

CB₁ and CB₂ Cannabinoid Receptor Antagonists Prevent Minocycline-Induced Neuroprotection Following Traumatic Brain Injury in Mice

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Traumatic brain injury (TBI) and its consequences represent one of the leading causes of death in young adults. This lesion mediates glial activation and the release of harmful molecules and causes brain edema, axonal injury, and functional impairment. Since glial activation plays a key role in the development of this damage, it seems that controlling it could be beneficial and could lead to neuroprotective effects. Recent studies show that minocycline suppresses microglial activation, reduces the lesion volume, and decreases TBI-induced locomotor hyperactivity up to 3 months. The endocannabinoid system (ECS) plays an important role in reparative mechanisms and inflammation under pathological situations by controlling some mechanisms that are shared with minocycline pathways. We hypothesized that the ECS could be involved in the neuroprotective effects of minocycline. To address this hypothesis, we used a murine TBI model in combination with selective CB₁ and CB₂ receptor antagonists (AM251 and AM630, respectively). The results provided the first evidence for the involvement of ECS in the neuroprotective action of minocycline on brain edema, neurological impairment, diffuse axonal injury, and microglial activation, since all these effects were prevented by the CB₁ and CB₂ receptor antagonists.

Keywords: cannabinoid system, diffuse axonal injury, microglial activation, minocycline, neuroprotection, traumatic brain injury

Introduction

Traumatic brain injury (TBI) produces severe physical and neuropsychological consequences and is the primary cause of death in young adults (World Health Organization 2006; Boto et al. 2009). Due to the huge impact of TBI on society, more than 700 clinical trials are currently recruiting or active (clinicaltrials.gov). However, an effective therapeutic agent for TBI remains elusive (Wang et al. 2006; Beauchamp et al. 2008; Elliott et al. 2011). Approximately 85–89% of TBI patients show nonopen trauma injuries caused by traffic accidents (Masson et al. 2001; Wu et al. 2008), so, closed-head trauma animal models provide useful tools of clinical relevance to mimic and investigate the majority of TBI cases in humans. In the present study, we have used the weight-drop method which is a direct, nonpenetrating, impact acceleration model (Cernak 2005) that causes a controlled closed-head trauma (Hellal et al. 2003; Homsí et al. 2009, 2010; Siopi et al. 2011; Siopi, Calabria, et al. 2012; Siopi, Llufríu-Dabén, et al. 2012; Siopi et al. 2013). The weight-drop method is one of the most

frequently used rodent models of head injury (Cernak 2005). It reproduces many symptoms found in head-injured patients, such as brain edema, hemorrhage, inflammation, cell death, diffuse axonal injury (DAI), and functional impairment (Homsí et al. 2009, 2010), what makes this procedure very suitable for studying a wide range of outcomes after TBI.

One of the hallmarks of TBI is edema formation that accounts for up to 50% of the mortality of TBI victims (Marmarou 2003). It occurs in the first few hours after TBI and persists up to 2 weeks (Hanstock et al. 1994; Barzó et al. 1997). In addition, TBI induces neurological impairment, behavioral alterations (Emilien and Waltregny 1996; see Bales et al. 2009 for review), DAI caused by uncontrolled inertia rotational forces (Vanezis et al. 1987; Iwata et al. 2004), and the activation of glial cells, particularly microglia (Davalos et al. 2005; d'Avila et al. 2012), which may contribute to the development of secondary damage in response to brain insults (Werner and Engelhard 2000; Lucas et al. 2006). Therefore, inhibition of these processes may result in neuroprotective effects.

Recent studies show that minocycline, a highly lipophilic derivative of the antibiotic tetracycline, suppresses microglial activation, reduces cerebral edema and lesion volume, and offers persistent neuroprotection in a TBI murine model (Homsí et al. 2009, 2010; Siopi et al. 2011; Siopi, Calabria, et al. 2012; Siopi, Llufríu-Dabén, et al. 2012). Minocycline exerts antiinflammatory, antiapoptotic, and antioxidative effects; however, the specific mechanism of action of this drug in the brain remains unclear (Sanchez Mejia et al. 2001; Chu et al. 2010; Plane et al. 2010). Some of the proposed mechanisms of minocycline-mediated neuroprotection are related to the inhibition of microglial activation (Tikka et al. 2001), the attenuation of the activation of the p38 mitogen-activated protein kinase (Tikka and Koistinaho 2001), or the reduction of proinflammatory cytokines (Markovic et al. 2011). Minocycline also has antioxidant activity (Kraus et al. 2005), reduces the production of nitric oxide (Romero-Perez et al. 2008), controls glutamatergic calcium signaling (Tikka and Koistinaho 2001; González et al. 2007), and inhibits the expression of the arachidonic acid metabolism enzyme, 5-lipoxygenase (Chu et al. 2010).

The endocannabinoid system (ECS) is comprised of the endogenous ligands (endocannabinoids), the enzymatic machinery, and specific membrane receptors and transporters (Piomelli 2003; Mackie 2006). Endocannabinoids mainly act through the

activation of specific metabotropic receptors coupled to Gai/o proteins (cannabinoid receptor type 1 and 2, CB1R and CBR2, respectively). The CB1R is the predominant cannabinoid receptor within the central nervous system and is highly expressed in brain regions involved in emotional processing, motivation, motor activation, and cognitive function (Herkenham 1992). It is expressed in neurons, astrocytes, microglia (Stella 2010), and oligodendrocytes (Molina-Holgado et al. 2002). CB2R is found in the brain under pathological conditions (Chin et al. 2008), and more recent studies have described its presence under normal physiological conditions as well (see Marco et al. 2011 for review). An ECS plays a key role in neuroprotection and neurotoxicity (Zhang et al. 2009; Fowler et al. 2010; Sarne et al. 2011), mediating compensatory and reparatory mechanisms under pathological situations, like neurodegenerative diseases (Micale et al. 2007), focal ischemic damage (Nagayama et al. 1999), stab wound lesions (López Rodríguez et al. 2011), and closed-head injuries (Panikashvili et al. 2001, 2006). Some of the mechanisms proposed to explain the neuroprotective actions of endocannabinoids appear to be shared by minocycline. These include the modification of calcium currents (García-Martínez et al. 2010; Sarne et al. 2011), the regulation of caspase-3 expression (Chen and Buck 2000; Sarker et al. 2000), the control of NF- κ B pathways (Nikodemova et al. 2006; Kozela et al. 2010), and the regulation of nitric oxide production (Esposito et al. 2001; Romero-Perez et al. 2008). In view of these common mechanisms, we hypothesized that the ECS could be involved in the neuroprotective effects of minocycline. To address this hypothesis, we studied the involvement of CB1R and CB2R in the effects of minocycline on edema and neurological impairment in a mouse TBI model. Moreover, we evaluated the activation of glial cells and the induction of DAI to define the connection between minocycline neuroprotective pathways and the ECS.

Materials and Methods

Animals

All experiments were performed on adult male Swiss mice (Janvier, Le Genet St Isle, France; 28–30 g body weight). Animals were group-housed (6 mice/cage) in a controlled temperature environment (22 ± 2 °C), under a 12 h light/dark cycle, with access to food and water ad libitum. Animal care and experiments were approved by the University of Paris Descartes Animal Ethics Committee (P2.MJT.049.08), and followed the French regulations and the European Communities Council Directive (86/609/EEC) on the protection of animals for experimental use.

Traumatic Brain Injury

The mouse model of closed-head injury was performed as previously described (Hellal et al. 2003; Homsí et al. 2009, 2010). Prior to the experiment, each mouse was randomly picked by the experimenter and instantly assigned to 1 of the 7 groups designed for the study. The mice were anesthetized with 2% halothane before being subjected to TBI. Closed-head trauma was induced by a 50-g weight dropped from a 36-cm height along a stainless steel rod, on the right frontal side of the head. This experimental paradigm creates DAI in the corpus callosum and a limited contra-coup lesion in the right hemisphere (orbitofrontal cortex and perirhinal cortex), accompanied with functional deficits and a 5–15% mortality rate within the first 5 min following the impact (Homsí et al. 2009, 2010; Siopi et al. 2013). Initially, we used a total of 60 animals and 49 of them sustained TBI. Among these latter mice, 4 individuals died after trauma, and these were the only subjects excluded from the study.

Pharmacological Treatments

Animals were randomly assigned to 1 of the 7 groups: Naive; TBI; TBI-AM251; TBI-AM630; TBI-Mino; TBI-AM251-Mino; TBI-AM630-Mino. The CB1R antagonist AM251 (1 mg/kg; Tocris Bioscience, Bristol, UK), the CB2R receptor antagonist AM630 (1 mg/kg, Tocris), or their vehicle [ethanol:cremophor:saline (1:1:18), Cremophor® EL Fluka, Sigma-Aldrich, Madrid, Spain] were intraperitoneally (i.p.) administered 1 h before the lesion. A cohort of animals undergoing this protocol was sacrificed 24 h after lesion by cervical dislocation and used for behavioral studies and brain edema assessment ($n = 12$ per group), and the other cohort was perfused intracardially 24 h after TBI and was used for immunohistochemistry studies ($n = 8$ per group).

The cannabinoid receptor antagonist and the doses used in this experiment have been previously described as receptor-specific and was able to block estradiol neuroprotective effects (López Rodríguez et al. 2011); furthermore, although there is a paucity of data on the half lives of these 2 antagonists in mice, some works suggest that the behavioral half life of AM251 in rats is approximately 22 h (McLaughlin et al. 2003). Thus, it is possible that the antagonists do indeed antagonize the effects of doses of minocycline administered up to 9 h later. Minocycline (minocycline hydrochloride; Sigma-Aldrich, Fallavier, France) or its vehicle (filtered PBS 0.01 M, pH 7.4) were i.p. administered at 5 min (90 mg/kg), and at 3 and 9 h (45 mg/kg) after TBI. This treatment protocol has been previously described to be the most effective in reducing edema, lesion volume, and microglial activation after TBI (Homsí et al. 2009, 2010) and to induce acute and persistent functional recovery in the same model of TBI (Homsí et al. 2009, 2010; Siopi et al. 2011; Siopi, Calabria, et al. 2012; Siopi, Llufríu-Dabén, et al. 2012).

Acute Neurological Assessment

The functional outcome was assessed by a person that was blind to the experimental groups. A cumulative score was designed based on a previous study (Flierl et al. 2009) and was performed at the peak of acute neurological impairment 24 h post-TBI (Girgis et al. 2013; Siopi et al. 2013). Five phenotypical cues (5 points) were evaluated, as well as the capacity of the animal to exit a circular arena within 2 min (3 points), adding up to a total score of 8 points (Girgis et al. 2013; Siopi et al. 2013). One point was attributed to every normal state of alertness, posture, and exploration, as well as to the absence of blepharoptosis and lack of stereotypic movements. Otherwise, 0 points were accorded.

The test was conducted in an open circular plastic arena (16 cm height and 30 cm diameter) that contained an exit aperture (2×2.5 cm). The animal was placed in the center of the arena and was allowed to explore freely for 2 min. The behavior of the animal was evaluated by its ability to exit the arena within 2 min after performing a few attempts, which were reflected by head-stretching movements at the exit aperture (3 points). A quotation of 2 points was attributed to the same performance when it surpassed the 2-min deadline. One point was attributed to a disinhibited behavior, depicted by an impulsive exit of the circle within 2 min without head-stretching. Finally, a score of 0 point was accorded when the animal did not leave the device nor made any attempts of exit within the 2-min time interval.

Evaluation of Cerebral Edema

Cerebral edema was evaluated by the measurement of the brain water content (BWC), as previously described (Hellal et al. 2003; Homsí et al. 2009; Siopi et al. 2013). Animals were sacrificed at the peak of cerebral edema occurred 24 h after TBI by cervical dislocation, and the brain was quickly and gently removed. We took a punch of tissue from the right injured hemisphere (3–0 mm from bregma), and it was immediately weighed in order to obtain the wet weight (WW) and heated at 100 °C for 24 h. Then, samples were weighed again to obtain the dry weight (DW). BWC was calculated as follows: $\% \text{H}_2\text{O} = [(WW - DW) / WW] \times 100$.

Tissue Fixation and Immunohistochemistry

For histological analysis, 24 h after brain injury, animals were anesthetized with i.p. sodium pentobarbital (100 mg/kg, Cevá Santé Animale, France) and perfused intracardially, first with 50 mL saline (0.9% NaCl) and then with 100 mL fixative solution (4% paraformaldehyde in 0.1 M

phosphate buffer). Brains were removed and immersed overnight at 4 °C in the same fixative solution and then rinsed with phosphate buffer. Coronal sections, 50 µm thick, were obtained using a Vibratome (VT 1000 S, Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was carried out on free-floating sections under moderate shaking. All washes and incubations were done in 0.1 M phosphate buffer pH 7.4, containing 0.3% bovine serum albumin and 0.3% Triton X-100. The endogenous peroxidase activity was quenched in a solution of 3% hydrogen peroxide in 30% methanol. Sections were incubated overnight at 4 °C with either a rabbit polyclonal antibody against beta-amyloid peptide (β-APP), a marker of DAI (diluted 1:1000, Invitrogen, Carlsbad, CA, USA), or a rabbit polyclonal antibody against Iba-1 (ionized calcium-binding adaptor molecule 1), a marker of microglia (diluted 1:2000; Wako Pure Chemical Industries, Japan). Next day, sections were incubated for 2 h with biotinylated goat anti-rabbit immunoglobulin G (diluted 1:300; Pierce, Rockford, IL, USA). After several washes, sections were incubated for 90 min with avidin-biotin-peroxidase complex (diluted 1:250; ImmunoPure ABC peroxidase staining kit, Pierce), and the reaction product was revealed with 2 µg/mL 3,3'-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Finally, sections were dehydrated, mounted on gelatinized slides, coverslipped, and examined with a Leitz Laborlux microscope (Leica Microsystems).

Morphometric Analysis

We examined every section obtained from 2.5 to -2 mm bregma (Paxinos and Watson 1998) in a blind code. Within these sections, the lesion was found in every group except in naïve animals. Sections from 8 animals in each experimental group were analyzed, all of them in the blind code. The number of Iba-1-positive cells within the area surrounding the lesion was counted with a morphometric grid under the ×10 objective in a defining area of 350 × 350 µm; this grid was placed in the screen of a TV monitor connected to the microscope. The morphology of Iba-1 immunoreactive microglia was assessed with the ×40 objective. Cells were classified in 5 morphological types: Type I, cells with few cellular processes (2 or less); Type II, cells showing 3–5 short branches; Type III, cells with numerous (>5) and longer cell processes and a small cell body; Type IV, cells with large somas and retracted and thicker processes; and Type V, cells with amoeboid cell body, numerous short processes, and intense Iba-1 immunostaining. Iba1-immunoreactive cells with large somas and retracted and thicker processes and cells with amoeboid cell body, numerous short processes, and intense Iba1 immunostaining were classified as reactive microglia (Diz-Chaves et al. 2012; see Fig. 1). For each animal, we analyzed a total of 100 cells, within 4 different slices in the ventral cortex of the right hemisphere surrounding the lesion, as well as in the “corpus callosum,” in the same region where we analyzed the DAI (interhemisphere area).

DAI was assessed by analyzing the surface density of β-APP-immunolabeled axons using a double square lattice test system defining an area of 80 × 80 µm on the grid C16 of Weibel (1979). The morphometric grid was placed in the screen of a TV monitor connected to the microscope. Four sections for each animal were examined using the ×40 objective, and the number of intersections of the lines of the grid with β-APP-immunoreactive material profiles was counted within the interhemisphere area of the corpus callosum.

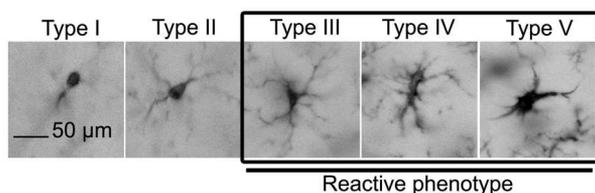


Figure 1. Microglia cells stained with Iba-1 marker and classified according to morphological aspects (Diz-Chaves et al. 2012). Highlighted, reactive phenotype (from Type III to V). Images at ×40 objective.

Statistical Analysis

Data were analyzed using a 3-way analysis of variance (ANOVA), with factors being TBI, cannabinoid receptor antagonists, and minocycline treatment. Data were not always normally distributed. Therefore, to satisfy the assumption of normality for the ANOVA, we transformed the data when necessary by the Neperian logarithm function. If transformed data were not normally distributed, nonparametric tests were used (Kruskal–Wallis and post hoc pair-wise comparisons with Mann–Whitney *U*-test). When appropriate, 3-way ANOVAs were followed by separate 2-way ANOVA and 1-way ANOVA split by the independent factors to further analyze the data. Post hoc comparisons were performed with a level of significance set at $P < 0.05$. For data that were normally distributed and homoscedastic, we used a standard parametric post hoc test (Bonferroni's test) and for those that were normally distributed, but nonhomoscedastic, we performed nonparametric post hoc comparisons (Games–Howell's test). Student's *t*-test was used when 2-group comparison was necessary. Statistical analyses were carried out with the SPSS 19.0 software package (SPSS, Inc., Chicago, IL, USA). Data are presented as mean + standard error of the mean (SEM).

Results

Minocycline-Induced Reduction of Brain Edema and Neurological Impairment After TBI Was Prevented by CB1R and CB2R Antagonists

Water content percentage and neurological score variations are represented in Figure 2. For water content, 3-way ANOVAs showed a significant effect of antagonists treatment ($F_{2,75} = 6.11$; $P < 0.05$) and a significant Antagonists × Minocycline interaction ($F_{2,75} = 4.61$; $P < 0.05$). Subsequent 2-way ANOVA split by TBI revealed a significant effect of antagonists treatment in injured animals ($F_{2,64} = 5.38$; $P < 0.05$). Final 2-way ANOVA split by antagonists showed a significant effect of minocycline in TBI animals ($F_{1,22} = 8.40$; $P < 0.05$). Post hoc comparisons revealed that the lesion induced an increase in the BWC when compared with naïve animals. The increase in edema was attenuated by minocycline treatment, and this antiedematous effect was prevented by both cannabinoid receptor antagonists.

Neurological score data were not normally distributed. Therefore, data were analyzed using the Kruskal–Wallis test, which revealed significant effects of TBI ($P < 0.001$), antagonists ($P < 0.001$), and minocycline treatment ($P = 0.001$). Subsequent post hoc Mann–Whitney *U*-tests revealed that TBI induced a significant decrease in the neurological score in every group except for the TBI-Mino group. The improvement in the neurological score induced by minocycline in TBI animals was prevented by both cannabinoid receptor antagonists.

Minocycline Reduces the Proportion of Iba-1 Microglia with the Reactive Phenotype After TBI That was Prevented by the Cannabinoid Receptor Antagonists

We analyzed the total number of Iba-1-positive cells in the ventral cortex of the right hemisphere surrounding the lesion (Fig. 3). Two-way ANOVA split by cannabinoid treatment revealed a significant effect of minocycline in the TBI group ($F_{1,12} = 5.83$; $P < 0.05$), but not in the animals treated with the antagonists. *T*-test analysis revealed that TBI induced an increase in the number of Iba-1-positive cells when compared with naïve animals ($t = 0.005$). Since the antagonists did not affect this parameter, we decided to analyze the proportion of microglia cells with a reactive phenotype.

The percentage of reactive microglia cells in the area surrounding the lesion is presented in Figure 4. The 3-way ANOVAs

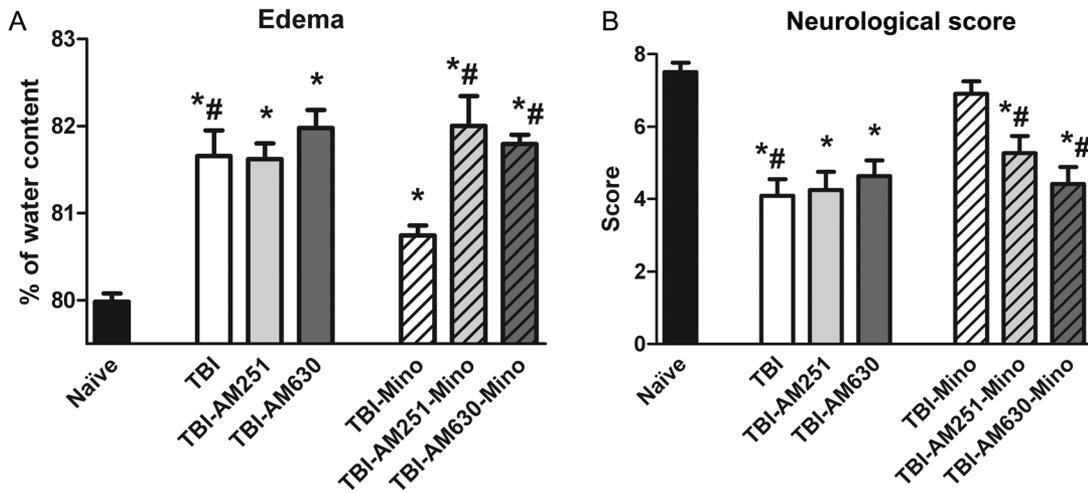


Figure 2. Effects of TBI, minocycline, and cannabinoid receptor antagonists on brain edema and neurological score, 24 h after lesion. Histograms represent the mean + SEM ($n = 12$) of the percentage of water (A) and of the neurological score (B). Naive, noninjured animals; TBI, traumatic brain injury; AM251, CB1R antagonist; AM630, CB2R antagonist; Mino, minocycline. Significant differences, $P < 0.05$: * versus naive; # versus TBI-Mino.

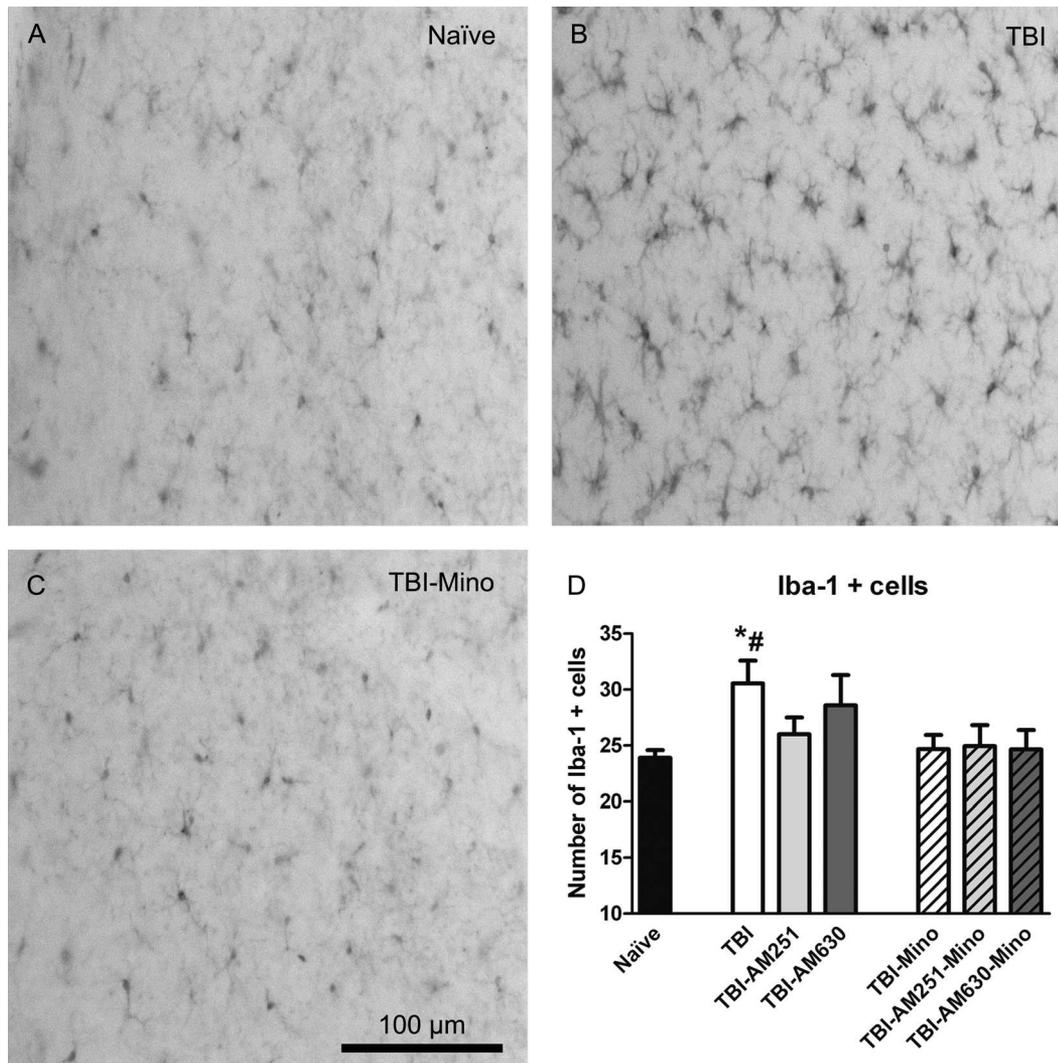


Figure 3. Effects of TBI, minocycline, and cannabinoid receptor antagonists on the number of microglial cells in the border of the lesion, 24 h after lesion. Representative examples of Iba-1-immunoreactive microglia cells in the injured brain area. (A) Naive; (B) TBI; (C) TBI-Mino; (D) TBI-AM251-Mino; (E) TBI-AM630-Mino; and (F) histogram representing the mean + SEM ($n = 8$) of the percentage of microglial cells with the reactive phenotype. Naive, noninjured animals; TBI, traumatic brain injury; AM251, CB1R antagonist; AM630, CB2R antagonist. Significant differences, $P < 0.05$: * versus naive; # versus TBI-Mino.

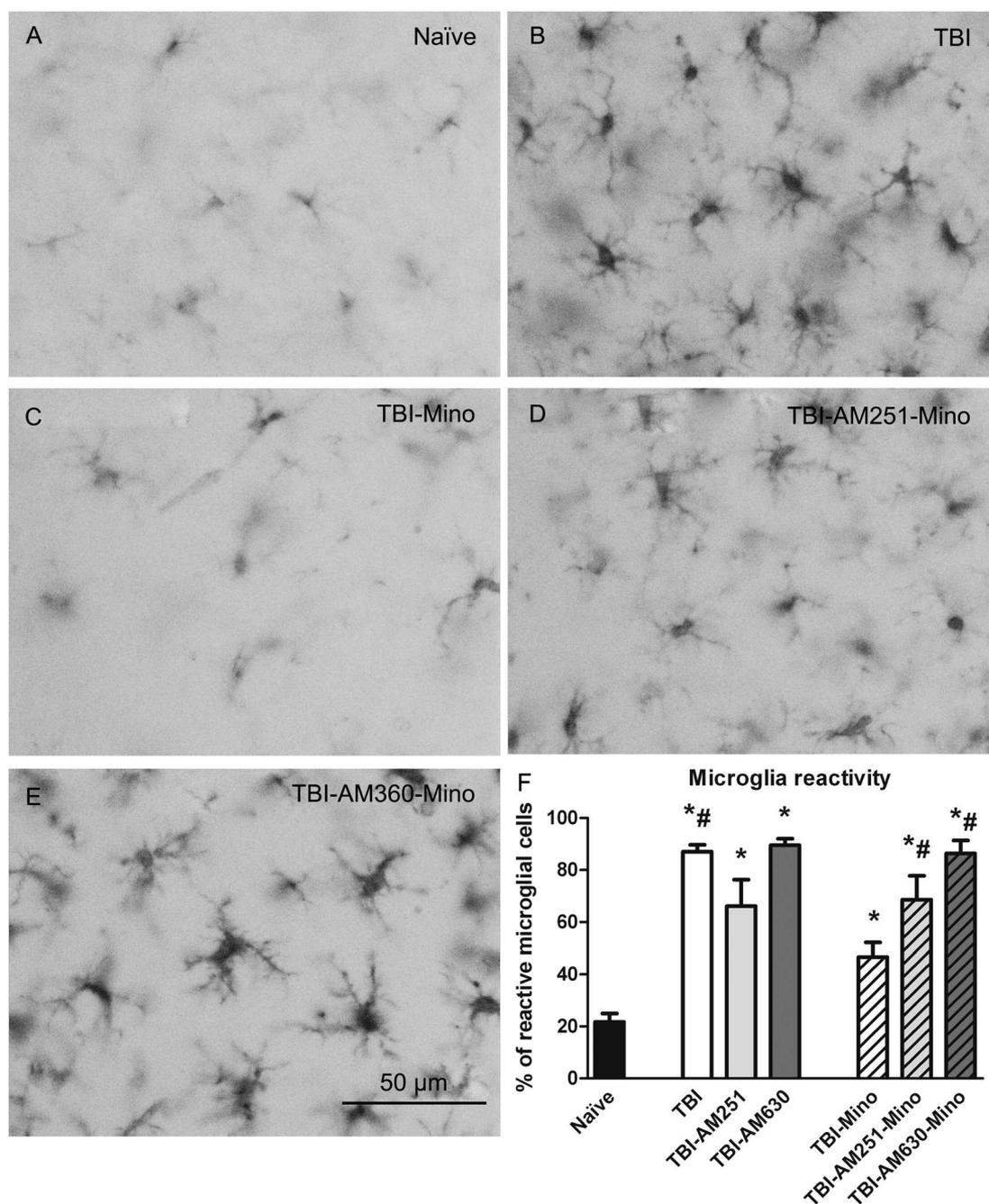


Figure 4. Effects of TBI, minocycline, and cannabinoid receptor antagonists on microglial reactivity in the border of the lesion, 24 h after lesion. Representative examples of Iba-1-immunoreactive microglia cells. (A) Naïve; (B) TBI; (C) TBI-Mino; (D) TBI-AM251-Mino; (E) TBI-AM630-Mino; and (F) histogram representing the mean + SEM ($n = 8$) of the percentage of microglial cells with the reactive phenotype. Naïve, noninjured animals; TBI, traumatic brain injury; AM251, CB1R antagonist; AM630, CB2R antagonist; Mino, minocycline. Significant differences, $P < 0.05$: * versus naïve; # versus TBI-Mino.

of the percentage of microglia cells with the reactive phenotype in the area surrounding the lesion revealed a significant effect of antagonists ($F_{2,43} = 9.01$; $P < 0.05$) and minocycline treatments ($F_{1,43} = 7.73$; $P < 0.05$), as well as a significant Antagonists \times Minocycline interaction ($F_{2,43} = 7.56$; $P < 0.05$). Subsequent 2-way ANOVA split by minocycline showed a significant effect of antagonists in minocycline-treated animals ($F_{2,19} = 10.18$; $P < 0.05$). Post hoc comparisons and Student's t -test revealed that the lesion induced an increase in the reactive morphology of microglial cells, that this increase was reduced by

minocycline treatment, and that the effect of minocycline was prevented by both cannabinoid receptor antagonists [AM630 ($P < 0.05$); AM251 ($t = 0.032$)].

Similar results were obtained for the percentage of reactive microglia cells in the corpus callosum (Fig. 5). Three-way ANOVAs revealed a significant effect of antagonists ($F_{2,22} = 7.034$; $P < 0.05$) and minocycline treatments ($F_{1,22} = 13.898$; $P < 0.05$), as well as a significant Antagonists \times Minocycline interaction ($F_{2,22} = 8.192$; $P < 0.05$). Post hoc comparisons revealed that the lesion induced an increase in the percentage of microglia

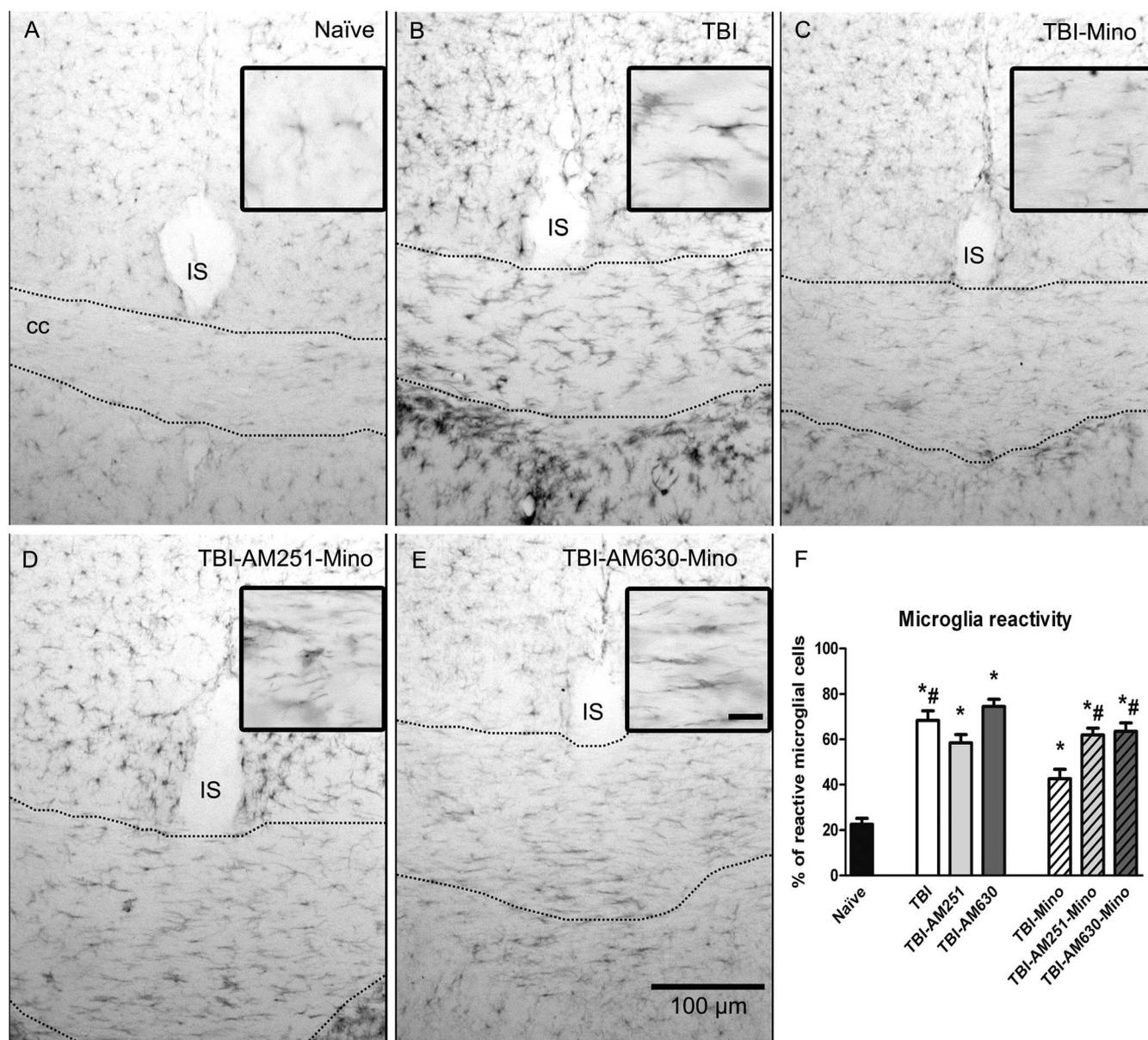


Figure 5. Effects of TBI, minocycline, and cannabinoid receptor antagonists on microglial reactivity in the corpus callosum, 24 h after lesion. Representative examples of Iba-1-immunoreactive microglia cells. (A) Naïve; (B) TBI; (C) TBI-Mino; (D) TBI-AM251-Mino; (E) TBI-AM630-Mino; and (F) histogram representing the mean + SEM ($n = 4$) of the percentage of microglial cells with the reactive phenotype. Naïve, noninjured animals; TBI, traumatic brain injury; AM251, CB1R antagonist; AM630, CB2R antagonist; Mino, minocycline; IS, interhemispheric space. Significant differences, $P < 0.05$: * versus naïve; # versus TBI-Mino.

cells with the reactive phenotype, that this increase was reduced by minocycline treatment, and that the effect of minocycline was prevented by both cannabinoid receptor antagonists.

Minocycline Reduces Diffuse Axonal Injury After TBI: CB2 Receptor Has a Relevant Role Modulating β -APP Accumulation

Results corresponding to the quantification of β -APP immunoreactivity are shown in Figure 6. The 3-way ANOVAs showed a significant effect of antagonists ($F_{2, 41} = 12.49$; $P < 0.05$) and an effect of minocycline treatment ($F_{1, 41} = 43.58$; $P < 0.05$). Subsequent 2-way ANOVA split by TBI revealed a significant effect of antagonist treatment in injured animals ($F_{2, 34} = 10.36$; $P < 0.05$) and an effect of minocycline treatment in injured

animals ($F_{1, 34} = 36.14$; $P < 0.05$). Finally, a 2-way ANOVA test split by minocycline showed a significant effect of antagonists in vehicle-treated animals ($F_{2, 16} = 8.07$; $P < 0.05$). Post hoc comparisons revealed an increase of β -APP immunoreactivity induced by TBI that was exacerbated by the CB2R antagonist AM630. Student's t -test comparisons showed a significant reduction of β -APP reactivity in TBI-Mino animals when compared with the TBI group ($t = 0.025$), whereas pretreatment with AM630 tended to counteract the effect of minocycline. This latter observation is clearly related to the exacerbating effect that the CB2 antagonist exerted "per se" in TBI animals, in the absence of minocycline treatment. The CB1R antagonist AM251 had no effect on the minocycline-induced attenuation of TBI-induced β -APP accumulation.

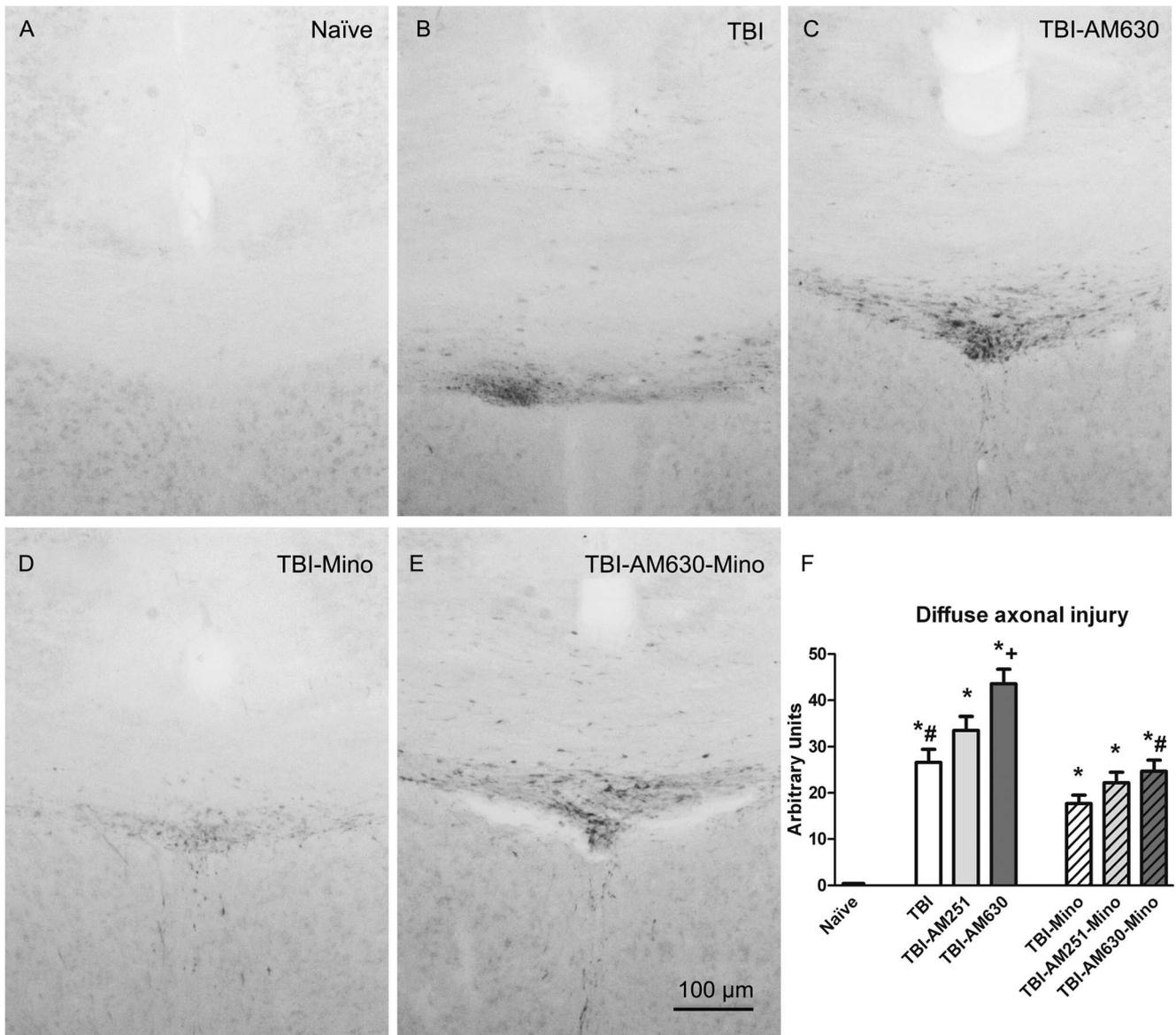


Figure 6. Effects of TBI, minocycline, and cannabinoid receptor antagonists on DAI, 24 h after lesion. Representative images of β -APP immunoreactivity in the corpus callosum. (A) Naive; (B) TBI; (C) TBI-AM630; (D) TBI-Mino; (E) TBI-AM630-Mino; and (F) histograms represent the mean + SEM ($n = 8$) of the surface density of β -APP-immunolabeled axons. Naive, noninjured animals; TBI, traumatic brain injury; AM251, CB1 receptor antagonist; AM630, CB2 receptor antagonist; Mino, minocycline. Significant differences, $P < 0.05$: * versus naive; # versus TBI-Mino; + versus TBI.

Discussion

In this study, we have assessed whether the endocannabinoid system is involved in the protective actions of minocycline after TBI in mice. We have used a murine weight-drop model that mimics some human symptoms after TBI. In agreement with previous studies, we have found that this model results in brain edema formation, neurological impairment, increase of microglial cell number, and activation and DAI. Administration of minocycline after TBI resulted in decreased edema formation, and improvement of neurological score, as well as in reduction of activated microglia confirming the previous reports (Tikka et al. 2001; Homsí et al. 2009, 2010; Siopi et al. 2011; Siopi, Calabria, et al. 2012; Siopi, Llufríu-Dabén, et al. 2012). Interestingly, the protective actions of minocycline were all prevented by both CB1R and CB2R antagonists, except for

the effect of minocycline in reducing the number of microglia cells in the border of the lesion. The latter indicates that CB1 and CB2 receptors are involved in the activation and reactivity of microglia, but not in the proliferation processes, at least in our model. Besides, there were not significant effects of either of these antagonists when administered alone in the majority of the studied parameters, except for the CB2R antagonist, when administered alone, enhanced the effect of TBI on diffuse axonal damage.

The mechanical forces associated with TBI produce hematoma, hemorrhage, contusion, injury in blood vessels, and a disruption in the blood-brain barrier, which increases cellular permeability and the entrance of water, causing edema formation (Chodobski et al. 2011). The impact induces the extravasation of red blood cells and activates the coagulation

cascade. Several studies have shown that coagulation factors can affect the function of microglia, triggering an inflammatory response around the injury and leading to the release of proinflammatory cytokines, such as TNF- α , IL-6 (Möller et al. 2000), and IL-1 β , which plays a crucial role in cerebral edema formation (Lazovic et al. 2005) by parenchymal cells (Soares et al. 1995; Royo et al. 1999). Minocycline may reduce edema formation by decreasing microglial activation and the release of proinflammatory cytokines, such as IL-1 β (Lazovic et al. 2005; Homsí et al. 2009). The present results show that the cannabinoid receptor antagonists inhibited the protective action of minocycline, suggesting that the activation of CB receptors may contribute to this effect of minocycline. Several previous data support this view. For instance, CB1R and CB2R agonists inhibit the production of proinflammatory interleukins (Klein et al. 2000; Smith et al. 2000; Molina-Holgado et al. 2002; Di Filippo et al. 2004; Kozela et al. 2010; Paulsen et al. 2011). Furthermore, the activation of CB2R controls the immune response of microglial cells, increasing their migration to the lesion area or reducing the production of harmful molecules, such as TNF- α or free radicals (Walter et al. 2003; Eljaschewitsch et al. 2006; Dirikoc et al. 2007). Particularly, in TBI models, the selective activation of CB2R leads to a reduction in the activated microglia (Elliott et al. 2011), demonstrating a pivotal role of this receptor in microglial activity and immune response.

The enhanced microglial reactivity caused by TBI may also contribute to DAI, since activated microglia trigger oxidative stress responses and excitotoxicity mechanisms that cause axonal damage (Hanisch and Kettenmann 2007). This possibility is supported by our findings in the corpus callosum, where microglial reactivity was increased by TBI and reduced by minocycline. These results suggest that the protective effect of minocycline on DAI may be, in part, related to the control of reactive microglia in the corpus callosum. In addition, our findings indicate that minocycline decreases the accumulation of β -APP in the corpus callosum caused by TBI. The majority of axonal injuries occur in the zones of transition between the gray and white matter of the brain, for example, the corpus callosum (Gennarelli 1994; Povlishock et al. 1994), and they can be detected by the accumulation of β -APP in the damaged axons (Roberts et al. 1994). This effect of minocycline may be related to the control of amyloid precursor metabolism, by the inhibition of β -site APP cleaving enzyme 1 activity and the decrease in NFkappa B levels (Ferretti et al. 2012). Our findings suggest that the activation of CB2R by endogenous ligands is also protective, since the CB2R antagonist, per se, enhanced the damage caused by TBI, increasing the β -APP accumulation in the corpus callosum. Some in vitro experiments in human tissue and cell lines (Tolón et al. 2009) have demonstrated that the activation of CB2 receptors with specific agonists (JWH-015) stimulates the phagocytosis of beta-amyloid deposits. Our findings also support the view that neuroprotective effects derived from selective CB2R activation may represent an avenue for further development of novel therapeutic agents in the treatment of TBI (Elliott et al. 2011). This protective action mediated by the ECS might involve the control of NFkappa B activity (Ferretti et al. 2012), the reduction of excitotoxicity and oxidative stress (Kozela et al. 2011), and the control of beta-amyloid phagocytosis (Tolón et al. 2009).

As for brain edema, microglial activation, and diffuse axonal damage, the protective action of minocycline on neurological impairment after TBI was prevented by both CB1R and CB2R antagonists. TBI produces cognitive impairments in humans (Wortzel and Arciniegas 2012) and in animal models (Rochat et al. 2010; Shenaq et al. 2012). For example, following TBI, humans have difficulty in remembering lists of numbers (Brooks 1972) or performing matching-to-sample tasks (McLean et al. 1983). In animal models, rats and mice show deficits in spatial learning and memory (Shenaq et al. 2012) and impulsive behavior (Rochat et al. 2010). Many of these dysfunctions are related to edema formation (Tominaga and Ohnishi 1989), and the improvement in neurological function produced by minocycline correlates inversely to brain edema (Homsí et al. 2009). Therefore, CB1 and CB2 receptors may contribute to the protective effect of minocycline on neurological impairment by decreasing brain edema.

In all the parameters that we have assessed, the cannabinoid receptor antagonists partially prevented the protective effects of minocycline. However, it seems that the CB1 receptor may be less involved in the protective effects of this drug. Microglial cells undergo a process of differentiation and activation after injury, and during these stages, the pattern of CB1 and CB2 receptor expression also changes. Cabral and Marciano-Cabral et al. (2005) have demonstrated that CB1 and CB2 receptors are found constitutively at very low levels in microglia; however, after activation microglial cells express high levels of CB2 receptor, while the expression of CB1 receptor remains at low levels. This difference in expression may explain why the CB1 receptor antagonist was less effective than the CB2 receptor antagonist in reducing the effects of minocycline in our studies.

In summary, our findings confirm that minocycline decreases brain damage caused by TBI and indicate for the first time, and that the activation of CB receptors is required for the neuroprotective actions of this compound. Further studies are necessary to determine at which molecular points the signaling of minocycline and CB receptors interact to exert neuroprotective actions.

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Notes

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