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EFEITO DO CANABIDIOL NA MODULAÇÃO DO SISTEMA ENDOCANABINOIDE EM MODELO PRÉ-CLÍNICO DE NEURÔNIO MADURO E DE DESENVOLVIMENTO NEURONAL

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharela em Biomedicina.

Orientador: Prof. Dr. Fábio Klamt

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RESUMO

O canabidiol (CBD) é um fitocanabinoide derivado da *Cannabis sativa* utilizado no tratamento de distúrbios neurológicos, incluindo epilepsias refratárias infantis. Há evidências positivas de seu uso, mas pouco se sabe sobre efeitos a longo prazo no sistema nervoso em desenvolvimento, já que é capaz de interagir com o receptor canabinoide 1 (CB1) do sistema endocanabinoide (SEC), que regula o neurodesenvolvimento. Neste trabalho, avaliamos níveis de expressão de enzimas e receptores do SEC - *CNR1* (Receptor canabinoide tipo 1), *CNR2* (Receptor canabinoide 2), *DAGLA* (Diacilglicerol lipase alfa), *MAGL* (Monoacilglicerol lipase), *NAPEPLD* (N-araquidonoil fosfatidiletanolamina fosfolipase-D) e *FAAH* (Amida hidrolase de ácidos graxos) - em resposta ao tratamento com CBD em neurônios maduros e no desenvolvimento neuronal. Para isso, células da linhagem de neuroblastoma humano SH-SY5Y foram diferenciadas em um fenótipo neuronal com ácido retinóico (AR) durante 7 dias. Para a avaliação dos efeitos do CBD em neurônios maduros, as células foram tratadas por 24h ao final da diferenciação. Já para a avaliação dos efeitos do CBD durante o desenvolvimento neuronal, o mesmo foi adicionado a cada troca de meio durante os 7 dias de diferenciação. Após os tratamentos, amostras foram coletadas em Trizol (Invitrogen) para extração de RNA de acordo com a orientação do fabricante. Em seguida realizou-se a síntese de cDNA que foi utilizado para a quantificação dos níveis de expressão dos genes por *RT-qPCR*, quantificados pelo método 2-ΔCt. Também avaliamos efeitos neuroprotetores / neurotóxicos do CBD e seus derivados sintéticos HUF-101, (-) -5′-DMH-CBD e HU-556 em células tratadas por 24h (neurônios maduros) ou durante a diferenciação neuronal e co-tratadas com 0,375 mM de Peróxido de Hidrogênio por mais 24h. Também avaliamos os efeitos neuroprotetores / neurotóxicos do CBD e seus derivados quando desafiados com 6-OHDA após as 24h do 7° dia de tratamento. Ambos os desafios foram avaliados a viabilidade celular por MTT e a produção de espécies reativas de oxigênio por DCF. Neste trabalho utilizamos uma dose de 0,1 μM de CBD e derivados sintéticos visto que demonstrou certa proteção. Os resultados indicam que neurônios maduros e em desenvolvimento respondem de forma diferente ao CBD tanto em nível celular quanto molecular. O CBD atua no sistema endocanabinoide (SEC), um sistema de mensageiros retrógrados, que produz endocanabinoides como 2-AG (2-araquidonoilglicerol) e AEA (Anandamida). Nosso trabalho apresentou uma redução na expressão da enzima que sintetiza AEA e houve um aumento na expressão da enzima que degrada AEA no modelo de desenvolvimento neuronal, e isso faz com que tenha uma menor disponibilidade de AEA que é um ligante de CB1. O efeito neuroprotetor não é o mesmo quando submetemos as células tratadas com CBD a um desafio mais intenso de H_2O_2 (Peróxido de hidrogênio), mas é neuroprotetor durante a diferenciação quando desafiado com 6-OHDA.

Palavras-chave: canabidiol; sistema endocanabinoide; epilepsia refratária; enzimas síntese e degradação; neuroproteção.

ABSTRACT

Cannabidiol (CBD) is a phytocannabinoid derived from *Cannabis sativa* used in the treatment of neurological disorders, including refractory childhood epilepsies. There is positive evidence for its use, but little is known about its long-term effects on the developing nervous system, as it is able to interact with the cannabinoid receptor 1 (CB1) of the endocannabinoid system (ECS), which regulates neurodevelopment. In this work, we evaluated expression levels of ECS enzymes and receptors - *CNR1* (Cannabinoid receptor type 1), *CNR2* (Cannabinoid receptor type 2), *DAGLA* (Diacylglycerol lipase alpha), *MAGL* (Monoacylglycerol lipase), *NAPEPLD* (N-arachidonoylphosphatidylethanolamine phospholipase-D) *and FAAH* (Fatty acid amide hydrolase) - in response to CBD treatment in mature neurons and in neuronal development. For this, cells of the human neuroblastoma lineage SH-SY5Y were differentiated into a neuronal phenotype with retinoic acid (RA) for 7 days. To evaluate the effects of CBD on mature neurons, cells were treated for 24h at the end of differentiation. For the evaluation of the effects of CBD during neuronal development, it was added to each change of medium during the 7 days of differentiation. After the treatments, samples were collected in Trizol (Invitrogen) for RNA extraction according to the manufacturer's instructions. Then, cDNA synthesis was performed, which was used to quantify gene expression levels by *RT-qPCR*, quantified by the 2-ΔCt method. We also evaluated neuroprotective / neurotoxic effects of CBD and its synthetic derivatives HUF-101, (-)-5′-DMH-CBD and HU-556 in cells treated for 24h (mature neurons) or during neuronal differentiation and co-treated with 0.375 mM of Hydrogen Peroxide for another 24h. We also evaluated the neuroprotective/neurotoxic effects of CBD and its derivatives when challenged with 6-OHDA after 24h of the 7th day of treatment. Both challenges were evaluated for cell viability by MTT and production of reactive oxygen species by DCF. In this work, we used a dose of 0.1 μM of CBD and synthetic derivatives as it showed some protection. The results indicate that mature and developing neurons respond differently to CBD at both a cellular and molecular level. CBD acts on the endocannabinoid system (ECS), a retrograde messenger system, which produces endocannabinoids such as 2-AG (2-arachidonoylglycerol) and AEA (Anandamide). Our work showed a reduction in the expression of the enzyme that synthesizes AEA and there was an increase in the expression of the enzyme that degrades AEA in the neuronal development model, and this causes a lower availability of AEA, which is a CB1 ligand. The neuroprotective effect is not the same when we subject the CBD-treated cells to a more intense challenge with H2O2 (Hydrogen Peroxide), but it is neuroprotective during differentiation when challenged with 6-OHDA.

Keywords: cannabidiol; endocannabinoid system; refractory epilepsy; enzymes synthesis and degradation; neuroprotection.

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1 INTRODUÇÃO COMPREENSIVA

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). Com o avanço de estudos, foi possível identificar seus compostos, isolá-los e identificá-los. Na resina de plantas fêmeas da *Cannabis* são encontrados aproximadamente 120 compostos lipossolúveis, os chamados fitocanabinoides (Morales et al., 2017). Todos fitocanabinoides são encontrados exclusivamente na *Cannabis* e seus principais componentes, o Δ9-tetrahidrocanabinol (Δ9-THC, principal composto psicoativo) e o canabidiol (CBD, não psicoativo) são produtos do metabolismo secundário de plantas a partir de um mesmo precursor, o canabigerol (Nachnani et al., 2021). O CBD é um dos fitocanabinoides mais estudados e tem sido utilizado no tratamento de crianças com epilepsia refratária (O'Connell et al., 2017). Embora existam muitas linhas de evidências positivas em relação ao uso clínico do CBD, não há evidências suficientes sobre os potenciais efeitos nocivos a longo prazo dos canabinoides no desenvolvimento do sistema nervoso central (SNC). O CBD é capaz de atuar direta e indiretamente sobre o sistema endocanabinóide (SEC) e pode perturbar os processos regulatórios mediados por esse sistema. Nos últimos anos, análogos sintéticos do CBD foram desenvolvidos para melhorar seus resultados benéficos e reduzir potenciais efeitos colaterais.

Os canabinoides estão sendo amplamente utilizados para diversas recomendações, embora a comprovação e aprovação pelo Food and Drug Administration (FDA) seja apenas para casos específicos. Portanto, ainda não se sabe a causa do CBD agir de maneira mais efetiva no cérebro em desenvolvimento do que no cérebro maduro.

1.1 FITOCANABINOIDES E SISTEMA ENDOCANABINOIDE

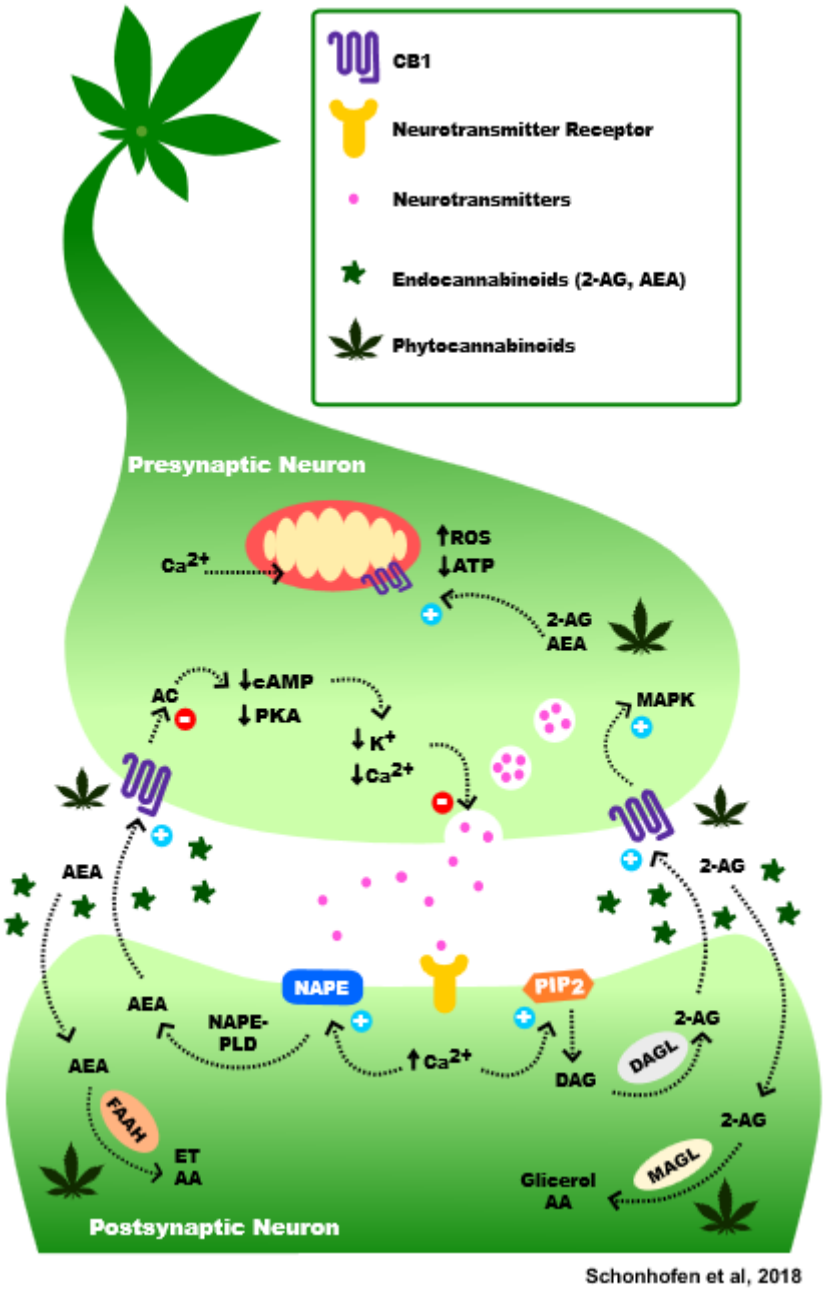
Os fitocanabinoides foram isolados pela primeira vez na planta *Cannabis sativa* do gênero *Cannabaceae* e demonstraram resultados promissores no tratamento de síndromes médicas (Russo, 2007). Estudos confirmam a presença de receptores canabinoides no cérebro de mamíferos e que respondem aos sinais dos compostos encontrados na *Cannabis sativa*, sendo denominados como o sistema endocanabinoide (SEC) (Gülk and Møller, 2020). Os endocanabinoides mais conhecidos são a N-araquidonoiletanolamida

(anandamida ou AEA) e o 2-araquidonoilglicerol (2-AG), importantes canabinoides endógenos do SEC (di Marzo and Piscitelli, 2015) que atuam no mecanismo de síntese e degradação do SEC (Pertwee et al., 2010), eles foram os primeiros a serem identificados e estudados (Luchicchi and Pistis, 2012; Pertwee and Ross, 2002), e também demonstram efeitos vantajosos em diversos mecanismos biológicos (Nigro et al., 2021). Esses endocanabinoides são mediadores endógenos e ativam alvos clássicos do SEC, como o receptor canabinoide tipo 1 (CB1) e o receptor canabinoide tipo 2 (CB2), quando acoplados à proteína G (Howlett et al., 2002). Os receptores CB1 são abrangentes no sistema nervoso central (SNC), diferentemente do CB2 que também é visto em algumas células do SNC, mas é mais comumente encontrado no sistema imune. No SNC, o CB2 é visto em células piramidais como a CA3 e CA2 do hipocampo, e tem participação fundamental na plasticidade sináptica (Skaper and Di Marzo, 2012; Stempel et al., 2016).

Os fitocanabinoides também agem sobre o SNC por meio do SEC, assim como os endocanabinoides, e possuem diferentes fins terapêuticos (Morales and Reggio, 2017). O SEC possui envolvimento nos estágios iniciais do desenvolvimento SNC e estudos recentes demonstram ação desse sistema na maturação neuronal (Galve-Roperh et al., 2009). Portanto, a sinalização endocanabinoide demonstra ação concludente em cérebros maduros e em desenvolvimento (Meyer et al., 2017) e isso demonstra a suscetibilidade e propensão em ocasionar possíveis problemas.

O SEC é um sistema de mensageiros retrógrados que, em resposta a neurotransmissão, produz endocanabinoides (AEA e 2-AG) sob demanda da membrana pós-sináptica. Os endocanabinoides agem nos receptores canabinoides no neurônio pré-sináptico reduzindo a liberação de neurotransmissores. A Figura 1 demonstra a sinalização do SEC e todos os componentes envolvidos (Schonhofen et al., 2018) (revisado por (Lu and MacKie, 2016)).

Figura 1 - Sistema endocanabinoide e síntese de endocanabinoides no neurônio pré e pós sináptico.



Representação do 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. FONTE: Schonhofen et al., (2018 Fig. 1).

A neurotransmissão tem como resultado o aumento do influxo de Ca^{2+} no neurônio pós-sináptico que sinaliza o início da síntese dos endocanabinoides por meio dos seus precursores na membrana plasmática. Os endocanabinoides atuam como mensageiros retrógrados após a liberação pelo neurônio pós-sináptico, ocasionando a ativação do CB1 no neurônio pré-sináptico e resultando em uma menor liberação de neurotransmissores na fenda sináptica. Todo esse processo é mediado pelo aumento do influxo de Ca^{2+} por consequência da neurotransmissão no neurônio pós-sináptico em que estimula a síntese de endocanabinoides por meio de seus prógonos presentes na membrana plasmática (Alger, 2002; Lu and MacKie, 2016; Velasco et al., 2012). Na membrana pós-sináptica, o AEA é sintetizado por meio da hidrólise intermediada pela fosfolipase D da N-araquidonoilfosfatidiletanolamina (NAPE-PLD), enquanto o 2-AG é gerado pela hidrólise intermediada pela diacilglicerol lipase do diacilglicerol (DAG), uma enzima chave na biossíntese do 2-AG (Reisenberg et al., 2012), decorrente do bifosfato de fosfatidilinositol (PIP2) localizado na membrana. O AEA e 2-AG quando se direcionam aos terminais pré-sinápticos acabam se propagando e ligam-se aos receptores CB1 pré-sinápticos unidos à proteína G, causando sua ativação. O resultado dessa ligação estimula e libera proteínas Gi/Go do CB1 que reduz a formação do AMP cíclico (AMPc) e posteriormente da proteína cinase A (PKA), isso ocorre por meio da inibição da adenilil ciclase (AC). Deste modo, canais de K^{+} são abertos e causam a hiperpolarização do terminal pré-sináptico, canais de Ca^{2+} são fechados e ocorre a redução da liberação de neurotransmissores que estão armazenados nas vesículas sinápticas (Mackie, 2008; Lévénés et al., 1998). Por fim, a AEA e 2-AG são catabolizadas após o seu retorno aos terminais pré ou pós-sinápticos por ação da amida hidrolase de ácidos graxos (FAAH), que age diretamente na quebra da AEA e é um promissor alvo terapêutico para tratar alguns distúrbios do SNC (Ulugöl, 2014), e pela monoacilglicerol lipase (MAGL), que age na quebra do 2-AG, liga o SEC após essa hidrólise e acaba fornecendo uma síntese pró-inflamatória (Mulvihill and Nomura, 2013).

O deslocamento dos endocanabinoides mediante a membrana plasmática na sua recaptação ainda é pouco distinguido, porém, alguns estudos demonstram resultados positivos com a modulação da AEA em que os seus efeitos parecem ser potencializados por meio do bloqueio farmacológico ocasionado pela sua degradação metabólica (Scherma et al., 2019), e sua locomoção pode ser mediada através de um transporte facilitado de membrana (Fowler, 2013). Outro meio de locomoção endógena dos endocanabinoides foi mostrado a partir de proteínas ligadoras de ácidos graxos (*fatty acid binding proteins* -

FABPs) que facilitam a degradação de AEA pelo transporte intracelular que facilita a dissolução do AEA, carregando-o para FAAH (Deutsch, 2016).

O principal componente do SEC nos neurônios é o receptor CB1, e sua ativação leva a ação da proteína cinase ativada por mitógeno (MAPK) que regula diversas atividades celulares, e esse mecanismo possivelmente afeta a plasticidade sináptica e o deslocamento celular do crescimento neuronal (Mechoulam and Parker, 2013). O CB1 está presente no córtex, hipocampo, amígdala, nas vias de saída dos núcleos da base e no cerebelo, além de ser enriquecido nesses mesmos locais. O tratamento com a *Cannabis sativa* pode afetar essas regiões, as quais são conhecidas pelo seu envolvimento comportamental e pode ter finalidade no auxílio a prevenção de doenças neurológicas (Mackie, 2005).

Entretanto, apesar de estudos demonstrarem a presença do CB1 na membrana plasmática, ele também está presente nas mitocôndrias cerebrais (mtCB1) onde é responsável pela alteração do ATP mitocondrial, espécies reativas de oxigênio, Ca^{2+}, metabolismo de neurotransmissores e a morte celular, e essa ativação do CB1 é mediada pelo acoplamento à proteína G, ocasionando efeitos significativos na difusão sináptica (Djeungoue-Petga and Hebert-Chatelain, 2017).

O SEC possui um papel importante no cérebro em desenvolvimento nos estágios iniciais do período embrionário (Meyer et al., 2017) e no SNC em desenvolvimento nas regiões neurogênicas que restam no cérebro adulto (zona subgranular do hipocampo e zona subventricular), e acaba exercendo um efeito moderador nas células progenitoras neurais em que age na sobrevivência, proliferação, diferenciação e migração dessas células por meio do CB1 (Díaz-Alonso et al., 2012; Harkany et al., 2007), e diante disso pode acabar influenciando na formação de tecidos adultos (Habayeb et al., 2008). Outro destaque importante da presença do SEC em regiões de desenvolvimento embrionário, é nas células-tronco hematopoiéticas e mesenquimais em que age como um mediador da proliferação e diferenciação, e pode afetar no desenvolvimento de diversos tecidos especializados adultos decorrentes de folhetos germinativos (Galve-Roperh et al., 2013).

Contudo, no cérebro maduro, a plasticidade sináptica é permeada pela sinalização retrógrada do SEC por meio da 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), ambos possuem mecanismos de aprendizagem e importância no desenvolvimento neural (Dow-Edwards and Silva, 2017). Além disso, estudos sugerem que no SNC maduro o SEC modula a ansiedade, depressão, recompensa, cognição, aprendizado e memória, assim como também a neurogênese (Mechoulam and Parker, 2013). A sinalização retrógrada do SEC

estimula a geração de crises epilépticas em uma ou mais regiões do cérebro, como também causa uma alta excitação neuronal (Ludányi et al., 2008). Dessa forma, estudos demonstram avanço da atividade de CBD em protótipos experimentais para fins terapêuticos, surtindo efeitos em crises epilépticas e epilepsia (Blair et al., 2015; Gaston and Friedman, 2017).

A modulação do SEC tem demonstrado implicações promissoras visto que endocanabinoides exógenos, como por exemplo o Δ9-THC, são psicotrópicos e causam muitos efeitos colaterais, deste modo, pesquisadores investigaram outros caminhos onde encontraram agonistas de CB1 periféricos restritos e/ou agonistas seletivos do receptor CB2 que reduzem os impactos indesejados do SNC mediado pelo receptor CB1 (Ulugöl, 2014). Estudos sugerem a existência de um antagonista do CB1 que, dependente do receptor CB1, causa uma excitabilidade neuronal exacerbada em células hiperexcitáveis e que ocasiona o aumento da frequência e duração de crises epilépticas (Deshpande et al., 2007). Portanto, é possível compreender que o bloqueio ou estímulo acentuado do SEC ocasiona desfechos inoportunos, ainda mais durante o desenvolvimento neuronal, portanto são necessárias pesquisas mais detalhadas.

Os fitocanabinoides, endocanabinoides e derivados sintéticos dispõem mecanismos que não estão agregados ao SEC, tais como canais iônicos clássicos, receptores, transportadores e enzimas demonstrados em estudos recentes (Ibeas Bih et al., 2015). Os derivados sintéticos foram desenvolvidos com o intuito de aumentar os efeitos benéficos e reduzir os efeitos colaterais de CBD (Schönhofen et al., 2018), levando a novas abordagens para melhores desempenhos de recursos terapêuticos (Morales and Jagerovic, 2021).

O CBD desempenha papéis fundamentais no SEC, como por exemplo na inibição do FAAH, fazendo com que o endocanabinoide seja regulado, ajustando sua disponibilidade por meio da diminuição da degradação de AEA (De Petrocellis et al., 2011), isso demonstra que ocorre um aumento da quantidade de AEA tecidual por ação do CBD. Por conseguinte, CBD e seus análogos sintéticos podem exercer efeitos farmacológicos favoráveis no tratamento de doenças graves (Bisogno et al., 2001).

1.2 PECULIARIDADES DO CÉREBRO EM DESENVOLVIMENTO

O cérebro humano é dividido em dois hemisférios: direito e esquerdo. Os hemisférios possuem compartimentos denominados lobos, os quais são intitulados como frontal, parietal, temporal, occipital e insular, e possuem um compartimento externo designado substância externa cinzenta, conhecido como córtex cerebral, e a substância branca. O lobo temporal possui uma estrutura denominada como hipocampo, a qual é amplamente estudada e discutida sobre os impactos de doenças que afetam seu funcionamento (Bui and M Das, 2022). A função primordial do hipocampo é a formação de memórias declarativas e é amplamente estudada devido ao impacto da epilepsia nessa estrutura (Wible, 2013).

A epilepsia é uma desordem neurológica crônica caracterizada por crises epilépticas espontâneas e recorrentes causadas por hiperexcitabilidade e hipersincronia dos neurônios do cérebro. Estas alterações ocorrem por anormalidades na reorganização sináptica e neurogênese, ou alterações intrínsecas das células neurais (Fisher et al., 2005, 2017). A epilepsia pode estar associada a comorbidades neurobiológicas, cognitivas, psicológicas e sociais, que podem afetar gravemente a qualidade de vida dos pacientes (Deuschl et al., 2020; Fisher et al., 2005, 2014). A epilepsia do lobo temporal com esclerose hipocampal é uma das causas mais frequentes de epilepsias refratárias (Blümcke et al., 2013). O hipocampo é especialmente vulnerável a *status epilepticus* (SE) que é mais comum de acontecer em crianças e adultos (Upadhya et al., 2018).

O cérebro de crianças passa por diversas etapas até o estágio adulto, possuindo diferentes desenvolvimentos durante o período pré-natal e pós-natal. Estudos indicam que a grande produção de neurônios é durante o período pré-natal em que ocorre uma grande migração para o córtex em desenvolvimento, e que a neurogênese prossegue dentro da zona subventricular durante a infância e fase adulta, porém o seu pico ocorre do nascimento até os 18 meses de idade, e a proliferação das sinapses acontece cerca de 20 semanas dentro do período gestacional. Os neurônios que foram produzidos seguem, eventualmente, um caminho até o hipocampo e bulbo olfatório, e acredita-se que há uma grande notoriedade dessas células gliais progenitoras neurais na estrutura principal dos primeiros circuitos neurais no cérebro após o nascimento (Semple et al., 2013; Houston et al., 2014). A diferença do cérebro em processo de amadurecimento começa a ficar evidente quando ocorre uma modificação nas áreas de substância cinzenta e branca em que o volume da região cinzenta diminui e a branca aumenta, e essa mudança durante a infância e a

adolescência acontece primeiramente em regiões cerebrais mais primitivas e por último em regiões filogeneticamente mais novas (Gogtay et al. 2004; Sowell et al. 1999a, b). A neurogênese acontece predominantemente na gestação, porém pode ter continuidade entre 2 a 5 anos de idade (Semple et al, 2013). Outro fator importante para um cérebro em desenvolvimento é a ação do glutamato, um neurotransmissor que age de modo excitatório no cérebro adulto, e atua inibindo a atividade excitatória do ácido gama-aminobutírico (GABA) durante o desenvolvimento por agir nos receptores metabotrópicos (van den Pol et al., 1998).

O ácido gama-aminobutírico (GABA), um neurotransmissor inibitório, atua de forma excitatória durante o desenvolvimento neuronal (Johnston et al., 2011) e favorece o desenvolvimento das sinapses, as quais evitam a morte neuronal. Diante disso, estudos demonstram que há uma grande equivalência da ação do GABA no cérebro fetal e após o nascimento, evidenciando que a ação excitatória desse neurotransmissor neste período deixaria o cérebro predisposto a crises epilépticas em períodos mais tardios (Dzhala et al, 2010). Por conseguinte, dos anos iniciais do nascimento até a vida adulta, ocorre uma intensa maturação do SNC e se algum distúrbio interferir durante esse estágio, poderá ocasionar alterações irreversíveis, como por exemplo uma crise epiléptica devido a intensa modificação da rede neural, podendo afetar nas emoções e problemas de memória e aprendizado (Dawson et al., 2014; Lee et al., 2004).

Enquanto isso no cérebro adulto, estudos demonstram que a zona subgranular, presente no giro denteado do hipocampo (Altman J. et al., 1965; Patzke N. et al., 2015), continua estimulando a neurogênese mesmo em um cérebro totalmente maduro e forma diversos novos neurônios diariamente que ficam alojados no giro denteado (Spalding et al., 2013). Entretanto, outros estudos demonstram resultados contrários em que não há comprovações suficientes da presença de neurogênese em adultos e idosos (Dennis et al., 2016; Knoth R et al., 2010). O giro denteado permanece desenvolvendo-se durante o período fetal e após o nascimento, mas sofre uma alteração durante a fase adulta, isso demonstra que as células presentes no hipocampo sofrem alterações caso sejam submetidas a diferentes situações de estresse em que há um aumento de glicocorticoides, ocasionando a liberação de glutamato e com isso impede a multiplicação de células granulares (Gould and Tanapat, 1999).

Portanto, os circuitos neuronais devem perdurar durante toda a vida, pois a sua ausência acarretaria em dificuldades no desenvolvimento estrutural do sistema nervoso ainda como embrião, visto que, no processo de amadurecimento da fase infantil até a fase adulta, o

nosso corpo passa por diversos estágios de mudança, remodelando nossa anatomia e incorporando novos neurônios. Além do mais, processos fisiológicos como movimentos corporais, lesões e o simples envelhecimento, causam estresse ao sistema nervoso, e os processos moleculares não devem perder suas propriedades particulares a cada desafio externo (Bénard and Hobert, 2009).

1.3 CBD E SEU MECANISMO DE AÇÃO

O canabidiol (CBD) foi isolado primeiramente pelo químico Roger Adams em 1940, mas apenas em 1960 que as suas estruturas foram completamente elucidadas (Burstein, 2015). A partir de 1964, o cientista Raphael Mechoulam, isolou e sintetizou o CBD, descobrindo seu potencial não psicoativo e uso para fins medicinais (Russo, 2011).

O receptor CB1 e CB2, diferentemente dos outros canabinoides, não possuem uma alta afinidade com o CBD, mas o CBD demonstra ser um modulador alostérico negativo não competitivo do CB1 (Laprairie et al., 2015) e modulador alostérico negativo para CB1R do Δ9-THC e do 2-AG, demonstrando uma gama de possibilidades de efeitos positivos para tratamentos por meio de uma modulação alostérica (Morales et al., 2016). Estudos demonstram ação efetiva do CBD em receptores transmembrana 7, canais iônicos e transportadores de neurotransmissores quando se comporta como agonista ou antagonista (Ibeas et al., 2015). Apesar de existir outros mecanismos de ação do CBD, outros fatores devem ser levados em conta, como seu nível de exposição e sua concentração (Gray and Whalley, 2020).

Endocanabinoides, como AEA, são inibidos moderadamente pelo efeito modulatório do CBD (Leweke et al., 2012), e sua ação pode ocasionar uma ampliação dos níveis de AEA, podendo sensibilizar os receptores canabinoides a ligarem-se ao CBD (Ibeas et al, 2015). O AEA, juntamente do 2-AG e os receptores CB1 e CB2, compõem o SEC, assim como as enzimas que atuam na síntese e degradação dos endocanabinoides (Campos et al., 2016). Os endocanabinoides podem interromper a saída de neurotransmissores como o GABA, dopamina, glutamato, serotonina (5-HT), norepinefrina e acetilcolina por consequência das suas atividades no CB1 a nível pré-sináptico (Pertwee and Ross, 2002; Szabo and Schlicker, 2005).

O CBD possui diversos alvos já descritos, dentre eles estão os receptores 5-HT que atuam no SNC mediante ao estímulo da serotonina, liberando neurotransmissores e hormônios. O receptor 5-HT1, presente na família do receptor 5-HT, trabalha juntamente com as proteínas Gi/Go e com a adenilato ciclase que causa a produção do AMPc. O receptor 5-HT1A, subtipo do receptor 5-HT1, está associado ao receptor acoplado à proteína G (GPCR) em que articula a neurotransmissão por meio dos canais de K^+ e Ca^{2+}, levando a uma inibição do canal de Ca^{2+} dependente de voltagem, uma ativação do canal de K^+ retificador de corrente de entrada e do canal de K^+ retificador de corrente de entrada ativado por proteína G (GIRK). Esses receptores, presentes em níveis pré e pós-sinápticos, atuam em processos importantes em que amenizam efeitos anticonvulsivantes (Raymond et al., 1999; Bevilaqua et al., 1997), como demonstra em um estudo recente de modelo experimental de camundongos machos, em que há uma administração de CBD e logo após é injetado uma dose de pentilenotetrazol (PTZ) para indução de crise epiléptica generalizada. Os resultados foram satisfatórios, demonstrando uma redução considerável na gravidade das crises epilépticas (Pelz et al., 2017).

Todavia, o CBD mostra maior afinidade pelos receptores 5-HT, receptores GPCRs não endocanabinoide e outros alvos, como enzimas e canais iônicos (Ibeas et al., 2015). Os receptores de adenosina (ARs) são GCPRs que são incitados pela adenosina endógena e estão envolvidos em diversos processos de doenças (Dal Ben et al., 2019). O CBD pode ativar os ARs (Gonça and Darıci, 2015) e atuar como antagonista do receptor acoplado à proteína G, que em humanos é codificado pelo gene GPR55, e também atua como agonista do receptor vanilóide 1 (TRPV1) e receptor vanilóide 2 (TRPV2), que são canais catiônicos que modulam diversos sistemas e podem estimular processos do metabolismo (Izzo et al., 2009), e atua como neuroprotetor por consequência da ativação do receptor ativado por proliferadores de peroxissoma gama (PPARγ) (Esposito et al., 2011; Scuderi et al., 2014).

O GPR55 é um receptor transmembrana 7 pelo qual o CBD tem alta afinidade (Gray and Whalley, 2020). Além de processos celulares, o GPR55 também está envolvido em diversos processos fisiológicos, sendo promissor na sua ação antiepiléptica e possui um efeito atenuante em que contrapõe a atividade do CB1 e bloqueia a transmissão de neurotransmissores (Ryberg et al., 2007), essa ação reforça sua efetividade no tratamento em pacientes epilépticos que possuem farmacorresistência (Jones et al., 2010). Estudos recentes em um modelo experimental de camundongo com síndrome de Dravet geneticamente induzida, o CBD foi administrado e reduziu consideravelmente a quantidade de crises epilépticas espontâneas e melhorou a hiperatividade causada pela doença, e a concentração

do CBD administrado durante o tratamento agudo foi de extrema importância para os resultados obtidos (Kaplan et al., 2017). Diante disso, os diferentes alvos do CBD devem ser melhor estudados e compreendidos para alvo terapêutico no tratamento contra a epilepsia.

1.4 EPILEPSIA REFRATÁRIA INFANTIL

1.4.1 Uso de CBD no cérebro em desenvolvimento

Os efeitos da *Cannabis* vêm sendo estudados e discutidos há muitos anos, mas ainda pouco se sabe sobre sua ação a longo prazo. No entanto, o seu uso terapêutico em doenças como ansiedade, depressão, esclerose múltipla, doença de Alzheimer, Parkinson e epilepsia demonstram resultados promissores, além de possuir um desempenho em aliviar dores e inflamações (Kogan and Mechoulam, 2007). Entretanto, o seu uso não pode ser indiscriminado, visto que a utilização em excesso causa dependência e psicose, assim como afeta funções cognitivas, tais como o aprendizado, memória e atenção. Sendo assim, seus efeitos deletérios foram elucidados em estudos pré-clínicos e clínicos (Poleg et al., 2019), como também seus efeitos benéficos em que valida ser um alvo terapêutico para doenças graves em modelos de estudos *in vivo* e *in vitro* (Kaur et al., 2016).

O Δ9-tetrahidrocanabinol (Δ9-THC) é o composto da *Cannabis* responsável pelos efeitos psicotrópicos (Fitzgerald et al., 2013) e age como um agonista incompleto dos receptores CB1 e CB2. Em contraponto, o CBD possui um menor alcance na interação com os receptores CB1 e CB2, mas possui efeitos positivos em várias funções cerebrais (Campbell et al., 2017).

O FDA é um órgão responsável pela autorização de inúmeros artigos ligados à saúde e bem-estar humano, e possui um regulamento em que reconhece e autoriza o uso de derivados da *Cannabis* para fins medicinais (FDA, 2019, fda.gov) (Tabela 1).

Tabela 1 - Produtos derivados da planta *cannabis* em que foi autorizado o uso terapêutico pediátrico pela FDA.

Canabinoide	Produtos derivados da *Cannabis*	Indicação aprovada pelo FDA	Ensaios clínicos para condições pediátricas do neurodesenvolvimento
CBD (derivado de plantas)	Epidiolex®	Crises epilépticas associadas a síndromes de Lennox-Gastaut ou Dravet em pacientes com 2 anos de idade ou mais;	TEA Espasmos Infantis Epilepsia Epilepsia Ausência na Infância Convulsões da Síndrome de Prader-Willi Síndrome de Lennox-Gastaut Síndrome de Dravet Epilepsia Intratável Síndrome de Sturge-Weber Síndrome de Rett Esquizofrenia
Dronabinol (THC sintético)	Marinol®, Syndros®	Anorexia, perda de peso em pacientes com AIDS; Náuseas e vômitos associados à quimioterapia do câncer;	
Nabilona (THC sintético)	Cesamet®	Náuseas e vômitos associados à quimioterapia do câncer;	
Nabiximols (1:1 - CBD e THC derivados de plantas)	Sativex®*	Espasticidade moderada a grave devido à esclerose múltipla*.	Paralisia cerebral

Canabinoides derivados da planta *Cannabis sativa* que são indicações aprovadas pelo FDA para tratamento de doenças neurológicas diante de resultados positivos em ensaios clínicos para condições pediátricas do neurodesenvolvimento. FONTE: FDA, 2019, fda.gov.

Apesar de muitos estudos demonstrarem efeitos positivos do uso do CBD *in vitro*, outros estudos trouxeram resultados importantes em que relatam dados provenientes do uso de CBD puro em pacientes jovens e crianças que sofriam de epilepsia refratária, e esses dados foram obtidos por meio dos pais que acompanharam o desempenho do tratamento (Porter and Jacobson, 2013; Maa and Figi, 2014; Hussain et al., Saade and Joshi, Press et al., 2015; Tzadok et al., Shannon and Opila-Lehman, Rosemergy et al., Crippa et al, 2016; Treat et al., Aguirre-Velázquez, 2017; Hausman-Kedem et al., Rajaraman et al., 2018; Knupp et al., 2019). A repercussão desses fatos salientaram a eficiência no combate a crises epilépticas, descartando efeitos colaterais, porém ainda pouco se sabe sobre seu efeito no neurodesenvolvimento e a padronização de protocolos de administração do CBD.

O site ClinicalTrials.gov, quando pesquisado sobre epilepsia refratária e o uso de CBD como meio de tratamento, apresenta cerca de 17 estudos de intervenção clínica mundiais em que estão recrutando pacientes de 0 a 17 anos e adultos de 18 a 64 anos, alguns que estão em andamento e outros que já estão finalizados. Diante disso, diversos estudos reforçam cada vez mais o uso benéfico e seguro de CBD em crianças com epilepsia refratária, porém apresenta efeitos adversos no neurodesenvolvimento de crianças e adolescentes (Campbell et al., 2017). Dessa forma, o CBD, seu mecanismo de ação e sua modulação no SEC devem ser mais aprofundados e esclarecidos.

1.5 JUSTIFICATIVA

A epilepsia refratária designa uma resistência severa a tratamentos e é diagnosticada quando não há uma resposta adequada ao uso de antiepilépticos prescritos para o controle da crise. Diante disso, estudos clínicos sugerem diferentes vias de tratamento, dentre elas o uso do CBD. Tratamentos com CBD têm apresentado efeito benéfico em crianças com epilepsia refratária, e modelos de estudo demonstram ação favorável do CBD quanto exposto à modulação do SEC. Desta maneira, o CBD deve ser melhor investigado sobre sua eficácia, mecanismo de ação, neuroproteção, neurotoxicidade e no seu impacto com a administração a longo prazo. Portanto, a liberação do CBD para tratamento em crianças deve ser bastante avaliada o seu risco e benefício, pois seu uso deve ser liberado apenas para crianças com diagnóstico de problemas graves. A preocupação é que com o uso do CBD para tratar esse tipo de doença, acabe gerando uma falsa ideia de que é um produto inerte que possa ser utilizado de forma irrestrita em doenças como ansiedade, transtorno do déficit de atenção com hiperatividade (TDAH) e outras doenças de comportamento infantil, visto que não possuem tantos resultados comprovados, e que segundo o nosso trabalho pode ser um problema.

1.6 OBJETIVOS

1.6.1 Objetivo geral

Verificar *in vitro* a expressão de enzimas e receptores do sistema endocanabinoide e de endocanabinoides em resposta ao tratamento com CBD, avaliando os níveis de expressão dos genes que codificam os receptores canabinoides e as principais enzimas de síntese e degradação dos endocanabinoides - CNR1 (CB1), CNR2 (CB2), DAGLA e MAGL (2-AG), NAPEPLD e FAAH (AEA) por RT-qPCR. Também foi avaliado efeitos neuroprotetores e neurotóxicos do CBD e seus derivados sintéticos: HUF-101, (-) -5′-DMH-CBD e HU-556.

1.6.2 Objetivos específicos

a) Realizar revisão bibliográfica do efeito do CBD, como também a interação do SEC, buscando elucidar os mecanismos de ação e abordar discussão de estudos clínicos;
b) Avaliar o efeito do tratamento com CBD em modelo pré-clínico de neurônio maduro (terminalmente diferenciados) e no desenvolvimento neuronal (durante a diferenciação neuronal):
    - sobre os níveis de expressão de genes que codificam enzimas e receptores do SEC por RT-qPCR;
    - efeito neuroprotetor / neurotóxico do CBD e seus derivados sintéticos quando desafiados com H_2O_2 e 6-OHDA, bem como o papel do receptor CB1 em seus efeitos.

2 ARTIGO CIENTÍFICO

Effect of cannabidiol on the modulation of the endocannabinoid system in a preclinical model of a mature neuron and neuronal development

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Abstract

The *Cannabis sativa* plant is known worldwide as a recreational drug that has been used for a variety of medicinal purposes. Its compounds were isolated and identified as phytocannabinoids, the best known being Δ9-tetrahydrocannabinol (Δ9-THC, main psychoactive compound) and cannabidiol (CBD, non-psychoactive). To date, there have been no documented adverse effects on the action of CBD in the treatment of diseases. In the present study, we evaluated the expression of enzymes and receptors of the endocannabinoid system (ESC) and of endocannabinoids in response to CBD treatment, evaluating the expression levels of genes that encode cannabinoid receptors and the main enzymes of synthesis and degradation of endocannabinoids - *CNR1* (CB1), *CNR2* (CB2), *DAGLA* and *MAGL* (2-AG), *NAPE-PLD* and *FAAH* (AEA) in a preclinical cell model of a mature neuron (terminally differentiated) and in neuronal development (during neuronal differentiation) of human neuroblastoma cell line SH-SY5Y. We also evaluated the neuroprotective/neurotoxic effect of CBD when exposed to a hydrogen peroxide (H_2O_2) and 6-0HDA challenge. We used a 0.1 μM dose of CBD as it has been shown to be protective. In the neuronal development model, there was a reduction in the expression of NAPE-PLD and an increase in the expression of FAAH, demonstrating interference in the synthesis and degradation of

anandamide (AEA), reducing its bioavailability. In the same model, we found neuroprotective effects in the use of 6-OHDA, different from H_2O_2. Cannabinoids are protective at lower concentrations or ineffective/toxic at higher concentrations in neuronal development. Therefore, careful use of CBD in child and young patients is necessary.

Keywords: Cannabidiol, Endocannabinoid System, Model of neuronal development, Neuroprotection

Introduction

The *Cannabis Sativa* plant is known for its medicinal purposes [1] and was discovered in China in 4000 BC [2]. Cannabis has fat-soluble compounds that are found in the resin of the female plant, and these compounds are called phytocannabinoids [3 - 4].

The most well-known and studied phytocannabinoids are Δ9-tetrahydrocannabinol (Δ9-THC, the main psychoactive compound in Cannabis) and cannabidiol (CBD, a non-psychoactive compound [5]. CBD is described as protective and therapeutic for many neurological conditions [6], with remarkable effects in children with refractory epilepsy, acting through several targets, including the endocannabinoid system (ECS) [7]. CBD also acts on the type 1 cannabinoid receptors (CB1), which has low affinity [8], and also at other points of the ECS, such as endocannabinoid synthesis/degradation enzymes [9].

The ECS is a retrograde messenger system [10] in which, in response to neurotransmission, endocannabinoids such as Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are produced on demand at the postsynaptic neuron membrane. These endocannabinoids will act on the synaptic cleft activating CB1 receptors (and eventually CB2 receptors, in some types of cells such as pyramidal neurons) present in the membrane of pré-synaptic neurons. This activation of CB1 leads to a reduction in the release of neurotransmitters by the presynaptic neuron, thus regulating the synapse [11]. The ECS is a highly modulable system during neuronal development, and acts through several signaling pathways, which demonstrates its importance to be able to direct different types of treatments [12 - 13]. The role of endocannabinoid signaling is critical to the functioning of many aspects of the mature and developing brain [14], in view of which any disturbance that occurs within the ECS can interfere with neural and neuronal development [15].

The human neuroblastoma lineage SH-SY5Y, being of human origin, is widely used for studies on neurodegenerative diseases because it is easier to be cultivated, allowing studies

on neurotransmission [16]. This lineage can be differentiated into a mature neuron phenotype by retinoic acid (RA) induction [17] and increases the sensitivity of cells to neurotoxins and neuroprotective mechanisms [18]. This *in vitro* model simulates the mature (adult) neuron model, when cells are treated after differentiation, and neuronal development (young) model, when they are treated during neuronal differentiation. A previous study by our group used this strain to verify the effects of CBD and it was observed that the neuronal development model was more sensitive to the effects of CBD and also to the subsequent challenge with redox-active neurotoxins [19]. Therefore, it is still necessary to verify the mechanisms involved and this project will elucidate the involvement of ECS components, the main target of cannabinoids, and the effects of CBD in these two models. In view of this, several studies increasingly reinforce the beneficial and safe use of CBD in children with refractory epilepsy, but it has adverse effects on the neurodevelopment of children and adolescents [20]. In this way, CBD, its mechanism of action and its modulation in the ECS should be further explored and clarified.

Experimental Procedures

Chemicals

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil). 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 and Trizol (Invitrogen) for MTT.

Cell culture, neuronal differentiation and treatments

In this work, we used cells from the human neuroblastoma lineage SH-SY5Y (ATCC, Manassas, VA, USA). These cells were cultured in a medium with a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 with the addition of 10% fetal bovine serum (FBS), 2 mM glutamine, 1000 U/mL penicillin, 1000 µg/ mL of streptomycin and 2.5 µg/mL of fungizone® (amphotericin B), and were kept at a temperature of 37°C in a humid

incubator with 5% CO_2. Cultivation was performed with a change of medium every three days and, after the cells reached a confluence of about 80%, trypsinization was performed to subject the cells to detachment by the presence of intercellular binding proteins. After trypsinization, the cells were centrifuged at 500 rpm for 4 minutes, discarding the medium and resuspending the pellet formed by the cells. Subsequently, the cells were plated in 25 cm² bottles or in 96-well plates. Cells need to go through 24 hours after plating and then neuronal differentiation occurs. Neuronal differentiation occurs by reducing FBS to 1% and adding 10 µM of retinoic acid (RA) to the culture medium for 7 days [17]. In the evaluation of treatments in the mature neurons model, after 7 days of neuronal differentiation, SH-SY5Y cells were treated with CBD or synthetic derivatives for 24 hours. The evaluation of the effects of cannabinoids on the neuronal development model occurred by co-administration of AR with CBD or synthetic derivatives from the beginning of differentiation. On the 7th day, the AR and CBD were replaced and, after 24 hours, the experiments were performed. On the 8th day, the experiments were performed as described below.

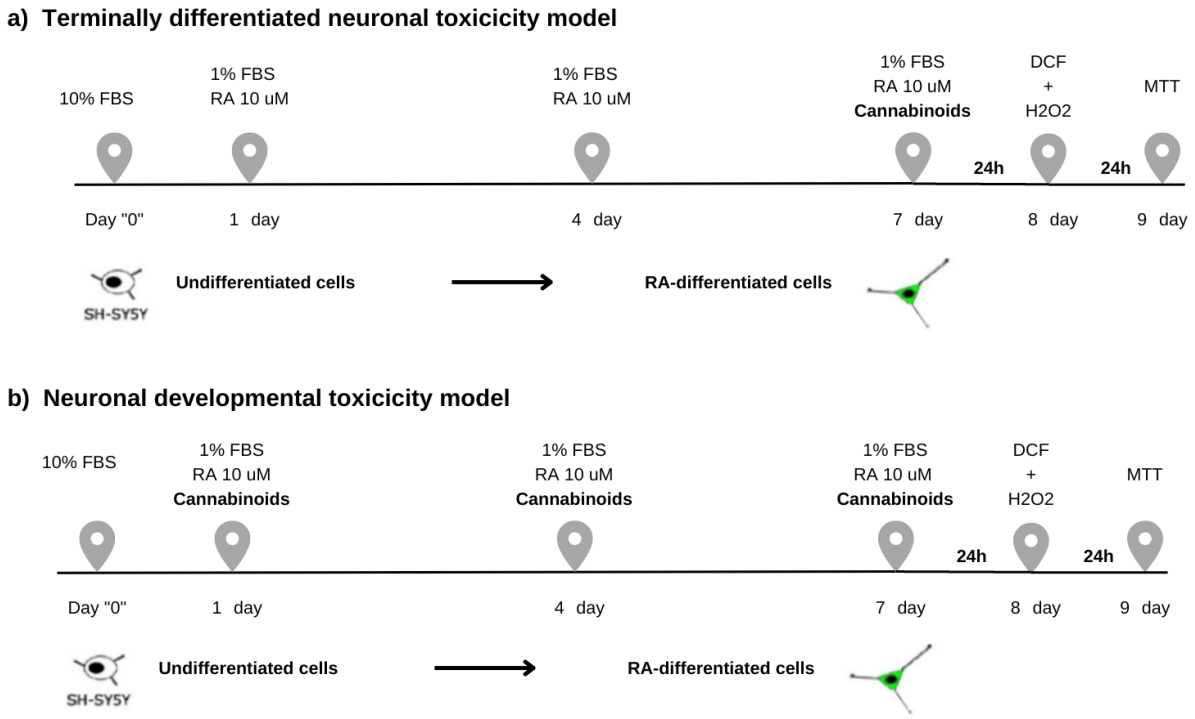
Neurotoxic/neuroprotective challenge

We used SH-SY5Y human neuroblastoma cells and performed the same cell culture and trypsinization protocol. Subsequently, the cells were plated in 96-well plates. 24 hours after plating, neuronal differentiation was initiated. The neuronal development model received treatment with CBD and its synthetic derivatives during 7 days of neuronal differentiation, whereas in the mature neuron model, CBD and its synthetic derivatives were administered on the 7th day of neuronal differentiation. After treatments with CBD or synthetic derivatives in the mature neuron and neuronal development model, the cells were subjected to a neurotoxic challenge with 375 µM of hydrogen peroxide (H_2O_2). Additionally, CBD-treated cells were also co-treated with 1,0 µM of CB1 antagonist (AM251) and challenged with 15 µM of 6-hydroxydopamine (6-OHDA). After treatment with the toxins, the cells were incubated at 37°C for an additional 24 hours. Afterwards, a colorimetric assay was performed to evaluate the cell viability of the cells by quantifying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from the reduction to a blue formazan product by dehydrogenases cell phones. Cells were incubated with 0.5 mg/ml MTT for 1h at 37°C. Then the medium was discarded and DMSO was added to solubilize the

formazan crystals. Absorbance was determined at 560 and 630 nm on a SoftMax Pro M5 Microplate Reader (Molecular Devices, USA). To evaluate the production of reactive oxygen species (ROS), the treatments with cannabinoids were performed as described above, and after 24 hours of treatment on the 7th day, the cells were incubated with dichlorofluorescein (DCF) solubilized in DMEM and Ham's F12 without addition of FBS for 1 hour at 37°C. Soon after, the medium was discarded and the cells were challenged with 375 μM of H_2O_2 and immediately followed by fluorescence reading in the SoftMax Pro M5 Microplate Reader (Molecular Devices, USA), with excitation at 485 nm and emission at 538 nm, with kinetics every 2 minutes and 30 seconds and a total time of 30 minutes.

The experiments were performed four times, which is commonly used as η (sample number) for neurotoxic/neuroprotective assessment. Results were expressed as percentage of untreated cells (mean ± SD value).

Fig. 1



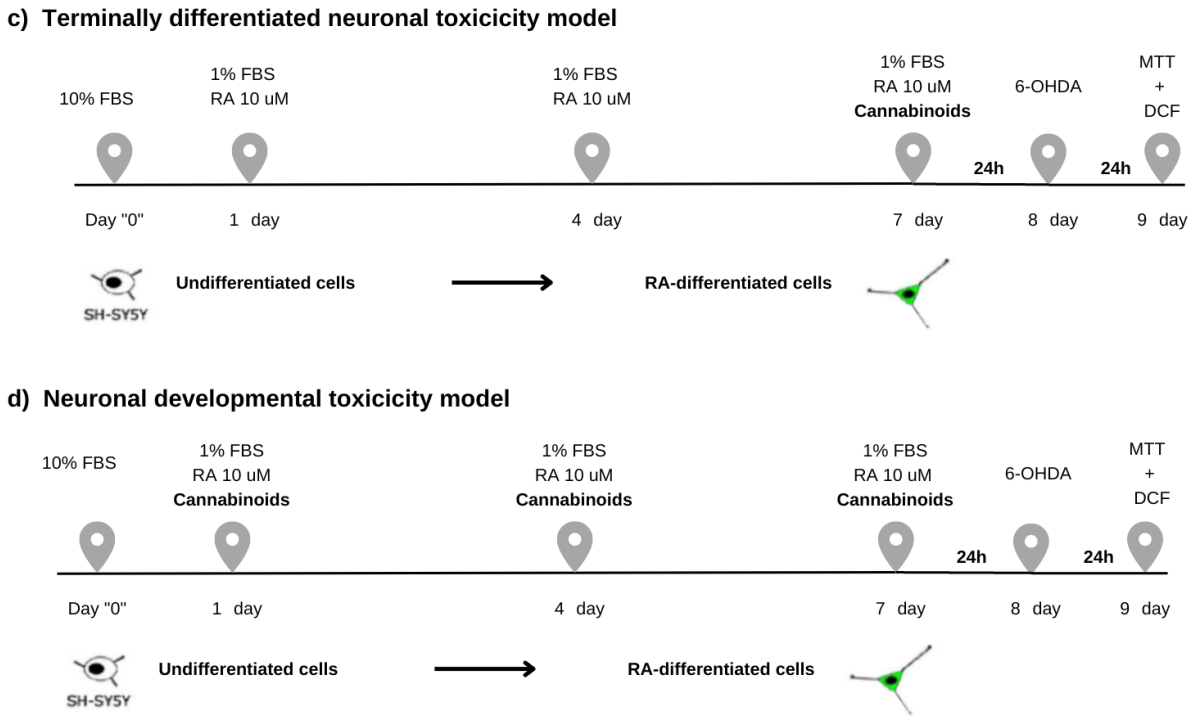


Fig. 1 Experimental models used for treatment with 0.1 µM CBD, HUF-101, (-)-5′-DMH-CBD and HU-556; (a) and (c) terminally differentiated neuronal model with addition of AR, being treated with CBD only on the 7th day, on the 8th day the challenge with H_2O_2 and 6-OHDA takes place. Still on the 8th day, the H_2O_2 DCF is performed. On the 9th day the MTT is performed; (b) and (d) model of differentiated neuronal development with addition of AR, being treated with CBD during the 7 days of differentiation, on the 8th day the challenge with H_2O_2 and 6-OHDA takes place. Still on the 8th day, the H_2O_2 DCF is performed. On the 9th the MTT is executed

Processing of RNA samples

After CBD treatments, the medium was removed and the cells were washed with PBS 1X to remove cellular *debris* and traces of medium and FBS. Total RNA was purified on Trizol (Invitrogen) according to the manufacturer's instructions. After extraction, the RNA was quantified by spectrophotometry (NanoDrop) and 2 µg of RNA was used for the synthesis of cDNA by reverse transcriptase, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's instructions.

RT-qPCR

The resulting cDNA samples were diluted 20x in DEPC-treated ultrapure water (Ambion®) and used for gene expression analysis by quantitative real-time PCR (RT-qPCR) using specific *primers* for each gene (table 1) with the *GoTaq® qPCR and RT-qPCR Systems kit* (Promega). For normalization, *primers* for the constitutive gene GAPDH – already used in our laboratory – were used to calculate the relative expression of $2^{-\Delta\Delta Cq}$. The experiments were performed six times, which is commonly used as η (sample number) for the evaluation of gene expression and were analyzed in triplicate.

Table 1

Gene	**Gene ID**	***Primer F* (5'- 3')**	***Primer R* (5'- 3')**
Diacylglycerol lipase alpha (2-AG synthesis)	*DAGLA*	TGTCACCCTCGGAATGGTTG	GGTTGTAGGTCCGCAGGTTA
N-arachidonoylphosphatidylethanolamine phospholipase-D (AEA synthesis)	*NAPEPLD*	CTTTAGCTCTCGTGCTTCACC	CGCATCTATTGGAGGGAGTTCA
Fatty acid amide hydrolase (AEA degradation)	*FAAH*	TGTCAACAGGTGGGCTCTTC	TTTCCAGCCGAACGAGACTT
Monoacylglycerol lipase (2-AG degradation)	*MAGL*	ACACCCAAGGCCCTCATCTTT	ACATGCTGCAACACATCCCT
CB1 (cannabinoid receptor type 1)	*CNR1*	TTCCTCACAGCCATCGACAG	TGCAGTTTCTCGCAGTTCCA
CB2 (cannabinoid receptor type 2)	*CNR2*	TGACCGCCATTGACCGATAC	TAGTGCTGAGAGGACCCACA

Table 1. List of primers that were used for RT-qPCR

Statistical Analysis

Data were computed and analyzed using GraphPad Prism 8. For RT-qPCR data, an unpaired t test with equal SD of at least six independent experiments performed in triplicates was performed (n=6). The results of neurotoxic challenges were analyzed by ordinary one-way ANOVA followed by Tukey's test of at least 4 independent experiments performed in 8 replicates (n=4). Differences were considered significant at $P < 0.05$.

Results and discussion

Dose curve

Initially, we performed a dose-response curve with concentrations of 0.01 to 2.5 μM of CBD in order to elucidate its neuroprotective effects [21] when administered in the SH-SY5Y model of human neuroblastoma in the mature neuron model (Fig. 2a) and in the neuronal development model (Fig. 2b).

Fig. 2

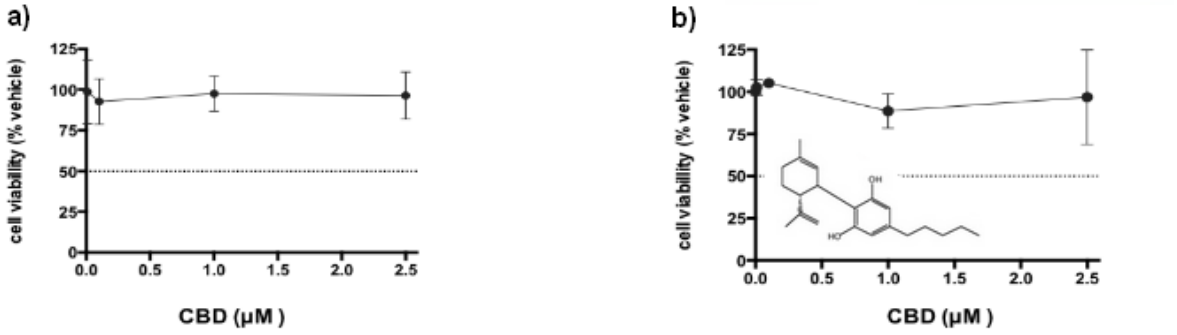


Fig. 2 Dose-response curve (0.01 to 2.5 μM) for CBD in the different experimental models: (a) mature neuron model and (b) neuronal development model

In the dose curve for CBD, no dose was toxic (it was not significant in any treatment). We chose 0.1 μM because previous data from the research group [19] the 2.5 μM dose sensitized the cells of these same models to the neurotoxic challenge. This result exemplifies why CBD has an inverted bell effect on the dose-response curve, as low doses are more effective [22 - 23]. Besides, previous unpublished data from our research group showed that

the 0.1 μM of CBD has a neuroprotective effect in the developmental model neuronal. Therefore, this was the concentration selected to be used in this work.

CBD effects on ECS modulation

Endocannabinoids and phytocannabinoids have several molecular targets in common and may be similar to some extent, as they have affinity for receptors, ligand-susceptible ion channels, and nuclear receptors [24 - 25]. GPR55, for example, can be activated by AEA and 2-AG, but little is known about this interaction [26 - 27]. However, CBD can act as an agonist and antagonist on GPR55 [28]. The action of CBD against the advancement of neurodegenerative diseases is still poorly understood, but it shows promising ways when it potentially acts as a neuroprotector when it activates PPARγ, which can also be activated in the presence of AEA and 2-AG [29 - 30]. In view of this, it is possible to understand that a modulation of the ECS is favorable for the advancement of treatments for diseases that affect the CNS.

Fig. 3

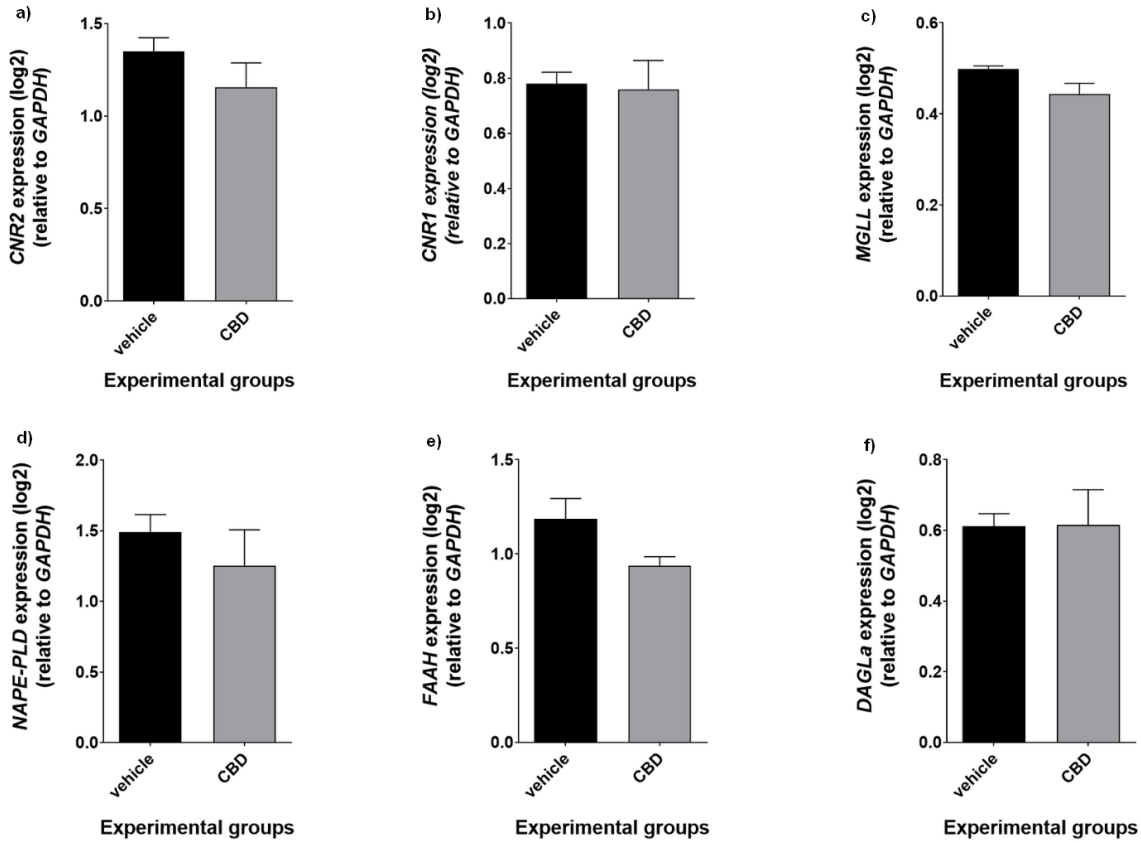


Fig. 3 RT-qPCR on genes encoding CB2 (a) and CB1 (b) receptors and the enzymes responsible for the 2-AG degradation (c), AEA synthesis (d), AEA degradation (e) and 2-AG synthesis (f) of the ECS in a mature neuron model. The results were analyzed by the unpaired t test with equal SD of at least six independent experiments performed in triplicate (n = 6).

Fig. 4

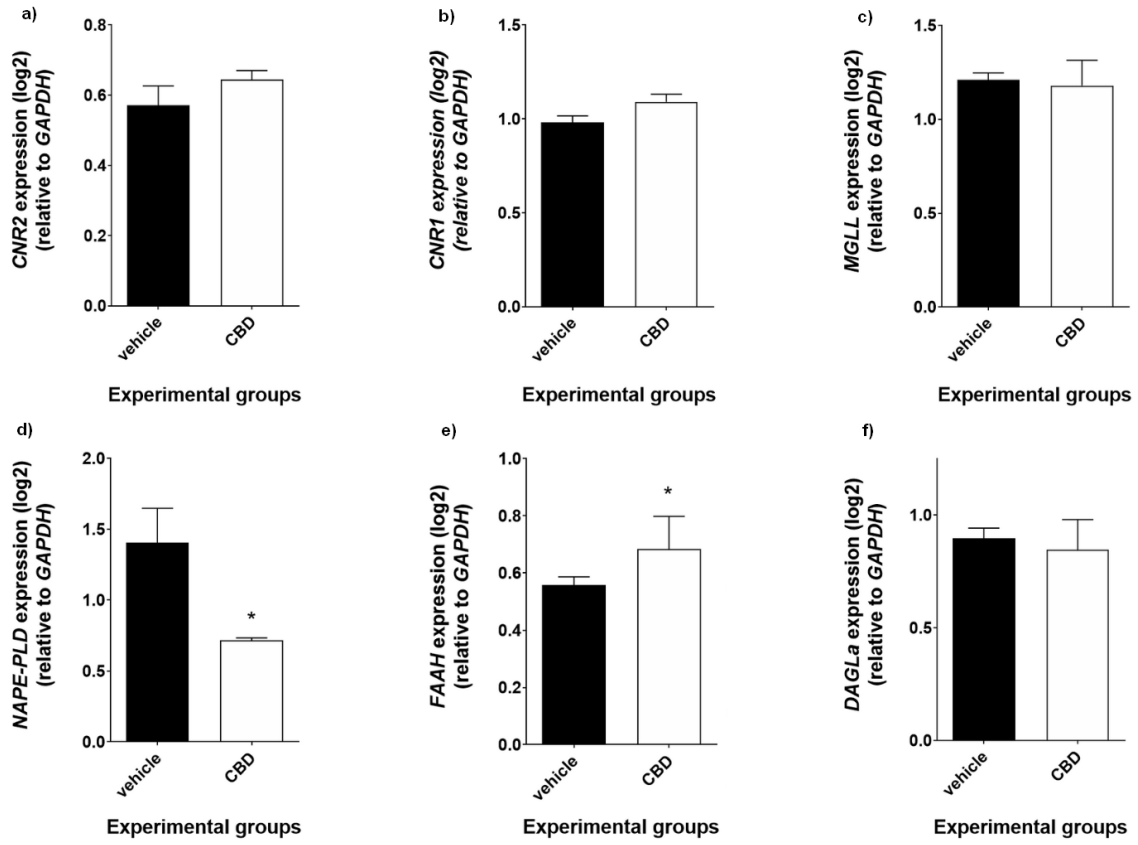


Fig. 4 RT-qPCR on genes encoding CB2 (a) and CB1 (b) receptors and the enzymes responsible for the 2-AG degradation (c), AEA synthesis (d), AEA degradation (e) and 2-AG synthesis (f) of the ECS in the model of neuronal development. The results were analyzed by the unpaired t test with equal SD of at least six independent experiments performed in triplicate (n = 6).

In our mature neuron model (Fig. 3) there was no influence of CBD on the expression levels of the analyzed genes. However, in the neuronal development model (Fig. 4) there was a significant difference, in which there is a decrease in NAPE-PLD expression levels (Fig. 4d) and an increase in FAAH expression levels (Fig. 4e) in response to treatment with 0.1 uM CBD. These two genes code for the endocannabinoid AEA synthesis and degradation

enzymes, respectively. The alteration in the expression levels of these enzymes probably leads to a decrease in the endogenous levels of AEA. This idea can be reaffirmed in the face of different models of recent studies in which CBD is administered to adolescent rodents, at a stage prone to manifest chemical dependence, in order to act as a potential therapeutic to prevent relapses in amphetamine users. When exposed to CBD, there is an excitability of the ECS, causing positive effects to prevent relapse. It was also possible to observe a change in synthesis and degradation similar to our work, where amphetamine reduced AEA neurotransmission [31]. Another study that reaffirms our data was performed in mice treated with THC and CBD, where brain samples were processed and analyzed in different regions, analyzing the effect of THC and CBD on the CNS. The results demonstrate the influence of CBD at the lipid level in the brain, and also on the activation of NAPE-PLD [32]. In view of this, it is possible to affirm that there is an interference of CBD in the synthesis and degradation of AEA and that this mechanism can be better elucidated when a lipidomics is performed, which may include the intervention of CBD in biological processes.

Therefore, we did not observed any difference in the gene expression of the CB1 receptor. CBD did not directly alter CB1 expression levels, but it did alter levels of enzymes that determine the bioavailability of one of its endogenous ligands: the endocannabinoid AEA.

Neurotoxic and neuroprotective effects of CBD and synthetic derivatives

In this work we tested CBD against a neurotoxin present in the nervous system, 6-OHDA. This neurotoxin is capable of gradually destroying catecholaminergic nerves in innervated tissues, and has been widely used as an experimental model in studies of Parkinson's disease [33]. We performed a neurotoxic challenge in cells treated with 0.1 μM CBD co-treated with CB1 antagonist, and in the mature neuron model there was no neurotoxic or neuroprotective effect (Fig. 5a). However, in the neuronal development model, the 0.1 μM dose of CBD was neuroprotective, but lost neuroprotection after addition of the CB1 antagonist, a evidence that this receptor participates in the neuroprotective effects of CBD (Fig. 5b).

Fig. 5

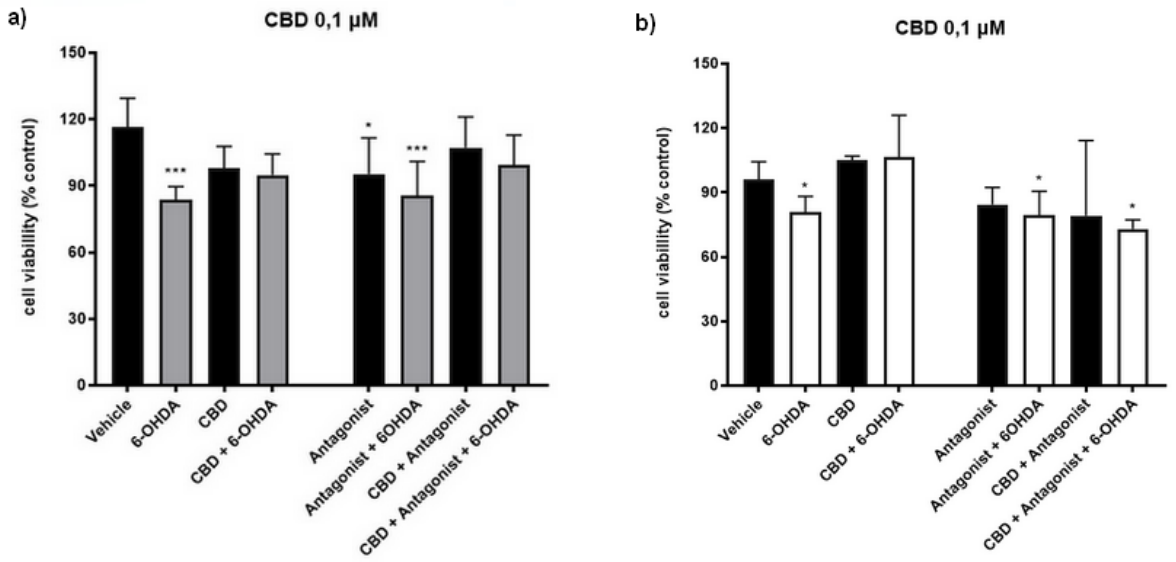


Fig. 5 Neurotoxic challenge in cells co-treated with modulators of CB1 activity and with 0.1 µM CBD in the mature neuron model (a) and in the neuronal development model (b). The results were obtained by ordinary one-way ANOVA followed by Dunnet's test of at least 4 independent experiments performed in quadruplicate (n=4)

Endocannabinoids are possibly acting on the tone of other synapses, as blocking CB1 by an antagonist will not necessarily have the same effect as removing endocannabinoids. Since AEA main signaling pathway is CB1, with the pharmacological effect of being a negative modulator of something, since if the receptor is active when bound to a ligand, CBD binds at another modulating site on the receptor, and this causes its activity to increase [34].

We also performed the neurotoxic challenge of the 0.1 µM treatment of CBD and its synthetic analogs, HUF-101, DMH and HU-556, in a more intense damage challenge with the LD_{50} of H_2O_2 (375 uM) (Fig. 6).

Fig. 6

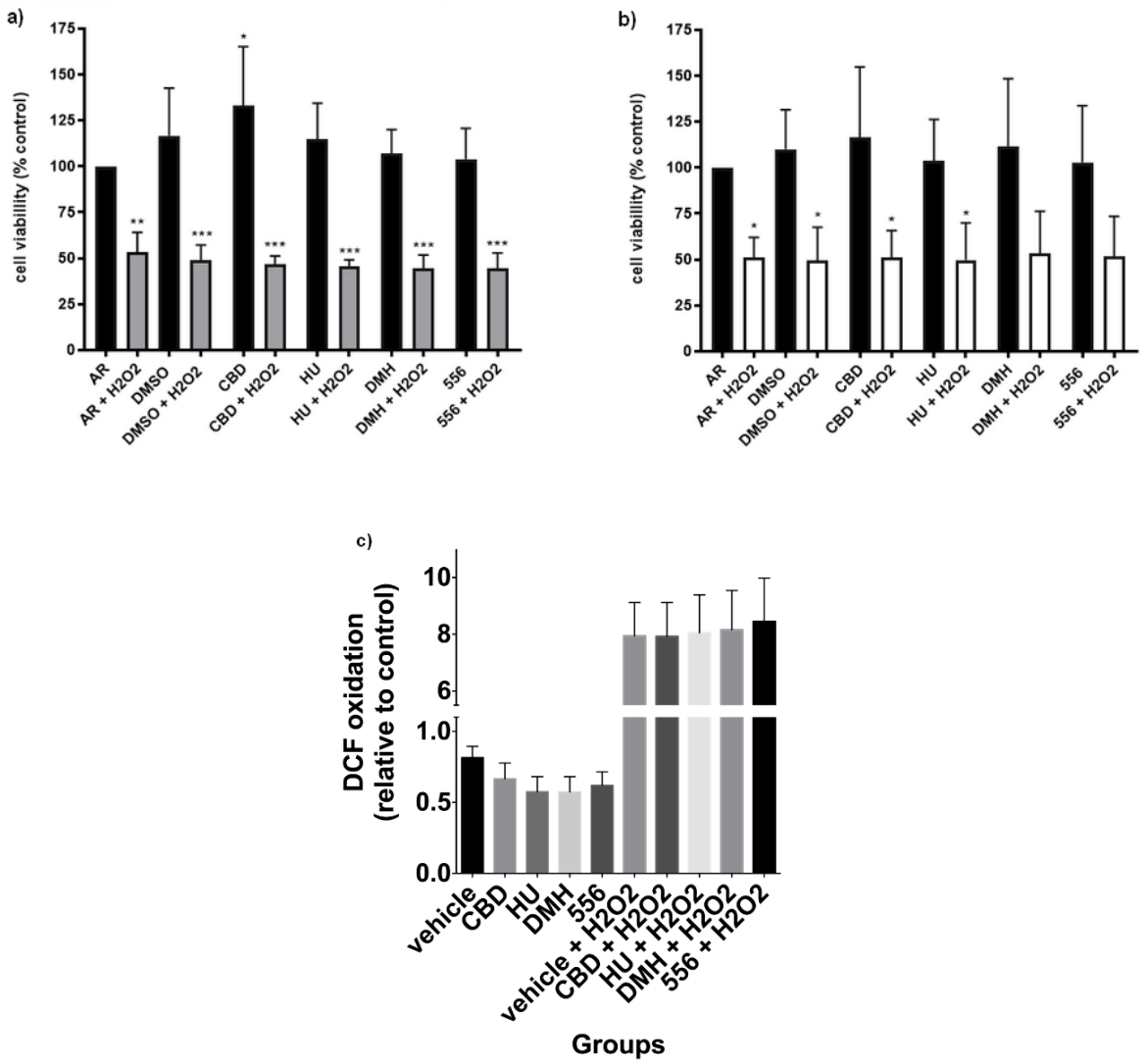


Fig. 6 Neurotoxic challenge of 0,1 μM treatment of CBD, HUF-101, DMH and HU-556 in mature (a) and developing (b) neuron models for analysis of neuroprotective/neurotoxic effects and for ROS production in both the treatment models (c). Results were obtained by one-way ANOVA followed by Dunnet's test of at least 4 independent experiments performed in 8 replicates (n=4)

In the neurotoxic challenge, there was no neuroprotective effect in both experimental models in the treatment with CBD and its synthetic derivatives (Fig. 6a, b). Also, no antioxidante effects were observed in the underdevelopment model (Fig. 6c) and in mature neurons (data not show). In the H_2O_2 LD_{50} challenge, the damage may have been too intense

and, consequently, it was not possible to detect any neuroprotection of CBD and its synthetic derivatives.

Therefore, CBD has a low affinity for CB1 [35 - 36] and its effects are observed only in some brain regions [37 - 38]. Thus, it is possible to affirm that CBD acts indirectly on the ECS, acting as an indirect agonist. This hypothesis is explained by the action of CBD in blocking the FAAH (enzyme that participates in the hydrolysis of AEA) in previous studies [35 - 39]. In our results, CBD reduced FAAH expression and increased NAPE-PLD expression, possibly reducing AEA levels, only in underdevelopment neurons. Therefore, CBD ends up modulating the expression levels of enzymes that regulate one of the endogenous ligands of CB1, the AEA, and the neurotoxic challenges with co-treatment with CBD and a CB1 antagonist showed that the neuroprotective effect of CBD is mediated by the receptor CB1 [40].

Conclusion

Our work demonstrates a difference between neuronal and mature neuron models of development. We saw a lower bioavailability of AEA, CB1 binder. Therefore, the effect we are seeing of CBD is not directly on the receptor, but on the availability of a CB1 receptor ligand. Bearing in mind that the ECS is involved in the development of the neuronal system, this can be a problem if CBD is administered at a time when there are a lot of developing cells, which can affect development. Thus, in our work we found effects of exogenous phytocannabinoids only in the modulation of developing neurons, as possibly occurs in the infant brain. Other studies have also shown similar results using other models at the lipid and protein level [26; 19]. Given this, still little is known about the effects of CBD on immature neurons.

Consequently, the release of CBD for treatment in children must be carefully evaluated its risks and benefits, as its use should only be released for children diagnosed with serious problems. The concern is that with the use of CBD to treat this type of disease, it ends up generating a false idea that it is an inert product that can be used unrestrictedly in diseases such as anxiety, attention deficit hyperactivity disorder (ADHD) and other diseases of childhood behavior, since they do not have so many proven results, and that according to our work can be a problem. Finally, more studies are necessary to assure the safety and efficacy of CBD and synthetic derivatives in periods of active neurodevelopment.

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Cinthia Borba Garofalo, Patrícia Schönhofen and Fábio Klamt. The first draft of the manuscript was written by Cinthia Borba Garofalo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available in the PUBMED (NIH) repository, [https://pubmed.ncbi.nlm.nih.gov/25108670/].

3 CONCLUSÕES E PERSPECTIVAS

Os endocanabinoides e fitocanabinoides possuem diversos alvos moleculares em comum e podem ser semelhantes até certo ponto, visto que possuem afinidade com receptores, canais iônicos suscetível a ligantes e receptores nucleares (Alexander, 2016; Di Marzo and Piscitelli, 2015). O GPR55, por exemplo, pode ser ativado pela AEA e 2-AG, porém ainda pouco se sabe sobre essa interação (Martínez-Pinilla et al., 2014; Zhao and Abood, 2013). No entanto, o CBD pode agir como agonista e antagonista no GPR55 (Ryberg et al., 2007). A ação do CBD contra o avanço de doenças neurodegenerativas ainda é pouco elucidado, mas demonstra caminhos promissores quando age potencialmente como neuroprotetor quando ativa o PPARγ, o qual também pode ser ativado na presença de AEA e 2-AG (Scuderi et al., 2014; O'Sullivan, 2007). Diante disso, é possível compreender que uma modulação do SEC seja favorável para o avanço de tratamentos de doenças que afetam o SNC. A síntese e degradação do AEA demonstra alterações em nosso modelo de desenvolvimento neuronal pelo aumento da NAPE-PLD e diminuição da FAAH. A alteração

nos níveis de expressão destas enzimas provavelmente leva a uma diminuição dos níveis endógenos de AEA. Esta ideia pode ser reafirmada diante de diferentes modelos de estudos recentes em que o CBD é administrado em roedores adolescentes, em um estágio propenso a manifestar dependência química, no intuito de agir como um potencial terapêutico para evitar recaídas em usuários de anfetamina. Quando expostos ao CBD ocorre uma excitabilidade do SEC, ocasionando efeitos positivos para evitar a recaída. Também foi possível observar uma alteração na síntese e degradação semelhante ao nosso trabalho, em que a anfetamina reduziu a neurotransmissão do AEA (Metz et al., 2022). Outro estudo que reafirmam nossos dados é de camundongos que receberam doses de THC e CBD, onde as amostras do cérebro foram processadas e analisadas em diferentes regiões, analisando o efeito do THC e CBD no SNC. Os resultados demonstram influência do CBD em nível lipidômico no cérebro, como em regular a NAPE-PLD (Leishman et al., 2018). Diante disso, é possível afirmar que há uma interferência do CBD na síntese e degradação do AEA e que esse mecanismo pode ser melhor elucidado quando for realizado uma lipidômica, podendo compreender a intervenção do CBD em processos biológicos.

Diante disso, não vimos diferença na expressão gênica do receptor CB1. O CBD não alterou diretamente os níveis de expressão do CB1, mas alterou níveis de enzimas que determinam a biodisponibilidade de um de seus ligantes endógenos: o endocanabinoide AEA. O SEC é receptor às enzimas de síntese e degradação, e o estímulo do SEC, por meio da modulação, demonstrou que o CBD durante o desenvolvimento neural leva a uma diminuição da síntese endógena, uma provável influência dos níveis de endocanabinoides e apresenta uma neuroproteção quando estimula o receptor CB1. Essa neuroproteção é dependente do estímulo do receptor porque quando ele é bloqueado, a neuroestimulação é perdida. Entretanto, ainda não se sabe se esses acontecimentos são paralelos, mas o que os resultados demonstram é que a neuroproteção é dependente da estimulação de CB1 quando ocorre a diminuição da síntese dos endocanabinoides. Por conseguinte, o CBD possui baixa afinidade pelo CB1 (Bisogno et al. 2001; Jones et al. 2010; Pertwee 2008) e seus efeitos são observados apenas em algumas regiões cerebrais (Breivogel et al. 2001; Thomas et al., 2007). Dessa forma, é possível afirmar que o CBD age indiretamente no SEC, atuando como um agonista indireto. Essa hipótese é explicada pela ação do CBD no bloqueio da FAAH (enzima que participa da hidrólise de AEA) (Bisogno et al. 2001; De Petrocellis et al. 2011). Portanto, o CBD acaba modulando os níveis de expressão de enzimas que regulam um dos ligantes endógenos do CB1, a AEA e que os desafios neurotóxicos com co-tratamento com CBD e um antagonista de CB1 mostrou que o efeito neuroprotetor do CBD é mediado pelo receptor CB1 (Zlebnik et al., 2018).

Os endocanabinoides vão agir no tônus de outras sinapses, pois o bloqueio do CB1 por um antagonista não necessariamente irá ocasionar o mesmo efeito do que retirar endocanabinoides, visto que tem como via de sinalização o CB1 tendo como efeito farmacológico ser modulador negativo de algo, pois se o receptor está ativo quando ligado a um ligante, o CBD se liga em outro sítio de modulação do receptor, e isso ocasiona o aumento da sua atividade.

Portanto, nosso trabalho demonstra uma diferença entre os modelos de desenvolvimento neuronal e neurônio maduro. Vimos uma menor biodisponibilidade de AEA, ligante de CB1. Por isso o efeito que estamos vendo do CBD não é diretamente sobre o receptor e sim sobre a disponibilidade de um ligante do receptor CB1. Lembrando que o SEC está envolvido no desenvolvimento do sistema neuronal e isso pode ser um problema se o CBD for administrado em uma época que haja muita célula em desenvolvimento, podendo afetar o desenvolvimento. Diante disso, no nosso trabalho encontramos produção exógena de fitocanabinoides apenas na modulação de neurônios em desenvolvimento como ocorre em cérebro infantil. Outros estudos também mostraram resultados semelhantes utilizando de outros modelos em nível lipídico e proteico (Metz et al., 2022; Leishman et al., 2018). Diante disso, ainda pouco se sabe sobre os efeitos do CBD em neurônios maduros.

Consequentemente, a liberação do CBD para tratamento em crianças deve ser bastante avaliada o seu risco e benefício, pois seu uso deve ser liberado apenas para crianças com diagnóstico de problemas graves. A preocupação é que com o uso do CBD para tratar esse tipo de doença, acabe gerando uma falsa ideia de que é um produto inerte que possa ser utilizado de forma irrestrita em doenças como ansiedade, transtorno do déficit de atenção com hiperatividade (TDAH) e outras doenças de comportamento infantil, visto que não possuem tantos resultados comprovados, e que segundo o nosso trabalho pode ser um problema.

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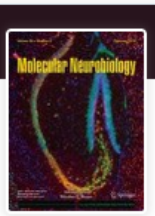

Molecular Neurobiology

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Submission guidelines

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Manuscript Submission

Manuscript Submission

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Always use footnotes instead of endnotes.

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References

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Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

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  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern

genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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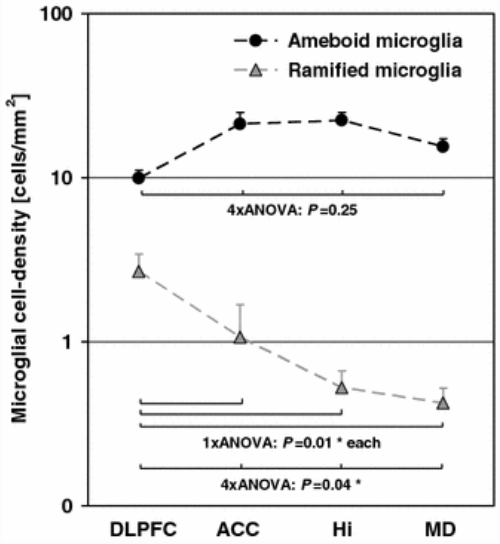

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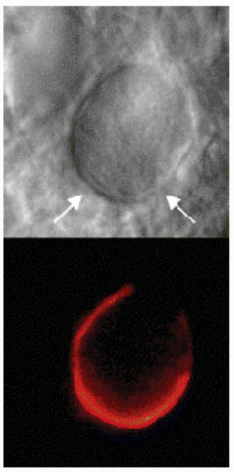

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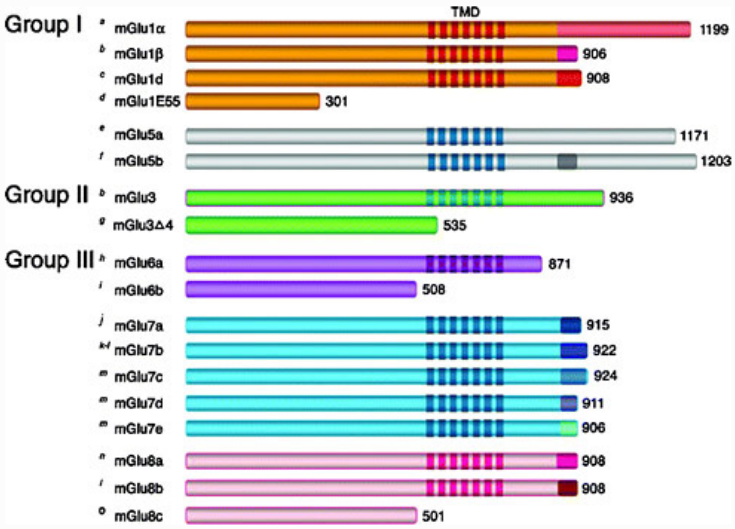

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publication, McNutt at all, PNAS February 27, 2018

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Summary of requirements

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• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

this study adhere to the tenets of the Declaration of Helsinki.

• The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

• Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

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Informed consent

All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. This is especially true concerning images of vulnerable people

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Sample statements for **"Consent to participate"**:

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