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Targeting the CB_2 receptor and other endocannabinoid elements to delay disease progression in amyotrophic lateral sclerosis

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Abbreviations: Amyotrophic lateral sclerosis (ALS); cannabidiol (CBD); cannabinoid receptor type-1 (CB_1); cannabinoid receptor type-2 (CB_2); diacylglycerol lipase (DAGL); fatty acid amide hydrolase (FAAH); fused in sarcoma (FUS); G-protein receptor 55 (GPR55); monoacylglycerol lipase (MAGL); *N*-arachidonoyl-phosphatidylethanolamine phospholipase D (NAPE-PLD); peroxisome proliferator-activated receptor (PPAR); superoxide dismutase-1 (SOD-1); TAR-DNA binding protein-43 (TDP-43); Δ^9-tetrahydrocannabinol (Δ^9-THC)

Abstract (words: 150)

Cannabinoids form a singular group of plant-derived compounds, endogenous lipids and synthetic derivatives with multiple therapeutic effects exerted by targeting different elements of the so-called endocannabinoid system. One of their therapeutic applications is the preservation of neuronal integrity exerted by attenuating the multiple neurotoxic events that kill neurons in neurodegenerative disorders. In this review, we will address the potential of cannabinoids as neuroprotective agents in amyotrophic lateral sclerosis (ALS), a devastating neurodegenerative disorder characterized by muscle denervation, atrophy and paralysis, and progressive deterioration in upper and/or lower motor neurons. The emphasis will be paid on the cannabinoid receptor type-2 (CB_2), whose activation limits glial reactivity, but the potential of additional endocannabinoid-related targets will be also addressed. The evidence accumulated so far at the preclinical level supports the need to move soon towards the patients and initiate clinical trials to confirm the potential of cannabinoid-based medicines as disease modifiers in ALS.

Keywords: Cannabinoids, endocannabinoid signaling system, CB_2 receptors, neurodegeneration, neuroprotection, amyotrophic lateral sclerosis.

BULLET POINT SUMMARY

What is already known:

- ALS is a neurodegenerative disorder with poor therapeutic development.
- Cannabinoids are pleiotropic compounds with promising neuroprotective properties.

What this study adds:

- Changes in endocannabinoid ligands, receptors and enzymes are evident in ALS patients and animal models.
- Targeting some of these elements with cannabinoids delays or attenuates disease progression in preclinical models.

Clinical significance:

- This preclinical evidence has recently moved to the clinical scenario to confirm these benefits in ALS patients.

Cannabinoids and neuroprotection

The discovery of cannabinoid receptors, endocannabinoid lipids and the machinery for their metabolism (synthesis, inactivation and precursor function; recently reviewed in Lu and Mackie, 2020) allowed in the 80s and 90s to solve an important unknown generated in the 60s, even earlier, with the chemical identification of the active constituents of the cannabis plant, so-called cannabinoids (Husnain et al., 2020). It served to identify the molecular mechanisms underlying the pharmacological action of plant-derived cannabinoids, in particular the major psychoactive compound, Δ^9-tetrahydrocannabinol (Δ^9-THC) present in cannabis plant (Mathew et al., 2020). These molecular mechanisms included as pharmacological targets some elements of an important intercellular signaling system, so-called endocannabinoid system, present in most of body cells and tissues, where it plays important regulatory functions aimed, among others, at the maintenance of cell and tissue homeostasis and integrity (Freitas et al., 2018). The endocannabinoid system was found to be particularly active at the CNS, but it is also present in numerous peripheral tissues (Joshi and Onaivi, 2019). In the CNS, the endocannabinoid system has been found to exert important regulatory functions in synaptic transmission, neuron-glia communication, glial activation, neural cell proliferation and differentiation, and protein homeostasis, among other important processes (Zou and Kumar, 2018), which support the promising position of the modulators of the endocannabinoid system for multiple CNS-related disorders which involve dysregulation in these processes (Cristino et al., 2020).

An important, and still relatively poorly explored, area for the development of cannabinoids as therapeutic agents in the CNS is the pathological aging, which includes numerous chronic progressive neurodegenerative disorders such as Alzheimer´s disease and other dementias, Parkinson´s disease, Huntington´s chorea, cerebellar ataxias, and amyotrophic lateral sclerosis (ALS) and other motor neuron diseases (Fernández-Ruiz, 2019). Given that aging is the major risk factor for these diseases and that the lifespan is expected to increase, in particular in developed countries, in the next decades, the prevalence of the neurodegenerative disorders is expected to significantly elevate with the risk to become an important biomedical challenge in the present century (Fernández-Ruiz, 2019), aggravated by the fact that there is not any available neuroprotective therapy for these disorders. Therefore, the search of new molecules with disease-modifying activity is one of the key challenges

demanded by patients affected by these neurodegenerative disorders (Fernández-Ruiz, 2019).

The pleiotrophic properties (broad-spectrum action) of cannabinoids provide these compounds an interesting pharmacological profile for limiting neurodegeneration, allowing a singular cannabinoid or a combination of different cannabinoids to modulate the common traits in neuronal deterioration and death, in other words, to be active against the numerous cytotoxic mechanisms that kill neurons and other neural cells in these disorders. This is something extremely relevant for neurodegenerative disorders in which the different neurotoxic events (e.g. excitotoxicity, oxidative stress, glial reactivity, local inflammation, abnormal protein accumulation, impairment of the lysosomal system, etc) cooperate to damage neural cells, so they need to be reduced with strategies based on polypharmacology, something that cannabinoids may provide (Fernández-Ruiz, 2019). For example, cannabinoids able to activate the cannabinoid receptor type-1 (CB_1) may normalize glutamate homeostasis then reducing excitotoxicity (Marsicano et al., 2003). The activation of CB_1 receptors can also induce autophagy then facilitating the elimination of protein aggregates (Costa et al., 2016). Such potential can be combined with the ability of these or other cannabinoids to target the cannabinoid receptor type-2 (CB_2) too, as well as to modulate the peroxisome proliferator-activated receptor-γ (PPAR-γ) or the G-protein receptor 55 (GPR55), three cannabinoid available receptors that have been found to may attenuate the glial reactivity then reducing local inflammatory events (Kallendrusch et al., 2013; Pistis and O'Sullivan, 2017; Aymerich et al., 2018). Lastly, using cannabinoid receptor-independent mechanisms (e.g. Nrf-2 signaling, scavenger action), certain cannabinoids may also reduce oxidative stress, which is also a relevant neurotoxic event in neurodegeneration (Fernández-Ruiz et al., 2013; Atalay et al., 2019). The relevance of this combination of effects is that they ultimately promote neuron survival in chronic neurodegenerative disorders (Fernández-Ruiz, 2019), a property that can be also extended to acute degeneration conditions (e.g. stroke, brain trauma, spinal injury) (Shohami et al., 2011; Arévalo-Martín et al., 2015; Kolb et al., 2019).

In this review, we will concentrate in one of these chronic neurodegenerative disorders, ALS, which has recruited an important attention in the last years (Bilsland and Greensmith, 2008; de Lago et al., 2015; Pryce and Baker, 2015; Giacoppo and Mazzon, 2016; Aymerich et al., 2018; Urbi et al., 2019). First, we will describe the changes that take place in the different

elements of the endocannabinoid signaling in those CNS structures more affected (e.g. spinal cord, brainstem, motor cortex) under the neurodegenerative conditions operating in ALS (see Table 1 for a summary). This information is certainly important as these changes (e.g. dysregulation in the endocannabinoid signaling) may contribute to the pathological processes in ALS, but also because changes in those endocannabinoid elements that may serve as pharmacological targets for the neuroprotective effect of certain cannabinoids may have an impact in these beneficial effects (Aymerich et al., 2018; Fernández-Ruiz, 2019). Second, we will collect the experimental evidence, obtained in animal and cell models of ALS, that support how the modulation of specific elements in the endocannabinoid signaling may preserve motor neurons from death and improve the neurological signs typical of this disease (e.g. motor weakness) (Aymerich et al., 2018; Urbi et al., 2019; see Table 2 for a summary). Lastly, we will review the clinical evidence collected so far, which is currently still too limited, and will discuss the future perspectives for the necessary traslational development of cannabinoid-based therapies (either for alleviating symptoms or delaying disease progression) from the animal studies to the ALS patients (Pryce and Baker, 2015; Giacoppo and Mazzon, 2016).

Cannabinoids and ALS

ALS is a progressive neurodegenerative disorder that predominantly affects the upper and lower motor neurons, which results in muscle denervation, atrophy and paralysis (Hardiman et al., 2011). Sporadic forms of the disease are the most abundant (Al-Chalabi and Hardiman, 2013). However, they are clinically and histopathologically indistinguishable from the different familial forms described so far, which involve mutations in genes such as: (i) *SOD1* encoding the key antioxidant enzyme superoxide dismutase-1 (SOD-1) (Kaur et al., 2016); (ii) *TARDBP* and *FUS* encoding the proteins TAR-DNA binding protein-43 (TDP-43) or fused in sarcoma (FUS), respectively, involved in pre-mRNA splicing, transport and stability (Butti and Patten, 2019; Xue et al., 2020); and (iii) *C9orf72* encoding a protein involved in intracellular trafficking in neurons, and other cell functions not completely understood (Renton et al., 2011; Gijselinck et al., 2018). These mutant genes (*SOD-1*, *TARDBP*, *FUS and C9orf72*) represent, among more than 25 ALS-related genes, most of the cases of familial ALS (Al-Chalabi and Hardiman, 2013; Gao et al., 2017; Kim et al., 2020), and they have been used to generate experimental models (transgenic mice overexpressing different human mutations) for the study of ALS (Van Damme et al., 2017). The disease still lacks an

effective treatment for symptoms and the disease progression, having the antiexcitotoxic agent riluzole (Rilutek®; approved in 1995) (Cheah et al., 2010; Geevasinga et al., 2016), the antioxidant agent edaravone (Radicava®; approved in Japan in 2015) (Bhandari et al., 2018), and the anti-inflammatory tyrosine kinase inhibitor masitinib (Kinavet-CA1®; designed as orphan drug in 2015) (Petrov et al., 2017), as the only therapies for the treatment of ALS patients, but all having a modest efficacy.

Preclinical studies that were initiated in 2004 explored cannabinoids and have situated these compounds as a promising disease-modifying therapy to be confirmed in clinical trials with ALS patients (Bilsland and Greensmith, 2008; Carter et al., 2010; Rossi et al., 2010; de Lago et al., 2015). These preclinical studies were conducted mainly in the G93A transgenic mouse that overexpresses a mutated form of SOD-1 and confirmed the potential of different cannabinoids able to reduce: (i) excitotoxicity (effects that depend on targeting CB_1 receptors); (ii) microglial activation and neuroinflammation (effects mediated by the activation of the CB_2 receptor and/or by modulating PPAR-γ/NFκB signaling or the GPR55 orphan receptor); and (iii) oxidative injury (effects that are receptor-independent and/or related to PPAR-γ/Nrf-2 signaling) (Fernández-Ruiz et al., 2015). These three important effects are presented in graphical form in Figure 1, with details on the cell compartments in which they appear to be exerted and the molecular mechanisms that appear to be involved for the preservation of motor neurons in ALS. Other mechanisms of action have been also proposed in the Figure 1, for example, that cannabinoids may enhance the trophic support exerted by astrocytes for neurons, as well as the contribution of cannabinoid effects on peripheral tissues (e.g. muscle, metabolic organs) that have been also associated with the pathogenesis of ALS (McDonald et al., 2020). Lastly, cannabinoids could be also useful to control the toxicity of protein aggregates (e.g. mSOD-1, TDP-43, FUS) in ALS, as cannabinoids have been found to enhance autophagy by inhibiting mTOR signalling (Das et al., 2019). However, this possibility is still pending to be investigated in experimental models of ALS (see below).

Endocannabinoid signaling in ALS

As mentioned above, the changes experienced by key endocannabinoid elements during the ALS pathogenesis is an important first aspect to discuss in this review, as these changes could

be critical for investigating the effects of cannabinoids exerted by targeting specific endocannabinoid elements, helping to adequately define the best pharmacological strategy in each disease phase. This objective has been investigated in two murine models of ALS (mSOD1(G93A) and TDP-43(A315T) transgenic mice), in samples from dogs affected by an ALS-like canine disorder (so-called degenerative myelopathy), and in samples from ALS patients. The focus of this work has been the analysis of endocannabinoid receptors (CB_1 and, in particular, CB_2) and enzymes (mainly the inactivating enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL)) (reviewed in Aymerich et al., 2018; see Table 1 for a summary). As in other disorders, these changes can be explained in two directions.

In one direction, some changes may reflect endogenous protective responses that should be enhanced from a pharmacological point of view. This is the case of the elevations found in anandamide and 2-arachidonoyl-glycerol, the two major endocannabinoids, in the spinal cord of SOD-1 transgenic mice (Bilsland et al., 2006; Witting et al., 2004). These elevations were associated with a parallel increase in the expression of *N*-arachidonoyl-phosphatidyl-ethanolamine-phospholipase D (NAPE-PLD), the enzyme that synthesize anandamide, in the spinal cord of SOD-1 mutant mice, but no changes in diacylglycerol lipase (DAGL), the enzyme that synthesizes 2-arachidonoyl-glycerol, and in the inactivating enzymes FAAH and MAGL (Moreno-Martet et al., 2014). The increase found in endocannabinoid levels in the spinal cord of these mice and its interpretation as an endogenous protective response have fuelled the idea that inhibiting endocannabinoid inactivating enzymes may be neuroprotective in ALS (Pasquarelli et al., 2017) (see below). In addition, as in other disorders (Fernández-Ruiz et al., 2007, 2015), CB_2 receptors experience an important up-regulatory response in the affected CNS structures in ALS. This was seen in the spinal cord of murine models of ALS at symptomatic stages, including SOD-1 mutant (Moreno-Martet et al., 2014; Shoemaker et al., 2007) and TDP-43 transgenic (Espejo-Porras et al., 2015, 2019) mice. The up-regulation was also visible in the spinal cord in the ALS-like canine neurodegenerative pathology (Fernández-Trapero et al., 2017), as well as in *post-mortem* tissues obtained in ALS patients, including spinal cords (Yiangou et al., 2006) and primary motor cortex (Espejo-Porras et al., 2018) (see Table 1 for a summary). This up-regulation appears to occur predominantly in astrocytes recruited at lesioned sites (spinal cords) in mutant SOD-1 mice (unpublished results) and TDP-43 transgenic mice (Espejo-Porras et al., 2015, 2019), dogs with degenerative myelopathy (Fernández-Trapero et al., 2017), as well as in *post-mortem* primary

motor cortex samples from ALS patients (Espejo-Porras et al., 2018) (see examples in Figure 2). This up-regulation occurring in astrocytes has been interpreted as a protective response aimed, for example, at facilitating astrocyte metabolic support to neurons or at enhancing the function of glial glutamate transporters to reduce the risk of excitotoxic events (see Figure 1; de Lago et al., 2015). It could also be related to a modulation of the astrocyte contribution to the so-called non-cell-autonomous pathogenic events occurring in ALS to kill motor neurons (Ouali Alami et al., 2018).

CB_2 receptor expression was up-regulated in activated microglial cells also recruited at lesioned sites in spinal grey and white matter areas in TDP-43 transgenic mice (Espejo-Porras et al., 2019) and in *post-mortem* spinal cord samples from ALS patients (Yiangou et al., 2006) (see examples in Figure 2). As in the case of astrocytes, the response of CB_2 receptors in microglial cells suggests that selectively targeting this receptor may have beneficial effects in the control of microglial toxicity for motor neurons, which is an extremely important event in ALS pathogenesis (see details below). Lastly, it is important to also mention that, in addition to astrocytes and activated microglial cells, CB_2 receptors have been also found in ALS in own upper and lower motor neurons using *post-mortem* tissues from patients (Espejo-Porras et al., 2018). Neuronal location of CB_2 receptors is still a matter of controversy given the problems of specificity of the CB_2 receptor antibodies (Atwood and Mackie, 2010). However, taking with the necessary caution, our data showed colocalization of CB_2 receptor immunofluorescence with neuronal markers in the primary motor cortex and spinal cord (Map-2 for cortical neurons and choline-acetyl transferase for spinal motor neurons, respectively) of ALS patients (Espejo-Porras et al., 2018). In addition, the immunoreactivity levels for the CB_2 receptor in both motor neurons in ALS were apparently similar than in control subjects, despite the marked deterioration experienced by these neurons in ALS which was confirmed in our study (Espejo-Porras et al., 2018).

In a second direction, these changes, rather than being merely an adaptative response of the endocannabinoid receptors and enzymes to the progressive deterioration of motor neurons, may have an instrumental value and contribute to the physiopathology of this disease. This was the interpretation in a study conducted in SOD-1 mutant mice in early presymptomatic phases at which authors described a down-regulatory response of CB_1 receptors in parallel to the elevation of glutamate receptors (Zhao et al., 2008). Given the role that CB_1 receptors play in the control of glutamate homeostasis, the reduction in these receptors found in SOD-1

mutant mice may predispose motor neurons to excitotoxic events then contributing to pathogenesis (see details below). Similar conclusions derived from a previous study conducted by Rossi and coworkers who described changes in the CB_1 receptor sensitivity in ALS compatible with risk of excitotoxic damage (Rossi et al., 2010). However, the changes in the expression of CB_1 receptors in animal models resulted to be inconclusive, as the down-regulation of these receptors found in that study (Zhao et al., 2008) was not validated in the same mutant mice in a further study (Moreno-Martet et al., 2014), although this was conducted in advanced phases in the progression of the pathological phenotype in SOD-1 mutant mice. Studies in an additional model, TDP-43 transgenic mice, described no changes in CB_1 receptor expression (Espejo-Porras et al., 2015). By contrast, studies conducted in the spinal cord of ALS patients reported apparent reductions in CB_1 receptors (Espejo-Porras et al., 2018).

Treatments with cannabinoids in preclinical models of ALS

As mentioned above, the pharmacological evidence in support of the idea that cannabinoids may be a promising neuroprotective therapy in ALS was collected first in the classic transgenic mouse that overexpress a mutated form (G93A) of SOD-1 (Raman et al., 2004; Weydt et al., 2005; Kim et al., 2006; Bilsland et al., 2006; Shoemaker et al., 2007; Rodríguez-Cueto et al., 2018; see Urbi et al. (2019) for a recent meta-analysis of these experimental studies), but recent studies have extended this evidence to the murine model based on the A315T mutation in TDP-43 protein (Espejo-Porras et al., 2019) (see Table 2 for a summary of all pharmacological evidence collected so far). The first studies were conducted by Abood and coworkers who observed that the administration of the phytocannabinoid Δ^9-THC at high doses delayed the onset of motor impairment and had a positive effect in the survival of these mice which was significantly prolonged (Raman et al., 2004). These effects were evident when the cannabinoid was administered before the onset of ALS signs, but they were also found despite the treatment was initiated after the appearance of symptoms (Raman et al., 2004). As regards to the mechanisms that underlie these effects, authors proposed a reduction in the oxidative stress and in the excitotoxic damage, as they also found that Δ^9-THC was effective in the reduction of both cytotoxic events in an *in vitro* study using spinal cord neuronal cultures (Raman et al., 2004). Similar results were reported with another phytocannabinoid, cannabinol, which, compared to Δ^9-THC, is significantly less

psychotropic (Weydt et al., 2005), with the non-selective cannabinoid agonist WIN55,212-2 (Bilsland et al., 2006; Shoemaker et al., 2007), and with synthetic compounds that selectively activate the CB_2 receptor, e.g. AM-1241 (Kim et al., 2006, Shoemaker et al., 2007). However, a recent study, also conducted in mutant SOD-1 (G93A) mice, has revealed that the effects of CB_2 receptor agonists in experimental ALS may be different depending on the stage of the disease progression, being detrimental in presymptomatic mice but changing to beneficial in later stages (Ouali Alami et al., 2018). These dual effects may depend on differences in the regulation exerted by astrocytes on the microglial activation, which have been mentioned before, in the different ALS stages (Ouali Alami et al., 2018). To explore the cellular and molecular bases underlying this dual effect of the CB_2 receptor represents one important objective to be investigated in coming years, a fact that will possibly reinforce the dual role that glial cells appear to exert in ALS in the control of motor neuron integrity, which includes both therapeutic effects and also contribution in the pathogenic events (see Izrael et al., 2020 for a recent review).

Additional studies have concentrated the focus in the TDP-43(A315T) transgenic mice in which the activation of CB_2 receptors with HU-308 improved motor behavior, preserved spinal motor neurons and attenuated astrocyte and microglial reactivity (Espejo-Porras et al., 2019; see details in Figure 3). Such effects were, in general, reproduced with the non-selective agonist WIN55,212-2 and reversed, as expected, by the blockade of CB_2 receptors (Espejo-Porras et al., 2019). However, certain contribution of CB_1 receptors in these effects of WIN55,212-2 cannot be discarded, as the co-treatment with rimonabant, a CB_1 receptor antagonist, attenuated some of these effects, in particular those reducing glial reactivity (Espejo-Porras et al., 2019). These results not only reinforced the interest of CB_1 receptor agonists, but also the elevation of endocannabinoid levels with FAAH inhibitors investigated in studies of genetic inactivation with positive results (Bilsland et al., 2006). Inactivation of the other major inactivating enzyme, MAGL, with the selective inhibitor KML29 in the same mutant SOD-1 mouse model also delayed disease onset and progression, and improved animal survival, probably through the reduction in pro-inflammatory cytokines and the elevation of BDNF in the spinal cord (Pasquarelli et al., 2017). These effects were also confirmed in additional *in vitro* studies (Pasquarelli et al., 2017).

These pharmacological studies have been paralleled by experiments using mice deficient in specific endocannabinoid receptors or enzymes. Thus, elevation of endocannabinoid levels

(mainly anandamide) obtained through the genetic ablation of FAAH enzyme also prevented the appearance of disease signs in the SOD-1 mutant mice (observed in animals having the SOD-1 mutation and the deficiency in FAAH in comparison with classic SOD-1 mutant mice), but it did not affect their survival (Bilsland et al., 2006). However, the genetic ablation of the CB_1 receptor had no effect on the onset of the disease in SOD-1 mutant mice, but it significantly extended life span (Bilsland et al., 2006). There is no data about the effects derived from the genetic ablation of CB_2 receptors in SOD-1 mutant mice, but the results of the pharmacological studies (Shoemaker et al., 2007) suggest that this may be a relevant target in ALS, so it is expected that SOD-1 mutant mice carrying a genetic deficiency in the CB_2 receptor gene should exhibit a significant worsening in their ALS phenotype. We have preliminary evidence that this happens in TDP-43 transgenic mice when they are crossed with CB_2 receptor knockout mice to generate double mutants, which, compared to TDP-43 transgenic mice exhibited an accelerated progression in the pathological phenotype (unpublished results), then stressing the relevance of the CB_2 receptor as a promising target for treating ALS.

Taken together, the different preclinical studies, using pharmacological treatments or genetically-deficient mice, suggest that cannabinoids may have neuroprotective effects in ALS that involve the activation of CB_1 and, in particular, CB_2 receptors, but also the contribution of cannabinoid receptor-independent antioxidant mechanisms. The role of glial CB_2 receptors in ALS is attractive given the important involvement of glial cells in ALS (Sargsyan et al., 2005; Turner et al., 2004) and the above-mentioned efficacy of CB_2 receptor agonists in the control of astrocyte support and microglial toxicity for neurons, in the case of ALS for motor neurons (Fernández-Ruiz et al., 2007, 2015). However, the control of glial functions, in particular microglial activation and toxicity, in ALS may be also dependent on additional receptors, other than CB_2 receptors, that may be also targeted by cannabinoids. This is the case of PPAR-γ receptors, whose activation with pioglitazone, a non-cannabinoid agonist of these receptors, was neuroprotective in the spinal cord of G93A mutant mice (Kiaei et al., 2005; Schütz et al., 2005). The effects of pioglitazone involved a reduction in both the activation of microglial cells and astrocytes and the expression of inducible nitric oxide synthase, NFκB and cyclooxygenase-2 (Kiaei et al., 2005; Schütz et al., 2005). Other studies involved the induction of heat-shock proteins in neuroprotective effects following PPAR-γ receptor activation (Park et al., 2007). Several cannabinoids (e.g. Δ^9-THC,

cannabinol, WIN55,212-2) investigated in the same model of ALS also has activity at the PPAR-γ receptor, so it is possible that at least part of their efficacy may be related to the activation of these receptors. In support of this possibility, we recently investigated VCE-003.2, a hydroxyquinone derivative of cannabigerol, which has activity at the PPAR-γ receptor without activating CB_1/CB_2 receptors (Rodríguez-Cueto et al., 2018). The study was conducted in mutant SOD-1 mice and demonstrated an important efficacy of VCE-003.2 in preserving spinal motor neurons, reducing glial reactivity and improving neurological defects in these mice (Rodríguez-Cueto et al., 2018).

Therefore, given that the cannabinoids that are beneficial in ALS encompass a broad range of endocannabinoid targets and mechanisms, and that the recently-licenced cannabinoid-based medicine Sativex® has this type of broad-spectrum profile (Wright, 2007), we wanted to evaluate a similar combination of phytocannabinoids in SOD-1 mutant mice (Moreno-Martet et al., 2014). In our hands, the treatment of post-symptomatic SOD-1 mutant mice with a Sativex®-like combination of phytocannabinoids preserved motor neurons in the spinal cord. Yet, this neuroprotective effect does not appear to be extended to the preservation of neuron-muscle synapse, as the beneficial effects found in neuronal survival did produce only a small delay in the onset of neurological symptoms but not an improvement in animal survival (Moreno-Martet et al., 2014). It is possible that different doses and/or ratios for the two major phytocannabinoids included in Sativex® may be necessary to enhance the benefits of this combination in SOD-1 mutant mice, for example, a better response may be obtained with a combination having higher Δ^9-THC and lower cannabidiol (CBD). It is also possible that this may happen with combinations of Sativex® with other approved (e.g. riluzole) or under investigation agents. These possibilities will have to be examined in coming years.

Treatments with cannabinoids in clinical studies with ALS patients

The possibility that certain cannabinoids may provide benefits in ALS has been also studied at the clinical level, although the number of clinical trials is still too reduced to get significant and reliable findings, thus stressing the urgent need of additional clinical investigation (Carter et al., 2010). First studies were exclusively observational (e.g. surveys) and based on ALS patients that self-medicated with cannabis (or were prescribed with cannabis formulations in dispensaries; Haug et al., 2016) for attenuating specific ALS-related symptoms (e.g. cramps,

spasticity, drooling; see Amtmann et al., 2004), but also searching more general effects that cannabis may provide to ALS patients (e.g. analgesia, bronchodilation, saliva reduction, appetite stimulation, and sleep induction; see Carter et al., 2010; Chiò et al., 2016). These observational studies have derived in a few number of controlled clinical trials. For example, a randomized, double-blind crossover trial conducted with oral Δ^9-THC studied its effects on cramps (Weber et al., 2010). Cramps are an important symptom experienced by ALS patients during the course of the disease that frequently remains refractory for most of the symptoms-relieving medications used in ALS (e.g. lioresal, dantrolene, clonazepam, gabapentin). The study showed that Δ^9-THC was well-tolerated but without positive effects on cramp frequency and intensity (Weber et al., 2010). These results should be, however, interpreted with caution due to the small number of patients recruited and the dose used (5 mg twice daily for 2 weeks) (Weber et al., 2010). Two additional studies indicated again good tolerability of Δ^9-THC in ALS patients and a non-significant attenuation on cramps and fasciculations (Gelinas et al., 2002; Joerger et al., 2012), although a high inter-individual variability was found in Δ^9-THC pharmacokinetics in one of these studies (Joerger et al., 2012). Given that nabiximols (Sativex®) have been approved for the treatment of spasticity in multiple sclerosis patients, and that spasticity is also an important determinant on the decline of the quality of life in ALS patients, a recent phase 2 clinical trial has investigated nabiximols in a group of these patients (Riva et al., 2019). The study revealed an acceptable profile of safety and tolerability, as well as positive effects on ALS-associated spasticity (Riva et al., 2019).

There are little clinical studies so far that have tried to demonstrate the potential of cannabinoids as disease-modifying therapies as largely supported by preclinical studies, so this hypothesis remains a major challenge for future research. According to the research in cannabinoid-based neuroprotective therapies conducted in experimental ALS, potential clinical studies should investigate the relevance of CB_2 receptor activation on astrocyte trophic support, microglial reactivity and neuroinflammation. It also involves certain CB_1 receptor-mediated effects that might contribute to attenuate excitotoxic damage as well as the contribution of other mechanisms/targets, for example, those related to the cannabinoid receptor-independent effects (perhaps related to PPAR-γ signalling) which underlie the effects of some phytocannabinoids, e.g. Δ^9-THC (Raman et al., 2004), cannabinol (Weydt et al., 2005), or the synthetic cannabigerol derivative VCE-003.2 (Rodríguez-Cueto et al., 2018).

This was the rationale for the research conducted with a Sativex-like combination of botanical extracts enriched in Δ^9-THC and CBD in post-symptomatic SOD-1 mutant mice (Moreno-Martet et al., 2014), although, as mentioned above, this combination provided a poor neurological recovery and no changes in animal survival, suggesting the need for additional cannabinoid combinations that demonstrate to be effective in preclinical models and that may be translated to the clinical scenario. In this respect, a clinical trial (the EMERALD trial) aimed at evaluating the efficacy of a standardized cannabis extract (with high CBD/low Δ^9-THC ratio) is presently in progress in Australia (Urbi et al., 2019). Additional support to the need of clinical trials comes from a case report, which demonstrated that the nutraceutical palmitoylethanolamide improved the clinical status of a sporadic ALS patient (Clemente, 2012). The benefits of palmitoylethanolamide in ALS have been furtherly investigated in other study conducted in a higher cohort of patients who experienced an improvement in the pulmonary function after being treated with this nutraceutical (Palma et al., 2016). Palmitoylethanolamide is a *N*-acylethanolamine that shares common metabolic pathways with the endocannabinoid anandamide, but only a few common pharmacological targets including PPARs and possibly GPR55 too (Petrosino and Di Marzo, 2017). Former studies had indicated that palmitoylethanolamide may activate CB_2 (or CB_2-like) receptors (Calignano et al., 1998), but further studies demonstrated that this effect is indirect and caused by its capability to be degraded by FAAH enzyme which results in the transient elevation of anandamide levels by the reduction in its degradation (Petrosino and Di Marzo, 2017).

Conclusions and future perspectives

The studies reviewed here are all concordant with the view that cannabinoid-based medicines may serve as novel therapy able to delay/arrest neurodegeneration in ALS. This is supported by the well-demonstrated capability of cannabinoids to correct glutamate impairment (excitotoxicity), to reduce oxidative stress, and/or to attenuate glial reactivity and local inflammatory events, and possibly also because of their capability to induce autophagy for the elimination of toxic protein aggregates. Special attention in this review has been paid to cannabinoids acting at the nuclear PPAR-γ receptors and, in particular, at the metabotropic CB_2 receptor, which experiences an important up-regulation in glial elements in lesioned sites of ALS patients and the experimental models. The experience collected so far on the

neuroprotective potential of cannabinoids in ALS has been predominantly obtained in animal or cellular models, but the number of clinical trials that have tried to confirm this potential in patients is still limited and focused more in the alleviation of specific ALS-related symptoms, rather than in the control of disease progression. Therefore, this last objective remains the major challenge for the future and it may be facilitated by the approval of the some cannabinoid-based medicines (e.g. Sativex®, Epidiolex®) that are currently available for clinical uses. In addition to their safety, already validated in the previous clinical studies, these approved formulations are based on cannabinoids having a broad-spectrum profile that appears to be highly adequate for ALS, a disease in which different cytotoxic mechanisms cooperate to damage motor neurons, so the therapeutic response should be based on the combination of neuroprotective properties. Therefore, it is expected that the issue recruits an important amount of clinical research in the following years that allow that the promising expectatives generated for these molecules progress from the present preclinical evidence to a real clinical exploitation.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019a,b,c,d)

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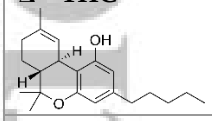
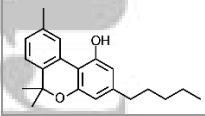
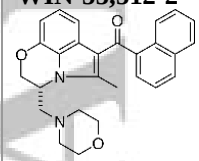
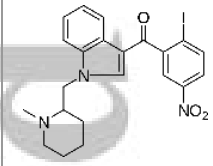
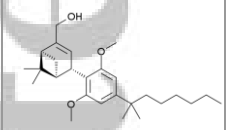
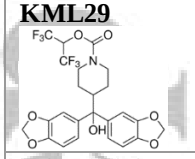
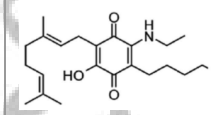
Table 1. Studies describing the changes in elements of the endocannabinoid system in tissues from ALS patients and animal models

Changes in endocannabinoid elements	Source	CNS tissues	Type of analysis	References
Endocannabinoids:				
Elevated AEA levels Elevated 2-AG levels	Mutant SOD-1 mice	Spinal cord	HPLC-MS	Bilsland et al (2006) Witting et al (2004)
Enzymes:				
Elevated NAPE-PLD mRNA levels No changes in DAGL, FAAH and MAGL mRNA levels	Mutant SOD-1 mice	Spinal cord	qPCR	Moreno-Martet et al (2014)
Receptors:				
Reduced CB_1 receptor protein levels No changes in CB_1 receptor mRNA levels Abnormal CB_1 receptor sensitivity	Mutant SOD-1 mice	Spinal cord	Immunofluorescence qPCR Neurophysiological recordings	Zhao et al (2008) Moreno-Martet et al (2014) Rossi et al (2010)
Reduced CB_1 receptor levels	Human	Spinal cord	Western blotting	Espejo-Porras et al (2018)
Elevated CB_2 receptor protein levels Elevated CB_2 receptor mRNA levels	Mutant SOD-1 mice	Spinal cord	Western blotting qPCR	Shoemaker et al (2007) Moreno-Martet et al (2014)
Elevated CB_2 receptor protein and mRNA levels No changes in CB_1 receptors	TDP-43 transgenic mice	Spinal cord	Immunostaining, Western blotting and qPCR	Espejo-Porras et al (2015 and 2019)
Elevated CB_2 receptor protein and mRNA levels No changes in CB_1 receptor levels	Dogs with degenerative myelopathy	Spinal cord	Immunostaining, Western blotting and qPCR	Fernández-Trapero et al (2017)
Elevated CB_2 receptor levels	ALS patients and controls	Spinal cord	Immunostaining	Yiangou et al (2006)
Elevated CB_2 receptor levels No changes in CB_1 receptor levels	ALS patients and controls	Primary motor cortex	Immunofluorescence and Western blotting	Espejo-Porras et al (2018)

Table 2. Preclinical studies investigating the neuroprotective effects of different cannabinoid compounds in ALS animal models

Cannabinoid compound	Target and strategy	Dose and route	Beneficial effects	Animal model	References
Δ^9 - THC 	CB_1 and CB_2 agonist GPR55 agonist	20 mg/kg i.p.	Delayed disease progression Extended lifespan	Mutant SOD-1 mice	Raman et al (2004)
CBN 	CB_1 and CB_2 agonist	5 mg/kg (osmotic pumps)	Delayed disease onset and progression	Mutant SOD-1 mice	Weydt et al (2005)
WIN-55,512-2 	CB_1 and CB_2 agonist	5 mg/kg i.p.	Delayed disease progression.	Mutant SOD-1 mice	Bilsland et al (2006)
			Delayed disease progression Increased lifespan	Mutant SOD-1 mice	Shoemaker et al (2007)
			Modest neuroprotective effects	TDP-43 transgenic mice	Espejo-Porras et al (2019)
AM-1241 	Selective CB_2 receptor agonist	0.3-3 mg/kg i.p.	Increased animal survival	Mutant SOD-1 mice	Shoemaker et al (2007)
		1 mg/kg i.p.	Delayed disease progression	Mutant SOD-1 mice	Kim et al (2006)
HU-308 	Potent and selective CB_2 receptor agonist	5 mg/kg i.p.	Improved motor function Preservation of motor neurons Reduction of glial reactivity	TDP-43 transgenic mice	Espejo-Porras et al (2019)
KML29 	MAGL inhibitor	8 mg/kg oral	Delayed disease onset and progression Increased animal survival	Mutant SOD-1 mice (low-copy model)	Pasquarelli et al (2017)
VCE-003.2 	PPAR-γ activator	10 mg/kg i.p.	Preservation of motor neurons Reduced astroglial reactivity Improved neurological defects	Mutant SOD-1 mice	Rodríguez-Cueto et al (2018)
Sativex-like combination of Δ^9-THC and CBD	CB_1 and CB_2 agonist PPAR-γ activator	40 mg/kg (20 mg/kg each compound) i.p.	Weak benefits in the progression of neurological deficits and in animal survival	Mutant SOD-1 mice	Moreno-Martet et al (2014)

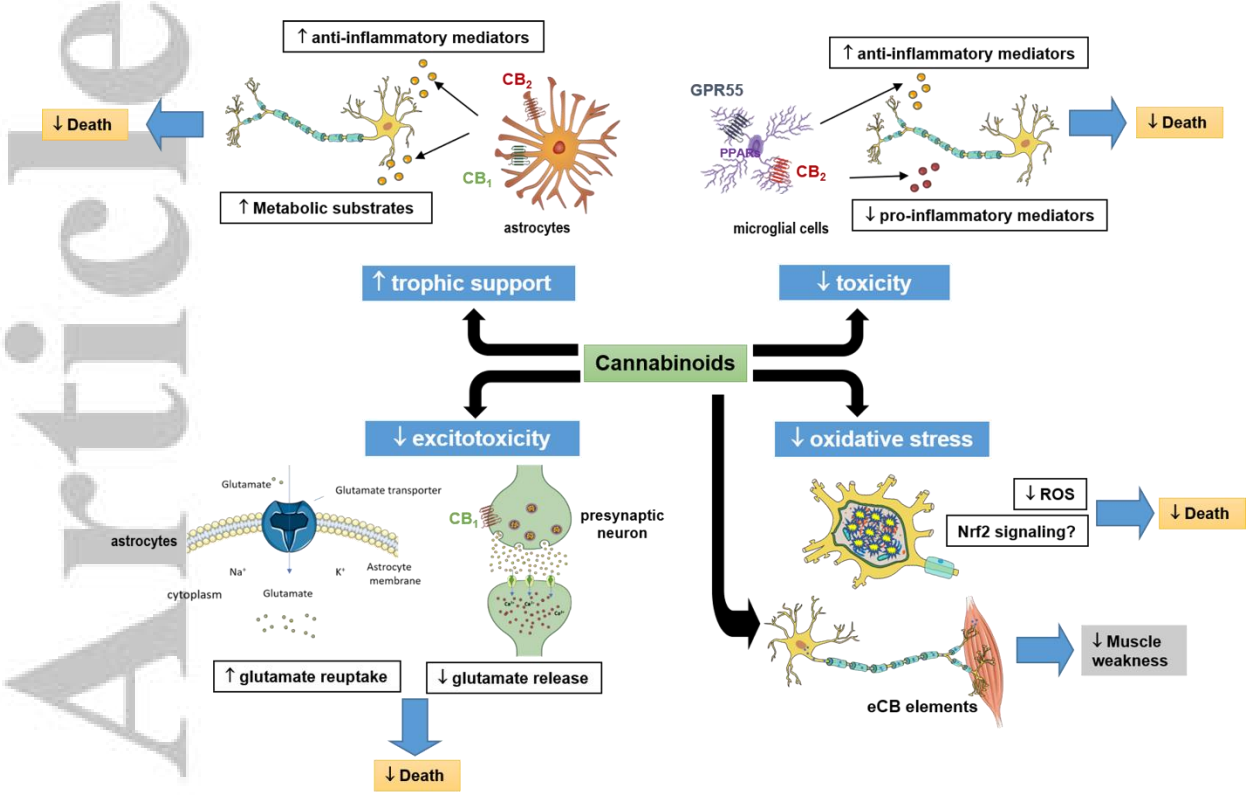


Figure 1.

Schematic summary of cellular and molecular mechanisms and cellular sites by which cannabinoids may provide neuroprotection in ALS in relation with the cytotoxic events involved in the pathogenesis.

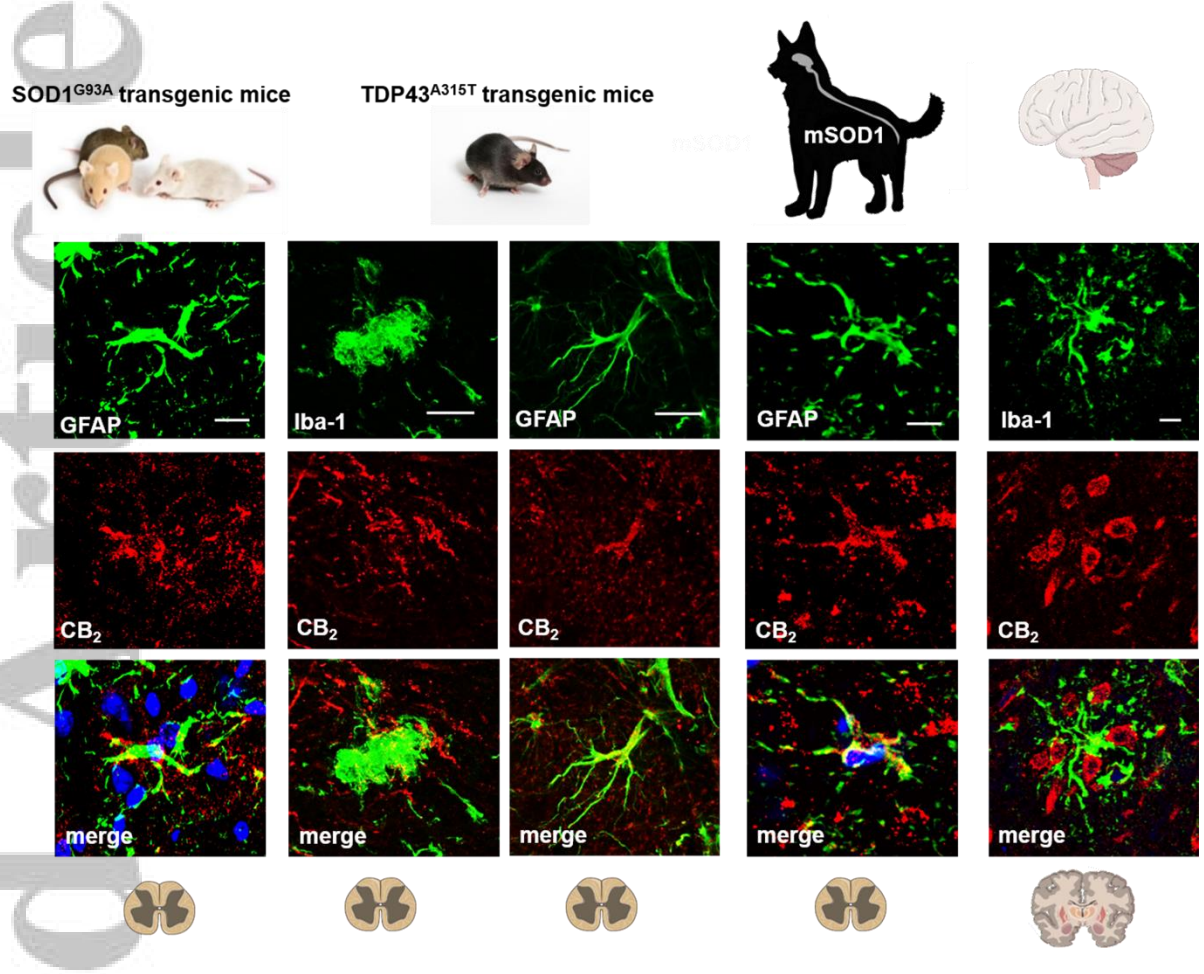


Figure 2.

Double immunostaining for the CB_2 receptor and markers of astrocytes and microglial cells supporting the up-regulatory response found in the CNS structures (spinal cord, primary motor cortex) lesioned in murine models of ALS (mSOD1(G93A) and TDP-43(A315T) transgenic mice), dogs with ALS-like degeneration, and in *post-mortem* samples from ALS patients (scale bar = 10 µm).

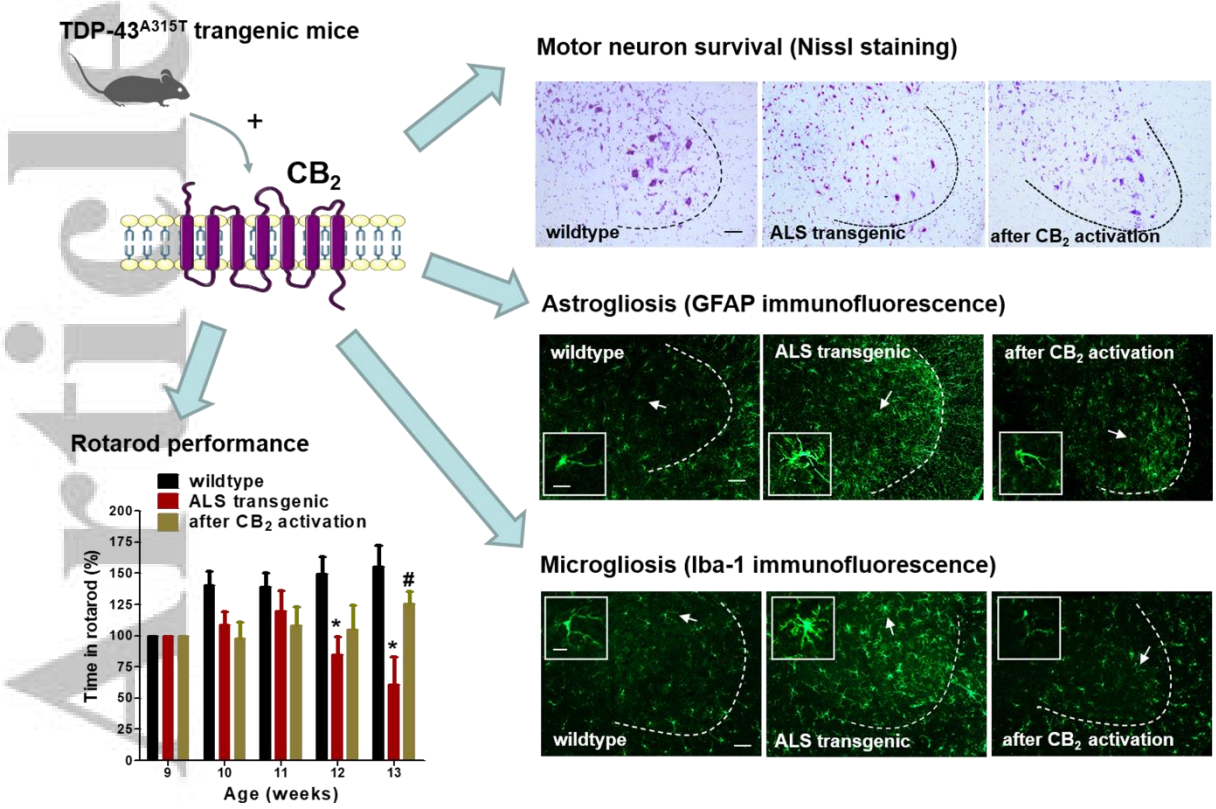


Figure 3.

Preservation of Nissl-stained motor neurons and reduced glial reactivity measured in the spinal cord, accompanied by improved rotarod performance, after the treatment with a selective agonist of CB_2 receptors in TDP-43(A315T) transgenic mice (scale bar = 100 µm; *p<0.05 *versus* wildtype, #p<0.05 *versus* ALS transgenic after data assessment with one-way ANOVA followed by the Bonferroni test).