Inactivation of the CB$_2$ receptor accelerated the neuropathological deterioration in TDP-43 transgenic mice, a model of amyotrophic lateral sclerosis

Carmen Rodríguez-Cueto$^{1,2,3}$ | Marta Gómez-Almería$^1$ | Laura García Toscano$^{1,2,3}$ | Julián Romero$^4$ | Cecilia J. Hillard$^5$ | Eva de Lago$^{1,2,3}$ | Javier Fernández-Ruiz$^{1,2,3}$

Abstract
The activation of the cannabinoid receptor type-2 (CB$_2$) afforded neuroprotection in amyotrophic lateral sclerosis (ALS) models. The objective of this study was to further investigate the relevance of the CB$_2$ receptor through investigating the consequences of its inactivation. TDP-43(A315T) transgenic mice were crossed with CB$_2$ receptor knock-out mice to generate double mutants. Temporal and qualitative aspects of the pathological phenotype of the double mutants were compared to TDP-43 transgenic mice expressing the CB$_2$ receptor. The double mutants exhibited significantly accelerated neurological decline, such that deteriorated rotarod performance was visible at 7 weeks, whereas rotarod performance was normal up to 11 weeks in transgenic mice with intact expression of the CB$_2$ receptor. A morphological analysis of spinal cords confirmed an earlier death (visible at 65 days) of motor neurons labelled with Nissl staining and ChAT immunofluorescence in double mutants compared to TDP-43 transgenic mice expressing the CB$_2$ receptor. Evidence of glial reactivity, measured using GFAP and Iba-1 immunostaining, was seen in double mutants at 65 days, but not in TDP-43 transgenic mice expressing the CB$_2$ receptor. However, at 90 days, both genotypes exhibited similar changes for all these markers, although surviving motor neurons of transgenic mice presented some morphological abnormalities in absence of the CB$_2$ receptor that were not as evident in the presence of this receptor. This faster deterioration seen in double mutants led to premature mortality compared with TDP-43 transgenic mice expressing the CB$_2$ receptor. We also investigated the consequences of a pharmacological inactivation of the CB$_2$ receptor using the selective antagonist AM630 in TDP-43 transgenic mice, but results showed only subtle trends towards a greater deterioration. In summary, our results confirmed the potential
of the CB₂ receptor agonists as a neuroprotective therapy in ALS and strongly support the need to progress towards an evaluation of this potential in patients.

**KEYWORDS**
amyotrophic lateral sclerosis, astrocytes, cannabinoids, CB₂ receptors, reactive microglial cells, spinal cord, TDP-43 (A315T) transgenic mice

1 | INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a chronic progressive neurodegenerative disorder derived from the deterioration and death of upper and lower motor neurons, leading to denervation, atrophy and paralysis of skeletal muscles (1–3). The death of motor neurons is the consequence of a combined action of excitotoxicity, oxidative stress, protein aggregation, glial reactivity, chronic inflammation, and other neurotoxic events (4–6). Most ALS cases are sporadic (7, 8), but familial cases have been identified that are associated with mutations in more than 25 genes. Superoxide dismutase-1 (SOD-1), TAR-DNA–binding protein-43 (TDP-43), fused in sarcoma (FUS), and C9orf72 are among the most frequently found gene products (5, 9–13). The variety of mutations in TDP-43, FUS, C9orf72 and other genes discovered over the past two decades have allowed ALS to be considered not as only one disorder, but part of a spectrum of disorders having motor and also cognitive deficits reminiscent of some types (i.e. tau protein-independent) of frontotemporal dementia (FTD) (14, 15). Despite the efforts addressed to develop effective treatments able to alleviate specific symptoms (e.g. cramps, spasticity) or to delay disease progression, ALS still lacks an effective therapy. The anti-excitotoxic agent riluzole (Rilutek®), approved in 1995, and the antioxidant compound edaravone (Radicava®), approved in 2015, are the only available medicines for patients, and both have only modest effect, Sativex® (26). Neuroprotection was also obtained and, to a lesser extent, the Δ⁹-THC:cannabidiol mixture, Sativex® (26). Neuroprotection was also obtained with synthetic cannabinoids such as the non-selective cannabimimetic WIN55,212-2 (27), the selective CB₂ agonist AM1241 (28, 29) and after the inactivation of the endocannabinoid-degradation enzyme fatty acid amide hydrolase (FAAH) (27). These last studies followed previous research that demonstrated elevations in the levels of the CB₂ receptor (30) and the endocannabinoids (27, 31), in the spinal cord in ALS patients and animal models, respectively.

The identification of new ALS-related genes during the last 15 years allowed the development of new experimental models, for example different transgenic models for TDP-43 protein (2, 32, 33), which represent important new tools for the study of ALS, being presently the most used alternative to the classic mutant SOD-1 mice. TDP-43 protein is encoded by *TARDBP* gene and is involved in pre-mRNA splicing, transport and/or stability [see Buratti (34) for a recent review]. Its dysregulation due to specific mutations or to impaired posttranslational modification is frequent in both familial and sporadic cases of ALS, which result in proteinopathy characterized by the accumulation of TDP-43 in the cytosol in the form of protein aggregates (35).

We recently published the first two studies investigating cannabinoids in the TDP-43 (A315T) transgenic mouse model (36, 37). In the first study, we recorded the damage of motor neurons in the spinal cord in relation with possible changes in endocannabinoid ligands, receptors and enzymes using animals at early symptomatic and advanced stages (36). The most relevant observation was the up-regulatory response found for CB₂ receptors in reactive microglial cells located in the spinal ventral horn (36), and activated astrocytes (37) of TDP-43 (A315T) transgenic mice. A similar CB₂ receptor glial response has been observed in most neurodegenerative disorders [reviewed in (38, 39)]. In the case of ALS, up-regulation of the CB₂ receptor in activated astrocytes has also been described in mutant SOD-1 mice (Espejo-Porras et al., unpublished results) and in a canine form of ALS, so-called degenerative myelopathy, which is also mutant SOD-1-dependent (40). Lastly, CB₂ receptor up-regulation in reactive microglial cells has been also found in *post-mortem* tissues of ALS patients (30, 41). In addition, CB₂ receptors were found in motor neurons in ALS patients, in spite of the fact that motor neurons degenerate during disease progression (41). Whether this also happens in animal models remains to be investigated.

Given the increase in glial CB₂ receptor expression in ALS animal models and patient samples, we examined the consequences of the activation of CB₂ receptors in the TDP-43 transgenic mice using the selective agonist HU-308, and the non-selective agonist WIN55,212-2 in the presence or absence of a selective CB₂ receptor antagonist. Our data confirmed an important neuroprotective effect mediated by the activation of the CB₂ receptor, reflected in a high level of preservation of spinal motor neurons, allowing the recovery of motor functions,
accompanying an important attenuation in the glial reactivity and toxicity (37). Similar beneficial effects with cannabinoids selectively activating CB2 receptors have been also described in preclinical models of other neurodegenerative disorders, including Alzheimer's disease (42, 43), Parkinson's disease (44), Huntington's disease (45, 46), and others [reviewed in (39)].

Given the promising pharmacological consequences that the activation of the CB2 receptor may have in ALS, the goal of the current study is to further confirm the relevance of the CB2 receptor as a potential target for developing a novel ALS therapy through investigating the consequences of its inactivation. To this end, TDP-43 (A315T) transgenic mice were crossed with knock-out mice for the CB2 receptor gene to generate double mutants, and examined progression of the pathological phenotype in comparison with TDP-43 transgenic mice with normal expression of the CB2 receptor. In a second experiment, we also investigated the consequences of chronic pharmacological inactivation of the CB2 receptor using the selective antagonist AM630 in TDP-43 transgenic mice.

2 | MATERIALS AND METHODS

2.1 | Animals, experiments and sampling

All animal experiments were conducted with two mouse colonies in C57BL/6J background: (i) Prp-hTDP-43(A315T) transgenic and non-transgenic littermate sibling mice bred in our animal facilities from initial breeders purchased from Jackson Laboratories (Bar Harbor, ME, USA); and (ii) CB2 receptor constitutive knock-out mice, generated at Genomic facilities (Lyon, France) from recombined mice bred with ubiquitous Cre-recombinase expressing mice, resulting in the deletion of the loxP-flanked (containing the entire exon 3, including the 3' UTR and knocked-in reporter) region [see details in (47)], they were provided by Julián Romero (Universidad Francisco Vitoria, Madrid, Spain). Both colonies were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1°C) with free access to high fat jelly diet (DietGel Boost, ClearH20, Portland, ME, USA), specific for TDP-43 transgenic mice (48), and water. All animal experiments were conducted according to local and European rules (directive 2010/63/EU), as well as conformed to ARRIVE guidelines. They were approved by the ethical committees of our university and the regulatory institution (ref. PROEX 059/16).

In a first experiment, Prp-hTDP-43(A315T) transgenic mice were mated with CB2 knock-out mice to generate the four genotypes to be investigated in this study: (i) wildtype mice with normal expression of the CB2 receptor (WT-CB2+/+); (ii) wildtype mice with genetic ablation of the CB2 receptor (WT-CB2−/−); (iii) TDP-43(A315T) transgenic mice with normal expression of the CB2 receptor (TDP-43-CB2+/+); and (iv) TDP-43(A315T) transgenic mice with genetic ablation of the CB2 receptor (TDP-43-CB2−/−). All animals generated were genotyped for the presence or absence of the transgene containing the TDP-43 (A315T) mutation (32) and the presence or absence of the CB2 receptor exon 3 (47). When animals in the four genotypes reached 4 weeks of age, they were subjected to analysis of animal weight gain and their performance in the rotarod test to detect muscle weakness, which was repeated weekly until the animals reached 17 weeks of age. Animals of the four genotypes were euthanized by rapid decapitation at two specific ages (in separate sets of this experiment): (i) 65 days of age, at which time only double mutants exhibited clear rotarod deterioration, and (ii) 90 days of age, at all TDP-43 transgenic mice were affected (36, 37). In a separate study, mice of the four genotypes were used to determine animal survival, using the following criteria to trigger euthanasia: (i) severe weight loss (>25%), (ii) animals having bristly hair, closed eyes, lethargy or immobility, (iii) paralysis in both hind limbs; and (iv) inability to walk and lack of response to manipulation.

In a second experiment, we treated non-transgenic and Prp-hTDP-43(A315T) transgenic male mice with the selective antagonist AM630 (Tocris Bioscience, Bristol, UK) at the dose of 3 mg/kg to selectively block CB2 receptors or vehicle (3.2% DMSO + 6.2% Tween-80 in saline solution), both administered i.p. The treatment was initiated when animals were 65 days old and prolonged daily up to the age of 90 days, the same treatment window used in our previous study (37) which extends from early symptomatic phases (around the 9th week of age) up to an advanced stage (around the 13th week of age). Animal weight gain, rotarod performance and clasping response to detect dystonia were recorded weekly (daily for weight) during the 3 weeks of treatment period (including a recording just before the first injection), and at the age of 90 days and at least 24 hours after the last treatment, all animals were euthanized by rapid decapitation.

In both experiments, animal spinal cords were rapidly removed after decapitation. The spinal samples (lumbar area) to be used for histology were fixed for one day at 4°C in fresh 4% paraformaldehyde prepared in 0.1 M phosphate buffered-saline (PBS), pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose solution for a further day, and finally stored at −80°C for Nissl staining and immunohistochemical analysis. The spinal samples (also lumbar area) to be used for biochemistry were collected and frozen by immersion in cold 2-methylbutane followed by storage at −80°C until qPCR analysis.

2.2 | Behavioral recording

TDP-43 (A315T) transgenic and wild-type mice were evaluated for possible motor weakness using the rotarod
test, using a LE8200 device (Panlab, Barcelona, Spain). Mice were exposed to a period of acclimation and training (first session: 0 r.p.m. for 30 seconds; second and third sessions: 4 r.p.m. for 60 seconds, with periods of 10 min between sessions), followed 30 minutes later by the assay. Mice were placed into the apparatus and the rotational speed was increased from 4 to 40 r.p.m. over a period of 300 seconds to measure the time to fall off. Mice were tested for 3 consecutive trials with a rest period of approximately 15 minutes between trials and the mean of the 3 trials was calculated.

2.3 | Clasping response

Dystonia was evaluated in mice when suspended by the tail for 30 seconds, so that their body dangled in the air facing downward. Animals were scored: 0 if the hindlimbs were consistently splayed outward, away from the abdomen; 1 if one hindlimb was retracted toward the abdomen; 2 if both hindlimbs were partially retracted toward the abdomen; 3 if both hindlimbs were entirely retracted and touching the abdomen. Mice were tested for 3 consecutive trials and the mean of the 3 trials was calculated.

2.4 | Real time RT-qPCR analysis

Total RNA was extracted from tissues using Trizol (Life Technologies, Alcobendas, Spain). The total amount of RNA extracted was quantified by spectrometry at 260 nm and its purity was calculated as the ratio between the absorbance values at 260 and 280 nm. RNA integrity was confirmed in agarose gels. DNA was removed and single-stranded complementary DNA was synthesized from 0.8 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at −20ºC until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, U.S.A.) to quantify mRNA levels for CB2 receptor (Mm00438286_m1), tumor necrosis factor-α (TNF-α) (Mm99999068_m1), and interleukin-1β (IL-1β) (Mm00434228_m1) using GAPDH expression (Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Expression levels were calculated using the 2−ΔΔCt method, but, for presentation, data were transformed to the percentage over the mean obtained in the wild-type group for each parameter.

2.5 | Histological procedures

2.5.1 | Tissue slicing

Fixed spinal cords were sliced with a cryostat at the lumbar level (L4-L6) to obtain coronal sections (20 μm thick) that were collected on gelatin-coated slides. Sections were used for procedures of Nissl-staining and immunofluorescence.

2.5.2 | Nissl staining

Slices were used for Nissl staining using cresyl violet, as described previously (49), which permitted to determine the effects of particular treatments on cell number. A Leica DMRB microscope (Leica, Wetzlar, Germany) and a DFC300Fx camera (Leica) were used to study and photograph the tissue, respectively. To count the number of Nissl-stained motor neurons (>400 μm²) in the ventral horn, high-resolution photomicrographs were taken with a 10x objective under the same conditions of light, brightness and contrast. Counting was carried out with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). At least 6 images per animal were analysed to establish the mean of all animals studied in each group. The morphology of motor neurons was analysed using high-resolution digital microphotographs taken with the 40x objective under the same conditions of light, brightness and contrast. For quantification, we use a minimum of 45 cells per condition on which several morphological parameters were analysed (area, perimeter, circularity and integrated density) using ImageJ software (U.S. NIH). In all analyses, data were transformed to the percentage over the mean obtained in the wild-type group for each parameter.

2.5.3 | Immunofluorescence

Slices were used for detection and quantification of choline-acetyl transferase (ChAT), glial fibrillary acidic protein (GFAP) or Iba-1 immunofluorescence. After preincubation for 1 hour with Tris-buffered saline with 1% Triton X-100 (pH 7.5), sections were sequentially incubated overnight at 4°C with the following polyclonal antibodies: (i) anti-ChAT (ref. AB144P, Abcam, Cambridge, UK) used at 1:100; (ii) anti-Iba-1 (ref. 019-19741, Wako Chemicals, Richmond, VI, USA) used at 1:500; or (iii) anti-GFAP (ref. Z0334, Dako Cytomation, Glostrup, Denmark) used at 1:200, followed by washing in Tris-buffered saline and a new incubation (at 37°C for 2 h) with an anti-rabbit or an anti-goat, as required, secondary antibody conjugated with Alexa 488 or 546 (Invitrogen, Carlsbad, CA, USA). A DMRB microscope and a DFC300Fx camera (Leica, Wetzlar, Germany)
were used for slide observation and photography. The same procedure used for Nissl staining was used here for measuring the mean density of immunolabelling in the selected areas. Again, all data were transformed to the percentage over the mean obtained in the wild-type group for each parameter.

### 2.6 Statistics

Data were assessed using one-way or two-way ANOVA followed by the Student-Newman-Keuls test or the Bonferroni test, as required, using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). Survival data were assessed using Log-Rank test and presented with a Kaplan-Meier analysis. A p value lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups were always ≥5.

### 3 RESULTS

#### 3.1 Genetic inactivation of the CB₂ receptor in TDP-43 (A315T) transgenic mice

Our first experiment consisted in generating double mutant animals overexpressing the A315T mutation of the TDP-43 protein and having genetic ablation of the CB₂ receptor. Genetic deletion of the CB₂ receptor accelerated the neurological decline reflected in a faster deterioration in the rotarod performance in double mutants that was already seen at 7 weeks of age (genotype: $F(3,395) = 101.1, p < 0.0001$; age: $F(13,395) = 0.747$, ns; genotype x age interaction: $F(39,395) = 2.61, p < 0.0001$; Figure 1), whereas this deterioration was not found in TDP-43 transgenic mice having a normal expression of the CB₂ receptor up to 11 weeks (Figure 1). In both cases and from the age at which the deterioration begins, this was progressively enhanced (showing apparently parallel

**FIGURE 1** Rotarod performance (analysed at the period of 4 to 17 weeks of age) and animal survival measured in TDP-43 (A315T) transgenic and wild-type male mice with normal or genetic ablation of the CB₂ receptor. Values for the rotarod performance are means ± SEM of 6-10 animals per group. Data were assessed by two-way analysis of variance followed by the Bonferroni test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ vs WT-CB₂⁺/⁺ mice; $^#p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.005$ vs WT-CB₂⁻/⁻ mice). Data for animal survival were presented as a Kaplan-Meier plot and assessed by Chi-square test.
patterns) in comparison with wildtype animals with normal or deficient CB2 receptor expression, which showed a progressive (and also apparently parallel) improvement in their rotarod performance (Figure 1). Despite the evident differences between TDP-43 transgenic mice having normal or ablated expression of the CB2 receptor [different onset age, different magnitude (probability levels)], these differences did not reach any statistical significance at any age (Figure 1).

We also analysed the animal survival in the four genotypes, data that are presented with a Kaplan-Meier curve (Figure 1). These data confirmed that the faster neuropathological deterioration seen in double mutants also led to a reduced survival in these mice (median survival = 113 days) compared again with TDP-43 transgenic mice with normal expression of the CB2 receptor (median survival = 176 days), whereas the median survival of the two wildtype groups was always >300 days. These differences reached significance (χ² = 6.899; p < 0.005 vs TDP-43 transgenic-CB2−/− mice).

Figure 2 shows the quantification of the number of Nissl-stained motor neurons, including representative images, in which the area analysed is marked by a dotted line (scale bar = 100 µm), in the lumbar ventral horn of the spinal cord in TDP-43 (A315T) transgenic and wild-type male mice with normal or genetic ablation of the CB2 receptor at two representative ages: 65 and 90 days. Values are means ± SEM of 5-7 animals per group. Data were assessed by one-way analysis of variance followed by the Student-Newman-Keuls test (***p < 0.005 vs WT-CB2+/− and WT-CB2−/− mice; ####p < 0.005 vs TDP-43 transgenic-CB2−/− mice).

TDP-43 transgenic mice) days of age. We found an earlier and greater loss of motor neurons, based upon quantification with Nissl staining (F(3,20) = 9.636, p < 0.001; Figure 2) and ChAT immunostaining (F(3,18) = 12.24, p < 0.0005; Figure S1), in the ventral horn of the spinal cord (lumbar level) of double mutants compared to the other three genotypes, in particular with the TDP-43 transgenic mice having normal expression of the CB2 receptor, at 65 days of age. However, the differences with this genotype disappeared at 90 days of age, as the TDP-43 transgenic mice with intact expression of the CB2 receptor also experienced a significant reduction in both Nissl stained- and ChAT immunolabelled-motor neurons (Nissl: F(3,23) = 33.636, p < 0.001; Figure 2; ChAT: F(3,23) = 28.14, p < 0.0001; Figure S1) at values similar to double mutants. While there was a trend towards a reduction in Nissl staining for CB2 receptor knockout mice having normal expression of TDP-43 at 65 days of age (Figure 2), this was not found with ChAT immunostaining (Figure S1) and disappeared at 90 days of age (Figure 2).

Analysis of glial reactivity (GFAP and Iba-1 immunostaining) in tissues from the four genotypes at the two ages showed a pattern similar to Nissl-stained and ChAT-immunolabelled motor neurons. At 65 days of age, we
found a more intense GFAP ($F(3,19) = 9.268, p < 0.001$; Figure 3) and Iba-1 ($F(3,19) = 4.426, p < 0.05$; Figure 4) immunoreactivity in double mutants compared to the other three genotypes, in particular with the TDP-43 transgenic mice with normal expression of the CB$_2$ receptor. At 90 days of age, the TDP-43 transgenic mice with intact expression of the CB$_2$ receptor also experienced a significant increase in both GFAP ($F(3,23) = 5.553, p < 0.01$; Figure 3) and Iba-1 ($F(3,21) = 5.089, p < 0.01$; Figure 4) immunoreactivity similar to the double mutants.

Expression of two proinflammatory cytokines, TNF-$\alpha$ and IL-1$\beta$ were also examined in the same spinal cord sections (Figure 5). These data demonstrated that both cytokines were significantly elevated in the lumbar spinal cords of TDP-43 transgenic mice at 65 days of age irrespective of the presence or absence of the CB$_2$ receptor (TNF-$\alpha$: $F(3,14) = 7.894, p < 0.005$; IL-1$\beta$: $F(3,16) = 5.65, p < 0.01$). The increase persisted at 90 days of age (TNF-$\alpha$: $F(3,20) = 6.108, p < 0.005$; IL-1$\beta$: $F(3,20) = 4.771, p < 0.05$; Figure 5), although, in the CB$_2$ receptor deleted mice, trended towards an increase without reaching statistical significance (Figure 5). We also analysed the expression of the CB$_2$ receptor in the same spinal cord samples, and found strongly elevated levels in TDP-43 transgenic mice with normal expression of this receptor, as described in previous studies (36), that were higher at 65 days ($F(3,18) = 6.338, p < 0.005$, Figure 5) compared to 90 days ($F(3,20) = 25.38, p < 0.0001$; Figure 5). As expected, CB$_2$ receptor-mRNA levels were not detectable in the two groups of animals having genetic ablation of this receptor (Figure 5).

### 3.2 Pharmacological inactivation of the CB$_2$ receptor in TDP-43 (A315T) transgenic mice

In a second experiment, we investigated whether the pharmacological inactivation of the CB$_2$ receptor using a chronic treatment with the selective antagonist AM630 (initiated in the early symptomatic period) in TDP-43 transgenic mice also resulted in an aggravation of the pathological phenotype of these mice. Our data, however, indicated that the chronic pharmacological inactivation of the CB$_2$ receptor produced much more limited effects, with only trends in some parameters. This was the case of the animal claspig
response, which is an index of dystonia (treatment: \( F(2,66) = 7.16, p < 0.005 \); time: \( F(3,66) = 4.54, p < 0.01 \); interaction: \( F(6,66) = 2.95, p < 0.05 \)), which tended to be apparently greater at all timepoints in transgenic mice treated with AM630, but without reaching statistical significance compared to transgenic mice treated with vehicle (Figure 6). Both transgenic groups (treated with vehicle or AM630) presented a statistically significant increase in the clasping response at 86 days of life compared to wildtype animals that apparently was greater in those treated with AM630 (Figure 6). Such trend towards an aggravation, in particular at the last timepoint analysed (higher \( p \) levels in the posthoc test compared to wildtype mice), was also seen in the rotarod performance (treatment: \( F(2,84) = 3.98, p < 0.05 \); \( p \) < 0.01; interaction: \( F(6,84) = 1.003, n s \); Figure 6). The analysis of the Nissl-stained motor neurons in the ventral horn of the spinal cords of these animals confirmed the absence of relevant differences between TDP-43 transgenic animals treated with vehicle or AM630, as the number of Nissl-stained motor neurons was reduced to a similar extent in both groups compared to wildtype animals (\( F(2,20) = 5.02, p < 0.005 \); Figure 7), and the same happened with the elevation of GFAP (\( F(2,15) = 8.34, p < 0.005 \); Figure 7) and Iba-1 (\( F(2,17) = 6.292, p < 0.001 \); Figure 7).

4 | DISCUSSION

This is a follow-up study aimed at further demonstrating the relevance of the CB₂ receptor as a promising neuroprotective target for developing a disease-modifying therapy in ALS. Such objective is supported by the results obtained in recent studies carried out by our group using selective activation of this receptor with HU-308 in TDP-43 transgenic mice (37), or generated by other groups using AM1241 in mutant SOD-1 mice (28, 29), the results of which have indicated that the CB₂ receptor is in a promising position to move to clinical studies in coming years. The objective of this study was to investigate the consequences of the chronic inactivation of the CB₂ receptor in the progression of the pathological phenotype in TDP-43 (A315T) transgenic mice, with the expectation that loss of CB₂ receptors would result in accelerated progression and/or more severe deterioration,
which may strengthen the relevance of this receptor as a neuroprotective target. Both genetic and pharmacological strategies were employed in this study.

We used a genetic approach comparing mice overexpressing the A315T mutation of the TDP-43 protein (32) with and without genetic deletion of the CB2 receptor. Our data provided solid evidence in support of the positive constitutive role played by the CB2 receptor in ALS, as its genetic inactivation significantly accelerated the development of the pathological phenotype. Evidence supporting this conclusion include: (i) an earlier onset of the neurological signs of the disease in the rotarod test, which were visible up to 4 weeks before (7 weeks of age) than the age at which they appeared in TDP-43 transgenic mice having normal expression of the CB2 receptor (11 weeks of age), (ii) premature mortality in double mutants compared to TDP-43 transgenic mice having normal expression of the CB2 receptor; and (iii) earlier death of motor neurons and microglial and astroglial activation, both already evident up to 4 weeks before in double mutants. These effects are interpreted as an earlier onset of the disease derived from the complete absence of the CB2 receptor by genetic ablation, which aggravates the prognosis as reflected in the animal survival data. However, our data do not suggest that the consequence of the CB2 receptor ablation was predominantly an increase in the magnitude of the neuronal death and the associated glial reactivity. This is supported by our observation of similar values in the death of motor neurons labelled with Nissl or with ChAT immunostaining at 90 days of age, and the same happened with the analysis of GFAP and Iba-1 immunostaining at 90 days of age, which showed similar elevated values for both TDP-43 transgenic genotypes. Therefore, we conclude that the major consequence of the absence of the CB2 receptor in the progression of the pathological phenotype of TDP-43 transgenic mice is that the neurological decline, the histopathological deterioration, and the mortality are all accelerated with respect to ALS mice that have a normal expression of this receptor.

Two observations, however, contrast with this finding. On one hand, analysis of two proinflammatory cytokines, TNF-α and IL-1β, found similar elevations in their expression in the TDP-43 transgenic genotypes at 65 days, an age at which glial reactivity was only evident in TDP-43 transgenic mice having gene ablation of the CB2 receptor. Additional research will be necessary to understand the paradoxical results in cytokines found at the two ages investigated. However, it is well-known that the induction of proinflammatory cytokines is an early event, even prior to elevations in glial markers Iba-1 and, in particular, GFAP (50–53). Therefore, a tentative explanation could be that the age-dependent peak experienced by these cytokines in the two TDP-43 transgenic phenotypes, although similar in extent, could occur earlier in the TDP-43 transgenic mice having no expression of the CB2 receptor. If this is true, the elevation of cytokines in these transgenic mice could occur before 65 days, although this has not been examined.

On the other hand, we identified some subtle differences in the intensity of Nissl staining and ChAT immunostaining in surviving motor neurons at 90 days between
both TDP-43 transgenic genotypes, suggesting that the absence of the CB$_2$ receptor may promote a greater deterioration in surviving motor neuron function at an age at which the loss of motor neurons appeared similar in both genotypes. Using a more precise analysis of morphological characteristics of the surviving motor neurons in both TDP-43 transgenic genotypes (results are presented in the Figure S2), we could detect lower Nissl staining intensity in neurons of TDP-43 transgenic mice with genetic ablation of the CB$_2$ receptor compared with the transgenic with normal receptor expression. This may be related to the greater chromatolysis (dilution of Nissl bodies) described in ALS patients (54) and experimental models of this pathology (55, 56). A parallel analysis using ChAT immunofluorescence led to similar conclusions, with a trend towards a greater decrease in immunoreactivity levels. This was associated with an apparent reduction in bouton-like structures with intense ChAT staining in TDP-43 transgenic mice with genetic ablation of the CB$_2$ receptor compared with the transgenic with normal receptor expression, despite the finding that the number of ChAT-positive motor neurons did not differ between both genotypes. These observations will require additional research to determine their roles in the accelerated pathogenesis elicited by the absence of the CB$_2$ receptor.

Coming back to the idea of an accelerated progression of the TDP-43-dependent pathology by the absence of the CB$_2$ receptor, we can add that this finding is indirectly supported by the results obtained in the second experiment conducted in this study, this time using a pharmacological rather than genetic inactivation. Our data in this experiment proved, in general, a very modest influence in the progression of the pathological phenotype, which can only be based on certain trends towards a greater behavioural deterioration with no significant differences in the histopathological analysis. The differences between both experimental approaches are obvious. The inactivation is more efficacious, complete and constant (from conception) using genetic ablation, whereas the pharmacological inactivation covers only a reduced window in the pathogenesis that includes from the early symptomatic to the advanced ages. In addition, the efficacy of the pharmacological blockade of the CB$_2$ receptor is affected by the progressive and time-dependent clearance of the antagonist following its daily administration, as well as by the expected regulatory responses tending to elevate receptor availability. Its capability to cross the blood-brain barrier could be also an important factor to be considered. Taking into account these differences, it is likely to consider that the consequences of the pharmacological inactivation concentrate more in transiently aggravating the pathological phenotype than in accelerating its progression, which
would fit with our data, and would support the idea that the therapeutic value of the CB₂ receptor in ALS would be more relevant at the onset of the disease and even at presymptomatic ages than in advanced stages.

5 | CONCLUSION

Therefore, the main conclusion of our study was that the complete absence of the CB₂ receptor using genetic inactivation in TDP-43 transgenic mice accelerated the appearance of the pathological phenotype. Such response should be necessarily related to the loss of the beneficial role played by this receptor in physiological events related to the maintenance of neuronal integrity, homeostasis and survival, in particular when this receptor is up-regulated in glial cells following damage [reviewed recently in (22)]. As found in our study and as it was expected, this up-regulation does not occur in TDP-43 transgenic mice having genetic ablation of the CB₂ receptor, which presumably may promote that glial cells in these mice may acquire soon a more harmful phenotype, then earlier facilitating the role of these glial cells in the so-called non-autonomous cell death operating significantly in ALS (57–59). However, in advanced stages of the disease, the genetic inactivation of the CB₂ receptor did not appear to significantly aggravate the magnitude of the pathological phenotype, as happened with experiments using pharmacological instead genetic inactivation of this receptor. In any case, our results confirm the relevance of targeting the CB₂ receptor for the development of a neuroprotective therapy in ALS and strongly support the need to progress towards a clinical evaluation of this potential in ALS patients.

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CONFLICT OF INTEREST

Cecilia Hillard is a member of the scientific advisory boards for Phytecs, Inc and Formulate Biosciences and has an equity share in Formulate Biosciences. The other authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

JFR and EdL contributed to study design, coordination and supervision. CH and JR contributed to development of CB₂ receptor-deficient mice. CRC, MGA and LGT contributed to generation of double mutants, pharmacological treatments, and behavioural, biochemical and histopathological analyses. CRC and JFR contributed
to statistical analysis of the data. JFR contributed to manuscript preparation with the revision and approval of all authors.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Javier Fernández-Ruiz https://orcid.org/0000-0002-4490-0604

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

FIGURE S1. Quantification of the number of ChAT-positive motor neurons, including representative images, in which the area analysed is marked by a dotted line (scale bar = 100 µm), in the lumbar ventral horn of the spinal cord in TDP-43 (A315T) transgenic and wild-type male mice with normal or genetic ablation of the CB₂ receptor at two representative ages: 65 and 90 days. Values are means ± SEM of 5-7 animals per group. Data were assessed by one-way analysis of variance followed by the Student-Newman-Keuls test (**p < 0.005 vs WT-CB₂−/+ mice; ###p < 0.005 vs TDP-43 transgenic-CB₂−/+ mice)

FIGURE S2. Quantification of Nissl staining density and ChAT immunoreactivity, including representative images [scale bar = 100 µm (detail = 50 µm)], in the lumbar ventral horn of the spinal cord in TDP-43 (A315T) transgenic and wild-type male mice with normal or genetic ablation of the CB₂ receptor at 90 days. Values are means ± SEM of 5-7 animals per group. Data were assessed by one-way analysis of variance (Statistics: Nissl staining: F(3,18) = 5.233, p < 0.01; ChAT immunostaining: F(3,17) = 51.37, p < 0.001) followed by the Student-Newman-Keuls test (*p < 0.05, **p < 0.005 vs WT-CB₂+/− and WT-CB₂−/− mice; ###p < 0.005 vs TDP-43 transgenic-CB₂+/− mice). Comparison of ChAT immunoreactivity between the two TDP-43 transgenic genotypes (data in right/bottom panel) was assessed by the Student’s t-test (###p < 0.005)

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