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Diazepam treatment reduces inflammatory cells and mediators in the central nervous system of rats with experimental autoimmune encephalomyelitis

Nicolás Fernández Hurst[a,b], Samanta R. Zanetti[c], Natalia S. Báez[d], Mario J. Bibolini[a,b], Cecilia Bouzat[c], German A. Roth[a,b,*]

[a] Departamento de Química Biológica "Ranwel Caputto", Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina
[b] Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, CONICET-UNC), Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina
[c] Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB, CONICET-UNS), Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina
[d] Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI, UNCCONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

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ABSTRACT

Benzodiazepines are psychoactive drugs and some of them also affect immune cells. We here characterized the inflammatory and infiltrating immune cells in the central nervous system (CNS) during the acute phase of experimental autoimmune encephalomyelitis (EAE) in animals treated with Diazepam. Also, we evaluated the expression of Translocator Protein (18 kDa) (TSPO), which is a biomarker of neuroinflammatory diseases. The results indicate that Diazepam exerts protective effects on EAE development, decreasing the incidence of the disease and reducing the number of inflammatory cells in CNS, with a concomitant decrease of TSPO levels in brain tissue and CNS inflammatory CD11b$^+$ cells.

1. Introduction

Multiple sclerosis (MS) a chronic, progressive inflammatory disorder of the brain and spinal cord is the most common neurologic disease of young adults (Goodin, 2014). Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics many of the clinical, biochemical and pathologic features of MS and is widely used to study the immunoneuropathological mechanisms of MS and to develop new therapies (Constantinescu et al., 2011; Simmons et al., 2013). Both, the human disease as the experimental model are T-cell mediated pathologies. Naive CD4$^+$ T cells are activated in periphery into pathogenic T helper 1 (Th1) and T helper 17 (Th17) cells that produce the proinflammatory cytokines interferon gamma (IFN-γ) and interleukin-17 (IL-17), respectively. These Th1 and Th17 activated CD4$^+$ T cells migrate across the disrupted blood-brain barrier and undergo reactivation by local and infiltrating activated antigen-presenting cells (microglia and macrophages), thus resulting in subsequent inflammatory processes. Activated CD4$^+$ T cells, blood infiltrating macrophages and local activated microglia act as important effectors of inflammation orchestrating the release of elevated levels of proinflammatory cytokines, chemokines and other inflammatory mediators (Murphy et al., 2010), that leads to damage of myelin, oligodendrocytes and neurons (Fletcher et al., 2010).

Benzodiazepines are psychoactive drugs used as sedatives, hypnotics, anxiolytics, anticonvulsants, and central muscle relaxants. Benzodiazepines enhance the effect of the γ-aminobutyric acid (GABA) neurotransmitter acting as positive allosteric modulators of the $GABA_A$ receptor (Griffin et al., 2013). These receptors are mainly located in neurons perfectly positioned to reduce excitability and inhibit the positive-feedback loop that constitutes excitotoxicity (Zhang et al., 2007). In recent years, several works proved that some benzodiazepines, including Diazepam (Dz), have an effect on immune cells (Massoco and Palermo-Neto, 2003; Wey et al., 2008). Dz presents an inhibitory effect on peripheral T cell function *in vivo* (Bento de Lima et al., 2010; Bibolini et al., 2011) and *in vitro* (Wei et al., 2010; Fernández Hurst et al., 2015a) by reducing cell proliferation, and proinflammatory cytokine

Abbreviations: APC, allophycocyanin; CFA, complete Freund's adjuvant; CNS, central nervous system; dpi, day post-induction; DTH, delayed-type hypersensitivity; Dz, Diazepam; EAE, Experimental autoimmune encephalomyelitis; FITC, fluorescein isothiocyanate; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MHCII, major histocompatibility complex class II; MS, multiple sclerosis; PBS, phosphate-buffered saline; PE, phycoerythrin; PerCP, peridininchlorophyll proteins; PET, positron emission tomography; TSPO, translocator protein 18 kDa

* Corresponding author at: Departamento de Química Biológica-CIQUIBIC, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Córdoba X5000HUA, Argentina.
E-mail address: garoth@fcq.unc.edu.ar (G.A. Roth).

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(IFN-γ and IL-17) production. Even more, Dz also affects innate immune cells as dendritic cells, macrophages and microglia, reducing the release of proinflammatory cytokines (TNF-α, IL-6, IL-12), inducible NO synthase expression, NO production, and CD40 cell expression when the cells are activated with LPS (Wilms et al., 2003; Bento de Lima et al., 2010; Fernández Hurst et al., 2015b).

Some benzodiazepines have an alternative peripheral binding site named translocator protein 18 kDa (TSPO), which is structurally and functionally different from the $GABA_A$ receptor and may produce different pharmacological effects (Papadopoulos et al., 2006). TSPO is a five-transmembrane domain protein localized primarily in the outer mitochondrial membrane. This receptor is expressed in abundance in platelets, immune cells, endothelium, vascular smooth muscle, bone marrow, endocrine cells, and to a lesser extent in the CNS, predominantly in glial cells (Venneti et al., 2006). It is involved in the regulation of mitochondrial cholesterol transport for the conversion to pregnenolone, a determinant step in steroidogenesis and neurosteroidogenesis, apoptosis, cell proliferation, differentiation, regulation of mitochondrial functions and immunomodulation (Papadopoulos et al., 2006; Bae et al., 2014; Gut et al., 2015). In the CNS, TSPO expression is restricted to astrocytes and microglia and it is present in very low levels under normal physiological conditions. However, by employing TSPO-binding chemicals as ^{11}C-PK11195 in a positron emission tomography (PET) technique, it was shown that TSPO levels increase in activated cells in inflammatory and neurodegenerative pathologies (Liu et al., 2014). Therefore, it is used as a biomarker in human disease pathologies including MS (Papadopoulos et al., 2006; Politis et al., 2012; Bae et al., 2014). Also, considerable evidence indicates that TSPO plays regulatory roles in various human diseases (Daugherty et al., 2013).

Previous work from our group showed that the administration of Dz to rats after EAE-active induction leads to a marked decrease of the disease incidence and of histological signs associated with the disease, a reduction of cellular reactivity and antibody response against the encephalitogenic myelin basic protein, and a positive effect on neurotransmission (Bibolini et al., 2011). These data indicate a beneficial role of the Dz treatment in preventing the development of EAE. We also showed the presence of neuroinflammation, with the concomitant activation of microglia and astrocytes concentrated in the frontal region of the cortex of EAE animals (Chanaday and Roth, 2016). However, infiltrating and inflammatory cell populations have not been investigated in the target organ of Dz-treated EAE animals.

We here characterize the main inflammatory and infiltrating cellular populations isolated from CNS of EAE animals treated with Dz. Also, we evaluated the levels of TSPO in brain tissue as a biomarker of neuroinflammation and as the possible receptor that mediates the action of Dz in our experimental model. Finally, we identified the main responsible cells involved in the changes of TSPO levels in CNS.

2. Materials and methods

2.1. EAE induction and experimental groups

Forty-five-day-old albino rats from a Wistar strain inbred in our laboratory for 40 years were used. All experiments were performed in accordance with international guidelines for animal care and the studies were approved by the Institutional Review Board and Ethical Committee (protocol 832/2015). Every effort was made to minimize both the number of animals used and their suffering. Animals were anesthetized with a mixture of xylazine and ketamine (10 and 65 mg/kg respectively, i.p.). Active disease was induced by intradermal inoculation in both hind feet with 8 mg whole myelin purified from bovine spinal cords in 0.5 ml of an emulsion consisting of 0.25 ml saline solution and 0.25 ml complete Freund's adjuvant (CFA, Sigma-Aldrich Co., St. Louis, MO, USA) (Bibolini et al., 2011). Control rats received 0.5 ml of the same emulsion without any antigenic preparation (CFA group). Dz (7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3H)-one) (Valium, Roche Internacional Limited, Montevideo, Uruguay) was dissolved in phosphate-buffered saline (PBS), and administered i.p. every day from 6 to 13 day post-induction (dpi) of the disease with 0.5 mg/kg/day (1.76 μmol/kg/day). Animals were weighted, and assessed daily for clinical signs of EAE and scored as follows: 0, no evident clinical signs; 1, flaccid tail; 2, hind limb weakness; 3, definitive hindquarter paralysis and urinary incontinence; 4, tetraparalysis; 5, moribund state or dead. The mean clinical score was defined as the sum of the highest clinical score achieved by each rat during the acute phase of the disease divided by the number of rats that developed the disease signs in that group. The disease index was calculated as the sum of the daily clinical score for each animal throughout the experimental period divided by the day of onset of EAE clinical symptoms × 100 (Staykova et al., 2002). The delayed type hypersensitivity (DTH) testing was performed at 9 dpi using myelin basic protein as antigen (Bibolini et al., 2011). At 13 dpi when the rats from the EAE group showed the maximum clinical signs of the disease (acute period) the animals were deeply anesthetized with CO_2 and perfused through the left cardiac ventricle with 60 ml of cold PBS.

2.2. Isolation of CNS-infiltrating cells

The brain, cerebellum, and spinal cord were aseptically dissected, cut into small pieces and digested with 1.5 mg/ml collagenase type IV (GIBCO Life Technologies, Carlsbad, CA, USA) and 50 μg/ml deoxyribonuclease (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS at 37 °C for 45 min. After mechanical and biological disruption, the homogenates were passed through a cell strainer (70 μm, BD Biosciences, San Jose, CA, USA), adjusted to 30% (v/v) Percoll (Sigma-Aldrich Co., St. Louis, MO, USA), overlaid onto 70% Percoll and centrifugated at 400 × *g* for 30 min at 4 °C. Cells were recovered from the 30%/70% Percoll interface and washed twice with RPMI 1640 (GIBCO Life Sciences, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (NATOCOR, Córdoba, Argentina). Viable cell number was determined by Trypan Blue exclusion.

2.3. Characterization of isolated cells from CNS tissues

CNS inflammatory cells (7.5×10^5) of each animal were incubated with FACS buffer (5 mM EDTA, 0.1% sodium azide, and 0.01% bovine serum albumin in PBS) for 10 min in cold. Subsequently the cells were cultured for 30 min with different mixtures of antibodies, anti-CD11b conjugated to phycoerythrin (PE) (AB_395562), anti-CD45 conjugated to biotin (AB_395569), anti-CD3 conjugated to biotin (AB_395542), anti-CD4 conjugated to FITC (AB_395547), anti-CD8a conjugated to peridinin-chlorophyll proteins (PerCP) (AB_397133) from BD Biosciences (San Diego, CA, USA), anti-CD45RA conjugated to biotin (AB_321426) and anti-major histocompatibility complex class II (MHCII) conjugated to fluorescein isothiocyanate (FITC) from ABDserotec, according to the manufacturer's instructions. The cells were washed with PBS and when appropriate incubated with streptavidin conjugated to allophycocyanin (APC) (AB_11140970) from eBioscience (San Diego, CA, USA). They were finally washed and resuspended in 100 μl of PBS. At least 50,000 events for each sample were acquired using a FACS Canto II cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo software version 5.7.2 and FlowJo version 10. The results were plotted using GraphPad program Prism, version 5.0. On one hand, the relative values correspond to the number of events obtained from a cell population in relation to the number of total events acquired by the flow cytometer. Absolute values correspond to the product between the relative value for the cell population and the number of total cells isolated from each organ from the same animal.

2.4. Intracellular cytokine determination by flow cytometry

CNS inflammatory cells (7.5×10^5) of each animal were cultured

for 5 h in 1 ml of complete RPMI medium with 50 ng/ml phorbol esters and 400 ng/ml ionomycin. After 2 h, monensin was added to a final concentration of 2 μM (Momcilovic et al., 2008). Cells were washed and proceed to the staining of surface antigens by incubation for 10 min in cold FACS buffer, washed and further incubated for 30 min with FITC-conjugated anti-CD4 antibody. Washed again and incubated for 20 min in fixation buffer, followed with permeabilization buffer with anti-IFN-γ conjugated to PE (AB_11097336) and anti-IL-17 conjugated to APC antibodies (AB_10830873) from ABDserotec, for 30 min. Finally, the cells were washed and resuspended in PBS. At least 50,000 events for each sample were acquired using a FACS Canto II cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with software FlowJo version 5.7.2.

2.5. Relative quantitation of TSPO RNA transcripts in frontal cerebral cortex

Brains were removed and frontal cortex defined as the frontal region of the isocortex from the Bregma 5.5 to 1.0 mm (Paxinos and Watson, 2007) was dissected and snap frozen in liquid nitrogen. The samples were stored at − 80 °C until processing. The tissues (50–100 mg) were placed in small bags free of DNase and RNase, frozen on dry ice and mechanically pulverized on an iron block using a porcelain pestle. The tissue samples were processed for total RNA extraction (Schmittgen and Livak, 2008). Briefly, total RNA was recovered by resuspension in 1 ml TRIzol reagent, and extracted with 0.2 ml of chloroform. The upper aqueous phase was incubated with equal volume of 2-propanol to induce RNA precipitation. After centrifuging at 12,000 × *g* for 15 min, the supernatant was discarded and the RNA was washed with 70% ethanol to finally be diluted in diethylpyrocarbamate treated water. The RNA was quantified by measuring the absorbance at 260 nm. Following DNAse treatment, 2 μg of total RNA was used for the synthesis of cDNA using Moloney murine leukemia virus reverse transcriptase and random primers (Promega, Wisconsin, USA) according to the manufacturer's specifications. Reverse transcription was performed in one cycle as follows: 6 min at 25 °C, 60 min at 37 °C, 18 min at 70 °C and 10 min at 4 °C. From the obtained cDNA, it was proceeded to the amplification of the fragments of interest in real-time PCR. The PCR primers fir analyzing TSPO mRNA expression were 5′ GGGAGGTTTCACAGAGGAGGCT 3′ (F), 5′·TCCACCAAAGCCCAGCCCAT 3′ (R); the primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level were 5′ TCACCACCATGGAGAAGGC 3′ (F), 5′ AGTGATGGCATGGACTGTGGTC 3′ (R); designed using the Primer Express software. For the real-time PCR reaction, 2.5 μl of the appropriate dilution of the obtained cDNA, 17.5 μl of the PCR master mix (Biodynamics, Argentina), and 30 pmoles of specific primers were used. Relative quantification by real-time PCR was performed using a Rotor-Gene 6000 (Corbett Research, Australia) with a program of 40 cycles of amplification. The final product of each reaction was analyzed by 2.5% agarose gel electrophoresis. Real-time PCR of the housekeeping gene allowed normalization of the expression of the TSPO gen and the relative quantification was obtained using the ΔΔCT method (Livak and Schmittgen, 2001).

2.6. Characterization of TSPO in isolated CNS inflammatory cells

Isolated CNS inflammatory cells obtained as indicated before were used for detection of TSPO by flow cytometry. The cells were labeled for surface molecules with anti-CD4 conjugated to APC-Cy7, anti-CD11b conjugated to PE, then incubated for 20 min in fixation buffer and subsequently for 1 h with a rabbit anti-TSPO polyclonal antibody (AB_2294015) from BioVision (Milpitas, CA, USA) prepared in permeabilization buffer. The cells were washed and incubate with a donkey anti-rabbit Ig secondary antibody coupled to Alexa 488 (AB_141708) from Invitrogen (dilution 1/1000) also prepared in permeabilization buffer. Finally, the cells were washed and resuspended in 100 μl of PBS. At least 50,000 events for each sample were acquired using a FACS Canto II cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using software version FlowJo 5.7.2.

2.7. Statistical analysis

Statistical analyses were performed using the computer based GraphPad Prism Program V5.0 (GraphPad Software Inc., San Diego, CA, USA). Data were plotted as mean ± SEM. A one-way ANOVA test with Newman-Keus multiple comparison test was used when three or more experimental groups were compared; and a paired *t*-test when two experimental groups were compared. P values < 0.05 were considered significant.

3. Results

3.1. Suppression of EAE by intraperitoneal administration of Dz

To study the ability of Dz as modulator of EAE development, a protocol of i.p. administration of 8 doses of 0.5 mg/kg body weight every day from 6 dpi was carried out. The effect of this benzodiazepine treatment was analyzed on the disease incidence and display of clinical signs (Table 1). The results showed a significant decrease in the incidence of the disease of Dz-treated group (approximately 50%), while in animals challenged for EAE treated with vehicle alone the incidence was 92%. As well as the disease severity of the Dz-treated EAE animals was significantly lower than the non-treated EAE ones (Fig. 1). To assess the state of the peripheral immune response *in vivo*, at 9 dpi the DTH reaction was performed with myelin basic protein. The results showed that the EAE animals treated with Dz had a significant lower inflammatory edema than the animals that received only the vehicle, regardless if they showed or not the characteristic clinical signs later. This result indicates that treatment with Dz decreases Th1 cell type response *in vivo*.

Table 1
Effect of diazepam treatment on EAE clinical signs.

Group	Disease incidence	Day of onset (dpi)	M.M.C.S.[a]	Length of disease (days)	Disease index[b]	DTH[c]
CFA	0/20	–	–	–	0	0.05 ± 0.01
Dz-CFA	0/10	–	–	–	0	0.03 ± 0.01
EAE	34/36	12.23 ± 0.23	2.50 ± 0.27	3.70 ± 0.29	47.3 ± 9.8	0.41 ± 0.01
Dz-EAE	13/26	12.14 ± 0.35	2.10 ± 0.26	3.40 ± 0.29	38.0 + 5.2	0.25 ± 0.02***

Treated animals were daily i.p. injected with 0.5 mg/kg/day (1.76 μmol/kg/day) of Diazepan (Dz) or vehicle alone from 6 to 13 days post-induction of the active EAE. The results are expressed as mean ± S.E.M. Significant differences of treated group *vs.* EAE group are indicated by ***$p < 0.001$.

[a] M.M.C.S., mean maximum clinical score is the sum of the highest clinical score (0–5) achieved by each rat during the acute phase of the disease divided by the number of rats that got sick in that group.

[b] The disease index was the sum of the daily clinical score for each sick animal throughout the experimental period divided by the day of onset of EAE clinical symptoms × 100.

[c] Delayed-type hypersensitivity reaction (DTH) was tested at 9 dpi. Animals were injected subcutaneously with myelin basic protein in the left ear and the vehicle in the right ear. Swelling was determined 24 h post-immunization and the results expressed as the difference of thickness (mm) between the left and right ear.

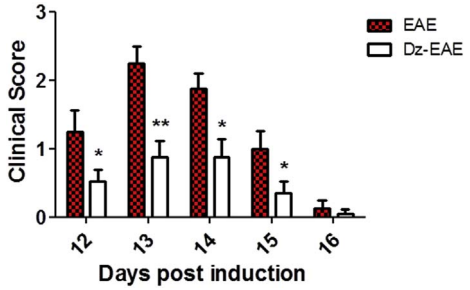

Fig. 1. Clinical scoring of rats with EAE. Data are shown as mean and standard error of the mean from EAE (red, EAE) and diazepam-treated EAE (white, Dz-EAE) (n = 8 and 17, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Effect of Dz on inflammatory CNS cells

Inflammation and tissue damage in the CNS of animals with EAE generally temporally correlates with clinical signs and histological alterations. Since it has been described that a large part of CNS damage is mainly caused by infiltrating leukocytes from circulating blood and activated microglia (Stromnes et al., 2008), the populations of inflammatory cells present in the CNS during the acute stage of the disease were quantified and characterized. To evaluate the effect of Dz treatment on the CNS inflammatory cell activation and infiltration, cells from brain, cerebellum and spinal cord were isolated of animals from the EAE experimental groups. As shown in Fig. 2A, a lower number of CNS infiltrating cells were found in Dz-EAE rats compared to sick untreated EAE animals. This effect was more noticeable on the nonsick Dz-EAE group. The expression of CD11b, CD45 and MHCII surface antigens was assessed by flow cytometry in the inflammatory cells isolated from CNS. Similarly, it was observed that Dz-EAE animals, principally the nonsick ones, showed a decrease in the absolute number of $CD11b^+$ cells, mainly characterized by monocytes, macrophages, and microglia (Miller et al., 2007) (Fig. 2B), but not in the relative values of this population (about 65%) among the different experimental groups (data not shown). When the MHCII molecule was analyzed in this cell population, it was observed that $CD11b^+$ cells showed lower levels of expression of MHCII in Dz-EAE animals (Fig. 2C, D). These results indicate a lower stage of antigen presentation in the CNS suggesting a consequent decrease of T cell reactivation at the target organ. In order to classify the $CD11b^+$ population in relation to their state of activation, the CD45 surface molecule was studied. This is a panleucocyte marker present in all leukocytes and whose levels of expression increase in activated $CD11b^+$ cells (Bibolini et al., 2014). Fig. 2E shows that the percentage of $CD11b^+$ $CD45^{hi}$ cells (mainly monocytes, macrophages and activated microglia) is significantly reduced only in the nonsick Dz-EAE animals compared to the other experimental groups. These results indicate that in animals that did not show the characteristic signs of the disease, this cell population was reduced. Within the $CD11b^+$ population, the percentage of the $CD11b^+$ $CD45^{low}$ cells, which mainly correspond to non-activated microglia, was higher in Dz-EAE animals, even more in the nsDz-EAE group (Fig. 2F). These results indicate that those animals treated with Dz, in which the drug prevented the development of the pathology, showed a reduction in cells with an activated phenotype ($CD45^{hi}$) within the $CD11b^+$ population and prevalence of nonactivated microglia ($CD11b^+$ $CD45^{low}$cells).

3.3. Nonsick Dz-treated EAE animals show fewer CNS infiltrating lymphocytes

Infiltrating inflammatory cells isolated from CNS at 13 dpi from all EAE experimental groups were incubated with the anti-CD3, anti-CD4, anti-CD8 and anti-CD45RA antibodies. The CD3 molecule is a surface marker of T lymphocytes present in $CD4^+$ and $CD8^+$ T cells. As shown

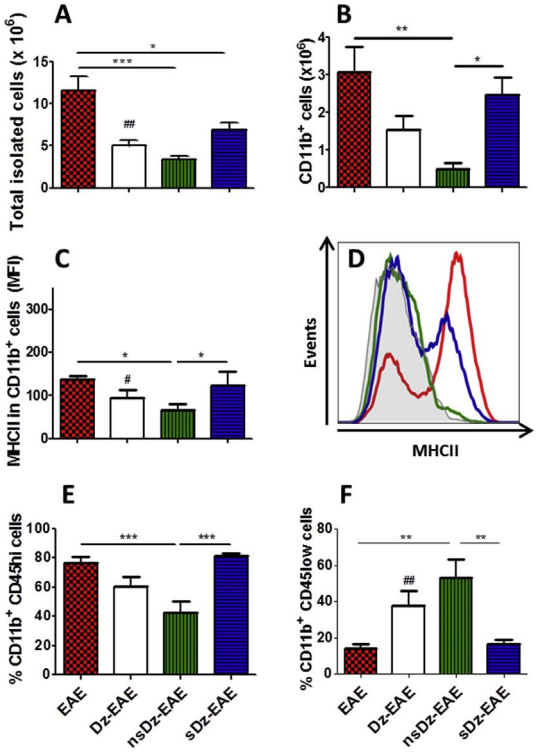

Fig. 2. Characterization of inflammatory cells in the CNS in diazepam-treated EAE animals. The graphic shows the absolute number of isolated inflammatory SNC cells from EAE (red, EAE), diazepam-treated EAE (white, Dz-EAE), nonsick (green, nsDz-EAE) and sick (blue, sDz-EAE) diazepam-treated EAE rats (A). Flow cytometry studies were performed on CNS isolated inflammatory cells and determined the absolute values of $CD11b^+$ cells (B), surface expression of molecule major class II histocompatibility complex (MHCII) within the $CD11b^+$ population (C and D) were expressed as mean fluorescence intensity. Changes in the phenotypic profile regarding to CD45 expression in the CNS $CD11b^+$ were determined and separated in two populations: $CD11b^+$ $CD45^{hi}$ and $CD11b^+$ $CD45^{low}$ cells in the different experimental groups (E and F, respectively). Bar charts are the result of the average of 8 animals per group. The significant differences correspond to $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ employing one-way ANOVA test with Newman-Keus multiple comparison test, and $\#p < 0.05$, $\#\#p < 0.01$ for paired Student's t-test when EAE and Dz-EAE groups were compared. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in Fig. 3A, the absolute number of CNS cells corresponding to the $CD3^+$ population is significantly reduced in nonsick Dz-EAE animals compare to untreated or sick Dz-treated EAE animals. When the two subpopulations within the T cells were evaluated, it was observed that the $CD3^+$ $CD8^+$ population corresponding to cytotoxic T lymphocytes was the minority and its absolute number was reduced in the nonsick Dz-EAE experimental group with respect to the sick EAE ones because of a lower number of infiltrating cells, while the relative proportions between the experimental groups were not modified (data not shown). The population of $CD3^+$ $CD4^+$ T helper cells showed a significant decrease in nonsick Dz-EAE animals compared to the other experimental groups (Fig. 3B). As for the B cell population ($CD45RA^+$ cells), it was found in very low proportions in CNS in the different experimental conditions (< 1%). The pathological features of MS and EAE are strongly associated with the invasion of $CD4^+$ T cells capable of secreting proinflammatory cytokines in the CNS that would be the main responsible for local inflammation in the acute period. These cells are capable of producing Th1 and Th17 cytokines, which are important for generating microglial activation, damage and chemotaxis of peripheral blood leukocytes (Fletcher et al., 2010). Encephalitogenic $CD4^+$ T cell populations producing pro-inflammatory cytokines (IFN-γ and IL-17) were evaluated among the CNS infiltrating cells of animals treated with Dz or vehicle after the induction of EAE. By flow cytometry, it was

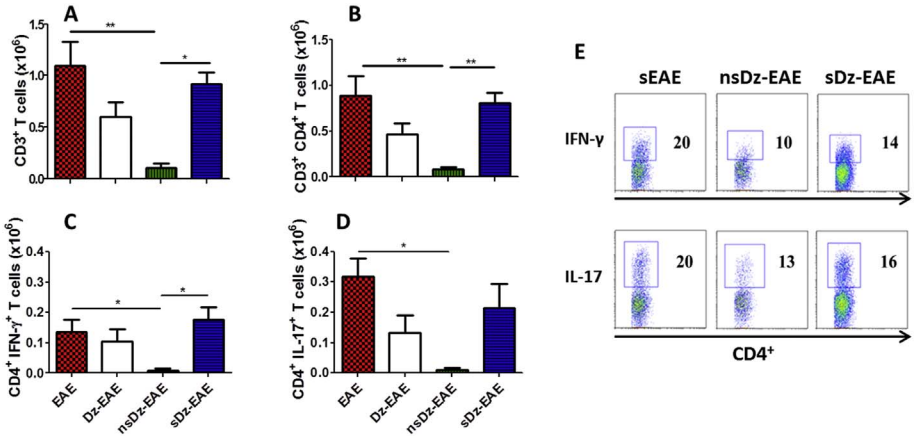

Fig. 3. Cytokine production by T cells infiltrating CNS of Diazepam-treated EAE animals. CNS infiltrating cells isolated at 13 dpi were labeled with anti-CD3, anti-CD4, anti-CD8, and anti-CD45RA antibodies and analyzed by flow cytometry in EAE (red, EAE), diazepam-treated EAE (white, Dz-EAE), nonsick (green, nsDz-EAE) and sick (blue, sDz-EAE) diazepam-treated EAE rats. The bars graphic shows the number of $CD3^+$ (A), and $CD3^+$ $CD4^+$ T cells (B). After that, cytokine production within the $CD4^+$ T cell population was determined: $CD4^+$ IFN-γ^+ and $CD4^+$ IL-17^+ T cells in the CNS (C and D, respectively). Dot plots (E) are representative of one experiment. Bar graphs represent the average of 6 animals per group. The significant differences correspond to: $*p < 0.05$, $**p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed that nonsick Dz treated animals showed a significant decrease in the absolute number of $CD4^+$ IFN-γ^+ and $CD4^+$ IL-17^+ T cells isolated from the CNS in relation to sick animals (Fig. 3C and D, respectively).

3.4. Levels of mRNA encoding the TSPO receptor in brain tissue

The levels of expression of TSPO transcripts were studied in tissue isolated from the frontal region of the cerebral cortex of different experimental groups, a region of the brain where our previous work evidenced the signs of neuroinflammation, with microglia and astrocyte concomitant activation (Chanaday and Roth, 2016). Fig. 4 shows that the transcript levels for TSPO significantly increased in the frontal cortex region of the EAE group compared to control animals. Animals challenged for the disease and treated with Dz that did not develop clinical signs showed intermediate values between sick EAE animals and control CFA group. Dz treatment did not induce changes in TSPO levels in the CFA group.

3.5. Levels of TSPO receptor in inflammatory infiltrating $CD11b^+$ cells isolated from CNS

Considering the data obtained regarding the expression of TSPO receptor in CNS tissue and the characterization of isolated CNS inflammatory cells, the presence of TSPO in the CNS $CD11b^+$ inflammatory cells was evaluated. As shown in Fig. 5, $CD11b^+$ cells isolated from the Dz-EAE group, principally from the nonsick subgroup, showed lower levels of expression of the TSPO protein respect to the EAE group (Fig. 5A, B). In animals from the EAE group, it was also evaluated de levels of expression of TSPO in $CD11b^+$ $CD45^{hi}$ and $CD11b^+$ $CD45^{low}$ cells. As expected (Bae et al., 2014; Politis et al., 2012), the $CD11b^+$ $CD45^{hi}$ population (macrophages and activated microglia) is the one that contributes to the high levels of TSPO in sick EAE animals (Fig. 5C, D).

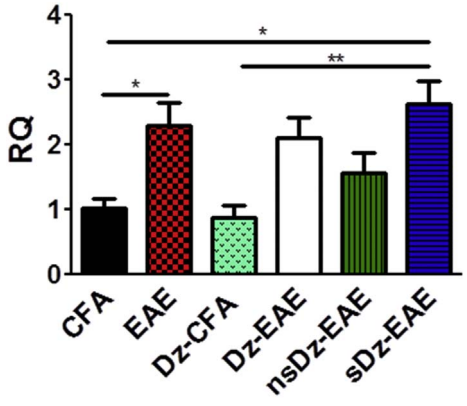

Fig. 4. Relative levels (RQ) of mRNA for the TSPO protein in the frontal region of the cerebral cortex of control (CFA), animals sensitized with whole bovine myelin to induce the disease (EAE), CFA treated with diazepam (Dz-CFA), diazepam-treated EAE (Dz-EAE) animals, nonsick (nsDz-EAE) and sick (sDz-EAE) subgroups of diazepam-treated EAE rats. The result corresponds to the mean of 3 animals per group. Significant differences are indicated as $*p < 0.05$; $**p < 0.01$.

3.6. Levels of TSPO in infiltrating $CD4^+$ T cells isolated from CNS

The distribution of TSPO receptor in $CD4^+$ T cells population isolated from the CNS of the different experimental groups was determined. The Dz-EAE group showed a tendency to present higher levels of expression of the TSPO protein in relation to the levels of this protein in EAE animals. Significant differences were observed between the nsDz-EAE group and sick EAE animals (treated and not treated) (Fig. 6A, B). There is very little evidence regarding the levels of TSPO in $CD4^+$ T cells and this cell population can be very heterogeneous in terms of its cellular functions (T helper1, T helper 2, T helper 17 or T

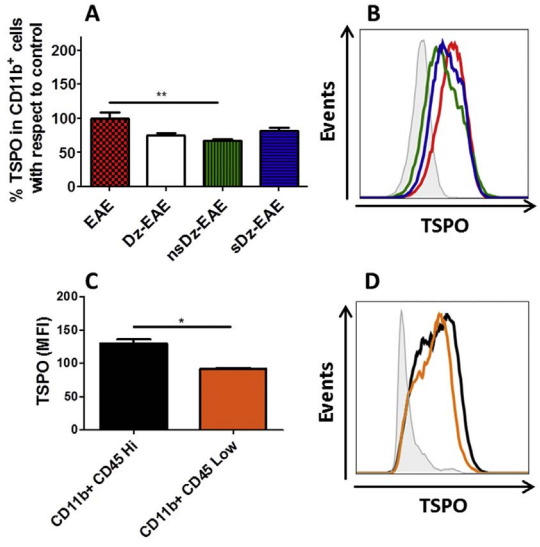

Fig. 5. TSPO levels in CD11b + cells isolated from CNS. Inflammatory cells were isolated, labeled with specific antibodies for the CD11b molecule, CD45, and TSPO, and then analyzed by flow cytometry. The bar chart shows the percentage of TSPO (A) in the total $CD11b^+$ population with respect to control (EAE animals). In figure C is represented the mean fluorescence intensity (MFI) of TSPO in the $CD11b^+$ $CD45^{low}$ and ^{high} subpopulations of EAE animals. The results correspond to 4 animals per group. Histograms of TSPO (B, D) are representative of an animal corresponding to each group. The shadowed gray line corresponds to the control condition using the secondary antibody in the absence of the primary. Significant differences are indicated as $*p < 0.05$; $**p < 0.01$.

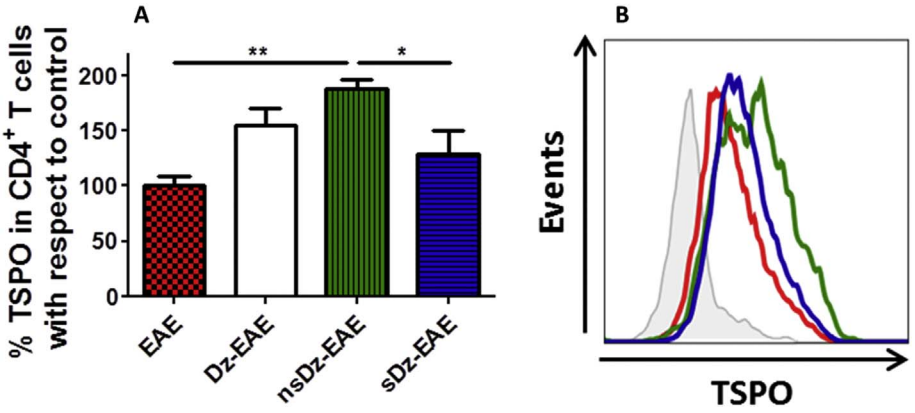

Fig. 6. TSPO levels in CD4 + T cells isolated from CNS. Inflammatory CNS cells were isolated and labeled with specific anti-CD4, and anti-TSPO antibodies, and then analyzed by flow cytometry. The bar chart (A) shows the percentage of TSPO on $CD4^+$ T cell population with respect to control (EAE animals). The results correspond to 5 animals per group. Histogram of TSPO (B) is representative of an animal corresponding to each group. The shadowed gray line corresponds to the control condition using the secondary antibody in the absence of the primary. Significant differences are indicated as $*p < 0.05$; $**p < 0.01$.

regulatory cells), so this result opens doors to a deeper study on its characterization.

4. Discussion

In animals challenged for EAE, Dz-treatment reduced its incidence by approximately 50%, attenuated the weight diminution indicative of the onset of clinical signs and reduced some parameters related to the severity of the disease. The results revealed that Dz treatment does not induce a uniform effect on animals challenged for EAE. Regarding to the development of the pathology we could speculate that there is a "threshold" in the immune cell activation that when it is exceeded, the animals develop the normal course of the disease. However, in half of the animals treated with Dz this threshold is not reached and a significant decrease in the number of CNS inflammatory cells and the absence of clinical signs are observed in our single-phase EAE model. In order to deepen about this behavior more studies should be performed before starting Dz treatment to determine the immunological background and levels of Dz receptors on immune cells of each individual animal that could account for this differential effect.

In previous works, we observed that Dz has a direct inhibitory effect on the proliferation, production of proinflammatory cytokines and activation of T lymphocytes isolated from the main lymphoid organ involved in the onset of the disease, which could be one of mechanisms that contribute to the beneficial effect previously observed with Dz *in vivo* during the EAE development (Bibolini et al., 2011; Fernández Hurst et al., 2015a). In this work, we characterized the main inflammatory cell populations in the target organ of the disease, the CNS (brain, cerebellum and spinal cord) and evaluated the levels of expression of TSPO in brain tissue and in the infiltrating and inflammatory isolated cell populations as a marker of neuroinflammation. We observed a lower number of infiltrating cells in rats treated with Dz that did not exhibit clinical signs of the disease than in sick EAE animals. The $CD11b^+$ population, mainly characterized by microglia, monocytes and macrophages (Miller et al., 2007), was the principal cell population isolated from CNS in all experimental groups. As expected, in nonsick Dz-EAE animals this population was reduced in absolute values, and presented lower levels of MHCII, a molecule involved in the antigenic presentation to T cells. We also revealed that the $CD11b^+$ $CD45^{hi}$ cells (monocytes, infiltrating macrophages and activated microglia) were less frequent in the CNS of nonsick Dz-treated EAE rats with a concomitant higher proportion of $CD11b^+$ $CD45^{low}$, which is a characteristic phenotype of non-activated resident microglia (Bibolini et al., 2014). When we evaluated the expression levels of TSPO in the $CD11b^+$ cell population and in the different subpopulations described subsequently, we evidenced that $CD11b^+$ cells isolated from the nonsick Dz-EAE group show significantly lower levels of TSPO protein. Even more, the high levels of TSPO in sick EAE animals were principally due to the higher levels of $CD11b^+$ $CD45^{hi}$ in this experimental group, whereas the $CD11b^+$ $CD45^{low}$ population (not activated microglia) exhibited lower levels. These results correlate with the data obtained for transcript levels of TSPO in tissue isolated from the frontal cortex region of sick EAE rats respect to CFA rats and they are also in line with previous reports in which an increment of binding sites for TSPO ligands were detected by PET in individuals with MS and other neuroinflammatory pathologies (Politis et al., 2012). The increase in TSPO levels in microglia under neuroinflammation was suggested as a mechanism of adaptive or compensatory response to the damage (Bae et al., 2014). It has also been postulated that TSPO overexpression could be involved in processes of regulation of cell death, cytokine production, free radical generation or microglial proliferation (Venneti et al., 2006). Therefore, TSPO has been proposed as an attractive therapeutic target and some TSPO ligands are currently under investigation as therapeutic means for promoting neuroprotection, axonal regeneration and modulating inflammation (Girard et al., 2012).

On the other hand, nonsick Dz-treated EAE animals showed intermediate levels of transcript for TSPO, suggesting that in this group the protective effect exerted by Dz during the development of the pathology results in less inflammation and associated cellular damage in the CNS. Our results for the $CD11b^+$ population suggest that nonsick Dz-EAE animals could show less reactivation of T lymphocytes in the CNS, with the consequent decrease in the recruitment of other inflammatory leukocytes, reduction in inflammation and associated damage.

We also studied the T lymphocyte population which has been described as the main responsible for the rupture of the blood-brain barrier, the first to access the CNS and to promote the recruitment of other leukocytes by the release of inflammatory mediators (Engelhardt, 2006). In our experimental model, we found a marked predominance of T lymphocytes with respect to B lymphocytes, more specifically, the subpopulation of $CD4^+$ T cells; which include the encephalitogenic T cells (IFN-γ and IL-17 producers). Dz treatment decreased the number of CNS infiltrating $CD4^+$ IFN-γ^+ and $CD4^+$ IL-17^+ T cells at the peak of the disease, proinflammatory populations involved in EAE and MS pathology (Murphy et al., 2010). When we evaluated the levels of expression TSPO in this cell population, we evidenced that infiltrating $CD4^+$ T cells isolated from nonsick Dz-EAE rats presented the highest levels. The increase in TSPO levels in this cell population is interesting to be explored in future work.

There is little bibliographical information regarding the effect of benzodiazepines for treatment of autoimmune diseases. Baclofen, tizanidine and gabapentin, and in some cases Diazepam, are used for symptomatic treatment of disability and symptoms (Tullman, 2013; Otero-Romero et al., 2016). Also, one reported case indicates that Dz in combination with Lorazepam exerts a beneficial effect on a patient with MS by relieving catatonic features (Hung and Huang, 2007). However, Dz treatment could be proposed to prevent MS and other autoimmune pathologies, such as rheumatoid arthritis, systemic lupus erythematosus, based on its action of reducing the release of pro-

inflammatory mediators and changing the cell phenotype (lymphocytes, dendritic cells, macrophages, microglia), to a non- inflammatory or tolerogenic one (Fernández Hurst et al., 2015a,b) and reducing the arrival of inflammatory cells to the target organ, as was demonstrated in our experimental model.

Although the mechanism by which Dz exerts its effects remains unknown, we can infer several possibilities. Dz can bind to the TSPO in the adrenal cortex and increase the production of glucocorticoids from the first step. Glucocorticoids are known for their modulatory or suppressive effects on the immune response (Massoco and Palermo-Neto, 2003; Bento de Lima et al., 2010). In addition, given its ability to cross the blood-brain barrier, Dz could access the CNS and stimulate the synthesis of neurosteroids by glia cells, mainly microglia, and modulate the activity of neurons and/or infiltrating leukocytes through the $GABA_A$ receptor described in both cell populations (Papadopoulos et al., 2006; Martín-García et al., 2007). Finally, we cannot omit the possibility that benzodiazepines exert a direct effect on cells of the immune system, as was previously observed (Fernández Hurst et al., 2015a,b).

In conclusion, we here show that Dz treatment decrease the incidence of the disease, a process that is accompanied by a lower number of infiltrating cells and a reduced inflammatory profile. We demonstrate that these events correlate with the TSPO levels, which are higher in brain tissue from the EAE sick groups, and that the main cellular populations involved are infiltrating macrophages and activated microglia.

Conflicts of interest

The authors report that there are no conflicts of interest.

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