroprotection against the neurotoxic effect redox-active neurotoxins in terminally-differentiated neurons. When SH-SY5Y cells undergoing neuronal differentiation were exposed to the same concentration of CBD, besides the lack of neuroprotection and antioxidant activity, CBD potentiated the neurotoxicity induced by all drugs tested [28]. These results suggest a possible hidden negative effect of CBD during neuronal development, reinforcing the observation that effective dosages for CBD and the resulting pathologies observed can vary widely according to the experimental model used. Thus, CBD presents both positive and deleterious effects in animal and cellular models of early stages of development.

As an alternative, recently, natural and synthetic derivatives of CBD are being synthesized and studied for a variety of purposes, most of them aiming to improve the potency, efficacy, or pharmacokinetic properties of CBD [45]. For instance, the conversion of oral CBD into $\Delta^9$-THC in an acidic environment (e.g. the stomach) is a concern, although it has not been observed *in vivo* thus far [46]. A novel CBD derivative, HU-444, is a potential novel drug which cannot be converted by acid cyclization into a $\Delta^9$-THC–like compound [47]. However, like natural occurring cannabinoids, different CBD derivatives vary in their pharmacological and therapeutic properties, such as receptor affinity, anti-oxidant properties, evidencing the need for a better understanding of their mechanism of action [36].

*In silico* approaches can be helpful in such mechanistic studies, through simulations of the interactions of a receptor with a potential ligand, which have been demonstrated to be effective and reliable in calculating some kinetic and thermodynamic parameters [48].

Herein, we evaluate CBD's and some of its synthetic derivatives' effects in mature (terminally differentiated neuronal toxicity model) as well as under differentiation (neuronal developmental toxicity model) neurons using the RA-differentiated human neuroblastoma SH-SY5Y cell line. Data will be discussed based on the direct and indirect interaction of compounds with CB1 using CB1 agonist/antagonist and *in silico* Molecular Docking experiments.

## Experimental Procedures

### *Chemicals*

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil). CB1 agonist, (±)-2-Methylarachidonoyl-2′-fluoroethylamide, and CB1 antagonist, AM251 and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cannabidiol (99.9%), and its synthetic derivatives HUF-101, 4'-fluoro-cannabidiol, (−)-5′-DMH-CBD, (-)-5'-Dimethylheptyl-cannabidiol, HU-556 (Patent protected), were diluted in ultrapure dimethyl sulfoxide (DMSO) to a final concentration of 0.1% of DMSO in treatments.

### *Cell Culture, Differentiation, and Treatments*

Exponentially growing human neuroblastoma SH-SY5Y cell line, obtained from ATCC (Manassas, VA, USA), was maintained at 37ºC in a humidified atmosphere of 5% $CO^2$. The

cells were grown in a mixture of 1:1 of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 2 mM of glutamine, 1000 U/mL penicillin, 1000 μg/mL streptomycin and 2,5 μg/mL of Fungizone® (amphotericin B). Medium was changed each 3 days and cells were sub-cultured once they reached 80% confluence. After 24h of plating, neuronal differentiation was triggered by lowering the FBS to 1% with the addition of 10 μM RA for 7 days, as previously established by our group [43]. In the seventh day of RA-induced differentiation, the SH-SY5Y cells were treated with each cannabinoid for 24h. For evaluation of cannabinoids' effects over neuronal development, each cannabinoid was co-administered with RA during the differentiation – in the seventh day, cannabinoids and RA were replaced and experiments were performed 24h after. For cell viability, cells were seeded in 96-wells plate at density of 2 x $10^4$ cells/well.

*Neurotoxicity/ Neuroprotection assay*

Cell viability was evaluated by the quantification of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases. At the end of the treatment, cells were incubated with 0.5 mg/mL of MTT during 1h at 37ºC. Then, medium was discarded and DMSO was added to solubilize the formazan crystals. The absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices, USA). After treatment or differentiation with selected concentrations of each cannabinoid, cells were washed with PBS and challenged with the toxin 6-Hydroxidopamine (6-OHDA, $LC_{50}$ = 30 μM or sublethal concentration = 15 μM), to evaluate the neuroprotective/neurotoxic features of the cannabinoids through MTT assay. The results were expressed in percentage of untreated cells (mean ± SD value).

*Total Radical-Trapping Antioxidant Potential (TRAP) and Total Antioxidant Reactivity (TAR)*

The non-enzymatic antioxidant capacity of each cannabinoid was assessed through the total radical-trapping antioxidant potential (TRAP) assay, which is based on the measurement of luminescence generated by luminol oxidation by AAPH (2, 20-azobis 2-amidinoprepane) decomposition, in glycine buffer (pH 8.6). After system stabilization (buffer, luminol and AAPH), different concentrations of CBD were added and the luminescence signal decreases proportionately to its antioxidant potential. The luminescence was monitored in a Wallace 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). For data analysis, a time per chemiluminescence curve was obtained and the relative ‘‘area under the curve’’ (AUC) in the recovery phase was used, as previously established [49, 50]. In order to evaluate not only the quantity of oxidants but also their reactivity, we used the total antioxidant reactivity assay (TAR), [49].

*Cannabinoid receptor modulation*.

In the seventh day of RA-induced differentiation, the SH-SY5Y cells were treated with 1.0 μM CB1 agonist - (±)-2-Methylarachidonoyl-2′-fluoroethylamide - or antagonist - AM251 - 15 min prior to the addition of each cannabinoid (0.01, 0.1, 1.0 and 2.5 μM) for 24h. For evaluation of cannabinoids’ effects over neuronal development, the same treatments were performed during the differentiation – in the seventh day, treatments and RA were replaced and neurotoxicity and neuroprotection experiments were performed 24h after.

Molecular Simulation Pipeline

The simulations were carried out through a pipeline comprising Docking - Molecular Dynamics - Docking approaches. Initially, ~1500 poses of each ligand at the CB1 receptor binding site were obtained via docking and optimized through 20 ns of molecular dynamics in order to adjust the geometries at the binding site. Following, a second docking round was performed using the optimized binding site as input, in order to obtain a more accurate interaction energy and calculated inhibition constant ($K_i$) for each ligand.

Structural Data

Coordinates of ligand molecules were retrieved from PUBCHEM and underwent a geometry optimization protocol, respecting the state of protonation at the physiological pH, through 10000 steps of the steepest descent algorithm protocol and the convergence tolerance set to $1x10^{-7}$. Protonation state were assessed with the help of both Avogadro and Marvin Sketch version 5.5.0.1 (Marvin Beans Suite – ChemAxon) codes, while geometry optimizations were performed using the former. The crystallographic structure of the human cannabinoid receptor CB1 linked to the Taranabant inhibitor at 2.6 Å resolution (PDB ID: 5U09) was used for the simulations. During the preparation of the receptor, the heavy atoms and the missing hydrogens were added respecting the state of protonation at physiological pH using PDB2PQR Web Server 2.1.1 (http://www.poissonboltzmann.org/docs/structures-ready) [51, 52], and the receptor prepared for docking in the Autodock Tools Suite [53].

Molecular Docking

The molecular docking was performed through the program Autodock4 [53, 54] after protocol validation through the redocking of Taranabant no CB1, as previously described [55, 56]. Docking procedure was repeated 10 times, resulting about 1500 poses (150 poses

per output). The procedure was set to use Lamarckian genetic algorithm (GA) [57], a GA with 25,000,000 energy evaluations per run, population size set to 150, and a maximum of 27,000 generations per run. At the end, resulting poses were clustered using a RMSD tolerance of 2.0 Å2 using Autodock Tools [53, 58]. The total energy of interaction and the calculated value of the inhibition constant were obtained through the analysis of clusters within the tolerance of 2 Å.

Molecular dynamics

Molecular dynamics simulations were performed using the code GROMACS v. 4.5.1 [59], adopting the explicit water model TIP3P and the force field CHARMM27 [60]. Chloride and sodium counter-ions were added to neutralize the system maintaining a final salt concentration of 0.15 mol/L. Before carrying out the MD simulations, total energy minimization was accomplished by combining the steepest-descent algorithm and the conjugate gradient method in sequence. This initial optimization was followed by equilibration steps where the temperature of the system was gradually increased to 310K. At the total, 20 ns of simulation time were performed for each complex CB1-ligand. After the equilibrium of the system, the final structure had its geometry optimized (energy minimization) and was used for the second molecular anchoring session.

*Statistical Analysis*

Data are expressed as percentage of untreated cells (control) (mean ± SD) from at least three independent experiments. For statistical analysis, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. Differences were considered significant at $P > 0.05$.

## Results and Discussion

*Experimental Design and Concentration selection*

The experimental design is presented in Fig. 1a. The RA-differentiated SH-SY5Y human neuroblastoma cell is an interesting *in vitro* model to access CBD's effects because many of the neurodevelopmental processes that occur *in vivo*, including cell differentiation and neurite outgrowth (as shown in Fig. 1b), can be accessed [28, 43, 44]. Thus, this cell lineage is suitable to study the effects of a drug or compound in mature neurons (terminally differentiated neuronal toxicity model), and during neuronal differentiation (neuronal developmental toxicity model).

As CBD is described as a potent antioxidant molecule, and since oxidative stress is related to neurodegeneration [61], a concentration-response curve of HUF-101 and (−)-5′-DMH-CBD (1.0, 2.5, 5.0 and 10.0 μM) was designed to find the concentration that presents high antioxidant potential (in a cell free approach) with concomitant low neurotoxicity to RA-differentiated SH-SY5Y cells, which was based on previous studies [62]. The results with CBD presented here were previously published [28] and are resumed so they can be compared to the synthetic derivatives analyzed. Data on CBD-treated RA-differentiated SH-SY5Y cells show that CBD does not significantly affect cell viability until 2.5 μM (Fig. 2a). Although by TRAP assay CBD was able to scavenge peroxyl radical only in the higher concentration (10 μM), TAR assay shows a significant antioxidant reactivity of CBD as low as 2.5 μM. (Fig. 2a). HUF-101 (Fig. 2b) and (−)-5′-DMH-CBD (Fig. 2c) also do not affect cell viability until 2.5 μM, and they are neurotoxic at 10 μM. By TRAP assay, HUF-101 was able to scavenge peroxyl radical in 5 and 10 μM, although by TAR it presented significant

antioxidant reactivity in concentrations as low as 2.5 μM. For (−)-5′-DMH-CBD, no significant differences were observed in antioxidant activity.

The HUF-101 concentration of 2.5 μM was selected for the following experiments since it is sublethal, and it presented antioxidant activity. In addition, we included 1 μM because, although it does not have antioxidant activity, it is also sublethal. For comparison purpose, although (−)-5′-DMH-CBD did not present antioxidant activity in any of the concentrations, we selected the same concentrations of HUF-101.

Our results regarding the concentration-response curve are in agreement with the known effect of CBD in humans as it follows an inverted U-shaped dose-effect curve pattern of effectiveness observed in many animal studies [63]. Here, the same occurs for the synthetic cannabinoids.

*Neuroprotective/Neurotoxic effects of CBD and it Synthetic Derivatives in Terminally Differentiated and During the Neuronal Differentiation of SH-SY5Y Cells*

The neuroprotective effects of each cannabinoid were evaluated by challenging pretreated cells with the LC50 of the redox-active neurotoxin 6-OHDA. This is one of the most used toxin in Parkinson's Disease experimental models [42, 64], and it is an analog of dopamine with similar structural characteristics and affinity to its transporter (DAT), that accumulates inside the dopaminergic neurons causing oxidative damage [65]. As RA-differentiated SH-SY5Y are dopaminergic-like neurons, they are more susceptible to the oxidative damage caused by 6-OHDA, which is dependent of the dopaminergic transporter (DAT) present only in differentiated cells [43, 44].

In terminally differentiated neurons, 1,0 and 2.5 μM of both HUF-101 (Fig. 3a) and (−)-5′-DMH-CBD (Fig. 3b) were not able to protect cells against 6-OHDA toxicity. However, when administered during the neuronal differentiation, both HUF-101 and (−)-5′-DMH-CBD at 2.5 μM increased the neurotoxic effects of 6-OHDA, while 1.0 μM was not able to protect cells. In previous results, 2.5 μM of CBD has also sensitized SH-SY5Y cells in a sublethal 6-OHDA-chalenge when treatment occurred during neuronal differentiation [28]. It corroborates other study in which CBD had no protective effect against 1-methyl-4-phenylpyridinium ($MPP^+$) toxicity at 0.01, 0.1, and 1.0 μM in differentiated SH-SY5Y [27]. However, CBD improved cell viability in response to *tert*-butyl hydroperoxide in PC12 rat pheochromocytoma cells and undifferentiated SHSY5Y cells, whereas it was not able to inhibit β-amyloid and $H_2O_2$ toxicity at 1.0 and 10.0 μM [26]. As in the concentration-response curve we only partially observed the proposed U-shaped effectiveness curve for CBD and its derivatives, and 1.0 μM and 2.5 μM of HUF-101 and (−)-5′-DMH-CBD presented negative results in cell challenged with 6-OHDA, lower concentrations of these compounds were included for the following neuroprotection/neurotoxicity evaluations. Also, lower concentrations of cannabinoids are frequently more protective than the higher ones [63]. In addition, a third synthetic CBD derivative, HU-556, was included in the next experiments.

Fig. 4 shows the concentration-response curves (0.01, 0.1, 1.0, and 2.5 μM), 24 h after the end of the treatment, in terminally differentiated SH-SY5Y cells in comparison to cells treated during the neuronal differentiation. In mature neurons, none of the cannabinoids concentrations altered cell viability. However, in the neuronal developmental neurotoxicity model, 2.5 μM of HUF-101, (−)-5′-DMH-CBD and HU-556 were neurotoxic, while no concentration altered significantly the cell viability for CBD. These results indicate that human neurons are more sensitive to cannabinoids when they are exposed during

differentiation, as previously shown for CBD by our research group [28]. As the $LC_{50}$ of 6-OHDA can be an extreme challenge for neurons, covering up possible protective effects, hereafter we evaluated neurotoxicity in SH-SY5Y treated with the same curve of each cannabinoid (0.01, 0.1, 1.0, and 2.5 μM) challenged with a sublethal concentration of 6-OHDA (15 μM) (Fig. 5).

For CBD, no concentration was able to increase the neurotoxic effects of 6-OHDA, in both toxicity models (Fig. 5a), as well as HUF-101 (Fig.5b) and (−)-5′-DMH-CBD (Fig. 5c). On the other hand, HU-556 increased 6-OHDA-mediated loss of viability in neurons treated with 2.5 μM during neuronal differentiation (Fig. 5d). Thus, it was neurotoxic at this concentration during development itself (Fig. 4) and when challenged with 6-OHDA. Our results might be attributed to the high antioxidant profile of CBD and HUF-101. Antioxidants are generally protective in low dosages, while in high concentrations they can lead to a redox imbalance and be as toxic as classical oxidants - when reactive species cause or significantly contributes to the progression of the disease, antioxidants can work even in higher dosages [66].

As discussed above, 6-OHDA mechanism of action is proposed to be mainly mediated through reactive oxygen species generation, and, as a consequence, by oxidative stress [44]. It is possible that a second redox-active compound, such as HUF-101 and CBD, will have an additive effect. Then, it is expected that this is the process that generates cell death observed in our experiments. Thus, a molecule with antioxidant potential *in vitro* (in a cell free system) would be expected to protect cells in culture against the oxidative injuries. However, in our results, no neuroprotection was found. It is possible that the concentrations used are still outside the protective threshold for these compounds. The experimental design may also have contributed to the lack of protection since the neurotoxic challenge was

performed after the treatment with the cannabinoids, which may not have been sufficient to generate an antioxidant effect against 6-OHDA.

However, in addition to lack of neuroprotection, there was a reduction in viability of cells treated with cannabinoids during differentiation (but not in those treated after differentiation), but the results shown so far do not allow us to define which are the mechanisms involved. We must also consider the bias of the time of exposure to cannabinoids that was higher for cells treated during differentiation, which should also influence the sensitization observed in this case. For (−)-5′-DMH-CBD, which has no antioxidant activity, and HU-556, which was not evaluated, other mechanisms must be involved. In addition, ECS signaling may be involved as CBD can antagonize the pharmacological effects of CB1 agonists [67] and the up-regulation of CB1 occurs in response to neuronal damage [27]. CB1-mediated neuroprotection occurs through inhibition of adenylyl cyclase, and further decrease of intracellular calcium during a neurotoxic event [68]. The expression of this receptor is increased in response to toxin exposure in differentiated SH-SY5Y, as an indicative of neuronal damage, which happens in disease process [27]. Yet, nigral CB1 activity was also increased in animal model for Parkinson's Disease [25]. However, the same study found that protective effect of CBD is unlikely to be mediated by the CB1 [25, 27].

In several studies both phytocannabinoids and endocannabinoids detain the development of early embryos through CB1 regulation [69–72]. Indeed, the ECS exerts a regulatory role on neural progenitor cell proliferation, differentiation and migration in the developing nervous system and in the neurogenesis of the adult brain [17].

In summary, 2.5 μM of each cannabinoid was neurotoxic when administered during neuronal differentiation - CBD when challenged with 6-OHDA (previous results [73]), and its

derivatives *per se* and when challenged with the $LC_{50}$ (for HUF-101 and (-) - 5'-DMH-CBD) or with a sublethal concentration of 6-OHDA (for HU-556). With this, we can conclude that the highest concentration tolerated by differentiated cells (2.5 μM) of these cannabinoids is neurotoxic during development. In addition, synthetic derivatives have neurotoxicity themselves as they have reduced cell viability even without the neurotoxic challenge. For all cannabinoids tested, concentrations that did not present toxicity *per se*, did not potentiate 6-OHDA toxicity. Taken together, our results suggest that these cannabinoids may be hazardous during neuronal development at 2.5 μM, being safer at lower concentrations. As the ECS signaling may be involved in the mechanisms of neurotoxicity observed, we further investigated the role of CB1 in such effects.

*ECS modulation – CB1 agonism and antagonism*

Although current evidence suggests that CBD only interact with the ECS *in vitro* at concentrations unlikely to be reached *in vivo* [39], it is reported also as a direct modulator of CB1 receptor. In the nanomolar range (below the reported affinity – *Ki* – for CBD to these receptors), CBD can antagonize the pharmacological effects of CB1 endogenous ligands [36].

At first, we tested the effects of 1 μM of the CB1 agonist ((±)-2-Methylarachidonoyl-2′-fluoroethylamide) and antagonist (AM251) alone in both cell models with and without 6-OHDA-chalenge (Fig. 6a). In mature neurons, CB1 agonist and antagonist did not alter the viability nor increased or protected neurons against 6-OHDA toxicity. When challenged with 6-OHDA, the CB1 agonist counteracts 6-OHDA effects, significantly enhancing cell viability in comparison to 6-OHDA alone. CB1 expression was already reported as increased in

response to toxin exposure in differentiated SH-SY5Y, as an indicative of neuronal damage, which happens in disease processes [27]. Thus, as CB1 agonist is protective, this receptor might be involved in the positive response to neurodegeneration processes.

So far, we verified that the CB1 agonist and antagonist alone do not alter cell viability. However, the CB1 agonist is neuroprotective during differentiation. We also verified that all cannabinoids can be neurotoxic, and they can increase 6-OHDA toxicity during neuronal differentiation at 2.5 μM. Thus, the following treatments aimed to investigate whether CB1 blockage or activation can affect the cell viability after a co-treatment with each cannabinoid (0.01, 0.1, 1.0, and 2.5 μM).

When co-treated with 2.5 μM CBD (Fig. 6b), the CB1 agonist increased cell viability in terminally differentiated neurons. In the neuronal developmental toxicity model, the CB1 agonist increased cell viability with 0.01 μM, and the CB1 antagonist reduced with 1.0 μM. When challenged with 6-OHDA, the CB1 antagonist potentiated the neurotoxicity of 6-OHDA in co-treatment with 1.0 μM (compared to 1.0 μM + 6-OHDA) and the agonist increased viability (was protective against 6-OHDA) in co-treatment with 2.5 μM of CBD.

As already discussed for CBD, although it has low affinity for CB1, it modulates ECS signaling, mainly through AEA reuptake and degradation [74]. It has also been described as a negative allosteric modulator of CB1, inhibiting cannabinoid agonist activity [38]. As HUF-101, (−)-5′-DMH-CBD, and HU-556 are CBD's derivatives, it is possible that they present the same effects over CB1. Besides, as CBD has many possible targets, other receptors and signaling pathways might be involved in such results and require further investigations.

Co-treatments with HUF-101 (Fig. 6c) in terminally differentiated neurons, no statistically significance was observed. However, when co-treated during neuronal

differentiation, the CB1 agonist was able to prevent the loss of viability caused by HUF-101 2.5 μM alone (Fig. 4). Furthermore, co-treatment of 0.1 and 1.0 μM HUF-101 with the CB1 antagonist reduced cell viability. In the neurotoxic challenge, co-treatment of 0.01 μM of HUF-101 and the CB1 antagonist prevented further loss of viability caused by 6-OHDA. However, at the concentration of 1.0 μM, both agonist and antagonist co-treatment led to a reduction in cell viability compared to 1.0 μM of 6-OHDA challenged HUF-101.

The synthetic CBD 4'-F-CBD (HUF-101) has been demonstrated to be considerably more potent than CBD in behavioral assays predictive of anxiolytic, antidepressant, antipsychotic and anti-compulsive activity in mice. Similar to CBD in a test used to detect anti-compulsive-like drugs effects, the anti-compulsive effects of HUF-101 depended on cannabinoid receptors, since both CBD and HUF-101 effects, are prevented by pretreatment with a CB1 antagonist. Presumably, the actions of this fluorinated CBD derivative, which parallel those of CBD, are based on the same proposed mechanisms [75].

HUF-101 induces analgesic effects at lower doses than CBD [76]. Also, HUF-101's and CBD's analgesic effects involve the activation of CB1 and CB2 receptors [76], which again corroborates our results, since CB1 activity modulation altered cell viability (Fig. 6c). As HUF-101 did not cause the typical psychoactive effects induced by CB1 agonists in mice [76], it probably targets other components of the ECS, which causes loss of cell viability in underdevelopment neurons at a high concentration. In a mouse model of neonatal sciatic nerve axotomy, HUF-101 also displays enhanced neuroprotective gliosis attenuating properties and comparing to CBD [77].

Regarding (−)-5′-DMH-CBD (Fig. 6d), in terminally differentiated neurons, no statistically significant result was observed. However, when co-treated during neuronal differentiation, the CB1 antagonist significantly reduced the viability with 0.1 and 1.0 μM (-)

- 5'-DMH-CBD. In addition, co-treatment with the CB1 agonist or antagonist was not able to prevent the viability loss caused by 2.5 μM (-) - 5'-DMH-CBD alone (Fig. 4). In the neurotoxic challenge, during neuronal differentiation, the CB1 agonist in co-treatment with 2.5 μM was able to prevent the loss of viability caused by 6-OHDA, and the CB1 antagonist potentiated 6-OHDA neurotoxicity when co-treated with 1.0 μM (-) - 5'-DMH-CBD (compared to 1.0 μM + 6-OHDA).

For the generation of the dimethyl-heptyl-CBD homologs, the introduction of the DMH alkyl chain in the (-)-DMH-CBD series did not alter the lack of CB1 and CB2 affinities [78]. (-)-DMH-CBD analogs have displayed anxiolytic, analgesic, anti-inflammatory, and antiproliferative effects in diverse assays [45]. Interestingly, (-)-7-OH-DMH-CBD exhibited potent inhibition of electrically-evoked contractions in the mouse vas deferens that was not mediated through CB1, CB2, TRPV1, opioid, or α2-adrenergic receptors [79, 80].

The dimethyl-heptyl-CBD derivative tested here, (−)-5′-DMH-CBD, is an AEA membrane transport inhibitor that is relatively metabolically stable. It displays some affinity for CB2 receptors but has only weak affinity for CB1 receptors and no activity over FAAH [78]. Here, we found changes in cell viability in the co-treatments of (−)-5′-DMH-CBD with CB1 agonist and antagonist. Thus, the effects demonstrated here might be due to inhibition of AEA transport, which causes loss of cell viability in underdevelopment neurons at a high concentration.

Regarding HU-556 (Fig. 6e), in terminally differentiated neurons, no statistically significant result was observed. However, during neuronal differentiation, co-treatment with CB1 agonist or antagonist were not able to prevent the loss of viability caused by HU-556 2.5 μM alone (Fig.4). Co-treated terminally differentiated neurons challenged with 6-OHDA, the CB1 antagonist increased cell viability in all dosages of HU-556. When co-treated with

HU-556 2.5 μM during neuronal differentiation, the CB1 agonist and antagonist potentiated the loss of viability caused by 6-OHDA. However, as HU-556 was already cytotoxic at 2.5 μM, it is possible that the observed enhanced cytotoxicity when challenged with 6-OHDA is due to HU-556 effects itself.

HU-556 is a new CBD derivative, protected by patent secrecy, with no previous published data. A similar compound, HU-444, showed *in vitro* anti-inflammatory activity (decrease of reactive oxygen intermediates and inhibition of TNF-α production by macrophages); *in vivo* it led to suppression of TNF-α production and amelioration of liver damage as well as lowering collagen-induced arthritis in mouse. HU-444 did not cause $\Delta^9$-THC–like effects in mice [47]. As both compounds were produced using CBD as the starting structure, and HU-444 is not a $\Delta^9$-THC–like compound, the effects demonstrated here for HU-556 might be due to other ECS targets, which causes loss of cell viability in underdeveloped neurons at a high concentration.

Since the agonist alone is neuroprotective (Fig. 6a) during the differentiation, and that the addition of the lower and higher concentration of CBD to the CB1 agonist leads to an increase in viability, it can be inferred that CBD is neuroprotective only in the presence of the CB1 agonist, but that this effect is probably due only to the agonist. It is also possible that at these concentrations CBD is already cytotoxic, although not detectable, through the inhibition of CB1, what is reversed by the addition of the CB1 agonist. Besides, CBD may be said to have some interaction with CB1 since the co-treatment of 1.0 μM CBD with the antagonist of this receptor reduced viability during differentiation, which did not occur for both when treated alone. Likewise, for HUF-101 and (-) - 5'-DMH-CBD, in general, co-treatment with the CB1 antagonist leads to reduced viability in treated cells during differentiation and the agonist led to increased viability at the highest concentration, allowing

to infer that an interaction between the receptor and these cannabinoids. For HU-556, on the contrary, co-treatment with the CB1 antagonist led to increased viability at all concentrations in mature neurons. During differentiation, in the highest concentration of HU-556, both the agonist and the antagonist led to potentiation of 6-OHDA neurotoxicity. Although antagonistic in comparison to other cannabinoids, the results obtained for HU-556 also suggest some type of interaction with the CB1 receptor. Another possibility is that the cannabinoids analyzed act through other targets in the ECS or even other systems.

Regardless individual differences among the cannabinoids tested, SH-SY5Y cells treated during the neuronal differentiation process presented an increased sensitivity to all challenges tested. It means that innocuous concentrations (for mature neurons) of cannabinoids can be harmful for immature neurons. As modulation of CB1 activity lead to altered cell viability in some of the treatments, our results suggest that the ECS may participate in the mechanisms of neurotoxicity / neuroprotection observed as well as in the mechanisms of action of the tested cannabinoids in a concentration-dependent manner. Besides, as the CB1 agonist is protective when challenged with 6-OHDA when administered during neuronal differentiation, an allosteric modulator would counteract this protection, what happens with 1 μM of each synthetic cannabinoid, but not with CBD, in the co-treatments during neuronal differentiation (Fig. 6). However, through this approach it is not possible to affirm that these cannabinoids are negative allosteric modulators of CB1, as already described for CBD [38]. For that, a blockage of the allosteric site would be more effective. Another possibility is that the cannabinoids analyzed act through other targets in the ECS or even other systems.

Molecular Simulations

As all cannabinoids we have tested were able to trigger CB1 receptor sensitization, we evaluated their in silico predicted affinity and energy of interaction for the orthosteric site of CB1 through molecular docking and molecular dynamics. Besides, AEA and 2-AG, endogenous CB1 ligands, were also analyzed for validation purposes. Our protocol consisted in a pipeline following three steps, as shown in figure 7a: 1) flexible docking of cannabinoids in a rigid human CB1 receptor structure; 2) molecular dynamics to improve the drug-receptor fit using results from step 1; 3) flexible docking of cannabinoids in the improved geometry of CB1 receptor from step 2. The original crystal and the adjusted receptor structure are shown in figure 7b. Figure 8 shows the docking of each cannabinoid with the adjusted CB1 receptor, in which it is observed the conformational changes resultant in the receptor. Of note, HU-556 leads to the most remarkable conformational adjust in the receptor, and this cannabinoid showed discrepant results in vitro in comparison with the other cannabinoids analyzed here.

Many laboratories have confirmed the original observation that AEA competes for binding to the CB1 receptor with $K_i$ values in the range of 50–100 nM [81], and there is substantial biochemical evidence that AEA is a partial agonist of the CB1 receptor [81]. The endocannabinoid 2-AG competes for binding to the CB1 receptor [82] with $K_i$ values significantly higher than for AEA (1.0 – 10 μM) [81] and is however a full agonist of this receptor.

Data from our *in silico* approach in comparison with experimental data are shown in table 1. Results from the analysis of interaction energy showed the compound AEA has a calculated $K_i$ of 144.14 nM, into the range observed experimentally, and showed a calculated interaction energy of is -9.33 kcal/mol. For 2-AG, the calculated $K_i$ was 1170 nM, also within the expected experimental range, and the calculated interaction energy was -

8.09 kcal/mol. Still, in accordance with experimental data, our results show a higher K for 2-AG in comparison to AEA. For CBD, the calculated $K_i$ (146,40 nM) was lower than previously described (2.0 – 10.0 μM [83]) and the energy of interaction was -9.32 kcal/mol. For HUF-101, the calculated $K_i$ value (381.28 nM) was larger than for AEA ($K_i$ = 144.12 nM), and the calculated interaction energy was -8.76 kcal/mol. This cannabinoid probably has low activity over CB1, since it has a high $K_i$, and has no Δ9-THC–like effects in other models [75], although an affinity for the receptor can also result in antagonism. Predicted Ki value for (−)-5′-DMH-CBD is very low (21.68 nM), similar to Δ9-THC values, and the energy of interaction of -10.46 kcal/mol. However, as already mentioned, (−)-5′-DMH-CBD previously showed weak affinity for CB1 with a $K_i$ >10μM [78]. For HU-556, the predicted $K_i$ value was 108,64 nM and energy of interaction was -9.5 kcal/mol. Although receptor binding affinity of several phytocannabinoids was reported to not explain their effects on neural cell culture [84], for all the cannabinoids tested here, the predicted $K_i$ are within the concentration curve used. Thus, these cannabinoids have predicted affinity for the orthosteric site of CB1, and since the agonist / antagonist of this receptor altered cellular viability in co-treatments, it can be inferred that there is probably a direct interaction. Despite such predicted affinity, as CBD can also reduce uptake and degradation of AEA [37], its synthetic derivatives can present the same properties. In this case, the administration of cannabinoids would increase AEA which activates CB1. Another predicted mechanism of action of CBD is the negative allosteric modulation, what means that CBD can reduce the activity of a CB1 ligand, and depended upon polar residues being present at positions 98 and 107 in the extracellular amino terminus of the CB1 receptor [38], However, it was not possible to evaluate the allosteric activity of CBD, since such amino terminus region of CB1 is to unstable and is not present in the structure of the CB1 crystal used for the predictions here presented.

## Conclusion

SH-SY5Y cells treated during neuronal differentiation were more sensitive to the analyzed cannabinoids, transforming innocuous concentrations in mature neurons into neurotoxic doses. In addition, in general, the CB1 agonist leads to increased viability, while the antagonist leads to a reduction.

The observed neurotoxic effects can be attributed to a possible redox imbalance caused in cells treated during differentiation with cannabinoids both alone and in challenges with 6-OHDA. However, as CB1 agonist and antagonist co-treatment alters these results, it is likely that ECS is involved in the neuroprotection/neurotoxicity mechanisms observed. In addition, both the effects of cannabinoids and ECS participation in these effects appear to be dose-dependent.

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**Figure Legends:**

**Fig.1** Protocol design in RA-differentiated SH-SY5Y cells. **a** RA differentiation protocol, treatments, and endpoints (arrows) in terminally differentiated (top) or during neuronal

differentiation (bottom) of SH-SY5Y cells. **b** Representative images with increased neurite outgrowth in RA-differentiated SH-SY5Y cells.

**Fig.2** Concentration selection. The left figure represents the cytotoxicity curve of cannabinoids in RA-differentiated SH-SY5Y cells. Cells were treated with each cannabinoid during 24 h, and cell viability was evaluated by MTT assay (as described in Fig. 1a, top). Next figure represents the *in vitro* total radical-trapping antioxidant potential (TRAP) – TRAP traces - followed by the AUC values and is expressed as a percentage of radical produced compared to vehicle (black bar). The right figure represents the total antioxidant reactivity (TAR) - expressed as a percentage of radical scavenging in comparison to vehicle (black bar). **a** CBD **b** HUF-101 **c** (−)-5′-DMH-CBD. Results are expressed as a percentage of untreated cells. * $P < 0.05$ in comparison with vehicle (one-way analysis of variance). Significant differences between groups are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences ($P<0.05$). Data are presented as mean± SD of four independent experiments carried out in triplicates (n = 4).

**Fig.3** Evaluation of neuroprotection of sublethal concentrations of cannabinoids against redox-active toxins over terminally differentiated human neuroblastoma SH-SY5Y cells (left), and during the RA differentiation of SH-SY5Y cells (right). **a** HUF-101 **b** (−)-5′-DMH-CBD. Results are expressed as a percentage of untreated cells. * $P < 0.05$ in comparison with vehicle. Significant differences between groups are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences ($P<0.05$) (one-way analysis of variance). Data are presented as mean± SD of four independent experiments carried out in triplicates (n = 4).

**Fig.4** The effect of a curve of cannabinoids over terminally differentiated human neuroblastoma SH-SY5Y cells (left), and during the RA differentiation of SH-SY5Y cells

(right). **a** CBD **b** HUF-101 **c** (−)-5′-DMH-CBD **d** HU-556. Results are expressed as a percentage of untreated cells. * P < 0.05 in comparison with vehicle (one-way analysis of variance). Data are presented as mean± SD of four independent experiments carried out in triplicates (n = 4).

**Fig.5** Evaluation of neuroprotection/neurotoxicity of a curve of cannabinoids against redox-active toxins over terminally differentiated human neuroblastoma SH-SY5Y cells (left), and during the RA differentiation of SH-SY5Y cells (right). **a** CBD **b** HUF-101 **c** (−)-5′-DMH-CBD **d** HU-556. Results are expressed as a percentage of untreated cells. * P < 0.05 in comparison with vehicle. Significant differences between groups are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences (P<0.05) (one-way analysis of variance). Data are presented as mean± SD of four independent experiments carried out in triplicates (n = 4).

**Fig.6** Evaluation of neuroprotection a curve of cannabinoids co-treated with 1 μM of CB1 agonist / antagonist against redox-active toxins over terminally differentiated human neuroblastoma SH-SY5Y cells (left), and during the RA differentiation of SH-SY5Y cells (right). **a** CB1 agonist and antagonist **b** CBD **c** HUF-101 **d** (−)-5′-DMH-CBD **e** HU-556. Results are expressed as a percentage of untreated cells. * P < 0.05 in comparison with vehicle. Significant differences between groups are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences (P<0.05) (one-way analysis of variance). Data are presented as mean± SD of four independent experiments carried out in triplicates (n = 4).

**Fig.7** Molecular simulations. **a** Simulation pipeline consisting in steps of docking followed by molecular dynamics. Each docking step generated ~1500 poses. Molecular dynamics step was performed during 20 ns. **b** Original crystal and adjusted CB1 receptor.

**Fig.8** Molecular docking of CB1 receptor and the synthetic derivatives of CBD. Molecules and molecular anchoring to CB1 active site.

**Table 1**. The total energy of interaction, calculated value of the inhibition constant ($k_i$) and experimental *Ki* (bibliography) for each ligand.

Fig 1

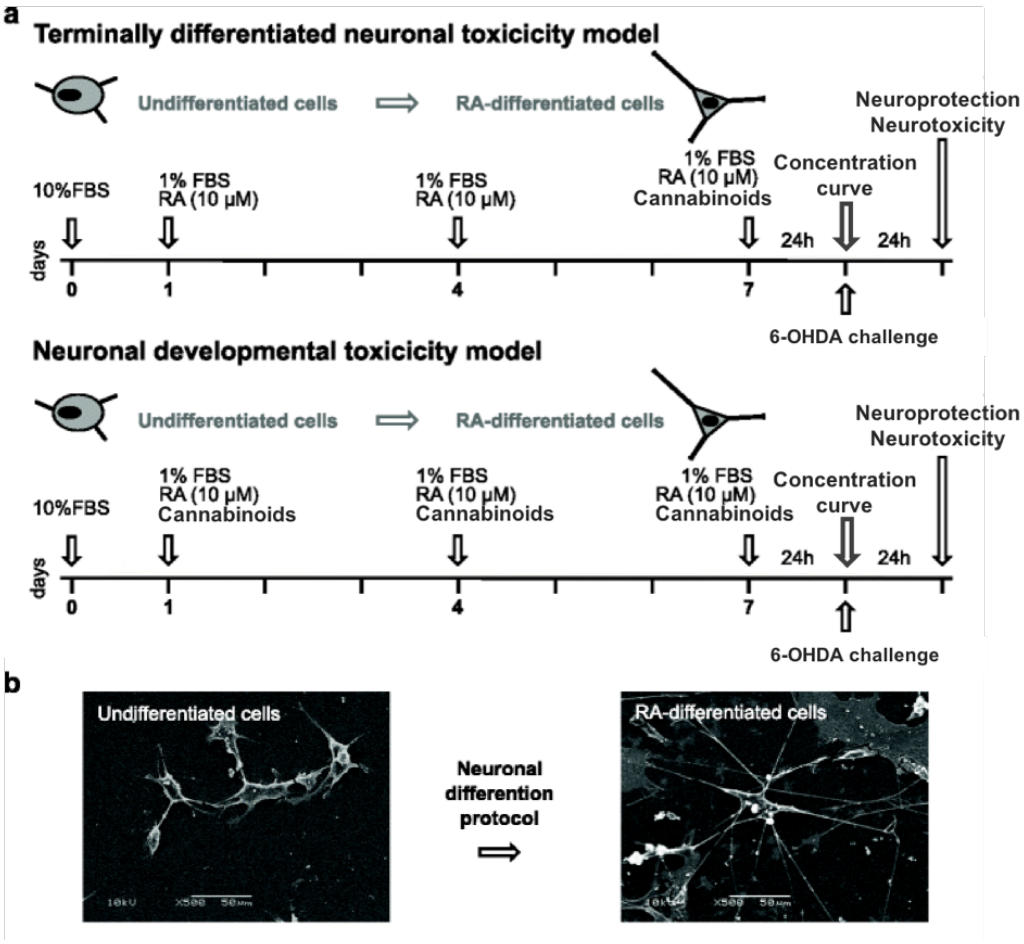


Fig 2

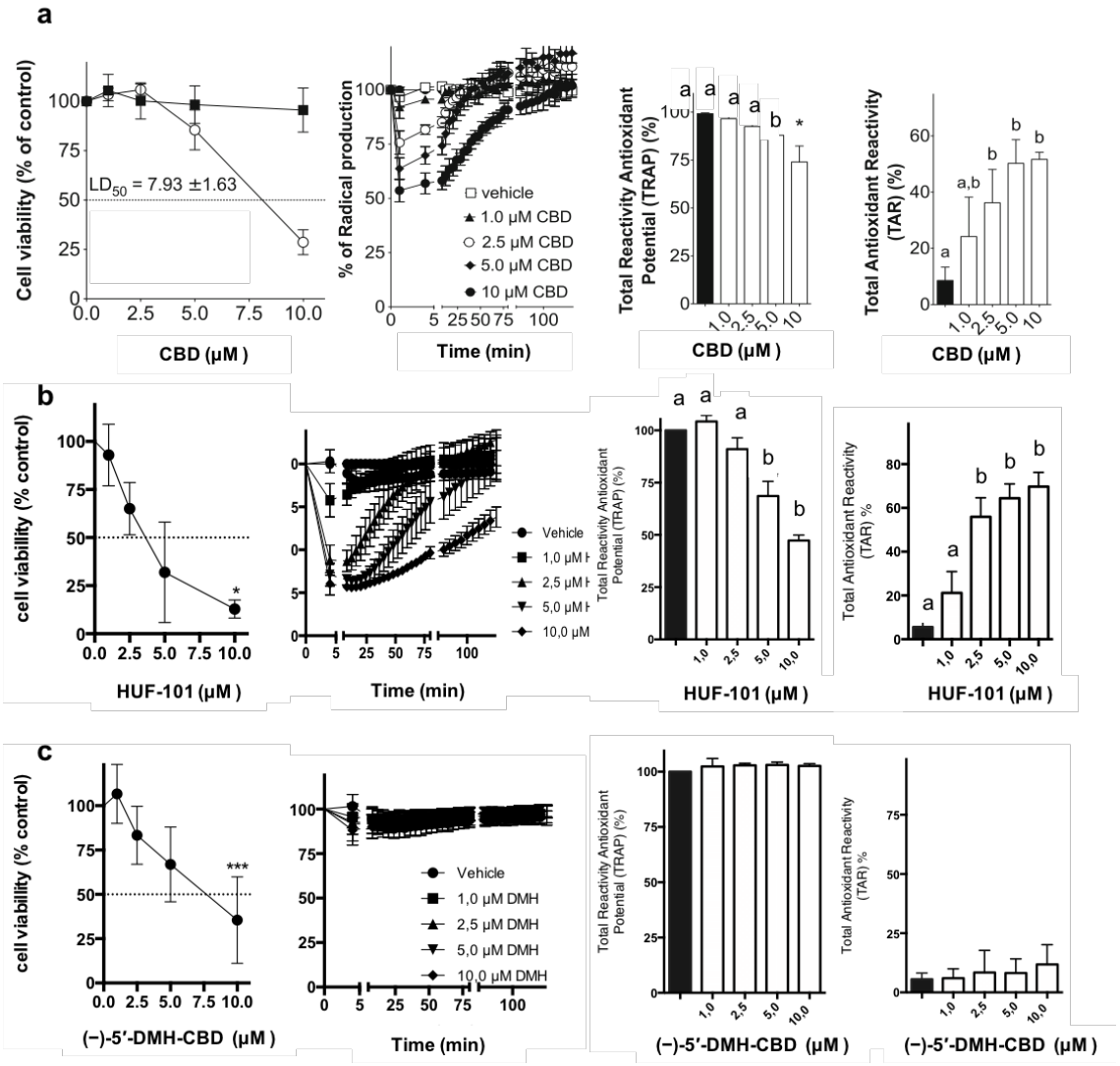

a
Cell viability (% of control)
LD50 = 7.93 ±1.63
CBD (μM )
% of Radical production
vehicle
1.0 μM CBD
2.5 μM CBD
5.0 μM CBD
10 μM CBD
Time (min)
Total Reactivity Antioxidant Potential (TRAP) (%)
CBD (μM )
Total Antioxidant Reactivity (TAR) (%)
CBD (μM )
b
cell viability (% control)
HUF-101 (μM )
Time (min)
Vehicle
Total Reactivity Antioxidant Potential (TRAP) (%)
HUF-101 (μM )
Total Antioxidant Reactivity (TAR) %
HUF-101 (μM )
c
cell viability (% control)
(−)-5′-DMH-CBD (μM )
Time (min)
Vehicle
1,0 μM DMH
2,5 μM DMH
5,0 μM DMH
10,0 μM DMH
Total Reactivity Antioxidant Potential (TRAP) (%)
(−)-5′-DMH-CBD (μM )
Total Antioxidant Reactivity (TAR) %
(−)-5′-DMH-CBD (μM )

Fig 3

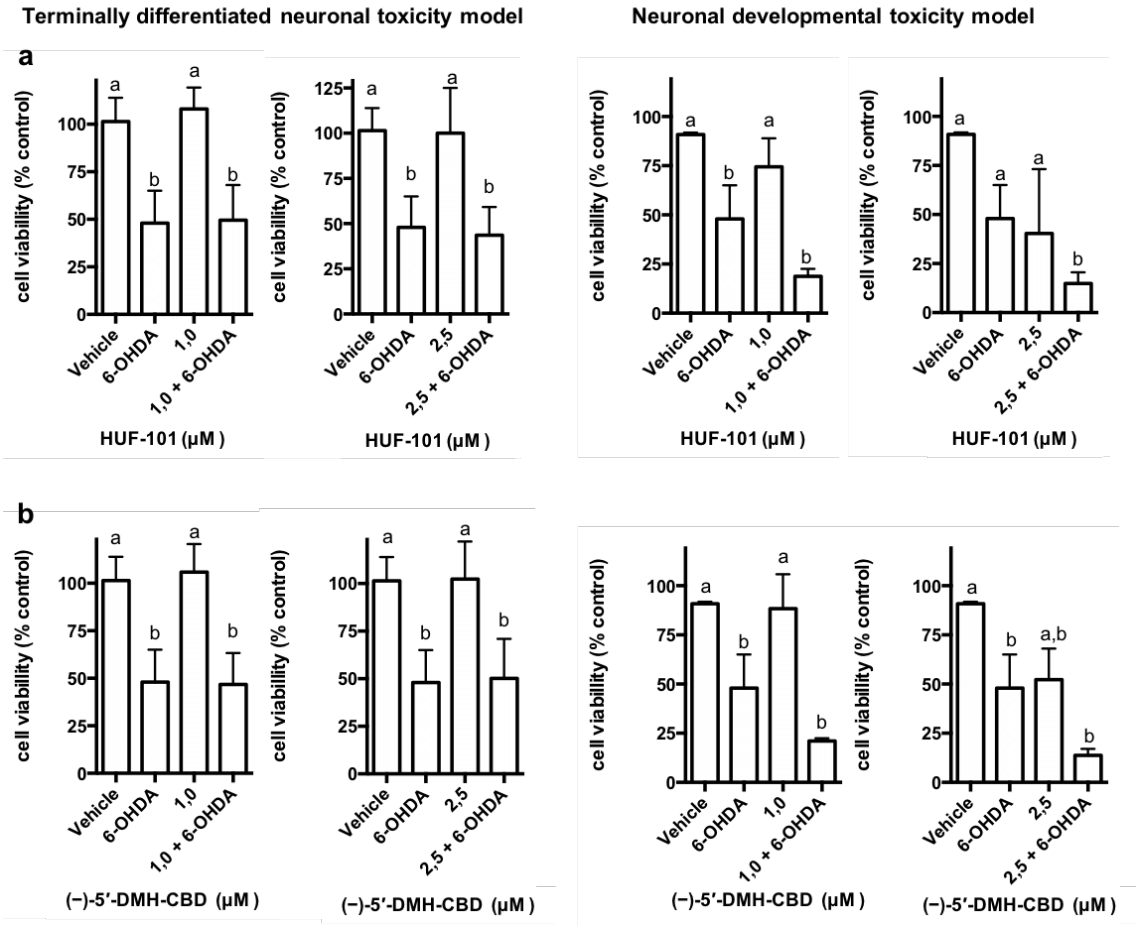

Terminally differentiated neuronal toxicity model
Neuronal developmental toxicity model
a
b
cell viability (% control)
Vehicle
6-OHDA
1,0
1,0 + 6-OHDA
2,5
2,5 + 6-OHDA
HUF-101 (μM)
(−)-5′-DMH-CBD (μM)

Fig 4

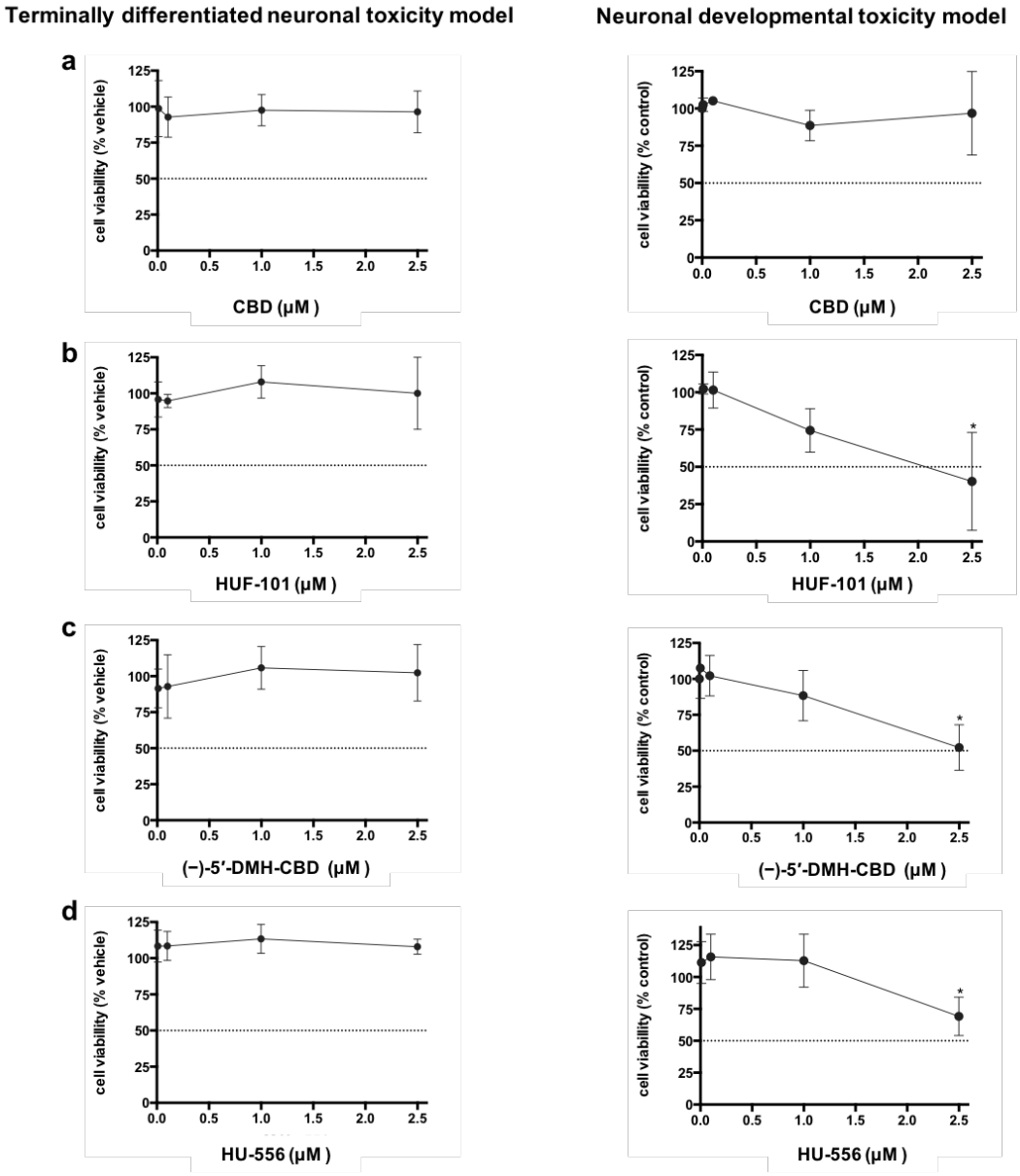
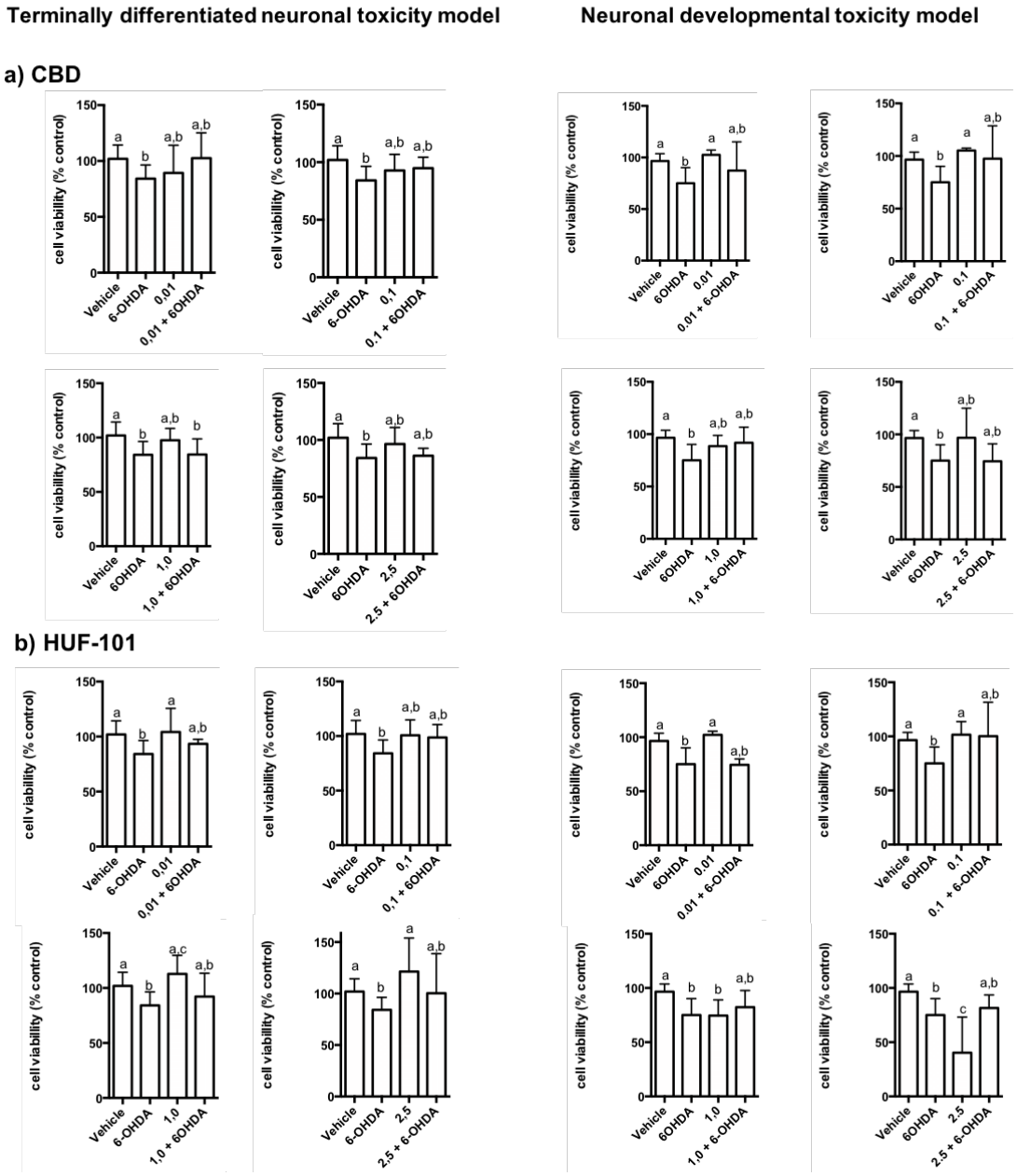

Terminally differentiated neuronal toxicity model
Neuronal developmental toxicity model
a
b
c
d
cell viability (% vehicle)
cell viability (% control)
CBD (μM )
HUF-101 (μM )
(−)-5′-DMH-CBD (μM )
HU-556 (μM )
0
25
50
75
100
125
0.0
0.5
1.0
1.5
2.0
2.5
*

Fig 5


Terminally differentiated neuronal toxicity model
Neuronal developmental toxicity model
a) CBD
b) HUF-101
cell viability (% control)
Vehicle
6-OHDA
0,01
0,01 + 6OHDA
0,1
0,1 + 6OHDA
1,0
1,0 + 6OHDA
2,5
2,5 + 6-OHDA

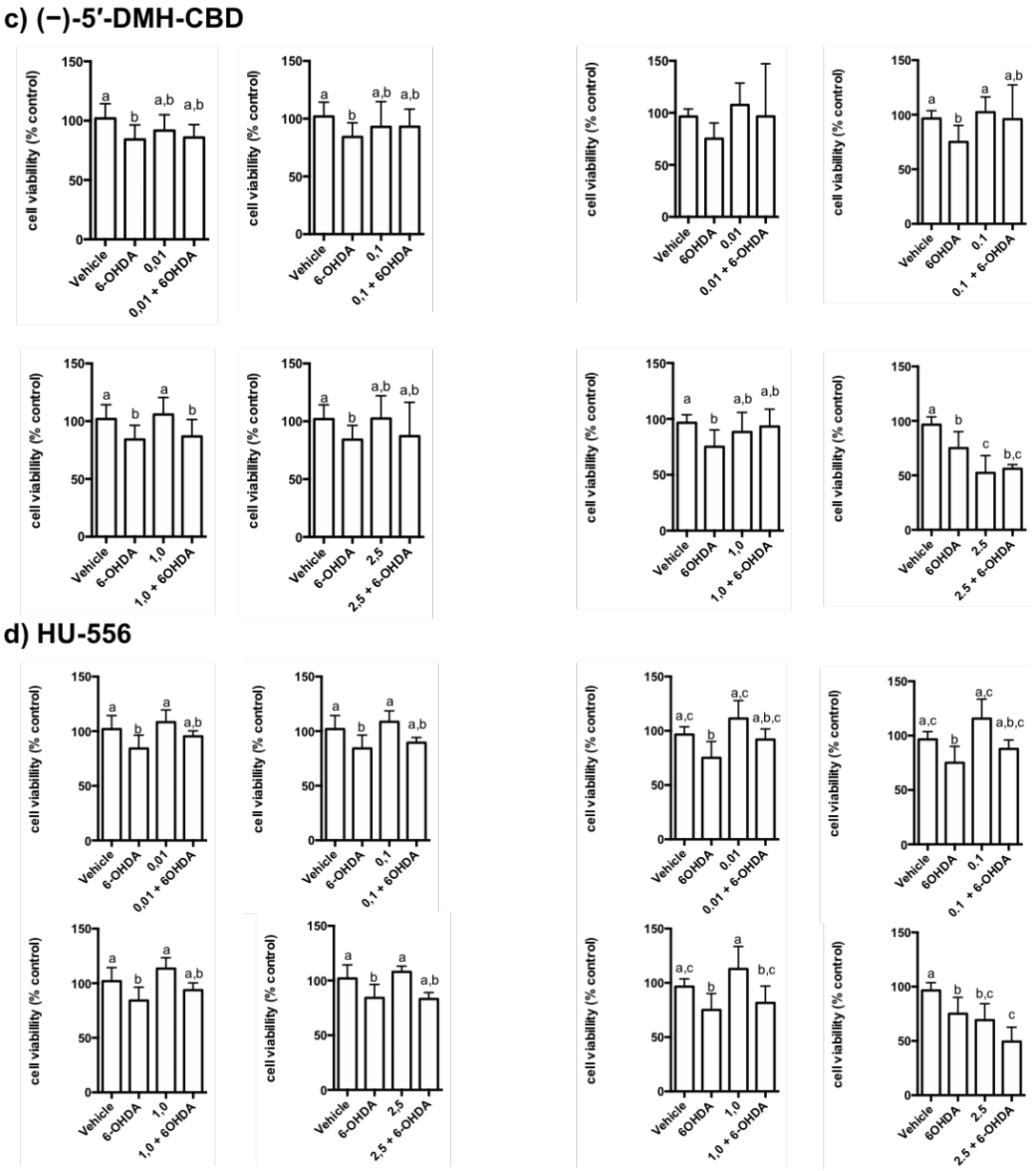
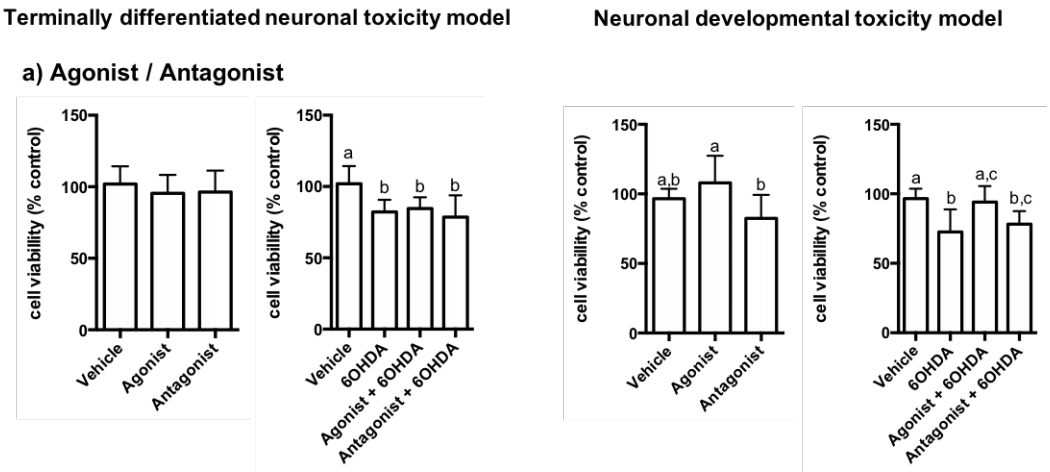
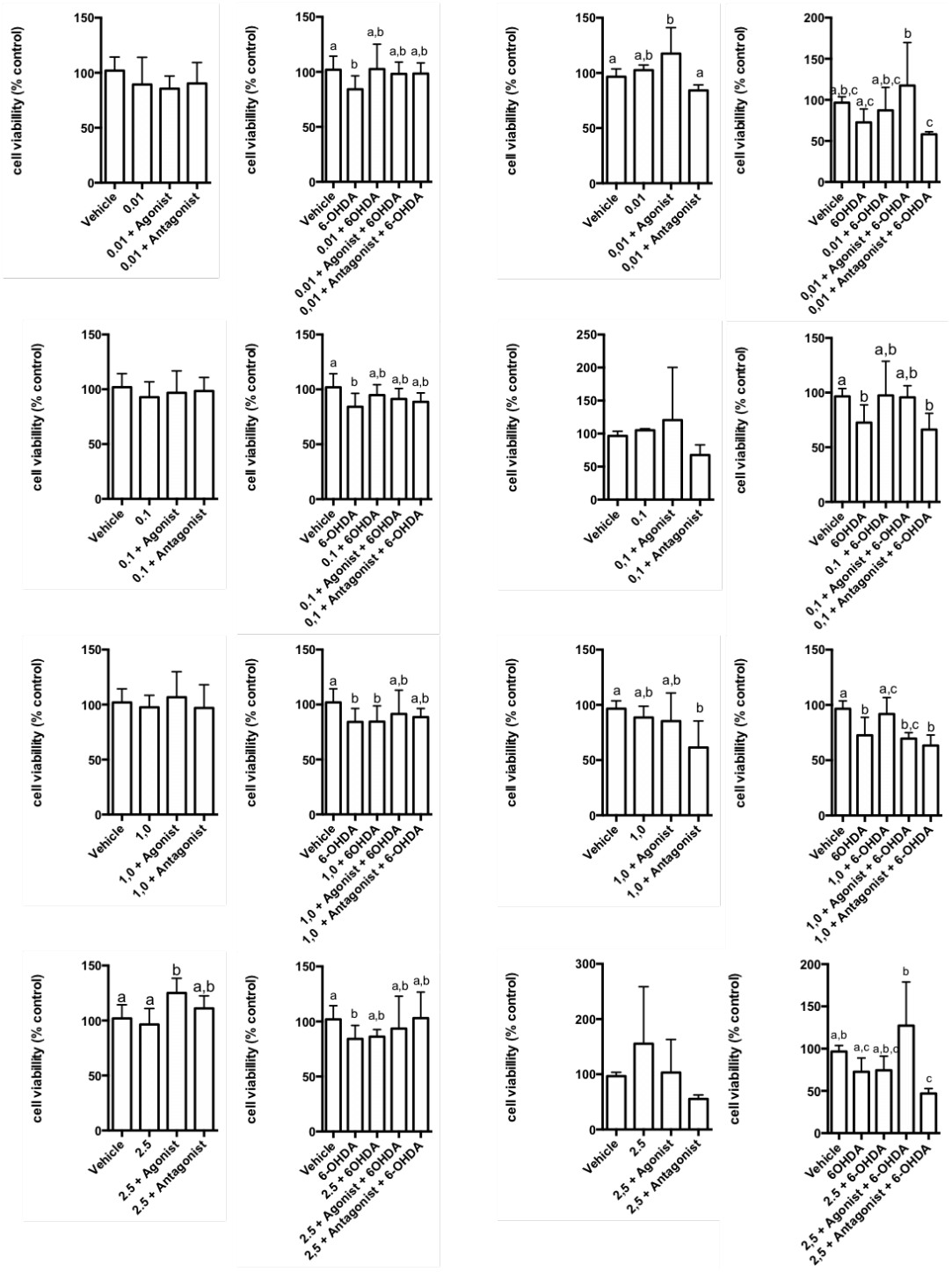

c) (−)-5′-DMH-CBD
d) HU-556
cell viability (% control)
Vehicle
6-OHDA
0,01
0,01 + 6OHDA
0,1
0,1 + 6OHDA
1,0
1,0 + 6OHDA
2,5
2,5 + 6-OHDA
0.01
0.01 + 6-OHDA
0.1
0.1 + 6-OHDA

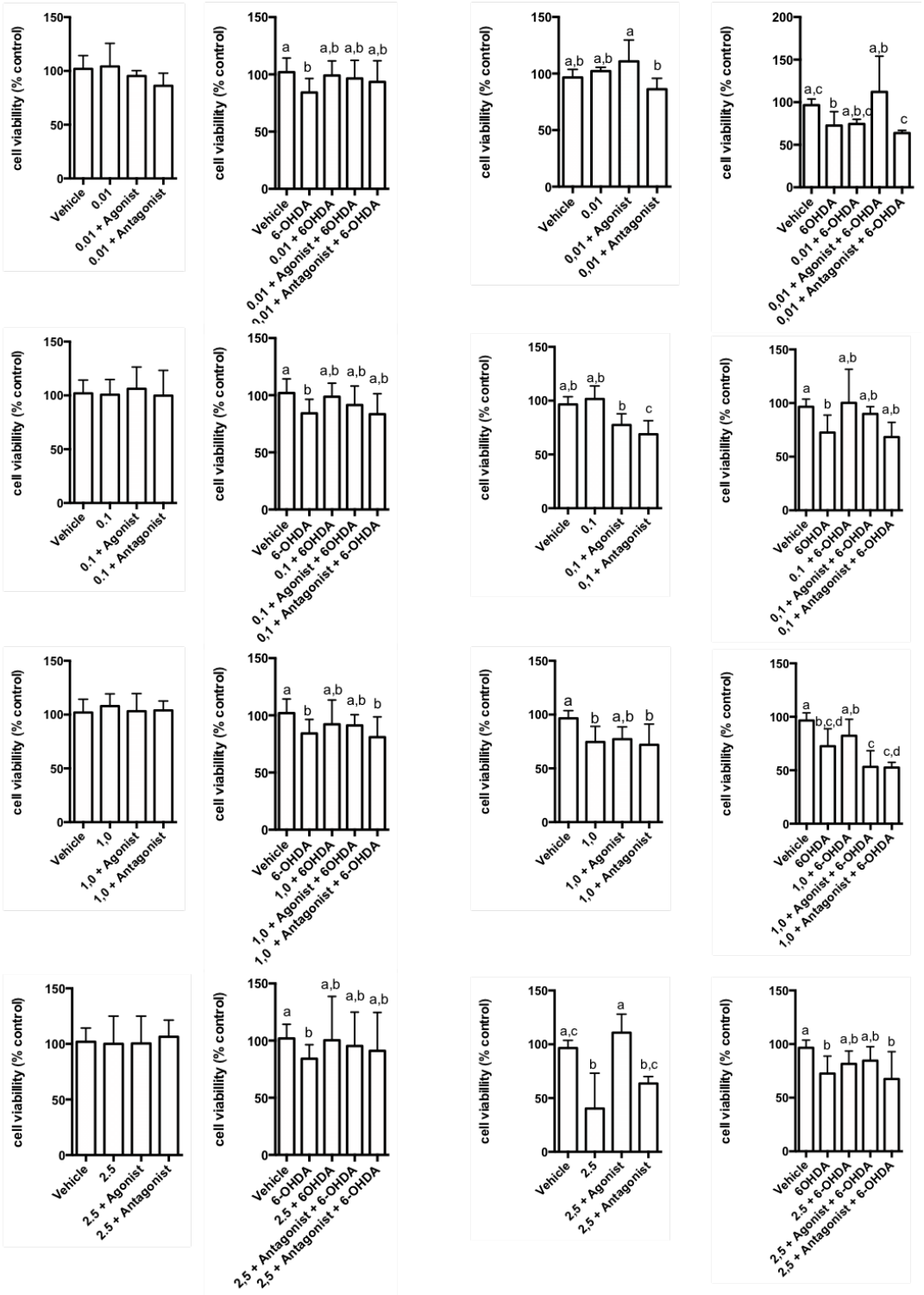
Fig 6


Terminally differentiated neuronal toxicity model
Neuronal developmental toxicity model
a) Agonist / Antagonist
cell viability (% control)
Vehicle
Agonist
Antagonist
6OHDA
Agonist + 6OHDA
Antagonist + 6OHDA

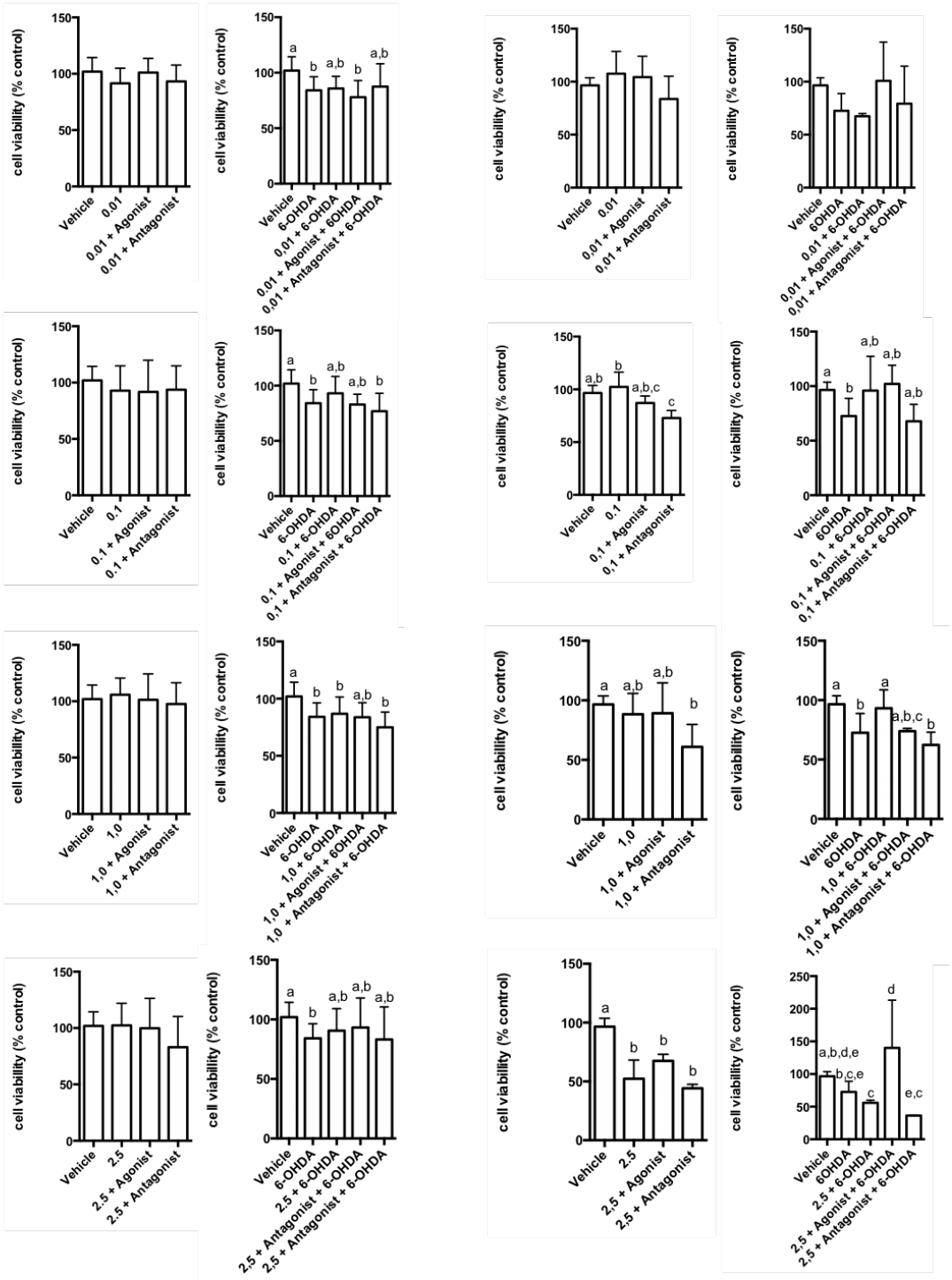
**b) CBD**


cell viability (% control)
Vehicle
0.01
0.01 + Agonist
0.01 + Antagonist
Vehicle
6-OHDA
0.01 + 6OHDA
0.01 + Agonist + 6OHDA
0,01 + Antagonist + 6-OHDA
Vehicle
0.01
0,01 + Agonist
0,01 + Antagonist
Vehicle
6OHDA
0,01 + 6-OHDA
0,01 + Agonist + 6-OHDA
0,01 + Antagonist + 6-OHDA
Vehicle
0.1
0.1 + Agonist
0.1 + Antagonist
Vehicle
6-OHDA
0.1 + 6OHDA
0.1 + Agonist + 6OHDA
0,1 + Antagonist + 6-OHDA
Vehicle
0.1
0,1 + Agonist
0,1 + Antagonist
Vehicle
6OHDA
0,1 + 6-OHDA
0,1 + Agonist + 6-OHDA
0,1 + Antagonist + 6-OHDA
Vehicle
1,0
1,0 + Agonist
1,0 + Antagonist
Vehicle
6-OHDA
1,0 + 6OHDA
1,0 + Agonist + 6OHDA
1,0 + Antagonist + 6-OHDA
Vehicle
1,0
1,0 + Agonist
1,0 + Antagonist
Vehicle
6OHDA
1,0 + 6-OHDA
1,0 + Agonist + 6-OHDA
1,0 + Antagonist + 6-OHDA
Vehicle
2.5
2.5 + Agonist
2.5 + Antagonist
Vehicle
6-OHDA
2.5 + 6OHDA
2.5 + Agonist + 6OHDA
2.5 + Antagonist + 6-OHDA
Vehicle
2.5
2.5 + Agonist
2.5 + Antagonist
Vehicle
6OHDA
2.5 + 6-OHDA
2.5 + Agonist + 6-OHDA
2.5 + Antagonist + 6-OHDA

## c) HUF-101


cell viability (% control)
Vehicle
0.01
0.01 + Agonist
0.01 + Antagonist
6-OHDA
0.01 + 6OHDA
0.01 + Agonist + 6OHDA
0,01 + Antagonist + 6-OHDA
0,01 + Agonist + 6-OHDA
0.1
0.1 + Agonist
0.1 + Antagonist
0,1 + Agonist + 6-OHDA
0,1 + Antagonist + 6-OHDA
1,0
1,0 + Agonist
1,0 + Antagonist
1,0 + Agonist + 6-OHDA
1,0 + Antagonist + 6-OHDA
2,5
2,5 + Agonist
2,5 + Antagonist
2,5 + Antagonist + 6-OHDA
2,5 + Agonist + 6-OHDA

**d) (−)-5′-DMH-CBD**


cell viability (% control)
Vehicle
0,01
0,01 + Agonist
0,01 + Antagonist
6-OHDA
0,01 + 6-OHDA
0,01 + Agonist + 6-OHDA
0,01 + Antagonist + 6-OHDA
0,1
0,1 + Agonist
0,1 + Antagonist
0,1 + 6-OHDA
0,1 + Agonist + 6-OHDA
0,1 + Antagonist + 6-OHDA
1,0
1,0 + Agonist
1,0 + Antagonist
1,0 + 6-OHDA
1,0 + Agonist + 6-OHDA
1,0 + Antagonist + 6-OHDA
2,5
2,5 + Agonist
2,5 + Antagonist
2,5 + 6-OHDA
2,5 + Agonist + 6-OHDA
2,5 + Antagonist + 6-OHDA

**e) HU-556**

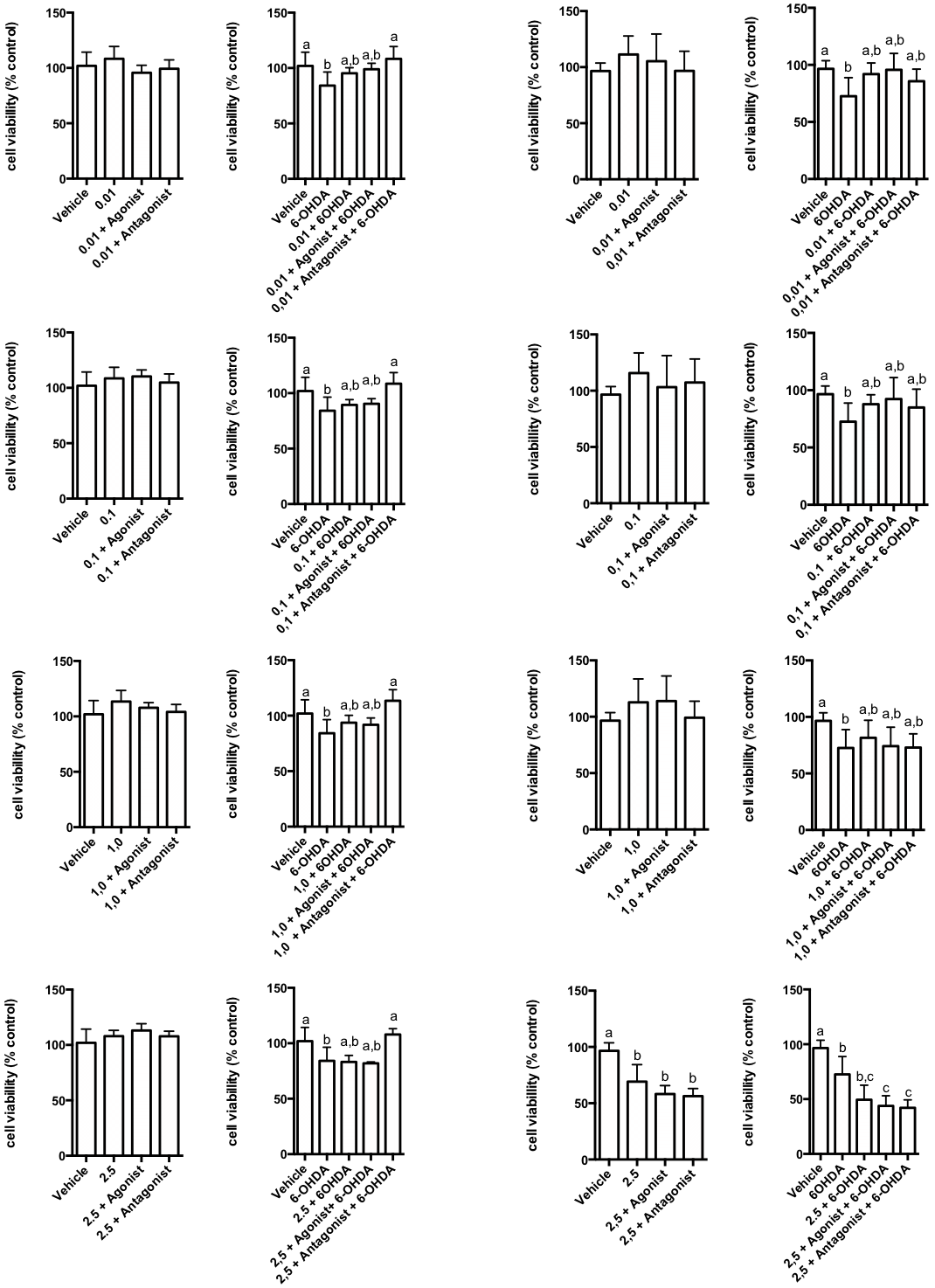

cell viability (% control)
150
100
50
0
Vehicle
0,01
0,01 + Agonist
0,01 + Antagonist
a
b
a,b
a,b
a
Vehicle
6-OHDA
0,01 + 6OHDA
0,01 + Agonist + 6OHDA
0,01 + Antagonist + 6-OHDA
0,1
0,1 + Agonist
0,1 + Antagonist
0,1 + 6OHDA
0,1 + Agonist + 6OHDA
0,1 + Antagonist + 6-OHDA
1,0
1,0 + Agonist
1,0 + Antagonist
1,0 + 6OHDA
1,0 + Agonist + 6OHDA
1,0 + Antagonist + 6-OHDA
2,5
2,5 + Agonist
2,5 + Antagonist
2,5 + 6OHDA
2,5 + Agonist + 6OHDA
2,5 + Antagonist + 6-OHDA
a,b
b,c
c

Fig 7

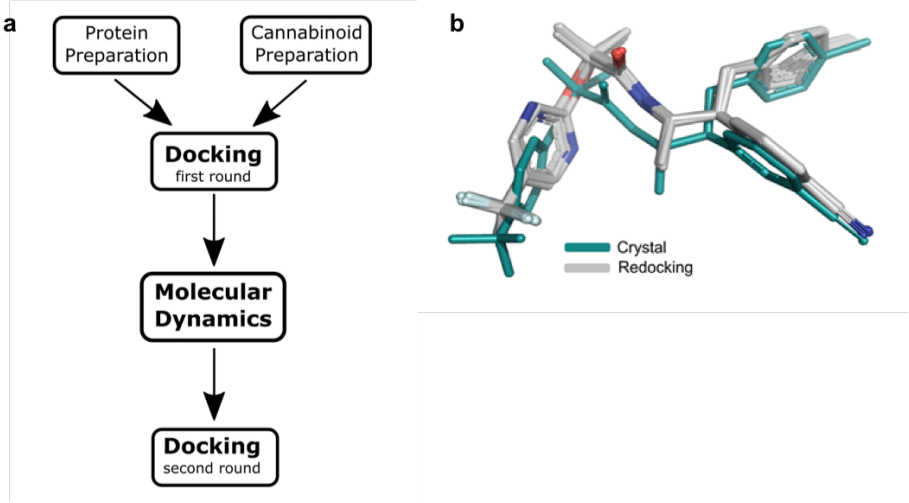

a
Protein Preparation
Cannabinoid Preparation
Docking
first round
Molecular Dynamics
Docking
second round
b
Crystal
Redocking

Fig 8

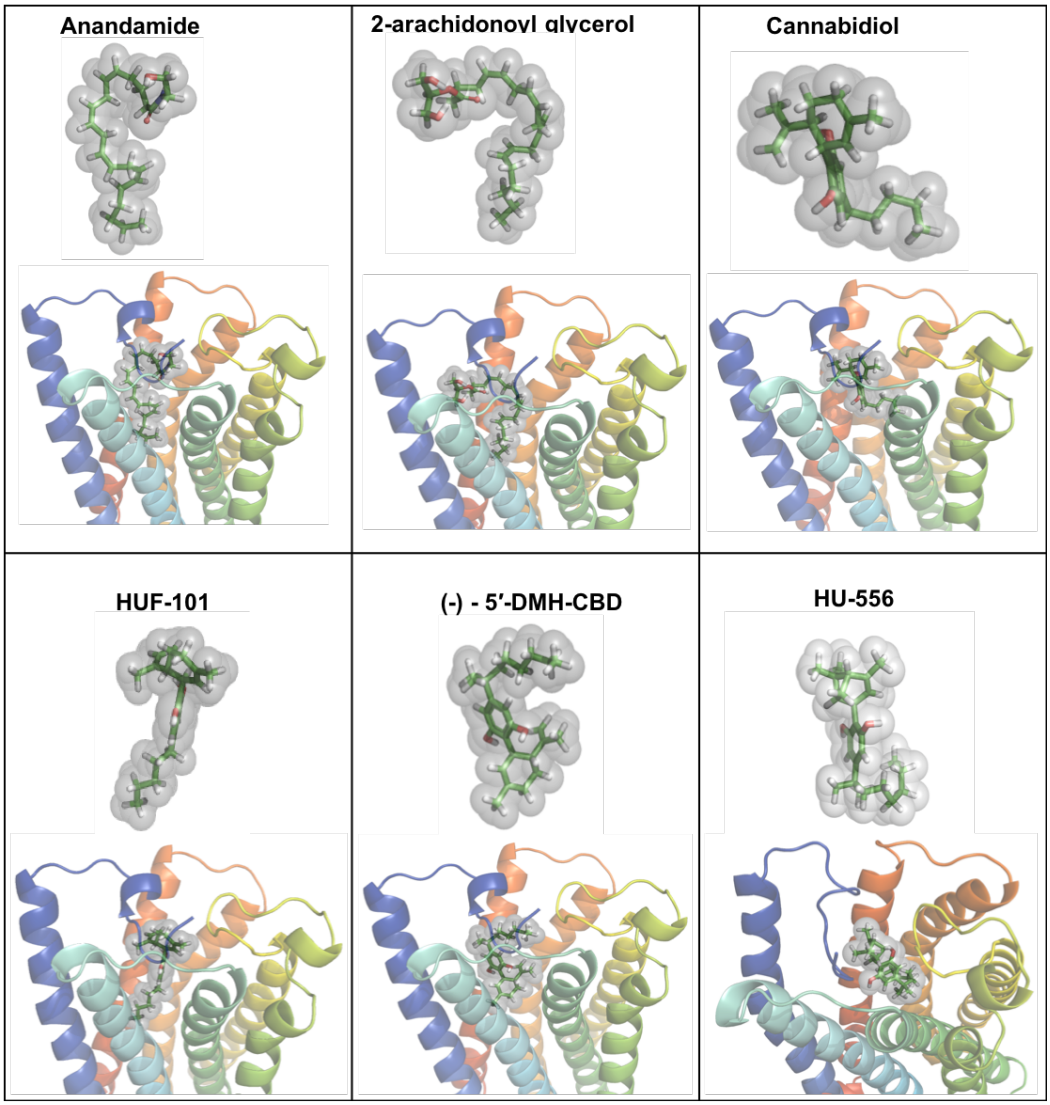

Anandamide
2-arachidonoyl glycerol
Cannabidiol
HUF-101
(-) - 5'-DMH-CBD
HU-556

**Table 1.** Energy of ligation, calculated and experimental Ki for each cannabinoid.

| ***Cannabinoid*** | ***Energy (Kcal/mol)*** | ***Ki (nM)*** | ***Experimental Ki (nM)*** |
|---|---|---|---|
| 2-arachidonoylglycerol | -8.09 | 1170.00 | 1000-10000 (Hillard, 2000); 3426.6 (McPartland et al., 2007) |
| Anandamide | -9.33 | 144.12 | 50-100 (Hillard, 2000) 239.20 (McPartland et al., 2007) |
| Cannabidiol | -9.32 | 146,40 | 2000 – 10000 (Pertwee and Ross, 2002) |
| HUF-101 | -8.76 | 381.39 | No experimental data |
| (−)-5′-DMH-CBD | -10.46 | 21.68 | >10000 (Bisogno et al., 2001) |
| HU-556 | -9.50 | 108.64 | No experimental data |

# Parte III

## 4. Discussão

Na introdução desta tese revisamos as diversas propriedades atribuídas ao CBD em diversas enfermidades, incluindo os numerosos estudos que atribuem a ele propriedades anticonvulsivantes (Campbell et al., 2017), talvez o uso mais proeminente do CBD atualmente, comprovado por estudos clínicos com pacientes de diversas idades que fizeram uso de CBD puro ou em extratos de *Cannabis* enriquecidos (Schonhofen et al., 2018). Mesmo que de uma forma geral os resultados obtidos sejam positivos, tais estudos também relatam efeitos adversos e mesmo ausência de efetividade, principalmente no uso de extratos de *Cannabis* enriquecidos com CBD, além da falta de acompanhamento a longo prazo.

Como o SEC, alvo direto ou indireto da ação de canabinoides como o CBD, tem um papel primordial no desenvolvimento SNC, o uso destes canabinoides em crianças em idades de intensa maturação do cérebro é preocupante.

Em humanos, a migração de neurônios imaturos ao longo da zona subventricular tem seu pico no início do período pós-natal até os 18 meses de idade. Enquanto a proliferação das sinapses começa por volta das 20 semanas de gestação, a densidade aumenta rapidamente após o nascimento, particularmente nos primeiros meses pós-natais, para atingir um nível aproximadamente 50% superior ao observado em adultos aos 2 anos de idade. Além disto, a neurogênese cortical ocorre predominantemente durante a gestação, mas pode continuar até os 2,5 anos de idade (Semple et al., 2013).

Portanto, os dois primeiros anos de vida humana correspondem a um período de intensa maturação do SNC. Neste período, perturbações como crises epilépticas recorrentes podem levar a danos irreversíveis devido a alterações nas redes neurais que favorecem a emergência de várias desordens neurológicas e psiquiátricas, incluindo

comportamento emocional, humor e cognição alterados (Dawson et al., 2014), bem como distúrbios na consolidação da memória e aprendizado (Lee et al., 2004).

Esse período (primeiros meses de vida) coincide com o início dos sintomas das síndromes epilépticas refratárias infantis, como a Síndrome de Dravet (Genton et al., 2011). Epilepsias refratárias infantis são caracterizadas por crises recorrentes, com alta frequência, e que se iniciam nos primeiros anos de vida e que não respondem a drogas antiepilépticas, sendo causadas por síndromes genéticas, traumas ou mesmo causas idiopáticas (Aneja and Jain, 2014). Devido a esta falta de resposta aos medicamentos convencionais, a busca por tratamentos que sejam capazes de reduzir ou mesmo eliminar as crises em crianças é de primordial importância, uma vez que essas crises comprometem o desenvolvimento e a qualidade de vida, além de aumentarem o risco de morte prematura (Genton et al., 2011).

Assim como perturbações causadas por enfermidades, pode-se inferir que drogas que afetem os sistemas reguladores da maturação do SNC, como o SEC, possam interferir no processo de diferenciação e migração de neurônios e outros tipos celulares, ou mesmo na formação e consolidação de vias e redes sinápticas. Os efeitos negativos da *Cannabis* e de alguns canabinoides isolados, como o $\Delta^9$-THC, já são bem conhecidos. Por exemplo, o consumo pré-natal de *Cannabis* pela mãe afeta o desenvolvimento do feto, enquanto que na infância, há impacto negativo nos resultados cognitivos ou comportamentais (Huizink, 2014). Os canabinoides também podem comprometer todos os estágios da memória incluindo codificação, consolidação e recuperação (Ranganathan e D'Souza 2006), devido a efeitos hiperativadores do sistema endocanabinoide que promovem LTD (Han et al., 2012). O consumo de *Cannabis* durante a adolescência também aumenta o risco de ocorrência de transtornos psicóticos, como a esquizofrenia, na vida adulta (Bossong and Niesink, 2010). No entanto, tais efeitos deletérios da *Cannabis* estão associados ao $\Delta^9$-

THC, e o CBD é capaz de contrapor ao menos parcialmente tais efeitos (Niesink and van Laar, 2013). Ainda assim, para o CBD isolado, ainda existem poucos dados em humanos (O'Connell et al., 2017).

Apesar dos inegáveis benefícios que o CBD parece trazer para crianças com epilepsias refratárias, sua possível ação sobre o desenvolvimento normal do SNC (através do SEC) pode levar a efeitos indesejados quando administrado em pacientes muito jovens. Por isso, como parte desta tese, foi realizada uma revisão bibliográfica acerca da efetividade e segurança do CBD em estudos clínicos, seus possíveis mecanismos de ação envolvendo o SEC e perspectivas terapêuticas. Tais informações geraram um artigo recentemente publicado, no qual opinamos que o CBD puro é mais recomendável em detrimento aos extratos de *Cannabis* (que contêm $\Delta^9$-THC) em pacientes infantis com epilepsia refratária (Schonhofen et al., 2018). Além disto, recomendamos que mais estudos clínicos com maiores períodos de tratamento e acompanhamento dos pacientes ainda são necessários, bem como a definição das doses e idades seguras para o uso do CBD (Schonhofen et al., 2018).

Ressaltamos também que os mecanismos de ação do CBD tanto no SNC saudável quanto em pacientes epilépticos ainda não estão totalmente esclarecidos. Apesar da provável baixa atividade direta sobre o receptor CB1, o CBD é capaz de alterar os níveis de canabinoides endógenos que atuam sobre este receptor (Lu and MacKie, 2016; Schonhofen et al., 2018). Além do SEC, o CBD atua em muitos outros alvos, uma lista que aumenta conforme as pesquisas com este canabinoide avançam, indicando que outros alvos ainda desconhecidos podem ter participação nos efeitos terapêuticos ou mesmo nos efeitos adversos do CBD.

Ainda que já existam estudos clínicos e que o CBD já tenha sido aprovado pelas autoridades de saúde para o tratamento de epilepsias refratária infantis, a elucidação dos

mecanismos de ação de uma droga ou composto ainda depende de estudos pré-clínicos, tanto em modelos animais quanto em nível celular.

O uso de modelos experimentais na ciência traz inúmeros benefícios, mas torna-se um grande desafio quando se pretende investigar o desenvolvimento do SNC. As diferenças entre os modelos animais e humanos devem ser consideradas quando se compara a idade de maturação do SNC tanto durante o desenvolvimento normal quanto em doenças que ocorrem durante esta maturação. Em geral, utiliza-se roedores como modelo animal, nos os quais o período crítico de sinaptogênese ocorre durante as primeiras três semanas de vida pós-natal, com seu auge durante a segunda semana (Semple et al., 2013). A densidade sináptica no córtex de ratos e camundongos é baixa na primeira semana pós-natal, seguida de um aumento abrupto a partir do PND10 (dia pós-natal, do inglês *post natal day*), para alcançar a equivalência com números adultos até PND30. Portanto, em roedores, o período entre o PND7 e PND21 é equivalente aos 2 primeiros anos de vida para humanos no que se refere a maturação do SNC. Ainda que nosso foco sejam esses primeiros meses de vida pós-natal, o cérebro humano continua a se desenvolver até a segunda década de vida (Bossong and Niesink, 2010; Mechoulam and Parker, 2013), com taxas mais baixas de neurogênese e migração celular. Em roedores, esta fase (final da adolescência) corresponde ao período entre o PND35 ao PND49 (Semple et al., 2013).

Apesar do uso crescente de CBD em crianças e adolescentes cujos cérebros ainda estão em desenvolvimento, a maioria dos estudos *in vitro* e *in vivo* usa células maduras ou modelos animais adultos e, portanto, não mimetizam peculiaridades do SNC juvenil. Pensando nesta lacuna experimental, nesta tese os efeitos anticonvulsivantes do CBD foram verificados em um modelo *in vivo* para maior susceptibilidade a crises epilépticas. Neste modelo, investigamos também o mecanismo de ação mais aceito atualmente para o

CBD no tratamento de crianças epilépticas, o aumento dos níveis de ativação do SEC (níveis de AEA e 2-AG). Além do modelo animal, o CBD e três de seus derivados sintéticos também foram testados em um modelo *in vitro* de neurônio humano maduro e em desenvolvimento. Em ambos, foi possível comparar os efeitos apresentados em fases iniciais e finais do desenvolvimento neuronal.

O modelo animal foi desenhado para mimetizar os aspectos clínicos de crianças epilépticas já que um episódio de hipóxia neonatal faz com que os animais tenham maior susceptibilidade a crises epilépticas induzidas farmacologicamente no futuro e que sejam mais refratários a anticonvulsivantes (Leonard et al., 2013). Lesões causadas por hipóxia-isquemia durante desenvolvimento podem levar à epileptogênese e existem vários modelos experimentais (roedores em PND7) que utilizam esta abordagem para testar possíveis tratamentos (Semple et al., 2013).

Em nosso modelo, camundongos foram submetidos à hipóxia global no PND7, que corresponde ao início da infância em humanos, e comparados com animais normóxicos. Foram avaliados a susceptibilidade a crises epilépticas e níveis hipocampais de endocanabinoides em animais com 15 dias de vida, equivalendo aos primeiros meses de vida humana, e 44 dias de vida, equivalendo ao final da adolescência humana, ou seja, uma idade em que o cérebro ainda se desenvolve em altas taxas e quando o cérebro já pode ser considerado maduro. Em seguida, avaliamos os efeitos da administração aguda de 10 mg/kg CBD sobre estes parâmetros.

Os animais neonatos hipóxicos foram mais suscetíveis a crises epilépticas induzidas e não apresentaram alterações nos níveis de endocanabinoides no hipocampo. Com 44 dias, não houve alterações comportamentais, mas a hipóxia já foi suficiente para aumentar os níveis de AEA no hipocampo. Corroborando nossos dados, AEA e/ou 2-AG geralmente

aumentam em doenças humanas e modelos animais para doenças, incluindo crises epilépticas induzidas por KA (Toczek and Malinowska, 2018).

Nosso modelo animal reproduziu o aumento da suscetibilidade a crises epilépticas na infância, mas não em adolescentes, o que está de acordo com a literatura, já que as crises epilépticas tendem a se tornar menos frequentes e menos graves após a infância (Genton et al., 2011).

Em nosso modelo, o CBD não protegeu contra crises epilépticas quimicamente induzidas e aumentou a susceptibilidade a crises em camundongos normóxicos no PND15. Isso significa que uma dose aguda baixa de CBD pode ser perigosa em animais saudáveis (que não passaram pelo evento de hipóxia neonatal) muito jovens e ineficaz para o modelo de doença. No entanto, em um estudo prévio, uma dose maior de CBD (100 mg / kg) foi protetora contra crises epilépticas quando administrada de PND21 a PND27 em um modelo genético de camundongo para a Síndrome de Dravet (Kaplan et al., 2017), o que pode sugerir um efeito específico da dose selecionada. Devemos levar em conta ainda que em estudos clínicos e em relatos de uso em pacientes, a dose utilizada e considerada segura de CBD é de cerca de 25 mg/kg/dia (Szaflarski et al., 2018), e que o tratamento geralmente se inicia em cerca de 5 mg/kg/dia. Assim, mesmo que a dose aqui utilizada seja comparativamente baixa, ela é ainda alta se considerarmos que ela representa o dobro da dose inicial, o que pode estar relacionado com a piora/ausência de proteção às crises quimicamente induzidas. Ademais, em um teste clínico recente com pacientes com Síndrome de Dravet, o CBD mostrou-se bem tolerado em doses de 5 a 20 mg/kg/dia, apesar de o período curto de tratamento (3 semanas) e da presença de efeitos colaterais (Devinsky et al., 2018b). Entretanto, os pacientes randomizados neste estudo tinham de 4 a 10 anos, uma idade em que o desenvolvimento neuronal já está mais consolidado.

*In vivo*, os efeitos benéficos do CBD em doses altas (100 mg / kg) estão correlacionados com a neurotransmissão GABAérgica aumentada, diminuição da razão de excitação / inibição e diminuição do potencial de disparo de neurônios excitatórios em resposta a estímulos fortes (Kaplan et al., 2017). Essas alterações na neurotransmissão GABAérgica são mimetizadas e ocluídas pela inibição do receptor GPR55 acoplado à proteína G ativada por lipídios, sugerindo que os efeitos do CBD são mediados, pelo menos em parte, por essa via de transdução de sinal (Kaplan et al., 2017). Uma diferença fundamental entre o cérebro imaturo e adulto é uma mudança de maturação nas ações de sinalização do neurotransmissor ácido gama-aminobutírico (GABA). A ativação do receptor GABAA em neurônios imaturos desencadeia a despolarização e a excitação, em comparação com o papel inibitório clássico desse sistema no cérebro adulto (Ben-Ari et al., 2012). É possível que o GABA excitatório no cérebro imaturo desempenhe um papel crucial em muitos processos de desenvolvimento, incluindo a diferenciação neuronal e a arborização dendrítica (Semple et al., 2013). Como o CBD parece exercer seus efeitos sobre neurônios GABAérgicos em um modelo genético para Síndrome de Dravet (Kaplan et al., 2017), o GABA excitatório em cérebros imaturos pode estar ligado aos efeitos terapêuticos do CBD. Entretanto, este neurotransmissor começa a agir como inibitório no final da segunda semana pós-natal no córtex de rato e em cerca de 40 dias pós-concepção no córtex humano (Ben-Ari et al., 2012), o que representa uma diferença importante entre o desenvolvimento neuronal humano e em modelos animais. Isso pode representar uma maior dificuldade em correlacionar os resultados obtidos em modelos murinos com as observações clínicas dos efeitos do CBD.

É importante ressaltar que em nossos resultados, o CBD foi capaz de aumentar os níveis de AEA apenas em animais PND15, o que associa níveis de hipocampo AEA aumentados a um aumento na susceptibilidade a crises epilépticas (em indivíduos

saudáveis). Em outras palavras, uma dose baixa de CBD pode levar a uma modulação dos endocanabinoides em cérebros ainda em desenvolvimento. Uma vez que a plasticidade sináptica e o desenvolvimento do SNC são dependentes da sinalização endocanabinoide, essa seletividade do CBD relacionada à idade pode representar um risco para sua administração em crianças com menos de 2 anos.

A modulação do SEC já demonstrou efeitos anticonvulsivantes em vários modelos, como revisado por Rosenberg et al (2017), através da inibição da recaptação de AEA por exemplo: a administração de um inibidor de recaptação de AEA reduziu a severidade, a duração das crises epilépticas induzidas por KA; outro inibidor de recaptação de AEA não exerceu efeito significativo sobre as crises epilépticas. Estes resultados sugerem que elevar os níveis dos endocanabinoides 2-AG e AEA pode ter um efeito anticonvulsivante (ou negligenciável) que pode ser pelo menos parcialmente mediado via mecanismos dependentes de CB1 (Rosenberg et al., 2017).

Além dos vários mecanismos pelos quais o CBD pode modular o SEC, recentemente tem sido proposto que o mesmo pode melhorar os níveis endocanabinoides por competição com AEA para ligação a proteínas de ligação a ácidos graxos (FABPs) que atuam como transportadores deste endocanabinoide facilitando sua degradação (Toczek and Malinowska, 2018). Assim, a ligação do CBD às FABPs inibe a degradação e eleva os níveis de AEA, o que pode ser um dos mecanismos pelos quais o CBD atua na epilepsia infantil (Deutsch, 2016). Ao contrário dos agonistas diretos dos receptores canabinoides, os fármacos que aumentam os níveis de AEA promovem um ótimo nível de ativação endocanabinoide, uma estratégia mais seletiva, pois visa principalmente locais onde a sinalização endocanabinoide já está ativada, o que também reduz os efeitos colaterais (Vilela et al., 2013).

Em animais hipóxicos e animais normóxicos, o CBD esteve presente em maior quantidade nos animais com 44 dias. Possivelmente porque, embora os neonatos sejam mais leves, seus hipocampos têm pesos semelhantes aos hipocampos dos adolescentes - de modo que recebem proporcionalmente menos CBD no hipocampo. Ou seja, os animais mais jovens apresentaram menos CBD no hipocampo e mesmo assim sofreram alterações significativas na susceptibilidade a crises epilépticas e nos níveis de AEA. Além disto, em a absorção de drogas administradas por via subcutânea ou intramuscular não é boa nos neonatos em função do baixo fluxo regional de sangue e da baixa reserva de massa muscular (Juárez-Olguín et al., 2014). Quanto ao próprio SEC, também há diferenças entre sua função e composição em células tronco neurais e neurônios imaturos em comparação com neurônios maduros – durante a diferenciação neuronal CB2 diminui e CB1 aumenta sua expressão (Galve-Roperh et al., 2013), o que pode alterar a resposta destas células a canabinoides.

Em conclusão, nossos resultados em camundongos reforçaram a hipótese da elevação dos níveis de endocanabinoides como mecanismo de ação do CBD mesmo em uma dose não associada a proteção contra crises. Reforçamos também a hipótese levantada na nossa revisão (Capítulo I) de que, durante fases de desenvolvimento neuronal, uma dose relativamente baixa de CBD é suficiente para modular o SEC. Além disso, este mecanismo foi aqui confirmado apenas para animais jovens, o que pode explicar a eficácia deste canabinoide no tratamento de epilepsias infantis, apesar de o CBD não ter sido protetor em nossos resultados, mas também pode significar um risco aumentado para o uso do CBD no período da infância.

A falta de efetividade pode ser explicada pela dose utilizada, já que o CBD tem apresentado efeitos positivos em outros estudos com dosagens diferentes. Em doses mais baixas, por exemplo, a administração de CBD em suínos neonatos submetidos à hipóxia-

isquemia teve um efeito protetor sobre os neurônios e astrócitos, preservando a atividade cerebral, prevenindo a ocorrência de crises epilépticas e melhorando o desempenho neurológico e comportamental dos animais tratados (Alvarez et al., 2008; Lafuente et al., 2011). Em um modelo *in vitro* para danos causados por hipóxia-isquemia em cérebros de recém-nascidos, o CBD também mediou a prevenção de morte celular por necrose e apoptose (Pazos et al., 2012). Nestes casos, a administração do CBD ocorreu imediatamente após o dano, o que difere de nossa abordagem na qual o CBD foi utilizado dias após o dano cerebral (hipóxia), além de ter sido avaliado apenas seu papel neuroprotetor e não seu potencial anticonvulsivante. Em dosagens mais altas, o CBD também já apresentou resultados positivos já mencionados – por exemplo, 100 mg/kg/dia em camundongos modelo para Síndrome de Dravet (Kaplan et al., 2017). Estes os resultados prévios indicam que o mesmo seria útil em estratégias de novas terapias ou como adjuvante em terapias já estabelecidas, como a hipotermia aplicada em cérebros de recém-nascidos isquêmicos (Pazos et al., 2012). Em testes clínicos, o CBD tem apresentado efeitos benéficos tanto na melhora da frequência e severidade das crises epilépticas, quanto nos aspectos comportamentais e cognitivos de pacientes jovens e adultos, apesar da presença de efeitos adversos, ausência de efeito terapêutico ou mesmo piora dos sintomas em alguns casos (Szaflarski et al., 2018).

Entretanto, tanto fitocanabinoides quanto endocanabinoides também são associados a efeitos negativos sobre o desenvolvimento, causando, por exemplo, interrupção do desenvolvimento de embriões através da regulação CB1 (MacCarrone et al., 2000; Paria et al., 1998; Wang et al., 1999). CB1, CB2 e os endocanabinoides têm seus padrões de expressão e atividade induzidos durante o desenvolvimento de células tronco embrionárias, e o bloqueio farmacológico desses receptores induz a morte destas células, sugerindo um papel fundamental do sistema endocanabinoide e de seus reguladores

endógenos e exógenos na sobrevivência de células tronco embrionárias (Jiang et al., 2007; Oh et al., 2013), bem como em células progenitoras neurais responsáveis pela neurogênese pós embrionária (Galve-Roperh et al., 2013).

Com base neste papel regulador do SEC no desenvolvimento neuronal, e nos resultados com CBD em neonatos, em que verificamos aumento dos níveis de AEA no hipocampo, utilizamos um modelo *in vitro* para analisarmos estes possíveis efeitos em nível celular. Com um modelo *in vitro*, é também mais rápida e econômica a realização de triagens de drogas de interesse (Bal-Price et al., 2008). No contexto do desenvolvimento neuronal, a linhagem de neuroblastoma humano SH-SY5Y surge como alternativa para avaliações de neurotoxicidade, já que pode ser diferenciada em fenótipo de neurônio dopaminérgico maduro (Lopes et al., 2017, 2010), como um modelo de toxicidade em neurônios maduros, e também pode ser usada para tratamentos durante a sua diferenciação, como um modelo de toxicidade durante o desenvolvimento neuronal (Schönhofen et al., 2015).

Além do CBD, 3 de seus derivados sintéticos também foram testados neste modelo: HUF-101, (−)-5′-DMH-CBD e HU-556. Nos últimos anos, análogos sintéticos do CBD foram desenvolvidos visando aumentar seus efeitos benéficos, mas reduzindo os efeitos colaterais. Aqui, os mesmos foram avaliados em ambos os modelos celulares (neurônio maduro e durante a diferenciação) e comparados ao CBD.

Os resultados iniciais com CBD – curva de concentrações e desafio neurotóxico com $CL_{50}$ de 6-OHDA – apresentados aqui foram publicados anteriormente (Schönhofen et al., 2015), e foram retomados para facilitar a comparação com os outros canabinoides. Considerando-se que o CBD é conhecido como um potente antioxidante e o estresse oxidativo está relacionado com os mecanismos de neurodegeneração (Schapira, 2008), e supondo-se que os derivados sintéticos apresentem um perfil antioxidante semelhante ao

CBD, uma curva de concentração de HUF-101 e (−)-5′-DMH-CBD foi projetada para encontrar a concentração que apresenta alto potencial antioxidante *in vitro* com baixa neurotoxicidade em células SH-SY5Y terminalmente diferenciadas.

A concentração de 2,5 μM de HUF-101 foi selecionada para os experimentos seguintes, uma vez que não foi neurotóxica e apresentou atividade antioxidante. Além disso, incluímos 1 μM já que, embora não tenha atividade antioxidante *in vitro*, também é subletal. Embora (−)-5′-DMH-CBD não tenha apresentado atividade antioxidante em qualquer uma das concentrações, selecionamos as mesmas concentrações que para o HUF-101, pois são também subletais. Previamente, para o CBD a concentração de 2,5 μM também havia sido selecionada (Schönhofen et al, 2015). Neste estudo, foi utilizada uma abordagem livre de células para a avaliação da capacidade antioxidante dos canabinoides. Em estudos prévios, o CBD levou ao aumento de defesas antioxidantes como a glutationa (GSH) (REF) o que não se pode verificar neste tipo de abordagem.

Nossos resultados em relação à curva concentração-resposta estão de acordo com o efeito conhecido do CBD em humanos, uma vez que segue o padrão de eficácia da curva dose-efeito em forma de sino observado em muitos estudos em animais (Zuardi et al., 2017). Aqui, o mesmo ocorre para os canabinoides sintéticos, pelo menos para as concentrações mais altas.

Os efeitos neuroprotetores de cada canabinoide foram avaliados por meio do desafio com o $CL_{50}$ da neurotoxina redox ativa 6-OHDA. A 6-OHDA é uma das toxinas mais usadas em modelos experimentais para DP (Gomez-Lazaro et al., 2008; Lopes et al., 2012), por ser um análogo da dopamina com características estruturais semelhantes a este neurotransmissor e com afinidade pelo seu transportador (DAT), que acumula nos neurônios causando estresse oxidativo e dano celular (Lehmensiek et al., 2006). Como o modelo celular utilizado aqui é de neurônio dopaminérgico, espera-se que a 6-OHDA seja

capaz de exercer seus efeitos clássicos (Lopes et al, 2017). No desafio neurotóxico, ambos canabinoides não ofereceram neuroproteção em células diferenciadas, mas 2,5 μM houve potencialização da toxicidadade da 6-OHDA em células tratadas durante a diferenciação.

Uma vez que as concentrações mais baixas da testadas aqui não reproduziram a curva de efetividade em forma de sino proposta para o CBD e seus derivados, e as concentrações selecionadas para o CBD, HUF-101 e (-) - 5′-DMH-CBD apresentaram resultados negativos no desafio com 6-OHDA, foram incluídas concentrações mais baixas para as avaliações de neuroproteção/neurotoxicidade a seguir, já que são frequentemente mais protetoras (Zuardi et al., 2017). A partir daqui os dados para o CBD são originais. Além disso, um terceiro derivado sintético do CBD, HU-556, foi incluído nos próximos experimentos e o próprio CBD foi avaliado em concentrações mais baixas.

Apenas no modelo de neurotoxicidade do desenvolvimento neuronal o HUF-101, (-) - 5′-DMH-CBD e HU-556 foram neurotóxicos na concentração mais alta (2,5 μM). Para o CBD, nenhuma concentração alterou significativamente a viabilidade celular. Estes resultados indicam que os neurônios humanos são mais sensíveis aos canabinoides quando expostos durante a diferenciação, como verificado previamente para as mesmas células (Schönhofen et al., 2015).

Como o $CL_{50}$ da 6-OHDA pode ser um desafio extremo para os neurônios e encobrir possíveis efeitos protetores dos canabinoides, avaliamos também a neurotoxicidade em SH-SY5Y tratadas com a mesma curva de cada canabinoide desafiados com uma concentração subletal de 6-OHDA. Apenas o HU-556 potencializou a perda de viabilidade causada pela concentração subletal de 6-OHDA em neurônios tratados com 2,5 μM durante a diferenciação neuronal.

Estes resultados podem ser atribuídos ao alto perfil antioxidante do CBD e HUF-101 – como o (-) - 5′-DMH-CBD não apresentou atividade antioxidante e o HU-556 não foi

avaliado, seus efeitos possivelmente se devem a outros mecanismos. Antioxidantes geralmente são protetores em doses baixas e podem levar a um desequilíbrio redox em altas doses, mas quando processos redox são importantes como causa ou progressão de uma doenças, os antioxidantes podem ser efetivos mesmo em altas doses (Gutteridge and Halliwell, 2010). Como discutido acima, o mecanismo de ação 6-OHDA, já verificado em células da linhagem SH-SY5Y, é mediado principalmente através da geração de espécies reativas de oxigênio e, por consequência, desequilíbrio redox (Lopes et al., 2017). Espera-se que este seja o processo que gera a morte celular observada em nossos experimentos. Assim, seria esperado que uma molécula com potencial antioxidante *in vitro* protegesse células em cultura contra os danos oxidativos causados. No entanto, em nossos resultados, isto não ocorreu. É possível que as concentrações utilizadas estejam ainda fora do limiar protetor para estes compostos. O desenho experimental também pode ter contribuído para a ausência de proteção já que o desafio neurotóxico foi realizado após o tratamento com os canabinoides, na ausência dos mesmos, o que pode não ter sido suficiente para gerar um efeito antioxidante contra a 6-OHDA. É possível ainda que a adição de um segundo composto redox-ativo possa ter um efeito aditivo à 6-OHDA, gerando um estado pró-oxidativo e levando à perda de viabilidade celular. Entretanto, como os testes antioxidantes foram realizados em uma abordagem livre de células, não é possível afirmar que o perfil antioxidante destes canabinoides se repete quando administrados em cultura de células.

Além disso, a sinalização endocanabinoide pode estar envolvida, pois a *up-regulation* do CB1 geralmente ocorre em resposta a danos neuronais (Carroll et al., 2012). De qualquer forma, além da falta de neuroproteção, houve uma redução mais acentuada da viabilidade em células tratadas com os canabinoides durante a diferenciação (mas não em nas tratadas após a diferenciação) na concentração mais alta, mas os resultados mostrados até agora não nos permitem definir quais são os mecanismos envolvidos. Além disto,

devemos considerar também o viés do tempo de exposição aos canabinoides que foi maior para as células tratadas durante a diferenciação, o que deve influenciar também na sensibilização observada neste caso. Outros possíveis alvos do CBD podem estar envolvidos nos efeitos observados, como o TRPV1, que já foi associado à neuroproteção contra o dano oxidativo causado pela 6-OHDA em ratos (Zhao et al., 2017).

Resumidamente, considerando todas as abordagens aqui utilizadas, 2,5 μM de cada canabinoide foi neurotóxico quando administrado durante a diferenciação neuronal - CBD quando desafiado com 6-OHDA (em resultados prévios (Schönhofen et al., 2015)), HUF-101, (−)-5′-DMH-CBD e HU-556 *per se* e quando desafiado com o $CL_{50}$ (HUF-101 e (-) - 5′-DMH-CBD) ou com uma concentração subletal de 6-OHDA (HU-556). Com isso, podemos concluir que a mais alta concentração tolerada pelas células diferenciadas (2,5 μM) destes canabinoides é neurotóxica durante o desenvolvimento, em resposta ao tempo de exposição aos canabinoides e / ou a interferências nos mecanismos de diferenciação neuronal (como o SEC). Além disto, os derivados sintéticos apresentam neurotoxicidade *per se* já que reduziram a viabilidade celular mesmo sem o desafio neurotóxico. Para todos os canabinoides testados, concentrações que não apresentaram toxicidade em si, também não potencializaram a toxicidade da 6-OHDA. Em conjunto, nossos resultados sugerem que esses canabinoides podem apresentar efeitos deletérios para o desenvolvimento neuronal, sendo possivelmente mais seguros nas concentrações mais baixas.

Além de não oferecer proteção contra crises epilépticas em nosso modelo animal, o CBD também pode apresentar efeitos indesejáveis e perigosos em testes clínicos (Szaflarski et al., 2018) e devem-se aplicar critérios de avaliação de riscos cuidadosamente elaborados antes que seu uso seja recomendado para crianças e adultos.

É importante lembrar que este estudo foi realizado utilizando-se uma linhagem celular que, apesar de humana, dotada de marcadores de neurônios diferenciados e de

apresentar morfologia estrelada típica de neurônios (Lopes et al., 2010), representa um único tipo celular. Com isto, não são consideradas relações intercelulares, o que pode ser um fator importante para a falta de reprodutibilidade *in vitro* de dados observados *in vivo*.

A utilização de experimentos *in vitro* para identificação e seleção de compostos perigosos ou protetores está baseada na premissa de que se um determinado composto que tem um efeito *in vitro*, ele também tem o potencial de alterar os mesmos parâmetros *in vivo*. Entretanto, para que fosse possível a aplicação direta de dados obtidos *in vitro* avaliação dos possíveis riscos ou potenciais de compostos, seria necessária uma compreensão mais completa dos mecanismos relacionados à expressão de toxicidade *in vivo*, o que ainda não é possível (Radio e Mundy 2008).

Lembrando que no modelo animal aqui apresentado o CBD modulou o SEC através do aumento dos níveis de AEA e ainda não reduziu a susceptibilidade a crises epilépticas quimicamente induzidas – levando inclusive a um aumento na susceptibilidade em animais normóxicos, podemos traçar um paralelo entre os modelos *in vivo* e *in vitro*. Assim, os animais neonatos tratados com CBD corresponderiam aos neurônios tratados durante a diferenciação. Os animais com 44 dias, seriam equivalentes aos neurônios terminalmente diferenciados. Ainda, o desafio com a toxina redox-ativa realizado nos tratamentos *in vitro* pode ser comparado à indução de crises epilépticas por fármacos em animais, uma vez que ambos podem causar desbalanço redox e neurodegeneração (Martinc et al., 2012).

Desta forma, nossos resultados *in vitro* corroboram os observados *in vivo*. Animais em uma idade de intenso desenvolvimento neuronal e neurônios tratados durante a diferenciação neuronal são mais sensíveis aos canabinoides. Em células tratadas durante a diferenciação, também foi possível observar que concentrações mais altas dos canabinoides, que apresentaram atividade antioxidante, sensibilizaram estas células contra a ação neurotóxica da 6-OHDA.

Como no modelo animal o CBD modulou o SEC e este sistema pode estar envolvido nos mecanismos de neurotoxicidade observados, para verificarmos se esse efeito se repete *in vitro* e se os derivados sintéticos do CBD têm ação semelhante, utilizamos um agonista e um antagonista de CB1 para modular o SEC em nosso modelo de toxicidade em neurônios maduros e o modelo de toxicidade durante o desenvolvimento neuronal. Utilizamos também uma abordagem *in silico* para predizer a energia de ligação e a afinidade destes possíveis ligantes ao receptor CB1, com base em suas estruturas químicas e em dados cristalográficos do receptor (Hua et al., 2017).

Como já discutido para o CBD, embora seja associado a uma baixa afinidade pelo CB1, ele modula a sinalização do SEC através da redução da recaptação e degradação de AEA (Deutsch, 2016). Ele também já foi descrito como modulador alostérico negativo de CB1, inibindo a atividade de agonistas deste receptor (Laprairie et al., 2015). Uma vez que em nossos resultados em células o agonista sozinho é neuroprotetor durante a diferenciação, e que a adição da concentração mais baixa e mais alta de CBD ao agonista de CB1 leva a um aumento de viabilidade, pode-se inferir que o CBD só é neuroprotetor na presença do agonista de CB1, mas que esse efeito provavelmente se deve apenas ao agonista. Além disto, o CBD não se comportou como modulador alostérico negativo de CB1 nestas concentrações, já que não inibiu o efeito do agonista nos desafios neurotóxicos em células co-tratadas durante a diferenciação. Como o agonista CB1 é protetor quando administrado durante a diferenciação neuronal e desafiado com 6-OHDA, um modulador alostérico negativo iria neutralizar essa proteção, o que acontece com 1 μM de cada canabinoide sintético nos co-tratamentos durante diferenciação neuronal desafiados com 6-OHDA (Fig. 6). Entretanto, por meio dessa abordagem, não é possível afirmar que esses canabinoides sejam moduladores alostéricos negativos do CB1, como já descrito para o CBD (Laprairie et al., 2015). Para isso, um bloqueio do sítio alostérico seria mais eficaz.

Ainda assim, pode-se dizer que o CBD tem alguma interação com o sítio ortostérico do CB1 já que o co-tratamento com 1,0 μM de CBD o antagonista deste receptor reduziu a viabilidade durante a diferenciação, o que não ocorreu para ambos quando tratados isoladamente. Da mesma forma, para o HUF-101 e para o (-) - 5′-DMH-CBD, de modo geral, o co-tratamento com o antagonista de CB1 leva a redução da viabilidade em células tratadas durante a diferenciação e o agonista levou ao aumento da viabilidade na concentração mais alta, permitindo inferir que pode haver uma interação entre o receptor e estes canabinoides sintéticos. Já para o HU-556, o co-tratamento com o antagonista de CB1 levou a um aumento da viabilidade em todas as concentrações em neurônios maduros. Durante a diferenciação, 2.5 μM de HU-556 em co-tratamento tanto com o agonista quanto o antagonista de CB1 resultou na potencialização da neurotoxicidade da 6-OHDA, o que pode ser atribuído ao próprio efeito citotóxico do HU-556 nesta concentração. Apesar de antagônicos em comparação aos outros canabinoides, os resultados obtidos para o HU-556 também sugerem algum tipo de interação com o receptor CB1.

O HUF-101 é consideravelmente mais potente que o CBD em ensaios comportamentais em camundongos, apresentando atividade ansiolítica, antidepressiva, antipsicótica e anticompulsiva. Os efeitos anticompulsivos do HUF-101 dependem dos receptores canabinoides, uma vez que os efeitos CBD e HUF-101 foram prevenidos pelo pré-tratamento com um antagonista de CB1 em camundongos (Breuer et al., 2016). Presumivelmente, as ações deste derivado fluorado de CBD, que são paralelas às do CBD, são baseadas nos mesmos mecanismos (Breuer et al., 2016). HUF-101 tem efeitos analgésicos em doses mais baixas do que o CBD (Silva et al., 2017). Essa maior potência explica nossos resultados com o HUF-101 sozinho ou em co-tratamentos em comparação com o CBD. Além disso, os efeitos analgésicos do HUF-101 e do CBD envolvem a ativação dos receptores CB1 e CB2 (Silva et al., 2017), o que mais uma vez corrobora nossos

resultados, já que a modulação da atividade CB1 alterou a viabilidade celular. Como HUF-101 não causou os efeitos psicoativos típicos induzidos por agonistas CB1 (Silva et al., 2017), provavelmente tem como alvo outros componentes do SEC, o que pode estar relacionado a perda de viabilidade celular em neurônios tratados durante a diferenciação quando em alta concentração. Em um modelo de camundongo para axotomia do nervo ciático neonatal, o HUF-101 também apresenta propriedades atenuantes da gliose em comparação com o CBD (Perez et al., 2018), apontando também para atividade anti-inflamatória deste canabinoide.

Para a geração dos homólogos dimetilheptil de CBD, a introdução da cadeia alquílica DMH na série (-) - DMH-CBD não alterou a falta de afinidade pelos receptores CB1 e CB2 em estudos prévios (Bisogno et al., 2001). Análogos do tipo (-)-DMH-CBD demonstraram efeitos ansiolíticos, analgésicos, anti-inflamatórios e antiproliferativos em diversos ensaios (Morales et al., 2017b). O derivado CBD dimetilheptil testado aqui, (-) - 5'-DMH-CBD, é um inibidor do transporte de membrana do AEA e é relativamente metabolicamente estável. Exibe alguma afinidade para os receptores CB2, mas tem apenas fraca afinidade para os receptores canabinoides e não tem atividade na FAAH (Bisogno et al., 2001). Em nossos dados, verificamos mudanças na viabilidade celular nos co-tratamentos do (-) - 5′-DMH-CBD com agonista e antagonista de CB1. Assim, os efeitos demonstrados aqui podem ser devidos à inibição do transporte de AEA, que causou perda de viabilidade celular em neurônios durante a diferenciação quando em alta concentração.

O HU-556 é um novo derivativo da CBD, protegido por sigilo de patente, sem dados publicados anteriormente. Entretanto, derivados da CBD com estrutura semelhante apresentaram alguns efeitos interessantes, como o HU-444, que apresentou atividade anti-inflamatória (diminuição dos intermediários reativos de oxigênio e inibição da produção de TNF-α pelos macrófagos) em modelos *in vitro*; *in vivo*, levou à supressão da produção de

TNF-α e melhora do dano hepático, bem como redução da artrite induzida por colágeno de camundongo. O HU-444 não causou efeitos do tipo $\Delta^9$-THC em camundongos (Haj et al., 2015). Como ambos são produzidos usando o CBD como material de partida, e o HU-444 não é um composto do tipo $\Delta^9$-THC, é esperado que atuem nos mesmos alvos do CBD. Assim, os efeitos aqui demonstrados para o HU-556 podem ser devidos a outros alvos do SEC, que causam perda de viabilidade celular em neurônios em diferenciação quando na mais alta concentração. Em nossos resultados, nos co-tratamentos com o agonista ou antagonista de CB1, o HU-556 apresentou resultados diferentes dos demais canabinoides, que foram muito semelhantes entre si.

Independentemente das diferenças individuais entre os canabinoides testados, os neurônios tratados durante a diferenciação apresentaram sensibilidade aumentada aos canabinoides e ao agonista e antagonista de CB1 (sozinhos ou em co-tratamentos) com ou sem o desafio com 6-OHDA. Isso significa que, para os neurônios imaturos, concentrações de canabinoides consideradas inócuas em neurônios maduros podem ser prejudiciais.

A expressão de CB1 é aumentada em resposta à exposição a toxinas em SH-SY5Y diferenciadas, como um indicativo de dano neuronal, o que ocorre nos processos de doença (Carroll et al., 2012). Assim, como o agonista de CB1 foi protetor em nosso modelo celular de desenvolvimento neuronal, este receptor pode estar envolvido na resposta positiva à neurodegeneração. De modo geral, pode-se dizer que há participação do CB1 nos mecanismos de neurotoxicidade e neuroproteção observados.

Como a modulação da atividade do CB1 altera a viabilidade celular em alguns dos tratamentos, nossos resultados sugerem que o SEC pode participar nos mecanismos de ação dos canabinoides testados. *In silico*, demonstramos que o *Ki* e energia de ligação destes canabinoides nos permitem inferir que pode haver afinidade pelo sítio ortostérico do CB1 e, como houve alterações na viabilidade celular com o agonista / antagonista desse

receptor, pode-se inferir que existe a possibilidade de uma interação direta entre esses canabinoides e o CB1. Embora tenha sido relatado que a afinidade de ligação de vários fitocanabinoides ao receptor não explica seus efeitos sobre cultura de células neurais (Rosenthaler et al., 2014), não podemos excluir esta possibilidade em nossos resultados. Ainda assim, em nossos dados *in silico,* os *Ki* preditos estão dentro da curva de concentração utilizada para os tratamentos em células para cada um dos canabinoides, nas concentrações mais baixas. Desta forma, os resultados quanto à neurotoxicidade observada para 2,5 μM de cada canabinoide se deve provavelmente a outros mecanismos que não a ação direta sobre o CB1 – outros pontos de modulação do ECS como a recaptação de AEA, outros sistemas de sinalização ou mesmo por mecanismos REDOX. Já nas concentrações mais baixas, 0,01 e 0,1 μM, de acordo com as predições para a afinidade por CB1, os efeitos desses canabinoides podem estar associados a este receptor, tanto por agonismo ou antagonismo. Quanto à possível modulação alostérica, nossa abordagem não é capaz de avaliar tal possibilidade, uma vez que a estrutura do cristal usado para modelagem do CB1 não possui a porção amino terminal, onde se encontra o sítio alostérico deste receptor.

Por fim, os dados aqui apresentados e discutidos apresentam relevância clínica, já que foram utilizados modelos experimentais mais adequados para o período de desenvolvimento alvo das síndromes epilépticas refratária infantis. Esses resultados apontam para um mecanismo de ação mediado pelo SEC e que levou a ausência de proteção ou mesmo piora das crises epilépticas em animais neonatos com idade correspondente aos primeiros anos de vida em humanos. Assim, consideramos que os derivados sintéticos aqui avaliados podem não ser seguros para crianças antes dos 2 anos de vida e necessitam ainda de muitos estudos sobre sua eficácia e segurança em modelos experimentais. Já CBD não apresentou toxicidade *per se*, o que pode significar que seja

mais seguro neste período, mas sensibilizou as células ao desafio neurotóxico quando administrado durante a diferenciação e não foi protetor contra crises epilépticas no modelo animal. Assim, mais estudos são necessários, principalmente para a definição de dosagens e períodos de tratamento seguros.

É importante reforçar ainda que, mesmo que o CBD apresente efeitos deletérios para o desenvolvimento do SNC, tais efeitos são provavelmente muito menos severos do que as sequelas resultantes das crises recorrentes causadas pelas síndromes refratárias infantis. Deste modo, o CBD continua sendo um bom candidato terapêutico. No entanto, é importante que se conheçam os efeitos adversos e os mecanismos de ação de um fármaco para que se possa explorar totalmente seu potencial, por exemplo, através do desenvolvimento de derivados sintéticos mais eficazes.

## 5. Conclusão

Nossos resultados em camundongo reforçam a hipótese do tônus endocanabinoide (níveis elevados de ativação deste sistema) como mecanismo de ação do CBD em um modelo animal com susceptibilidade aumentada a crises epilépticas, podendo ser comparado à epilepsia refratária infantil. Além disto, durante fases de desenvolvimento neuronal, no período pós-natal equivalente aos 2 primeiros anos de idade humana, uma dose aguda baixa de CBD é suficiente para modular o SEC, o que neste estudo, foi associado à falta de efetividade ou piora na susceptibilidade a crises.

*In vitro*, neurônios tratados durante a diferenciação foram mais sensíveis aos canabinoides analisados, transformando concentrações inócuas em neurônios diferenciados em concentrações neurotóxicas. Os efeitos neurotóxicos observados podem ser atribuídos a um possível desequilíbrio redox causado em células tratadas durante a diferenciação com canabinoides tanto isoladamente quanto em desafios com a 6-OHDA. No entanto, como o co-tratamento agonista e antagonista CB1 altera estes resultados e os canabinoides testados possuem certa afinidade pelo CB1 *in silico*, é provável que o SEC esteja envolvido nos mecanismos de neuroproteção/neurotoxicidade observados.

Por fim, os resultados obtidos nos modelos *in vivo* e *in vitro* corroboram entre si, já que indivíduos e neurônios imaturos foram mais sensíveis aos canabinoides e à modulação do SEC. Isto representa um grande avanço para pesquisas que visam o desenvolvimento do SNC, uma vez que o modelo *in vitro* utilizado aqui reproduz ao menos parcialmente os resultados no modelo animal. Com isto, além de efeitos benéficos, possíveis riscos à saúde poderiam ser identificados antes que estes compostos cheguem à utilização por humanos. A relação risco / benefício seria então avaliada de acordo com a necessidade de cada paciente e com os comprovados efeitos terapêuticos do canabinoide em questão.

Com isto, concluímos que o uso do CBD e de seus derivados sintéticos em humanos é ainda controverso, principalmente durante o desenvolvimento do sistema nervoso, sendo necessárias mais pesquisas em modelos experimentais adequados. Ainda, a associação do tratamento com CBD ao aumento de susceptibilidade a crises em neonatos não susceptíveis à crises reforça nossa hipótese de que a modulação do SEC pode representar riscos à saúde em indivíduos muito jovens.

## 6. Perspectivas

Como vimos, este trabalho mostrou que tanto o modelo de célula diferenciada quanto o de célula em diferenciação são eficientes para triagem de compostos, gerando de dados importantes para a classificação destes compostos como neuroprotetores / neurotóxicos e posterior aplicabilidade em estudos *in vivo*. Por isso, estes modelos podem ser utilizados para avaliação de diversos compostos com possíveis ações em células neuronais humanas. Para a complementação deste trabalho, pretendemos avaliar o potencial antioxidante dos co-tratamentos dos canabinoides com o agonista e o antagonista de CB1 e ainda avaliar aspectos da morfologia celular correlacionados ao desenvolvimento neuronal, como densidade e extensão de neuritos.

No modelo animal, futuramente serão utilizadas dosagens mais altas de CBD para tratamento agudo e também para tratamento crônico em animais neonatos e adultos. Ainda, serão avaliadas as taxas de neurogênese e neurodegeneração no hipocampo destes animais e realizaremos testes comportamentais para avalição de ansiedade, motricidade e cognição. Além disto, este modelo animal poderá ser utilizado para avaliações das capacidades anticonvulsivantes de outros canabinoides.

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