Dopaminergic Receptors as Neuroimmune Mediators in Experimental Autoimmune Encephalomyelitis

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Research Article

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Abstract

The dopaminergic system plays an essential role in maintaining homeostasis between the central nervous system (CNS) and the immune system. Previous studies have associated imbalances in the dopaminergic system with the pathogenesis of multiple sclerosis (MS). Herein, we investigate the protein levels of dopaminergic receptors (D1R and D2R) in different phases of the experimental autoimmune encephalomyelitis (EAE) model. We also examined if the treatment with pramipexole (PPX) – a dopamine D2/D3 receptor-preferring agonist – would be able to prevent the EAE-induced motor and mood dysfunction, as well as the underlying mechanisms of action. Our findings showed that D2R immunocontent upregulated in the spinal cord of EAE mice 14 days post-induction. Moreover, D1R and D2R immunocontent was significantly increased in the lymph nodes during the chronic phase of EAE. Also, during this phase, oxidative damage in the spinal cord and striatum of EAE animals was significantly higher. During the pre-symptomatic phase, axonal damage in the spinal cord of EAE mice could already be found. In addition, PPX treatment failed to inhibit EAE progression and anhedonic-like behavior. Otherwise, PPX inhibited the depressive-like behavior. Relevantly, PPX oral treatment downregulated IL-1β levels and increased BNDF content in the spinal cord after EAE induction. Altogether, it is possible to conclude that D2R participates in crosstalk between CNS and immune system during autoimmune and neuroinflammatory response induced by EAE, mainly in the acute and chronic phase of the disease. Relevantly, a dopamine D2/D3 receptor-preferring agonist mitigated depressive-like behavior induced by EAE, which offers a new possibility for treating depressive symptoms in MS patients.

1. Introduction

Multiple sclerosis (MS) is an autoimmune and chronic disease of the central nervous system (CNS) recognized as the most common neuroinflammatory cause of non-traumatic neurological disability arising in young adults between 20 and 40 years of age [1, 2]. Indeed, MS affects more than 2.5 million worldwide, mainly women (ratio of 3:1 over men) [2, 3]. It is a neurodegenerative disorder characterized by chronic demyelination with concomitant oligodendrocyte death, axonal and neuronal loss [4, 5]. Although it is an idiopathic disease, MS seems to result from a complex and dynamic interaction between deregulation of immune homeostasis, genetic predisposition, and environmental factors. The risk factors for the disease development include infections, especially Epstein-Barr virus (EBV) [6], lower levels of vitamin D [7], smoking [8], childhood obesity [9], and variations in the HLA-DRB1 gene [10].

The pathophysiology of MS possibly comprises crosstalk between the innate and adaptive immune system, which allows the activation of potentially autoreactive $CD4^+$ T lymphocytes in the periphery against protein constituents within the myelin sheath [11, 12]. The immune cells – mainly Th1 and Th17 – migrate across the blood-brain barrier (BBB), triggering the emergence of demyelinating plaques within the white and grey matter of the CNS, which consequently harms the synaptic transmission [13–15]. In turn, the spatial distribution of lesions in the CNS is closely related to symptomatology. Clinical manifestations comprise visual and sensory disturbances, urinary and intestinal system disorders, fatigue, weakness, ataxia, and cognitive deficits [16]. Nevertheless, the clinical course of MS is quite

variable and difficult to predict, and its symptoms are heterogeneous making it a polysymptomatic disease [17]. So far, there is not an isolated mechanism underlying the development of autoimmune diseases and MS seems to derive from an unfortunate combination of the breakdown of immune tolerance mechanisms and a vicious circle of inflammation and degeneration [18–20]. It comprises, for example, oxidative stress [13], ion channel dysfunction [21], NLRP3 inflammasome activity [22], cytotoxic mechanisms, cytokine-mediated immune responses, and activation of plasma cells and B cells [23]. In this context, some evidence in the literature supports the role of neurotransmitters in the pathogenesis of MS [24], among them dopamine (DA) [25]. Dopamine is a monoaminergic neurotransmitter [26] produced in the *substantia nigra*, ventral tegmental area, and hypothalamus of the brain [27] with an essential role in the modulation of sexual behavior, memory, learning, voluntary movement [28, 29], motivation, and reward [30]. Besides its role as a neurotransmitter, DA also exerts multiple functions in peripheral tissues [31]. It has the ability of mediating the crosstalk between CNS and immune system, since immune cells can synthesize and utilize dopamine as an autocrine/paracrine transmitter, which gives it an immunomodulatory character [32, 33].

Previous findings reported that preventive treatment with bromocriptine, a D2-dopamine receptor agonist, via subcutaneous pellet implantation significantly reduced the severity of experimental allergic encephalomyelitis (EAE) in rats [34]. However, an open pilot study conducted in 18 diagnosed patients with MS demonstrated that treatment with bromocriptine (2.5 mg/day for 1 year) was ineffective in inhibiting disease progression in humans [35]. However, Lieberknecht and colleagues showed that the preventive treatment of C57BL/6 mice with pramipexole (PPX) (D2-like (D2/3) receptor agonist) administered at 1 mg/kg, i.p., day 0–40 post-immunization (p.i.) abolished the development of EAE. Moreover, the effects of dopamine on T cells, for example, are pretty dynamic since they are subject to the immune activation *status* of the cell as well as the types of DA receptors expressed and the local levels of this neurotransmitter [28]. In this way, the dopaminergic signaling could vary between pro- or anti-inflammatory pathways [36, 37]. Therefore, due to disagreements in the literature about dopaminergic receptor-mediated signaling during the autoimmune response in EAE and MS, herein, we investigated the role of DA receptors, mainly D1R and D2R, during a different phase of development of EAE in C57BL/6 mice. Furthermore, we also performed additional experiments to characterize the oxidative stress levels and the axonal damage in different phases of EAE development.

2. Material And Methods

2.1. Animals

The experiments were performed in female C57BL/6 mice (20–35 g, 6–10 weeks of age) obtained from the Universidade Federal de Santa Catarina, and experiments were performed in the Laboratory of Autoimmunity and Immunopharmacology. Mice were kept in groups of four to six animals per cage, maintained under controlled temperature (22 ± 2°C) with a 12 h light/dark cycle (lights on at 07:00 a.m.), and given free access to food and water. Animals were acclimatized to laboratory settings for at least 1 h before testing and were used only once throughout the experiments. Behavioral experiments were

performed during the light cycle of the day (07:00 a.m. – 05:00 p.m.) in a soundproof room. All behavioral data were measured manually, and the observer was fully blinded to the experimental protocol for all tests. Mice were randomly assigned before treatment or behavioral evaluation. All experimental procedures in this study were strictly performed with relevant ethical regulations, including the National Institute of Health Guide for the Care and Use of Laboratory Animals [38] and were approved by the Animal Ethics Committee of the Universidade Federal de Santa Catarina (CEUA-UFSC, protocol number: 3914220319).

2.2. EAE induction

Female C57BL/6J mice were immunized subcutaneously in both flanks with 200 µg of myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) (EZBiolab, New Jersey, USA) emulsified in Incomplete Freud's Adjuvant (Sigma Chemicals, St. Louis, MO, USA), which was supplemented with 500 µg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). On days 0 and 2, 300 ng of *Pertussis* toxin was administered intraperitoneally (i.p.). At the end of the experiment, animals were euthanized by decapitation followed by spinal cord and striatum removal. The tissues were snap frozen or fixated in 4% formalin solution for posterior analysis.

2.3. Treatment protocol

The animals were given PPX (Aché Laboratórios Farmacêuticos, Guarulhos, SP, Brazil) – 1 mg/kg, once a day (i.p.), dissolved in saline – according to Lieberknecht and colleagues [39], between 0- and 40-days post-induction of EAE for the preventive treatment and 15- and 40- for therapeutic treatment.

2.4. Forced swimming test (FST) and sucrose preference test (SPT)

The forced swimming test (FST) is used to evaluate depressive-like behavior in rodents. The animals were placed into an inescapable transparent water tank while the time spent in an escape-related mobility behavior was registered [40]. Another way to assess the depressive-like behavior is the sucrose preference test (SPT), a reward-based test used as an indicator of anhedonia that consisted of giving two bottles of choice, one containing a sucrose solution (200 mL, 1%) and another one containing filtered tap water. First, each animal habituated to be housed individually and had access *ad libitum* to two bottles containing only tap water for 48 h. Then, one of the bottles was changed for a 1% sucrose solution for 24 h [41] and to avoid a place preference, the bottles were placed swapped after the first 12 h. The bottles were weighed before and after the test to determine the amount of solution consumed, and the consumption was calculated as the percentage of sucrose solution consumed relative to the total amount of liquid drunk [42]. This behavioral method was adapted from Pazini et al. (2017) [42].

2.5. Bielschowsky's silver staining

The spinal cord samples were extracted, weighed, and stored in a buffered fixing solution (PBS/Formalin 4%) at 4ºC to perform morphometric analyses. After this process, they were removed from the fixative solution, washed in deionized water for 30 min, progressively dehydrated in an increasing series of

alcohols; diaphanized with xylol for 30 min (3x); bathed in paraffin at 56°C for 30 min (3x) and included in paraffin blocks. Subsequently, the samples were cut into 10 µm thick sections in a microtome. The sections obtained were mounted on slides previously treated with 1% gelatin solution and remained for 24 h in an oven at 56°C and, posteriorly dewaxed and hydrated in deionized water, 3 times, for 3 min. The sections were placed in ammoniacal silver in the dark at 37ºC for 20 min, keeping the solution for later use, and washed again in deionized water, 3 times, for 2 min. Ammonium hydroxide (28–30%) was added dropwise to the silver nitrate solution (20%) while stirring sufficiently to dissolve the precipitated content. Then, another 2 drops of ammonium hydroxide solution (28–30%) were added for effective silver solubilization. Working solution (0.2 mL of 37% formaldehyde, 12 mL of distilled water, 12.5 µL of 20% nitric acid, and 0.05 g of citric acid) was added to the silver hydroxide solution and the samples remained in this solution for 10 min until they became black. After this, the samples were washed with 0.1% ammonium hydroxide solution, 3 times, for 2 min, and then with distilled water 3 times for 2 min. In the next step, the samples were conditioned in auric chloride solution for 15 min, fixed in sodium thiosulfate, washed in distilled water for later dehydration in alcohol and diaphanization with xylene, cleaned, and mounted in assembly medium. The slides were analyzed using Olympus® AX 70 light microscope using 40x, 100x, and 200x magnifications with a video camera attached to the microscope to capture Hitachi® VK-C150 images [43].

2.6. Western blotting

Western blotting analysis was performed according to described by Lieberknecht et al. [39, 41]. The spinal cord, striatum, and inguinal lymph nodes samples were homogenized in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM sodium fluoride, 1 % protease inhibitor cocktail, 100 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 1 % Triton X-100, and 10 % glycerol). Samples (60 µg protein/track) were subjected to SDS-PAGE, electrotransferred to a nitrocellulose membrane using a semi-dry blotting device (1.2 mA/cm2; 1.5 h), and probed overnight with primary antibodies anti-GPx (Abcam, Cambridge, MA, USA; dilution 1:1000), anti-D1R, anti-D2R (sc-31479 and sc-5303, respectively. Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-BDNF/Pro-BDNF (Abcam, Cambridge, MA, USA; dilution 1:1000), and β-actin as a loading control (Cell Signaling, Danvers, MA, USA; dilution 1:1000). Membranes were blocked by incubation with 5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). Membranes were incubated with appropriate peroxidase-conjugated secondary antibodies for 1 h and visualized using the enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T (10 mM Tris, 150 mM NaCl, 0.1 % Tween-10, pH 7.5). Densitometric analysis was performed using the Image Lab software v.4.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA) [39, 44].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Spinal cord was collected at the end of experiment and homogenized in lysis buffer (0.05% Tween 20, 0.1 mM benzethonium chloride, 10 mM EDTA, 0.5% bovine serum albumin, 0.4 M NaCl, 0.1 mM PMSF, and 2.0 µg/ml aprotinin). Homogenates were centrifuged at 3000 g, at 4°C, for 10 min, and the supernatant

was collected and stored at − 80°C until use. The levels of interleukin-1β were evaluated using an ELISA kit from R&D Systems (Minneapolis, MN, USA) and performed according to the manufacturer's protocol.

2.8. Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were evaluated using one-way ANOVA, followed by Neuman-Keuls or Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant. For data analysis, the GraphPad Prism 8.2.1 software (GraphPad Software Inc., USA) was used. The animals were randomized, and the analysis was blinded.

3. Results

3.1. Protein levels of dopaminergic receptors – D1R and D2R – in the spinal cord and peripheral lymphoid organs of EAE mice

The immune and nervous systems are tightly integrated through a bidirectional pathway, which allows neural pathways to regulate peripheral immunity, and conversely, immune mediators to affect neuronal activity [45–48]. Different immune cells express receptors for neurotransmitters released by neurons such as DA [49], with a pivotal role for neuroimmune communication [50]. First, we investigated the immunocontent of dopaminergic receptors in the peripheral lymphoid organs of EAE mice – where T and B cells are initially activated upon induction – during the chronic phase of EAE development [51]. Herein, we observed a significant increase in the protein levels of both receptors – D1R and D2R – in the lymph nodes of EAE mice when compared to the naïve group ($p < 0.05$; Fig. 1A, B). The immunocontent of dopaminergic receptors in the spinal cord of mice exposed to EAE was also evaluated – since the autoreactive T cells begin infiltrating the CNS in the lumbar region – in different phases of the disease development, such as *i)* induction (up to day 7 post-induction); *ii)* acute (between days 7 and 14 post-induction), and *iii)* chronic (from day 30 post-induction upwards) [51, 52]. Our results showed that there was no statistically significant difference related to the protein levels of D1R in the spinal cord of EAE mice *versus* naïve animals in the different phases of the experimental model (Fig. 2A). However, during the acute phase, the spinal cord of immunized mice showed a significant increase in the immunocontent of D2R when compared to the control group ($p < 0.05$; Fig. 2B).

3.2. Oxidative parameters and axonal degeneration in central tissues during induction, acute and chronic phase of EAE

The spinal cord has extensive projections of dopaminergic neurons involved in the modulation of sensory, motor, and autonomic functions [53]. Previous reports demonstrated that excess DA levels – not degraded through catabolic enzymatic pathways – can induce neuronal death mainly due to ROS generation in the auto-oxidation of L-DOPA and DA [54, 55]. In this set of experiments, we characterized the levels of oxidative stress and the activity of the antioxidant system 7, 15, and 40 days after EAE induction. Our data showed that GPx levels were significantly increased in the spinal cord of EAE mice 7 days post-induction compared to naïve animals ($p < 0.01$; Fig. 3A). In contrast, GPx was significantly

downregulated 40 days after EAE induction (chronic phase) ($p < 0.01$; Fig. 3A). Additionally, the enzymatic activity of GPx was significantly decreased in the spinal cord of EAE mice compared to the naïve group during the acute and chronic phase of the disease ($p < 0.01$; Fig. 3B).

The striatum integrates motor behavior, cognition, emotion, and limbic information processing [56, 57]. According to Gentile and colleagues, the striatum of EAE mice showed impairment of DA neurotransmission characterized by an imbalance in dopaminergic receptors signaling [58]. Afterward, we also evaluated the levels of oxidative stress and the activity of the antioxidant system in the striatum. Our data demonstrated that the levels of GPx in the striatum of EAE mice were significantly increased 7- and 14-days post-induction when compared to the control group (naïve) ($p < 0.01$; Fig. 3D). Surprisingly, during the chronic phase of the disease (40 days p.i.), the striatum of the EAE group showed a significant decrease in the levels of GPx compared to naïve animals ($p < 0.01$; Fig. 3D), as well as the enzymatic activity ($p < 0.01$; Fig. 3E). Next, we performed DCF fluorescence to measure ROS in both tissues. Our data demonstrated a significant reduction in ROS levels in the spinal cord of EAE animals 7 days post-immunization ($p < 0.01$; Fig. 3C). However, 15- and 40-days post-immunization, EAE animals had an increase in the production of ROS in the spinal cord when compared to the naïve group ($p < 0.05$) (Fig. 3C). Meanwhile, in the striatum of EAE mice, we identified a significant increase in the levels of ROS compared to the control group (naïve) 40 days post-induction (chronic phase of EAE) ($p < 0.01$; Fig. 3F).

In order to characterize the axonal damage during the development of EAE, the results of Fig. 3 illustrate that EAE induced significant axonal damage from 7 days p.i. ($p < 0.05$), with greater damage after 30 days p.i. ($p < 0.01$) when compared to the naïve control group (Fig. 3G, H).

3.3. Effect of therapeutic treatment with PPX, dopaminergic D3/D2R agonist, on the progression of EAE

As aforementioned, there is still no consensus in the literature about the effects of dopaminergic agonist drugs in treating MS and EAE [34, 35, 39]. Lieberknecht and colleagues provided evidence about the beneficial effects of PPX (administered from day 0 to 40 p.i.) in preventing EAE-induced motor symptoms [39]. In another set of experiments, we assessed the effect of therapeutic treatment with PPX in EAE-induced mice from day 15 to 40 post-induction. Figure 4 illustrates that the therapeutic treatment with PPX failed to inhibit the progression of EAE (Fig. 4A), confirmed by analyzing the area under the curve (AUC) (Fig. 4B). Furthermore, the animals treated with PPX had significantly higher scores when compared to the EAE group, which received vehicle (Fig. 4C). Therefore, it is possible to conclude that the therapeutic treatment with PPX could not ameliorate the progression of EAE.

3.4. The effect of PPX on depressive-like behavior in mice with EAE

Studies had provided direct evidence about dopaminergic dysfunction in clinical manifestations of MS and EAE, including the occurrence of depressive symptoms [58–60]. Considering these findings, we

investigated if PPX would modulate the depressive-like and anhedonic-like behavior in EAE mice. Mice were given PPX (1 mg/kg, i.p.) from days 0 to 11 and 12 when they were subjected to the SPT and FST, respectively. Our results showed that PPX treatment significantly reduced the immobility time compared with the untreated EAE group ($p < 0.05$; Fig. 5A). Conversely, no differences between the groups were observed when the mice were submitted to SPT (Fig. 5B).

3.5. PPX modulates the protein levels of BDNF and pro-inflammatory cytokine IL-1\varvecβ in EAE mice

Gentile and colleagues reported that IL-1β impairs dopamine neurotransmission in the striatum of EAE mice triggering behavioral changes [58]. Considering that exogenous administration of IL-1β peripherally produces depressive-like symptoms [61, 62], we evaluated if PPX could modulate the IL-1β levels in the spinal cord of EAE mice, justifying its antidepressant-like effect observed in the FST (Fig. 5A). Herein, EAE mice showed a significant increase of IL-1β immunocontent when compared to the naïve group ($p < 0.001$) (Fig. 6A). Meantime, PPX treatment (1 mg/kg, once a day, i.p., during 40 days after EAE induction) significantly reduced the amount of IL-1β in the spinal cord ($p < 0.001$). According to Qu and co-authors, preventing the loss of the brain-derived neurotrophic factor (BDNF) – the most prevalent growth factor in the CNS – in the spinal cord of EAE mice has an essential role in mitigating the severity of the disease [63]. Considering that preventive PPX administration prevented EAE development, we further assessed whether PPX effectively modulates the levels of BDNF in the spinal cord of EAE mice. Interestingly, 40 days post-induction EAE mice showed downregulation of BDNF protein levels in the spinal cord when compared to the control group (naïve) ($p < 0.01$), an effect abolished by PPX treatment ($p < 0.01$; Fig. 6).

4. Discussion

MS is the most prevalent chronic neuroinflammatory disease of the brain and spinal cord [64]. This study aimed to investigate mechanisms by which peripheral and central inflammatory signals are integrated, mainly dopaminergic receptors' role in different phases of the EAE model. Firstly, our data demonstrated enhanced levels of D2R immunocontent in the spinal cord of EAE mice during the acute phase of disease development. In accordance with our finding, the expression of D2R on brain-resident or infiltrating immune cells in EAE was already showed to be significantly increasing [65]. Moreover, in the work by Robichon et al. (2021) no significant reduction in the immunocontent of D1R on macrophages and monocytes was reported [65], which differs from our findings since we did not identify differences regarding the immunocontent of D1R in the spinal cord of EAE mice. Therefore, it is possible to hypothesize that the activation of D2R, mainly during the acute phase of EAE, could increase the severity of the disease, conferring to this receptor a pro-inflammatory characteristic. Additionally, 30 days post-induction, the immunocontent of both dopaminergic receptors in the lymph nodes of EAE mice was significantly increased. In accordance, peripheral blood pro-inflammatory monocytes obtained from MS patients showed an exacerbated level of all dopamine receptors [66]. Considering this, we can assume that D1R also plays a pro-inflammatory role in maintaining this autoimmune disorder. Consistent with

these findings, D5R-deficient mice (belonging to the dopamine D1-like receptor family) exhibited a delayed onset of the disease compared with WT mice [67]. According to the same authors, the depletion of D5R in dendritic cells decreased Th17 cells infiltrating into CNS [67]. It demonstrates that activating the D1-like receptor family triggers the activation of pro-inflammatory signaling pathways and modulates the autoimmune response. In addition, dendritic cells-derived dopamine stimulates D5R-signalling and inhibits STAT3 phosphorylation promoting an inflammatory response during EAE development [66], as well as favors selectively the production of both IL-23 and IL-12 [67], which influence the development of Th1 and IL-17–producing Th17 cells [68]. However, it is essential to highlight that the D1-like receptor family can play a dual role in EAE. According to Osorio-Barrios and colleagues, D5R-mediated signaling may vary depending on the cell type involved and the stage of the disease [36]. The authors suggested that D5R-signaling in CD4 + T-cells potentiates T-cell activation and exerts a pro-inflammatory role favoring EAE development [36]. While in the opposite way, D5R-signaling in Treg cells favoring Treg-mediated suppressive response in late stages of the disease [36]. Consequently, the increased protein levels of D1R in the lymph nodes of the EAE group found in the present study – particularly in the chronic phase – could be related to maintenance of autoimmune response or an attempt to suppress the immune response through Treg cells activation. Therefore, further studies are needed to identify the cause of upregulation of dopaminergic receptors in the CNS after EAE-induction, especially D1R.

In order to characterize the oxidative stress during different phases of EAE, we evaluated the levels of GPx – a crucial antioxidant enzyme – and DCF in the spinal cord and striatum of EAE mice. The imbalance in the antioxidant defense system has been implicated in the pathogenesis of MS. Herein, our findings demonstrated that the levels of GPx are downregulated in the spinal cord and striatum of EAE mice in the chronic phase of the experimental model. In agreement with our results, Conde and colleagues also reported a significant reduction in the protein levels of GPx in the brain and spinal cord of rats with EAE, 65 days post-immunization [69]. Additionally, the levels of GPx were reduced in the blood of patients with MS [70]. Nevertheless, we also identified increased GPx levels during the acute phase of EAE (7 days post-induction). In accordance with these results, a previous study reported that thirteen genes were upregulated in acute and chronic MS lesions, among them GPX1 (GPx coding gene) [71]. The demyelinated acute lesions obtained from patients with MS also showed upregulation of superoxide dismutase 1 and 2 (SOD1 and SOD2) and heme oxygenase (HO), which play an essential role in the antioxidant system, suggesting an adaptive defense mechanism that aims to restore the levels of ROS, reducing cell damage related to its accumulation [72]. We also demonstrated a significant increase in the oxidative damage in the spinal cord and striatum of EAE mice during the chronic phase of the EAE model. ROS are naturally produced within biological systems and play an essential role as signaling molecules in a wide variety of physiological processes [73]. However, the disruption of redox homeostasis promotes a vicious circle of inflammation and degeneration [74]. The intensity of DCF fluorescence corresponds to the detection of reactive oxygen and nitrogen species. In this way, our data agree with a previous work, which reported increased levels of inducible nitric oxide synthase (iNOS) in the spinal cord of mice submitted to EAE between 32 and 67 days post-immunization [75]. According to Espejo and colleagues, during the chronic phase of EAE, the brain, cerebellum, brain stem, and spinal cord of immunized mice

showed intense oxidative damage characterized by immunoreactivity of iNOS, nitrotyrosine, and malondialdehyde (MDA) [76]. In contrast, we also identified a significant reduction in the fluorescence intensity of DCF, only in the spinal cord of mice, in the initial phase of EAE development (7 days post-immunization). Different from our findings, Hasseldam and colleagues showed an increased reactive oxygen species burden in the brain tissue 5 and 7 days after EAE induction in rats, without significant differences in the spinal cord tissue [77]. However, the increased immunocontent of GPx during the induction phase of the disease could justify the ROS reduction. Indeed, further studies are needed to give us a deeper understanding on these findings. Our next step was to characterize the axonal damage in the spinal cord of EAE mice during the disease development. The data presented here identified a significant reduction in axonal density in the spinal cord of animals submitted to EAE 7 days post-induction. It suggests axonal damage in the pre-symptomatic phase of the disease when no macroscopic motor disturbance is evident. In consonance, the axonal damage not only occurs in lesioned areas but also normal-appearing white matter. According to DeVos and colleagues, the axonal damage characteristic of MS-related lesions is similar to those detected in other neurological conditions unrelated to demyelination, such as amyotrophic lateral sclerosis (ALS) [78]. From this, it is possible to hypothesize that the axonal damage occurs independently of the demyelination process. Additionally, although axonal damage can be observed already in the acute phase of EAE, there is no correlation between axonal damage and the degree of neurological disability [79]. However, according to the authors, the number of symptomatic attacks in EAE mice was correlated with axon loss and neurological impairment in the chronic phase of the disease development [79]. In this way, our work offers additional evidence on damage to neurons in the dorsal horn of the spinal cord and subsequent appearance of sensory disturbances preceding the emergence of EAE-induced motor deficits.

Posteriorly, we showed that therapeutic treatment with PPX, a dopamine D2/D3 receptor-preferring agonist, failed to prevent EAE progression. In turn, our findings support previous evidence, which reported the ineffectiveness of bromocriptine (2.5 mg/day) in inhibiting MS progression in humans [35]. Even though this evidence supports a negative relation between dopamine agonists and MS, some studies have described benefits related to bromocriptine administration in Lewis rats submitted to EAE [34, 80]. Similarly, Lieberknecht and colleagues also demonstrated that the preventive administration of PPX modulated the inflammatory response induced by EAE induction [39]. Additionally, it is important to highlight it has already been shown that the antagonism of D2-like receptors also induced a worsening of EAE symptoms in mice [81]. In another way, the use of atypical antipsychotic agents, including risperidone and clozapine (potent antagonists of a wide range of neuroreceptors, including dopamine and serotonin receptors), have been investigated in mouse models of MS [65, 81, 82]. The preventive treatment of EAE mice with risperidone (serotonin 2A and C (5-HT2A and 5-HT2C) and D2R antagonist) (3 mg/kg/day) decreased disease severity [81]. However, the authors highlighted that the immunomodulatory effect mediated by risperidone is not due to the antagonism of the dopaminergic receptors. In this case, risperidone appears to have an effect similar to that induced by dopamine [81]. Another recent work provided new evidence about the mechanisms underlying the immunomodulatory properties of antipsychotic agents [65]. According to Robichon and colleagues, the authors observed a

significant downregulation of D1R and D5R and up-regulation of D2R on microglia and CD4+-infiltrating T cells [65]. Clozapine treatment upregulated the protein levels of D1R and D2R, suggesting that this drug could modify dopaminergic pathways in MS [65]. A recent clinical trial suggested that risperidone and clozapine were beneficial for reducing neuroinflammation induced by MS [83]. Thus, it needs further investigation since the patients with progressive MS (PMS) may experience increased sensitivity to these drugs, which means that the dosing schedule used to treat schizophrenia may not be suitable for them [83]. Altogether, our data suggest that D2R plays a dynamic and dual role in the pathogenesis of MS since the preventive treatment with PPX inhibited EAE progression. At the same time, the therapeutic administration did not show the same effect confirming that dopaminergic receptors play an essential role in the development/progression of EAE and MS.

Depressive disorders have a high incidence among patients with MS, negatively influencing their quality of life [84]. The present study also showed that PPX significantly decreased the EAE-induced depressive-like behavior but did not affect the anhedonic-like behavior. Previous works have demonstrated that PPX exhibits promising responses for treatment-resistant depressed patients [85] and LPS-induced depressive-like behavior [41]. In the same way, a randomized trial compared PPX treatment with an established antidepressant (sertraline) in patients with Parkinson's disease (PD) without motor complications and identified that PPX ameliorated depression in PD [86]. As aforementioned, inflammatory cytokines contribute to depression’s development [87]. In this context, some evidence demonstrated a relationship between the activity of the immune system and persistent alterations in the neurotransmitter system leading to psychological symptoms [88, 89]. The repeated administration of PPX for 7 days completely prevented LPS-induced depression-like and anhedonic-like behavior in mice [41]. In this work, the authors showed that PPX modulated the levels of pro-inflammatory cytokine IL-1β and 3-nitrotyrosine [41, 90, 91]. The same author suggested that the antidepressant-like effect of PPX was due to immunomodulatory properties since the administration of the dopaminergic antagonists haloperidol and sulpiride was unable to prevent the antidepressant-like effect of PPX [41]. Taken together, PPX seems a viable pharmacological strategy, which can become an adjuvant for the treatment of depressive disorder related to MS and other neurological diseases.

IL-1β is a pro-inflammatory cytokine expressed preferably by innate cells, including macrophage, NK cells, monocytes, and neutrophils, which plays a key role in the pathogenesis of major depressive disorder [92, 93] and mood disturbances in MS patients. According to Gentile and colleagues, IL-1β-driven dysfunction of dopaminergic signaling triggers mood alterations in EAE mice [58]. Here, our findings indicated that PPX decreased the levels of IL-1β in the spinal cord of EAE mice. Previous evidence have reported the immunomodulatory properties linked to PPX [39, 41, 94, 95]. PPX inhibited the production of inflammatory cytokines (IL-17, IL-1β, and TNF) in peripheral lymphoid tissue and re-established reactive oxygen species production in the spinal cord and striatum of mice after EAE induction [39]. Furthermore, PPX decreased the levels of IL-1β in the hippocampus of mice submitted to LPS-induced depressive-like behavior [41]. Altogether, it is possible to conclude that dopamine D2/D3 receptor-preferring agonist possibly mediates its antidepressant-like effect through downregulation of IL-1β immunocontent. BDNF, a neurotrophic factor that plays a critical role in neuronal and oligodendroglial growth and survival, has a

potential benefit in reversing neurodegenerative diseases [96, 97]. Therefore, preventing its loss in the spinal cord of EAE mice may mitigate the severity of the disease [63]. Afterward, our findings showed that PPX increased the protein levels of BDNF in the spinal cord of mice immunized with EAE, supporting the beneficial effect of PPX preventive treatment [41]. In agreement, other pieces of evidence suggest neuroprotective effects of PPX on the protein levels of BDNF in cell cultures [98, 99]. Surprisingly, PPX did not affect the BDNF levels in the hippocampus, frontal cortex, and striatum of C57BL/6 mice [100]. A subsequent study reported that PPX influences BDNF signaling in limbic structures in the preclinical model of PD [101]. Herein, we suggest that PPX, when administered preventively, possibly interacts with BNDF signaling to induce its neuroprotective effect in EAE, although additional protocols are necessary to confirm these hypotheses.

5. Conclusion

Dopaminergic signaling has emerged as an essential neuroimmune-mediator in the communication between immune and CNS, promoting homeostasis [102]. Consequently, imbalances in the dopaminergic system affect both innate and adaptive immunity contributing to the development of neurodegenerative diseases, including MS. Herein, we suggested the existence of a differential immunocontent of dopaminergic receptors according to the stages of EAE development (induction, acute and chronic phase). It could justify the divergences in the literature related to the dual effects of dopamine agonist treatments. Our data confirmed that axonal damage precedes EAE-induced motor deficits and intense oxidative stress in the spinal cord and striatum of EAE mice, mainly during the chronic phase of the disease. In this way, we hypothesize that the administration of dopamine agonists, such as PPX, only offers advantages if performed preventively. During acute and chronic phases of EAE, dopaminergic receptors-mediated signaling seem to acquire a pro-inflammatory character, which explains, at least in part, the beneficial effects obtained with the administration of antipsychotic agents in the EAE model [65, 81]. Additionally, we also demonstrated that PPX could represent an exciting strategy and adjuvant for treating depression in MS patients.

Declarations

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Authors' Contributions

ECDG and VL performed all experiments. ECDG analyzed and interpreted the data and was a major contributor in writing the manuscript. VVH, BDR, FAF, ALSR and DFM contributed to the methodological

analysis and manuscript editing. RCD designed this study, edited and wrote the manuscript. All authors read and approved the final manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article, and are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Competing Interests

The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate

All experimental procedures in this study were strictly performed with relevant ethical regulations, including the National Institute of Health Guide for the Care and Use of Laboratory Animals [38] and were approved by the Animal Ethics Committee of the Universidade Federal de Santa Catarina (CEUA-UFSC, protocol number: 3914220319).

Moreover, the number of animals and the intensity of the noxious stimuli used were the minima necessary to demonstrate consistent effects. All of the experimental procedures were conducted according to the guidelines of CONCEA and CEUA/UFSC, based on the principles of the 3Rs (Replacement, Reduction, and Refinement).

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Code Availability

Not applicable

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Figures

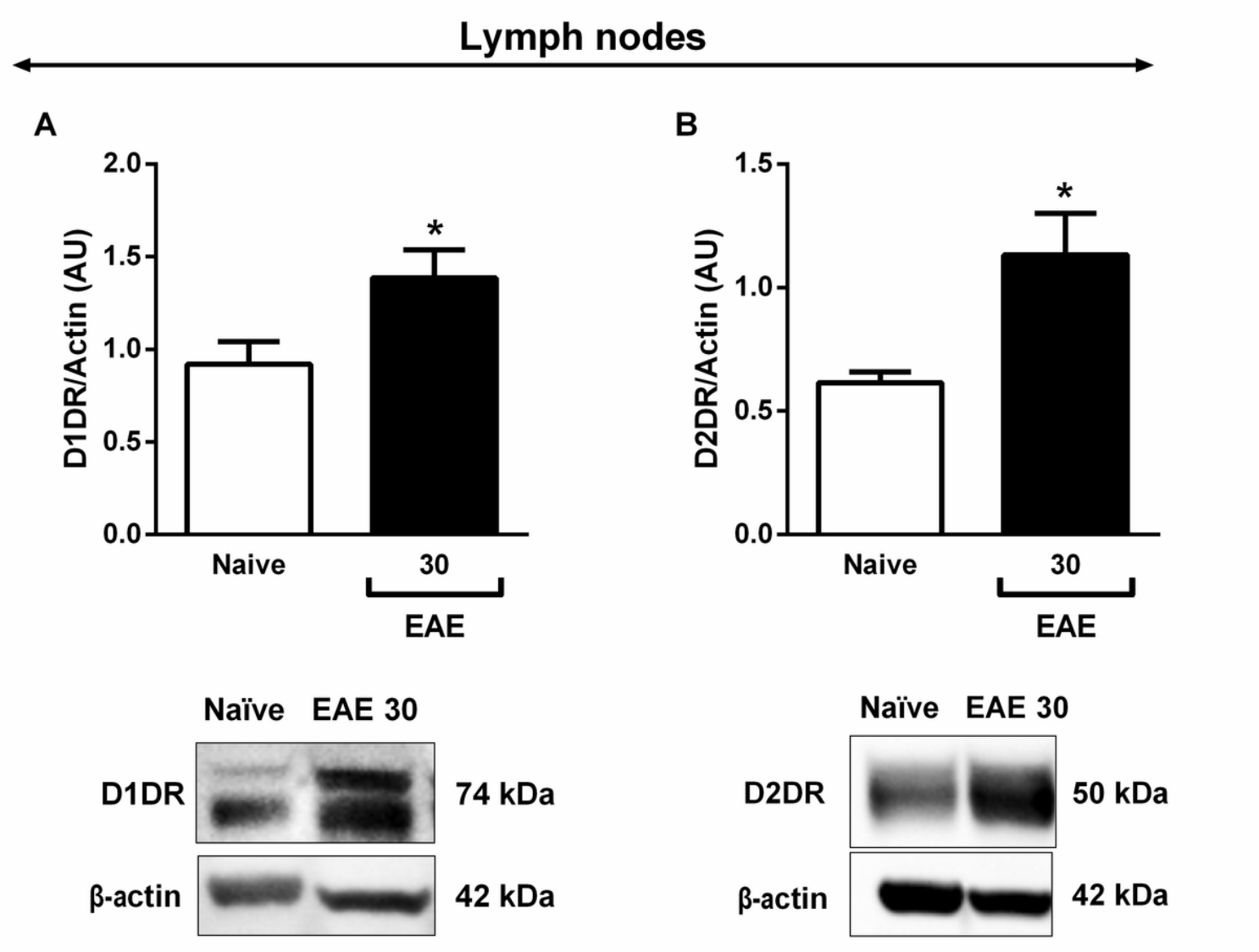

Figure 1

EAE-induced upregulation of dopaminergic receptors expression in the lymph nodes of mice during the chronic phase of the disease. Panel A: D1DR representative immunoelectrophoresis with densitometry. Panel B: D2DR representative immunoelectrophoresis with densitometry. Each column represents the mean ± SEM of 5 animals/group. Asterisks indicate the levels of significance compared to the naive group. *$p<0.05$ using one-way ANOVA followed by Bonferroni's post hoc test.

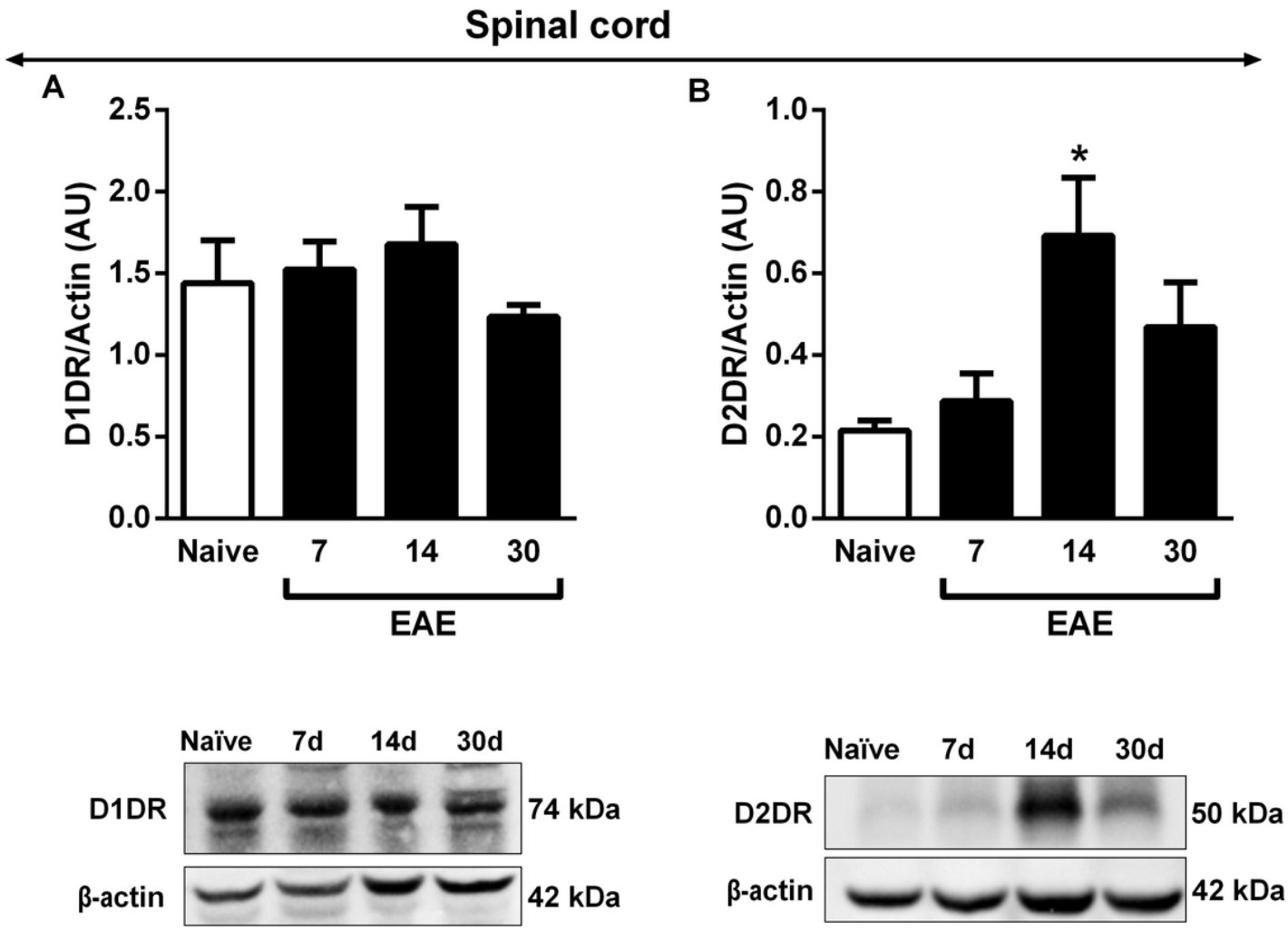

Figure 2

EAE-induced upregulation of D2R immunocontent in the spinal cord 14 days post-induction. Panel A: D1DR representative immunoelectrophoresis with densitometry. Panel B: D2DR representative immunoelectrophoresis with densitometry. Each column represents the mean ± SEM of 5 animals/group. Asterisks indicate the levels of significance compared to the naive group. *$p<0.05$ using one-way ANOVA followed by Bonferroni's post hoc test.

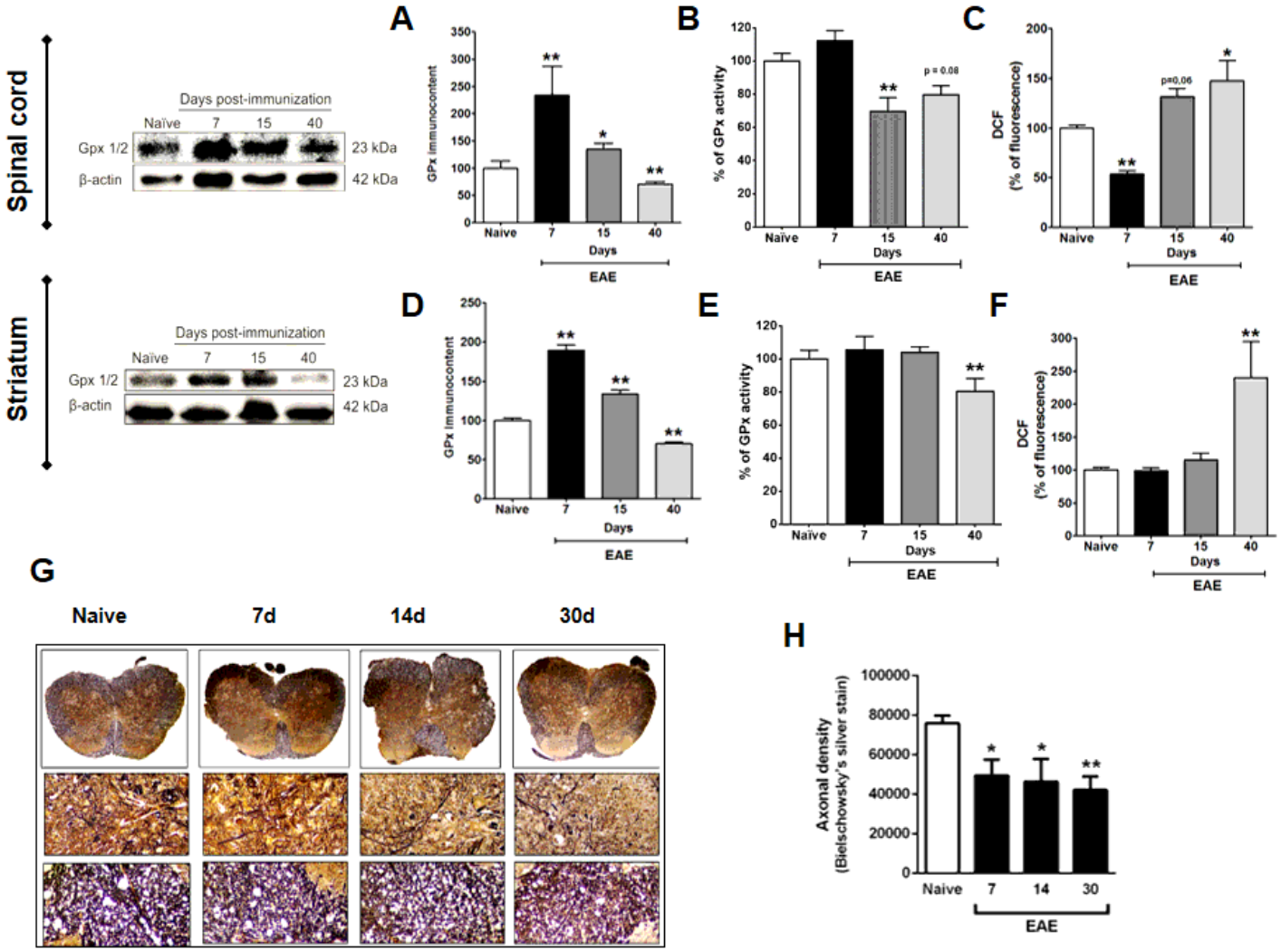

Figure 3

Oxidative parameters and axonal damage in central tissues during induction, acute and chronic phase of EAE. Each column represents the mean ± SEM of 12 animals/group. Asterisks indicate the levels of significance compared to the naive group. *p<0.05, **p<0.01 using one-way ANOVA followed by Newmann Keuls post hoc test.

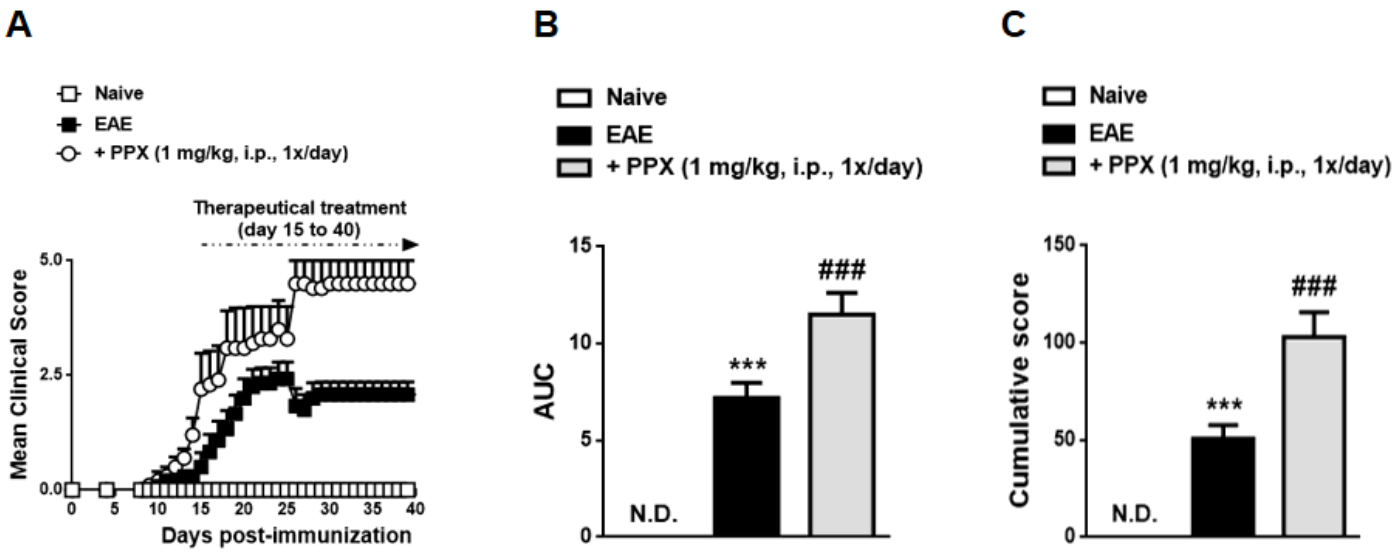

Figure 4

Therapeutic treatment with PPX (D2-like (D3/2) receptor agonist) increased the severity of EAE-induced symptoms. Each column represents the mean ± SEM of 12 animals/group. Asterisks indicate the levels of significance compared to naive group ***p<0.001; ###p<0.001 versus EAE-group using one-way ANOVA followed by Newmann Keuls post hoc test.

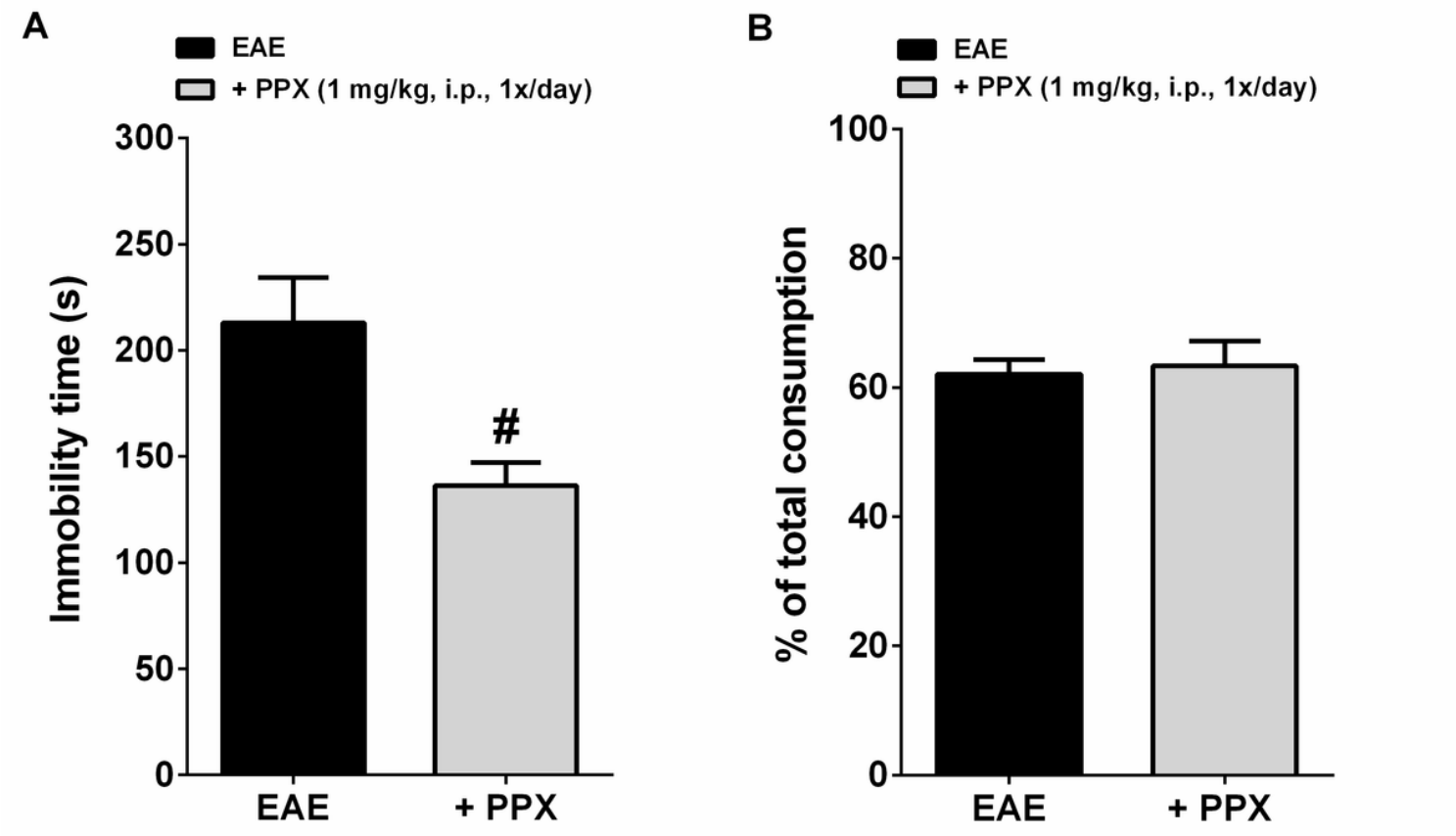

Figure 5

The effect of PPX on depressive like-behavior induced by EAE. Each column represents the mean ± SEM of 12 animals/group. #p<0.05 versus EAE-group using one-way ANOVA followed by Newmann Keuls post hoc test.

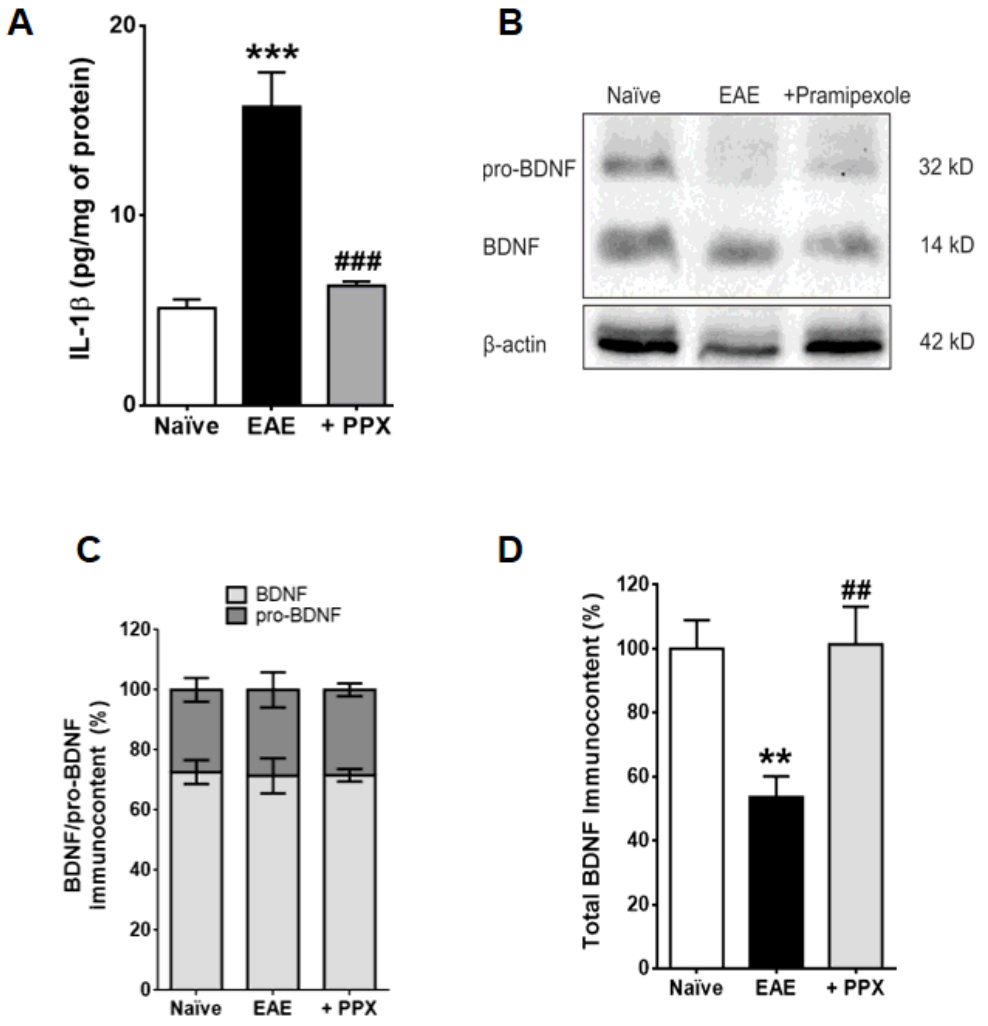

Figure 6

PPX modulated the levels of pro-inflammatory cytokine IL-1β and neurotrophic factor BNDF in the spinal cord of EAE mice during the chronic phase of disease development. Each column represents the mean ± SEM of 12 animals/group. Asterisks indicate the levels of significance compared to naive group **p<0.01, ***p<0.001; ##p<0.01 and ###p<0.001 versus EAE-group using one-way ANOVA followed by Newmann Keuls post hoc test.