Neuroprotection by the cannabis-related products, cannabidiol and cannabigerol, and their associated mechanisms of action

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Abstract

The discovery and characterization of the endocannabinoid system (ECS) brought out years of research focusing on two aims. The study of its participation in the physiopathology in several diseases, including neurodegenerative disorders, and as a direct or indirect target for treating these disorders by cannabinoids or phytocannabinoids (i.e., specific compounds present in the *Cannabis sativa* plant). Preclinical evidence and some clinical data have shown the therapeutic potential of the most relevant phytocannabinoids, Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD), but less for cannabigerol (CBG). In the present review, we summarized data focused on the therapeutic potential of CBD and CBG as neuroprotective agents. This property appears to be exerted by the direct or indirect activation of targets within the ECS and also by mechanisms non-mediated by the ECS. We provide information which could be useful for future CBD and CBG applications in human neurodegenerative diseases treatment.

Keywords: neurodegenerative diseases, endocannabinoid system, phytocannabinoids, cell survival, neuroprotective agents

Abbreviations

2-AG	2-arachidonoyl glycerol
5-HT1A	serotonin receptor subtype
A2A	adenosine receptors subtype
AEA	anandamide
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CBD	cannabidiol
CBG	cannabigerol

CBGA	cannabigerolic acid
CNS	central nervous system
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
H_2O_2	Hydrogen peroxide
MAGL	monoacylglycerol lipase
PPARs	peroxisome proliferator-activated receptors
TRP	transient receptor potential
Δ9-THC	Δ9-tetrahydrocannabinol

Introduction

1. Cannabis sativa and phytocannabinoids

Cannabis sativa is an ancient and particular plant that has been used both medicinally and recreationally for centuries. Phytocannabinoids belonging to the chemical class of terpenophenols have been isolated from this plant. The Δ9-tetrahydrocannabinol (Δ9-THC) is the most representative phytocannabinoid of the plant, which is responsible for the psychotomimetic-like effects of marijuana a recreational form use of cannabis (Gaoni and Mechoulam 1964;). Cannabidiol (CBD) is another abundant phytocannabinoid with several differences to Δ9-THC. CBD does not produce the psychotomimetic-like effects induced by Δ9-THC. It is well-tolerated, with low toxicity, and exhibits a broad spectrum of therapeutic properties (Navarrette et al 2021). On the other hand, some reports have begun to focus on cannabigerol (CBG), the biosynthetic precursor of all phytocannabinoids in the plant. Due to the low concentration achieved at cannabis flower harvest time, CBG is less studied than CBD or even Δ9-THC. Nowadays, it is known that some strains, which accumulate higher levels of the acidic form of CBG (i.e., CBGA), have reduced activity of the three major enzymes that transform the CBGA into the other cannabinoids (Fellermeier et al. 2001).

2. Endocannabinoid System (ECS)

Cannabinoid receptors (CB1 and CB2), endocannabinoids (anandamide, AEA, and 2-arachydonoil glycerol, 2-AG), the enzymes responsible for their biosynthesis and degradation (i.e., fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL) and transporters, constitute the ECS (Zou and Kumar 2018). Several reports have provided information about the participation of the ECS in multiple physiological functions, such as energy balance, appetite stimulation, blood pressure, pain relief,

nausea and vomiting control, perception, reward, memory, and learning and immune response (Pacher et al 2006). In pathological conditions, changes in receptor expression (CB1 and CB2) or in levels of AEA and 2-AG have been demonstrated (Zou and Kumar 2018). All these data support the idea that the modification of endocannabinoid levels (e.g., inhibiting the FAAH or MAGL enzymes) or cannabinoid receptors expression could be helpful strategies to treat different pathologies (Pacher et al 2006). Even though phytocannabinoids can mediate some of these beneficial properties acting on the ECS (e.g., on CB1 and CB2), other targets have been identified which are independent of this system (Morales et al 2017).

3. Neurodegenerative processes

During the last 20 years, the therapeutic potential of natural products capable of increasing neuron survival has been investigated (Fei and Fei 2020). This knowledge has provided the basis for developing novel therapies for neurodegenerative disorders. Although neurodegenerative diseases differ in their clinical and neuropathological characteristics, all share a common morphological base. They are characterized by the progressive loss of specific and vulnerable neuronal populations in the central nervous system (CNS). Neuronal death in neuropathologies involves several cellular processes, e.g., metabolic and mitochondrial dysfunction, increased oxidative stress, defects in the proteasome system and protein aggregation, iron and calcium metabolism changes, excitotoxicity, and inflammation (Azam et al 2021). Thus, therapeutic strategies including antioxidants, anti-inflammatory, and anti-excitotoxic agents, apoptosis inhibitors, autophagy enhancers, or neurotrophic factors, appear as potential treatments. There is strong evidence showing that components belonging to the ECS diminish with aging (at least in some regions of the brain), such as cannabinoid receptors density, levels of endocannabinoids, or levels and activities of the metabolizing enzymes. Also, some changes associated with specific

neurodegenerative diseases have been reported (Di Marzo et al 2015). This scenario has placed the ECS in a promising position for the search of effective treatments.

4. Neuroprotective property of CBD and CBG

Phytocannabinoids have been proposed as promising neuroprotective agents in neurodegenerative diseases due to the beneficial property derived from their pleiotropism and the ability to activate multiple pharmacological targets on the ECS and also, outside this signaling system (Stone et al 2020). The therapeutic potential of CBD as a neuroprotective agent has been reported in several preclinical and clinical studies related to neurodegenerative disorders (Campos et al 2016). Fewer studies have been focused on the therapeutic benefits of CBG and particularly on its neuroprotective potential (di Giacomo et al 2020; Valdeolivas et al 2015). Preclinical and clinical studies have shown that CBD exerts neuroprotective effects in a variety of ways for the treatment of epilepsy, multiple sclerosis, Parkinson's disease, Alzheimer's disease and other systemic diseases caused by degeneration or abnormalities of the CNS. It was Hampson and colleagues in1998 who firstly reported the *in vitro* neuroprotective effects of CBD using the primary cultures of cortical neurons exposed to toxic concentrations of glutamate. They found that the neuroprotection by CBD was not inhibited by the antagonism of CB1 and CB2 receptors, suggesting an ECS non-mediated mechanism for CBD action (Hampson et al 1998). After this work, several preclinical studies using neuronal cultures treated with different neurotoxins or *in vivo* models of neurodegenerative diseases, confirmed the neuroprotective effect of CBD (Kim et al 2021; Martin-Moreno et al 2011). In contrast, the evaluation of CBG as a neuroprotective agent is much less reported. Recently, a preclinical study reported that the CBG pre-treatment of the NSC-34 cellular line before the injury, was able to attenuate apoptosis signaling and was proposed as an effective neuroprotective agent (Valeri et al 2022). Another study has shown that CBG pre-treatment reduces the loss

of cell viability induced by the medium of LPS-stimulated macrophages in NSC-34 cells (Gugliandolo et al 2018). Moreover, some studies have shown comparative results between CBD and CBG. di Giacomo and colleagues (2020) observed how CBG resulted in a more effective agent than CBD to protect the hypothalamic Hypo-E22 cells from the oxidative stress induced by the hydrogen peroxide (H_2O_2). Besides, we have recently reported the neuroprotective property of CBD and CBG in cerebellar granule cell cultures subjected to two insults involving oxidative stress (H_2O_2) and mitochondrial dysfunction (rotenone). We found that both phytocannabinoids were more effective in attenuating the rotenone-induced neurotoxicity (Echeverry et al 2020). In addition, there are *in vivo* studies showing the beneficial effect of CBG in experimental models of neurodegenerative diseases. For example, CBG was neuroprotective in mice modeling Huntington's disease, a clinical condition characterized by the degeneration of nerve cells in the basal ganglia (Valdeolivas et al 2015). Another report using a synthetic quinone derivative of CBG, namely VCE-003, or a second-generation synthetic quinone derivative VCE-003.2, has shown neuroprotective potential in this animal model (Díaz-Alonso et al 2016). This finding helps to propose all these compounds to reduce the severity of neurologic illnesses, such as Huntington's disease (Díaz-Alonso et al 2016), amyotrophic lateral sclerosis (Rodríguez-Cueto et al 2018), Parkinson's disease (Burgaz et al 2021), and multiple sclerosis (Granja et al 2012). Taking into account that all these processes can involve excitotoxicity, oxidative stress, glial reactivity/inflammatory events, or protein aggregations, the neuroprotective action of CBD and CBG might be exerted by the combination of different capabilities involving the inhibition of the mentioned processes (Aymerich et al 2018). Actually, several beneficial actions as antioxidant, anti-inflammatory, anti-excitotoxicity, modulation on Ca^{2+} homeostasis and metabolic activities have been reported for CBD and CBG. While all these activities may explain their neuroprotective property, the interaction with multiple biological targets are reported underlying this property.

5. Mechanisms of action of CBD and CBG as neuroprotective agents

The therapeutic properties of CBD or CBG appear to be exerted by the direct or indirect activation of critical targets within the ECS. Besides, some other receptors not belonging to the ECS mediate the beneficial effect of CBD or CBG. The activation of CB1, CB2, and GPR55 (i.e., G protein-coupled receptor), and the endocannabinoids tone (i.e., transporter, FAAH and 2-AG degradation enzyme), are the main reported ECS-mediated mechanisms. On the other hand, the ECS non-mediated mechanisms include the interaction of CBD and CBG with the transient receptor potential (TRP) channels, adenosine receptors, serotonin receptors, and peroxisome proliferator-activated receptors (PPARs). The antioxidant capacity of CBD and CBG is also included in this type of mechanisms since it is a crucial activity to prevent cellular dysfunctions involving oxidative stress. All these sites of action are outlined in Figure 1.

5.1. ECS-mediated mechanisms

5.1.1. CB1 and CB2 receptors and endocannabinoids tone

The direct or indirect activation of CB1 and CB2 receptors produces a dose-dependent decrease in cellular cAMP levels and modulation of intracellular Ca^{2+} and K^{+} levels, followed by a decreased release of neurotransmitters and the activation of the mitogen-activated protein kinase (MAPK) signaling pathways, including ERK, c-Jun N-terminal kinase (JNK), and p38. All these proteins are involved mainly in regulating cell proliferation, cell control, and cell death (Zou and Kumar 2018). Interestingly, CB1 receptors were described as expressed primarily on the neuron plasma membrane; however, Marsicano et al (2008) have reported that CB1 receptor is also expressed in the mitochondrial membrane of neurons (mtCB1R). Some studies have shown that the activation of brain mtCB1R reduces mitochondrial respiration and ATP production and induces protective effects on neurons in brain ischemic injury models (Bénard et al

2012). This evidence suggests that mtCB1R may be a potential novel target for neuroprotective therapies. Activation of CB2 receptor has been associated with the canonic pathway for the anti-inflammatory effects of most cannabinoid agonists and, thus, the neuroprotective capacity (Pacher et al 2008). For many years, CB2 receptor has been seen as the peripheral receptor of the ECS; however, current research has identified the presence of brain CB2 receptors in microglial cells and neurons. Anti-inflammatory effects of CBD have been related to the control of microglial cell migration (see ahead; Martín-Moreno et al 2011).

Direct action of CBD and CBG through CB1 receptors is questioned, given the low affinity for the orthosteric site (Turner et al 2017). Several studies have demonstrated that CBD acts as a negative allosteric modulator of CB1, modifying the potency and efficacy of orthosteric ligands without activating the receptor (Tham et al 2019). Also, while CBD is reported as an inverse agonist/antagonist of CB1 receptor, it seems to act as a partial agonist of CB2 receptor (Tham et al 2019), although another study showed that CBD is a negative allosteric modulator on this molecular site (Martínez Pinilla et al 2017). CBD can also exert an indirect agonism of the CB1 receptor by inhibiting both the FAAH enzyme and the AEA transporter (Howlett et al 2011). Both actions elicit an increase in AEA levels and, consequently, the CB1 receptor activation (Howlett et al 2011). Since the inhibitory effect on FAAH is widely accepted, a recent report suggests that FAAH does not serve as a target for CBD in humans (Elmes et al 2015); therefore, this fact deserves more research.

On the contrary of CBD, CBG has been reported as a partial agonist or agonist of CB1 and CB2 receptors, although with low affinity (Navarro et al 2020;). Moreover, a human cell culture study showed negligible binding affinity of CBG for CB1 and CB2 receptors (Granja et al 2012). In turns, CBG is able to inhibit the AEA uptake, affecting the endogenous endocannabinoid tone (Pagano et al 2022), it does not inhibit the FAAH enzyme, but inhibits MAGL, the 2-AG degradative enzyme at high concentrations, which apparently could be clinically irrelevant (Pagano et al 2022).

5.1.2. GPR55 receptors

CBD has a high affinity for GPR55, acting as an antagonist. GPR55 is a G protein-coupled receptor involved in proliferation, differentiation and cytoskeletal modulation (Marichal-Cancino et al 2017). It was reported that CBD elicited anti-inflammatory effects in experimental models of genetically-induced Dravet syndrome (Kaplan et al 2017) and experimental Parkinson's disease (Celorrio et al 2017). In contrast, there is no information about the action of CBG on GPR55 so far (Ryberg et al 2007).

5.2. ECS non-mediated mechanisms

Nowadays, it is known that most of the pharmacological effects induced by CBD, and a few by CBG, cannot be explained entirely through their action on targets within the ECS in certain pathophysiological conditions. Some other molecular targets, localized outside the ECS, participate in the mechanisms of action of these phytocannabinoids (Figure 1).

5.2.1. TRP channels

Among the classical functions (i.e., thermosensation, mechanosensation, and chemosensation) associated with TRP channels, several studies have shown that this molecular site also regulates neuronal excitability, intracellular Ca^{2+} and Mg^{2+} homeostasis, cell proliferation, and differentiation (Nilius and Voets 2004). Importantly, these receptors also have a crucial role in inflammatory processes (Silverman et al 2020). Binding and functional studies have demonstrated that CBD and CBG modulate different subfamilies of TRP channels, i.e., activateTRPA1 and TRPV1–4 subtypes, and block TRPM8 subtype channels with relatively minor differences in affinity (Muller et al 2019). There are several studies that suggest that these receptors are responsible for the neuroprotective action of CBD and CBG (Cassano et al 2020; Muller et al 2019).

5.2.2. *Equilibrative nucleoside transporter 1 and adenosine receptors*

Studies have shown that CBG and CBD have significant anti-inflammatory effects, including attenuating cytokine release and decreasing the activation of immune cells, an effect observed in both *in vitro* and *in vivo* assays (Granja et al 2012). Inhibition of the adenosine uptake by CBD is also related to its anti-inflammatory effect. CBD competitively inhibits ENT1 (i.e., equilibrative nucleoside transporter 1), leading to an increase in extracellular adenosine and a consequent neuroinflammation reduction (Carrier et al 2006). This effect seems to involve the activation of adenosine A2A receptors (Aden et al 2003), since the A2A receptor antagonist SCH58261 abolished the effect of CBD (Castillo et al 2010). Thus, it was suggested that the activity of CBD on A2A receptors is a consequence of its ability to inhibit ENT1, which results in an indirect activation of A2A receptors (Carrier et al 2006). Unlike CBD, CBG behaved as a potent A2A receptor agonist in *in vitro* experiments (Cascio et al 2010).

5.2.3. 5-HT1A receptors

Within the fifteen-subtype receptors for serotonin (5-HT) described to date (Rojas and Fiedler 2016), it has been shown that exogenous cannabinoid ligands interact with the subtype 5-HT1A (i.e., inhibitory GPCR). A pioneer *in vitro* study done by Russo and collaborators demonstrated that CBD could facilitate 5-HT1A-mediated neurotransmission by the agonism of this receptor (Russo et al 2005). More recent findings indicated that CBD interacts with an allosteric site of the 5-HT1A receptor (Rock et al 2012). Interestingly, Mishima and colleagues (2005) explored the role of the 5-HT1A receptors in the neuroprotective effects of CBD, using the paradigm of middle cerebral artery occlusion in mice. These authors observed that WAY100135 (i.e., 5-HT1A receptor antagonist) pretreatment was able to inhibit the effect of CBD, suggesting the involvement, at least in part, of 5-HT1A receptors in the neuroprotective effects of CBD against cerebral ischemia (Mishima et al 2005). Regarding CBG, some crucial differences have been reported compared with CBD. CBG seems to be a

moderate 5-HT1A receptor antagonist (Cascio et al 2010), given that CBG antagonized, in a competitive manner, the effect of the classical 5-HT1A selective agonist, R-(+)-8-hydroxy-2-(di-n-propylamine) tetralin (8-OH-DPAT) in the [35S]GTPγS binding assay (Cascio et al 2010). We recently reported the neuroprotective effect of CBG, using a neural cell culture exposed to two insults involving oxidative stress (H_2O_2) and mitochondrial dysfunction (rotenone). In our experiments, we specifically found that the neuroprotective effect elicited by CBG against rotenone (but not by CBD) was significantly reduced by the 5-HT1A receptor antagonist (WAY100135; Echeverry et al 2020). Although this study showed for the first time the dependence of the 5-HT1A receptor in the mechanism of action of the neuroprotective effect of CBG, more research is needed to understand if this phytocannabinoid modifies the 5-HT neurotransmission to exert this beneficial action.

5.2.4. Peroxisome proliferator-activated receptors

Evidence indicates that CBD or CBG impact on 5-HT1A receptors, adenosine uptake and A2A receptors (Cascio et al 2010), and also on other pharmacological targets. Among them, the nuclear receptors of the PPAR family (i.e., PPARγ) are very relevant in the anti-inflammatory effect of phytocannabinoids, especially CBD (O'Sullivan 2016; Sonego et al 2018). The activation of PPARs inhibits the transcription of pro-inflammatory genes and cytokines such as TNF- alpha IL-1beta and IL-6, thus preventing the NF-kappa B signaling pathway (Figure 1). The transcription factor NFκB plays a crucial role in regulating inflammation and oxidative stress leading to neuronal death, which explains why PPARs have been suggested as possible targets for neuroprotection (O'Sullivan 2016). Results obtained by both *in vitro* and *in vivo* experimental models of multiple sclerosis (Giacoppo et al 2017), ischemic stroke (Hind et al 2016), and Parkinson disease (Dos-Santos-Pereira et al 2016), showed the implication of PPARγ in the anti-inflammatory effects of CBD (Hind et al 2016; Sonego et al 2018). In the same direction, CBG and some CBG derivatives (e.g., VCE003 and

VCE003.2) regulated neuroinflammation processes through PPARs (García et al 2018; Granja et al 2012). *In vitro* modeling studies of phytocannabinoids have suggested that CBG is a dual PPARα/γ agonist (D'Aniello et al 2019).

5.2.5. Antioxidant action

In addition to the afore mentioned mechanisms involving molecular sites in the CNS, several authors have attributed the neuroprotective capacity of cannabinoids, especially CBD, to their antioxidant action. Throughout this property, CBD is capable to restore the normal balance between oxidative events and antioxidant endogenous mechanisms that are frequently disrupted in neurodegenerative disorders (Gandhi and Abramov 2012; Hampson et al 1998). The structural characteristics of CBD and CBG, mainly the hydroxyl groups of the phenol ring, give them a powerful direct antioxidant capacity (Borges et al 2013). This has been shown in an *in vitro* assay used to evaluate free radical scavenger capacity, where CBD and CBG showed higher antioxidant activity than Trolox, the reference compound (Dawidowicz et al 2021).

In *in vitro* neuron culture assays, CBD and CBG have shown neuroprotection against diverse insults which induce oxidative stress (Echeverry et al 2020; Gugliandolo et al 2018). The exact underlying mechanism of protection is already unknown, but some studies have shown that CBD decreases oxidative stress markers such as the malondialdehyde (Costa et al 2007). Also, CBD is a regulator of the expression of nitrotyrosine and the inducible isoform of nitric oxide synthase (iNOS), thus promoting the reduction of the production of reactive oxygen species (ROS) (Esposito et al 2007). However, some controversial results have been reported. In comparison with potent and classical antioxidant natural products, like butylhydroxytoluene (Ryan et al 2009) or quercetin (Echeverry et al 2020), CBD shows a lower neuroprotective effect on cultured neurons against H_2O_2. In turns, the efficacy of CBG in reducing oxidative stress and apoptosis was demonstrated in an *in vitro* model of neuroinflammation (Gugliandolo et al 2018). The increased activities of

catalase and SOD-1, and higher levels of GSH, induced by CBG appear to be a key mechanism for the beneficial effects of this phytocannabinoid in *in vivo* models of Huntington's disease (Valdeolivas et al 2015). Some authors suggest that CBD and CBG exert their antioxidant action in an indirect manner, i.e., through molecular targets associated with the redox system such as Nrf2 factor; although more information is needed. While CBD regulates Nrf2 in microglia (Juknat et al 2013), immunostaining assays of Nrf-2 and qRT-PCR analysis of Nrf-2-dependent genes failed to prove any CBG effect (Valdeolivas et al 2015).

Applications to Other Areas

This chapter reviews the preclinical evidence supporting CBD and CBG as neuroprotective agents to be potentially applied in neurodegenerative disorders. The preclinical evidence included here shows that CBD and CBG are compounds that can modulate a range of pharmacological targets as well as exhibiting antioxidant capability (Borges et al 2013). These features, along with their lipophilicity, make them promising therapeutic candidates for the treatment of CNS disorders. Moreover, some other preclinical studies suggest that the neuroprotective effects of CBD and CBG may be helpful to treat mood disorders or schizophrenia. Moreover, high levels of comorbidity between neurodegenerative and neuropsychiatric disorders, as well as common features, including degenerative and inflammatory processes, have been described in conditions like depression or schizophrenia (Wee et al 2016). Accumulating data from postmortem and brain imaging studies revealed morphological changes in the brain of patients with these mental disorders, such as ventricle enlargement, volumetric reduction, attenuation of neuronal viability and atrophy, or loss of neurons and glial cells in particular cortical and limbic brain regions (Hunsberger et al 2009). In this context, cannabinoids, especially CBD and CBG, have emerged as a new class of

drugs with potential effects on a broad range of neurodegenerative and psychiatric disorders (Campos et al 2016).

Mini-Dictionary of Terms

Allosteric binding site: is a domain topologically distinct from the orthosteric site of a receptor that can bind to small molecules or other proteins in order to modulate receptor activity.

Free radical scavenger: refers to substances which can donate an electron to a free radical, thus inactivating the radical oxygen species. Antioxidants which are able to inhibit the oxidation process are known as free radical scavengers.

Orthosteric binding site: refers to the active site of a receptor that can bind the endogenous substance.

Oxidative stress: is defined as imbalance between the production of reactive oxygen species and the antioxidant defense system, which leads to lipid peroxidation, protein oxidation and/or DNA damage.

Pleiotropic effect: refers to the effect induced by a compound acting throughout several molecular targets, leading to elicit different cellular responses.

Polypharmacology phenomenon: refers to many effective drugs or compounds, used in diverse therapeutic areas, acting on multiple rather than single targets.

Psychotomimetic-effect: is defined as drug-related alterations in mental activity that causes changes in mood, behavior, or perception.

Key Facts

- Neurodegenerative diseases are characterized by progressive loss of specific and vulnerable neuronal populations in the CNS.
- There are no effective therapeutic options for patients with neurodegenerative disorders; however, cannabinoids seem to be a promising strategy.
- Cannabidiol (CBD) is one of the most abundant extracts from Cannabis sativa.
- CBG is the biosynthetic precursor of all phytocannabinoids in the plant.
- CBD and CBG seem to have multiple therapeutic benefits without psychotomimetic-like effects.
- Our focus of interest is to study the role of different receptors which underly the neuroprotective effect of CBD and CBG.

Summary Points

- An emerging polypharmacological action-based therapeutic strategy has been explored in neurodegenerative diseases.
- A comprehensive review of the mechanisms of action of CBD and CBG as neuroprotective agents, including those mediated- and non-mediated by the ECS.
- Although they are structurally similar, CBD and CBG differ in their pharmacological profile.
- Relevance to continue studying the therapeutic potential of natural products to treat neurodegenerative diseases.
- Advantage for the use of CBD and CBG: both compounds have a low affinity for CB1 receptors, which results in the removal of the psychotropic effects triggered by the agonism of this site.

- Preclinical evidence supports the therapeutic application of CBD and CBG in neurodegenerative disorders; however, more clinical research is mandatory.

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Figure 1 Mechanisms of action of CBD and CBG

Legend to Figure 1:

Possible targets responsible for the neuroprotective effects of cannabidiol and cannabigerol. Diagram shows the main ECS-mediated mechanisms (left) and ECS-non-mediated mechanisms (right). *Created with BioRender.com*