Neuroscience 199 (2011) 421–428

INHIBITORY ROLE OF DIAZEPAM ON AUTOIMMUNE INFLAMMATION IN RATS WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

M. J. BIBOLINI,[1] N. L. CHANADAY,[1] N. S. BÁEZ, A. L. DEGANO, C. G. MONFERRAN AND G. A. ROTH*

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, CONICET-UNC), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

Abstract—Glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the CNS, and both may be involved in the neuronal dysfunction in neurodegenerative conditions. We have recently found that glutamate release was decreased in isolated synaptosomes from the rat cerebral cortex during the development of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis. In contrast to control animals where GABA induced a decrease in the evoked glutamate release, which was abolished by picrotoxin (a $GABA_A$ antagonist), synaptosomes from EAE rats showed a loss in the inhibition of the glutamate release mediated by GABA with a concomitant diminution of the flunitrazepam-sensitive $GABA_A$ receptor density. We have presently further evaluated the relevance of the GABAergic system in EAE by treating rats challenged for the disease with the GABA agonist diazepam. Administration of diazepam during 6 days starting at day 6 or 11 after EAE active induction led to a marked decrease of the disease incidence and histological signs associated with the disease. Cellular reactivity and antibody responses against the encephalitogenic myelin basic protein were also diminished. Beyond the effects of diazepam on the autoimmune, inflammatory response, we report also a positive effect on neurotransmission. Treatment with diazepam inhibited the previously described reduction in glutamate release in the frontal cortex synaptosomes from EAE animals. These data suggest that an endogenous inhibitory GABAergic system within the immune system is involved in the diazepam effect on EAE and indicate that increasing GABAergic activity potently ameliorates EAE.

Key words: autoimmunity, diazepam, multiple sclerosis, GABAergic system, neurotransmitter release.

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the human CNS, the most common neurologic disease of young adults (Trapp and Nave, 2008). Although its etiology is unknown, the primary insult in MS is mainly attributed to an autoimmune attack against myelin components, involving progressive accumulation of inflammation, and posterior neurodegeneration that leads to axonal and neuronal dysfunction within the CNS (McFarland and Martin, 2007; Dutta and Trapp, 2011). A well-accepted model that mimics many of the clinical and pathological features of MS is experimental autoimmune encephalomyelitis (EAE). This pathology can be induced in genetically susceptible animals by a single injection of CNS antigens homogenized in an adequate adjuvant (Ludwin, 2006). Wistar rats develop a monophasic course (acute stage, 11–13 days post-induction, dpi) characterized by ataxia and hind limb paralysis associated with weight loss and fecal and urinary incontinence. Affected animals show a spontaneous neurological improvement 2–4 days after the onset of the disease regaining the full ability to walk by 17–18 dpi (Slavin et al., 1996). Nevertheless, in spite of the well-defined histopathology of MS and EAE, the mechanisms that contribute to neurological deficits remain unclear. In this respect, there is a growing recognition of the importance of axonal and cortical over the known white matter changes in the genesis and evolution of the lesions (McFarland and Martin, 2007; Vercellino et al., 2007).

Glutamate is the major excitatory neurotransmitter in the CNS, and its concentration at the synaptic cleft is finely regulated by multiple mechanisms, which include the glutamate–glutamine cycle and the activity of glutamate uptake and transport mechanisms. The central role played by glutamate receptors in mediating excitotoxic neuronal death in stroke, epilepsy, trauma, and MS has been well established (Bolton and Paul, 2006; Centonze et al., 2010). However, the glutamatergic release, crucial in information intake and information processing within the brain (Vereker et al., 2000), has been poorly explored for this disease. Using the EAE model, we described a diminution on the calcium-dependent glutamate release in isolated nerve terminals of the cerebral cortex from EAE animals, which was coincident with the onset of the clinical signs (Vilcaes et al., 2009). These results strongly support the fact that cortical region could contribute to clinical symptoms and disease progression as well as provide novel insights into the molecular events that may affect the normal neuronal function during the course of the disease in EAE and possibly in MS.

GABA is the major inhibitory neurotransmitter balanced with glutamate in the CNS. GABA-mediated synaptic inhibition is essential for normal brain function, neuronal activity, information processing and plasticity, network synchronization, and in disease (Huang, 2009). $GABA_A$ recep-

[1] Both authors contributed equally to this work.

*Corresponding author. Tel: +54-351-4334168; fax: +54-351-4334074.
E-mail address: garoth@fcq.unc.edu.ar. (G. A. Roth).

Abbreviations: APCs, antigen-presenting cells; CFA, complete Freund's adjuvant; Con A, concanavalin A; dpi, days post-induction; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MNC, mononuclear cells; MS, multiple sclerosis; PBS, phosphate-buffered saline; SI, stimulation index; 4AP, 4-aminopyridine.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.08.076

Table 1. Effect of diazepam treatment on EAE clinical signs

Group	Diazepam (mg/kg/d)	Days of treatment	Disease incidence (%)	MMCS[a]	Day of onset (dpi)	Length of disease (d)	Disease index[b]
CFA	None	No	0/20	0	0	0	0
CFA	0.10	6–11	0/10	0	0	0	0
CFA	0.10	11–16	0/10	0	0	0	0
EAE	None	No	17/20 (85.00)	2.50±0.27	12.00±0.36	4.77±0.54	68.00±7.97
EAE	0.05	6–11	7/12 (58.33)	2.89±0.56	13.14±0.49	5.71±1.02	94.50±27.95
EAE	0.10	6–11	6/20 (30.00)	2.50±0.29	13.00±0.71	4.75±0.48	55.82±8.86
EAE	0.10	6–16	0/4 (0.00)	0	0	0	0
EAE	0.10	11–16	10/20 (50.00)	2.14±0.40	12.00±0.50	5.14±0.34	61.64±13.51

Animals were injected i.p. with the indicated amount of diazepam at the indicated days post-induction of the EAE with bovine myelin in CFA. The results are expressed as mean±SEM.

[a] MMCS, 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 animal throughout the experimental period divided by the day of onset of EAE clinical symptoms×100.

tors are perfectly positioned to reduce excitability and inhibit the positive-feedback loop that constitutes excitotoxicity (Zhang et al., 2007). In this regard, we have recently demonstrated that, in contrast to controls, synaptosomes from EAE rats showed a loss in the inhibition of the glutamate release mediated by GABA. Furthermore, we observed a decreased flunitrazepam-sensitive $GABA_A$ receptor density in synaptosomes from symptomatic EAE rats (Cid et al., 2011). These results indicate that both glutamatergic and GABAergic neurotransmission are impaired in symptomatic EAE animals and could contribute to clinical symptoms and disease progression. Interestingly, MS has been associated with diminished serum levels of GABA and its synthetic enzyme, glutamic acid decarboxylase (Demakova et al., 2003). Moreover, studies using the EAE model have shown that the concentration of GABA and glutamic acid decarboxylase activity in blood, as well as the uptake of [^{3}H]GABA by spinal cord synaptosomes, were also reduced in EAE animals (Gottesfeld et al., 1976).

These observations prompted us to explore the impact of agents that can modulate GABA action in the pathology of EAE. Among them is diazepam, a classical benzodiazepine that binds to a central benzodiazepine receptor increasing the effects of GABA by enhancing chloride conductance (Drugan and Holmes, 1991). Therefore, in order to explore the role of GABA neurotransmission in the pathogenesis of EAE, we conducted *in vivo* studies examining the effects of diazepam on the development of EAE in Wistar rats.

EXPERIMENTAL PROCEDURES

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 and institutional guidelines for animal care, and the protocol was approved by the local institutional review committee for animal studies (Exp. No. 15-99-40426). 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.), and the 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) (Degano and Roth, 2000). In some experiments, other group of rats received 0.5 ml of the same emulsion without any antigenic preparation (CFA group). Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3H)-one) (Glutasedan, Northia Medicinales, Argentina) was dissolved in phosphate-buffered saline (PBS) and administered i.p. at a concentration of 0.05 mg (1 ml)/kg/d or 0.10 mg (2 ml)/kg/d during 6 or 12 consecutive days (see Table 1). 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; and 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 9 or 12 days after challenge, and then the animals were sacrificed at 12–14 dpi when the rats from the EAE group showed the maximum clinical signs of the disease (acute period). Rats were anesthetized again with xylazine and ketamine and bled to death by cardiac puncture. Inguinal lymph nodes were aseptically removed and placed in ice-cold RPMI 1640 medium. Other set of animals were processed for histological studies.

Histopathology

Animals from each group at the disease acute period (14 dpi) were deeply anesthetized and intracardiacally perfussed with ice-cold PBS and then with 4% paraformaldehyde in PBS, pH 7.4. Segments from the lumbar region (L1–3) of the spinal cords were removed and post-fixed in buffered 4% paraformaldehyde for 24 h. These tissues were embedded in paraffin, cut into transverse sections (5-μm thick), and stained with hematoxylin–eosin according to standard procedures. Six step-sections were examined for each animal with an Axiovert 200 microscope (Carl Zeiss, Thornwood, NY, USA). The histological findings were graded according to the extent of leukocyte infiltrates in the white matter. The severity of inflammation was scored from 0–4 as follows: 0, no

inflammatory cells; 1, inflammatory cells limited to meninges or submeninges; 2, inflammatory cells in meninges and perivascular spaces; 3, perivascular and mild parenchymal infiltrates; and 4, several and large perivascular and parenchymal inflammatory infiltrates.

Immunological T-cell determinations

For DTH reaction determination at 9 and 12 dpi, the animals were injected intradermally in the right ear with 40 μl of 3 mg/ml myelin basic protein (MBP) in PBS, and 40 μl PBS in the left ear (negative control), both sterile solutions. The ear thickness was determined in a blind fashion with a digital caliper. The results are expressed as the difference in mm between the thickness of the right ear and the left ear measured 24 h after the injection.

For the measurement of the proliferative response, mononuclear cells (MNC) aseptically isolated from inguinal lymph nodes were cultured by triplicate in 96-well flat-bottom plates essentially as previously indicated (Degano and Roth, 2000). Briefly, 3×10^6 cells/ml were cultured in a total volume of 200 μl/well of RPMI 1640 medium with 75 μg/ml of PBM or 1 μg/ml concanavalin A (Con A) for 72 h. When indicated, 2.5 μg/well of diazepam was also added to the cultures. Each well was pulsed with 20 μl of medium containing 1 μCi of [^{3}H]TdR during the last 18 h of culture, then the cells were harvested onto fiberglass filters, and the radioactivity incorporated was counted using standard liquid scintillation techniques. The results from the proliferation assays are expressed as stimulation index (SI), defined as the ratio between mean counts per minute (cpm) of antigen-stimulated culture/mean cpm of the unstimulated culture, considering a SI$\geq$2 as positive response.

Antibody determinations

Total anti-MBP antibodies were determined by a Western blot technique (Roth and Obata, 1991). Polyacrylamide gels were prepared in 12% concentration without lanes, and 200 μl of sample (300 μg of lyophilized bovine myelin) was planted along the surface. The electrophoretic run was performed in the presence of sodium dodecyl sulfate at a constant voltage of 120 mV. The separated proteins were subsequently electrotransferred to a nitrocellulose membrane, using a constant current of 350 mA for 50 min. The membranes were stained with 0.29% Ponceau-S and cut into strips 0.5-cm wide, which were blocked with a 5% solution of skim milk powder in PBS at 4 °C overnight. The sera of animals were diluted in PBS buffer with 0.05% Tween20 and 1% bovine serum albumin. Each nitrocellulose strip was incubated with 600 μl of serum at 4 °C with constant shaking all night. They were then washed with 0.05% Tween20 in PBS, and the membranes were incubated overnight at 4 °C with the corresponding monoclonal antibody followed by the HRP-conjugated anti-mouse IgG diluted 1/500. Finally, the strips were washed, and the color reaction was carried out with 0.05% 4-Cl-naphthol and 0.01% hydrogen peroxide in PBS with constant agitation. The reaction was stopped by adding distilled water, the nitrocellulose strips were left to dry, and then they were scanned and analyzed with the computer program GELPRO.

Preparation of synaptosomes and glutamate release assay

The frontal cortex was isolated from the different groups when the animals of EAE control group showed the clinical signs (6–24 h after onset, 12–13 dpi). The synaptosomes were purified on discontinuous Percoll gradient as described previously (Vilcaes et al., 2009). Synaptosomes that sedimented between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES, pH 7.4, before centrifugation at 27,000$\times$g for 10 min at 4 °C. The pellets thus formed were resuspended in 5 ml of HEPES buffer medium, and the protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). For glutamate release assays, the synaptosomal pellets were stored on ice and used within 3–4 h. Glutamate release from cerebrocortical synaptosomes was monitored on-line, using an assay that employs exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative decarboxylation of the released glutamate. Then, the generated NADPH was detected fluorometrically (Sihra et al., 1992; Vilcaes et al., 2009). Briefly, synaptosomal pellets were resuspended in HEPES buffer medium and incubated in a stirred and thermostated cuvette maintained at 37 °C in a FluoroMax-P Horiba Jobin Yvon spectrofluorimeter (Horiba Ltd, Kyoto, Japan). After 3 min, 1 mM $NADP^+$, 50 U/ml glutamate dehydrogenase, and 1.2 mM $CaCl_2$ were added. Then, after 5 min of incubation, 3 mM 4-aminopyridine (4AP) was added to stimulate glutamate release. Traces were calibrated by the addition of 4 nmol of glutamate at the end of each assay. Data points were obtained at 1-s intervals.

Data analysis

The results were expressed as the mean$\pm$SEM. The levels of glutamate were analyzed using the one-way or two-way analysis of variance (ANOVA). Whenever ANOVA indicated significant effects ($P<0.05$), a pair-wise comparison of means by Newman–Keuls test (NK) was carried out. In all cases, the assumptions of the analysis of variance (homogeneity of variance and normal distribution) were attained. In all statistic analysis, a $P<0.05$ was considered to represent a significant difference between groups.

RESULTS

Clinical evaluation of EAE

The clinical analysis of the different groups of animals sensitized to induce EAE is summarized in Table 1. As previously shown (Scerbo et al., 2009), about 85% animals of the EAE control group treated with vehicle alone exhibited the characteristic clinical signs of the disease. The rest of the animals manifested a subclinical state of disease with histological alterations and immunoreactivity to MBP (Figs. 2, 3, and 4). Treatment with 0.05 mg/kg/d (low dose) or 0.10 mg/kg/d (high dose) of diazepam markedly diminished the incidence of EAE to 60% (low dose, 6–11 dpi), 30% (high dose, 6–11 dpi), 50% (high dose, 11–16 dpi), and 0% (high dose, 6–16 dpi). However, the analysis of disease indexes revealed that sick EAE rats from the diazepam-treated groups showed similar onset, severity, and length of the disease as sick animals from the vehicle-treated EAE control group.

The disease was also associated with a marked body weight loss (Slavin et al., 1996). Rats from the vehicle-treated EAE group began to lose weight just before the onset of the clinical signs (10 dpi), and the body weight loss continued during the acute phase reaching a maximum of 10–20%. The EAE animals treated with diazepam lost less body weight than rats from the vehicle-treated EAE group (Fig. 1). After the acute period, all the animals began gradually to regain body weight. There were no differences in body weight among the different CFA groups, indicating that diazepam has no effect *per se* on this parameter (data not shown).

In order to get some insight about the mechanism of diazepam effect on EAE incidence, we evaluated several

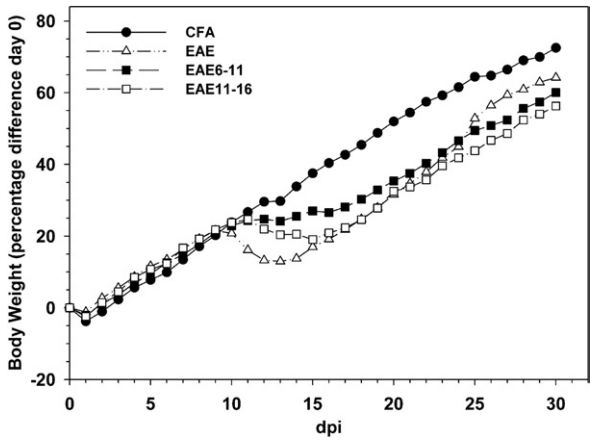

Fig. 1. Comparison of the body weight variations determined as the percentage difference with respect to day 0 of rats from CFA-injected animals, EAE animals, and EAE rats treated with 0.10 mg/kg/d diazepam during 6–11 dpi or 11–16 dpi.

immune parameters in animals injected with myelin to induce EAE that developed or did not develop clinical signs after treatment with 0.10 mg/kg body weight/day of diazepam during 6–11dpi (induction stage) or 11–16 dpi.

Diazepam treatment decreased the histological lesions within the CNS

Examination of histological sections from the lumbar region of the spinal cords from the vehicle-treated EAE animals stained with hematoxylin–eosin showed marked inflammation (Fig. 2A). Tissue sections from these rats revealed numerous inflammatory cells infiltrated in the meninges, perivascular spaces, and throughout the parenchyma (Fig. 2C), and the histopathological scores were indistinguishable between animals that developed or did not develop clinical signs. However, the spinal cord sections from non-sick animals that received diazepam showed a significant decrease in the extent of inflammatory infiltration with respect to vehicle-treated animals (Fig. 2A). Representative histological sections from CFA animals and vehicle- or diazepam-treated EAE groups are depicted in Fig. 2B–E. These results indicate that the absence of clinical symptoms in the non-sick diazepam-treated rats is associated with the inhibition of inflammatory infiltrates in CNS.

Effect of diazepam on the MBP-specific T- and B-cell responses

In order to evaluate whether the incidence of EAE in animals treated with diazepam correlates with a decreased immunological response to myelin antigens, we analyzed the cellular immune response to the encephalitogenic MBP. Both, DTH and EAE, are mediated by antigen-specific $CD4^+$ T cells of the Th1 cytokine phenotype, suggesting a causal link between EAE and DTH responsiveness (Cua et al., 1995). Fig. 3A shows that DTH reaction to MBP determined 9 days after the EAE challenge was significantly lower in diazepam-treated rats with respect to the respective vehicle-treated EAE control group. At 12 dpi, lower DTH values were only observed in the group treated from 6 to 11 dpi. However, when the results were separated into sick and non-sick animals (Fig. 3B), the inhibitory effect of diazepam on the DTH reaction was more evident.

Proliferation of MNC was tested *in vitro* at the acute (12 dpi) stage of the disease. Similar positive levels (SI>2) of proliferation in response to MBP were observed in MNC from EAE animals and sick diazepam-treated rats (Fig. 4A). On the contrary, the MBP-induced MNC proliferation was abrogated in treated non-sick animals. Interestingly,

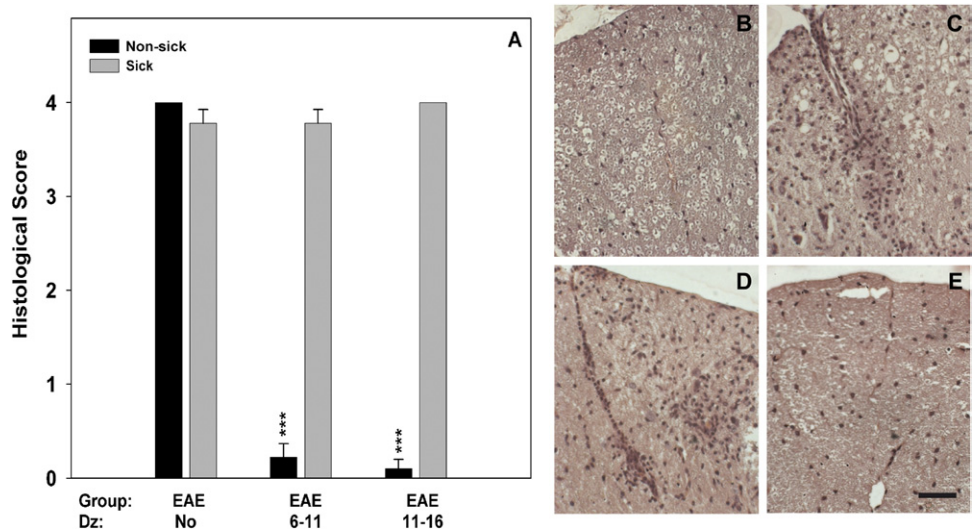

Fig. 2. Histology of spinal cord sections. Sections from the lumbar region (L1–3) of spinal cords were obtained at the acute period, embedded in paraffin and stained with hematoxylin–eosin. (A) Inflammatory cell infiltration scored as described in Experimental procedures. Values are expressed as the mean±SEM of four animals examined per group. Significant differences of treated group vs. vehicle are indicated by *** $P<0.001$. Representative histological sections of (B) CFA rat, (C) sick EAE control rat, (D) sick EAE rat treated with diazepam from 6 to 11 dpi, and (E) non-sick EAE rat treated with diazepam from 6 to 11 dpi. Scale bar: 50 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

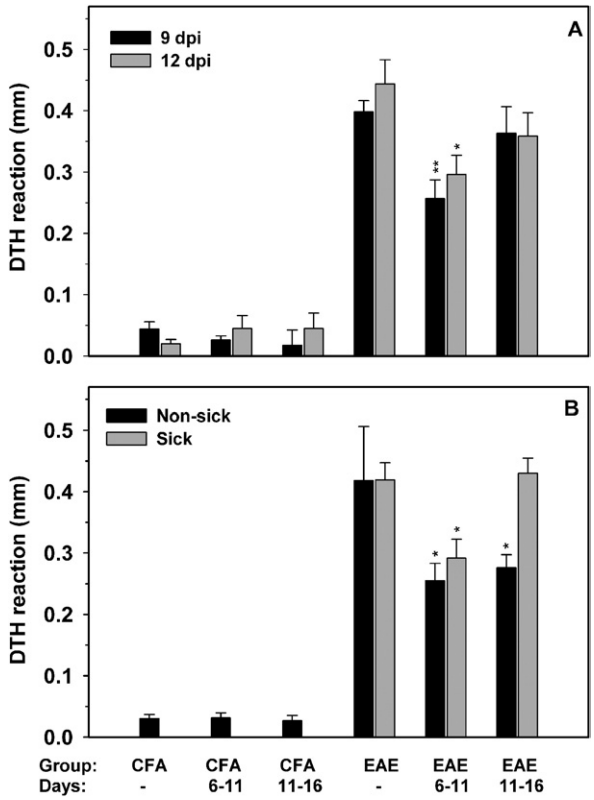

Fig. 3. Effect of diazepam on DTH response. (A) Rats were treated as indicated, and DTH was tested at 9 or 12 dpi. Animals were injected s.c. with MBP in the left ear and the vehicle in the right ear. Swelling was determined 24 h post-immunization and the results expressed as the difference of thickness (mm) between the left and right ear. (B) The obtained results independent of the testing day were grouped in sick or non-sick animals. Data correspond to the mean value±SEM of three experiments performed each with four animals per group. Significant differences of treated group vs. vehicle are indicated by * $P<0.05$, ** $P<0.01$.

we observed similar levels of MNC proliferation in response to Con A among all groups analyzed (Fig. 4B), indicating that the diazepam action involves the inhibition of antigen-specific responses.

Some studies have suggested a possible role of B cells and antibodies in the induction and immunoregulation of EAE (Rivero et al., 1999). In this respect, we evaluated the humoral response by analysis of total serum IgG antibodies to MBP. The data presented in Table 2 indicate that levels of total IgG anti-MBP antibodies in sera from the diazepam-treated animals during 6–11 dpi were lower than in sera from vehicle-treated animals. When rats were treated with diazepam during 11–16 dpi, the total anti-MBP antibodies, although lower, were not significantly different from the EAE animals. The Th1-like immune response has been associated in rats with the switching of immunoglobulins secretion towards IgG2b, and the IgG1 isotype has been associated with a Th2-like response (Mosmann and Sad, 1996; Rivero et al., 1999; Degano et al., 2004). The anti-MBP IgG isotype analysis showed no significant differences among the different animal groups, indicating that there is no shift of the type of immune response in animals that were treated with diazepam compared to the vehicle-treated animals.

Glutamate release of the frontal cortex synaptosomes isolated from EAE animals

We studied several preparations of frontal cortex synaptosomes from control CFA rats, vehicle-treated EAE animals, and EAE rats treated with diazepam that developed or did not develop clinical signs. The release of glutamate was evoked by addition of 4AP. 4AP is a potassium channel blocker, which has been shown to destabilize the plasma membrane potential of the synaptosomes, with an increase of cytoplasmic free Ca^{2+} concentration by opening voltage-gated Ca^{2+} channels, allowing them to fire spontaneous, tetrodotoxin-sensitive, action potentials capable of triggering exocytotic release of glutamate (Tibbs et al., 1989). Thus, this repetitive firing induced by 4AP mimics more closely the physiological mechanism of terminal depolarization than other inductors. In control nerve terminals from non-treated CFA rats, this release reached 18.81±0.59 nmol glutamate/mg protein/7 min in the presence of 1.2 mM $CaCl_2$. As previously shown, in the nerve terminals from normal rats, the total glutamate release evoked by 4AP in the presence of Ca^{2+} had no significant differences with respect to the CFA rat synaptosomes

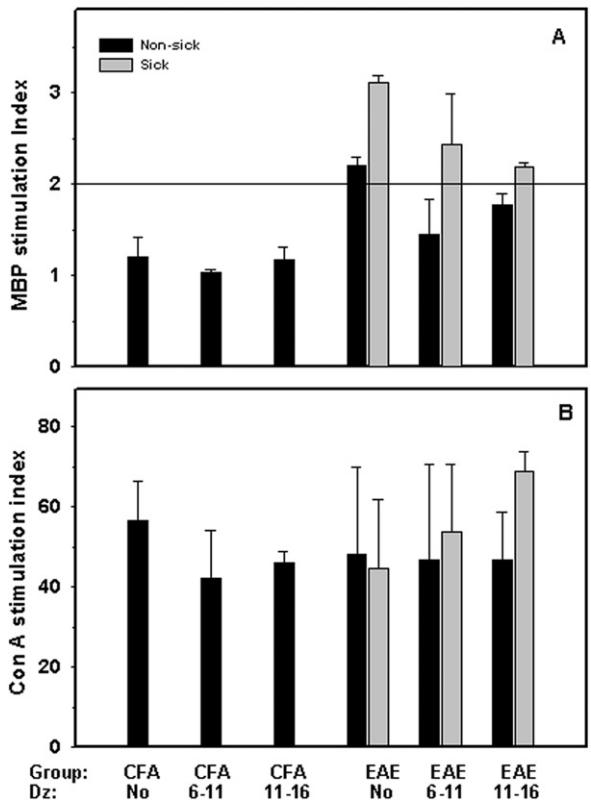

Fig. 4. Effect of diazepam on MBP-specific T- and B-cell responses. (A) Specific MBP-, (B) Con A-proliferative response indicated as stimulation index (SI). MNC were isolated from inguinal nodes of animals under different treatments during the acute period (12–14 dpi). Data correspond to the mean value±SEM of three experiments performed each with four animals per group.

Table 2. Specific IgG subclasses of anti-MBP antibodies in sera from control and diazepam-treated groups

Group	Diazepam (mg/kg/d)	Days of treatment	*n*	MMCS	Anti-MBP antibodies			
					Total IgG	IgG2b	IgG1	IgG2b/IgG1 (Th1/Th2)
CFA	None	No	3	0	2.89±0.30	10.83±3.59	9.25±1.38	1.13±0.32
EAE	None	No	2	0	87.58±10.26	95.25±3.63	92.55±5.76	1.01±0.10
EAE	None	No	6	2.87	93.61±15.66	147.17±9.77	100.68±19.12	1.40±0.29
EAE	0.10	6–11	4	0	26.55±5.18*	124.32±5.68	98.52±3.15	1.18±0.14
EAE	0.10	6–11	3	2.33	28.57±6.91*	150.67±9.58	112.58±17.66	1.45±0.24
EAE	0.10	11–16	4	0	60.69±11.73	138.48±13.29	106.12±13.36	1.37±0.14
EAE	0.10	11–16	3	2.67	65.99±12.92	145.85±13.07	120.29±21.51	1.20±0.17

Immunoblotting analysis of IgG (sera diluted 1:1000), IgG1, and IgG2b (sera diluted 1:200) reactivity against MBP.
Data are expressed as arbitrary units and correspond to the mean value±SEM of the indicated number of animals per group.
Significant differences of treated group vs. sick EAE animals non-treated with diazepam are indicated by * $P<0.05$.

(Vilcaes et al., 2009). In addition, no significant differences were observed between these groups and the diazepam-treated CFA animals.

Synaptosomes from vehicle-treated sick EAE rats showed a significant decrease in the total release of glutamate evoked by 4AP (13.82±1.58 nmol/mg protein/7 min, −26.53% with respect to the vehicle-treated CFA control). Similar results were obtained in synaptosomes from the diazepam-treated sick EAE animals. Conversely, the glutamate release in synaptosomes derived from non-sick diazepam-treated EAE animals was similar to the respective control CFA groups (Fig. 5). These results indicate that diazepam treatment also reverses the defect in neurotransmission we described in EAE rats.

DISCUSSION

The present data show that diazepam treatment applied after the induction of EAE strongly decreased the susceptibility to the disease and spinal cord histological lesions. A key event in the development of EAE is the activation and proliferation of encephalitogenic cells in peripheral lymph nodes (Pedotti et al., 2003). Therefore, in order to shed light about the mechanism underlying EAE inhibition mediated by diazepam, we analyzed whether this effect was associated with changes in the immune response against myelin antigens. Here, we demonstrated that the diazepam-treated rats elicit a significantly lower cellular response to the encephalitogenic protein MBP. Thus, we detected a reduction in the DTH reaction to MBP *in vivo*, (Fig. 3) and an inhibition of lymph node MNC proliferation *ex vivo* (Fig. 4). Further analysis of the cellular immune response showed that MNC from protected diazepam-treated rats proliferated in the presence of Con A in a similar way as cells from sick EAE rats, indicating that the suppression of the disease would not involve total anergy of the cellular response. The immunization to induce EAE stimulated also a humoral response to MBP. The total anti-MBP IgG antibody levels were decreased in diazepam-treated rats, but anti-MBP IgG isotypes analysis showed similar IgG2b/IgG1 ratios among all the studied animals. Therefore, the resistant state of the diazepam-treated animals to develop EAE could not represent an impaired Th1 response with a concomitant shift to a Th2-type milieu, but a decrease in the global encephalitogenic immune response. In line with these results, we also observed that diazepam inhibited the antigen-specific proliferation of lymph node MNC when added *in vitro* (data not shown). The inhibition of the neuropathological signs mediated by diazepam was greater when the treatment was applied during the inductor phase of the encephalitogenic immunoresponse (6–11 dpi) than during the immune effector stage (11–16 dpi).

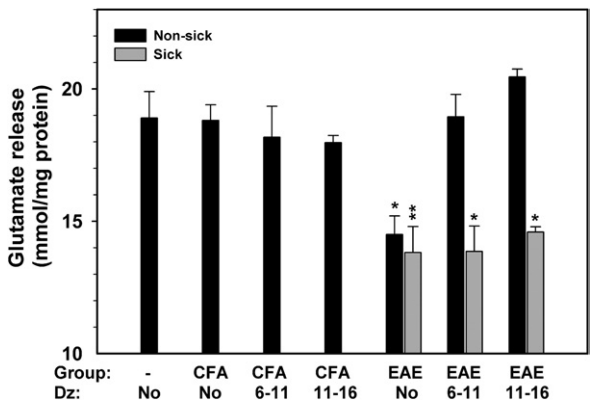

Fig. 5. 4-aminopyridine–evoked total glutamate release from rat frontal cortex synaptosomes. Synaptosomes were purified from normal, control (CFA groups), and EAE animals treated with diazepam as indicated and sacrificed at 11–12 dpi, and glutamate release was evoked by the addition of 3 mM 4-aminopyridine and assayed by on-line fluorometry. Results represent means±SEM of four independent animals per group. Glutamate release from diazepam-treated or not treated EAE rats was significantly different from the corresponding CFA animals, * $P<0.05$, ** $P<0.01$, two-way ANOVA.

In our study, increasing GABAergic activity ameliorated the manifestations of EAE, but treated rats that got sick were clinically no different from control EAE rats. These results suggest that the observed effect of diazepam occurred at least in part through a direct effect of the GABAergic agents on the development of reactive immune factors. Although the effect of GABA in the CNS is well known (Huang, 2009), recent data demonstrated that GABA and its receptors are also present and functional in the immune system. Thus, it has been shown that a subset of $GABA_A$ receptor subunits are expressed by T cells;

GABA at relatively low concentrations have a dramatic inhibitory effect on effector T-cell development and function both *in vitro* and *in vivo*, apparently by arresting T-cell cycling (Tian et al., 2004). More recently, it has been reported that antigen-presenting cells (APCs) also express functional GABA receptors and respond electrophysiologically to GABA. There is an endogenous GABA secretion by both APCs and T cells, and functional GABA channels on macrophages (Bhat et al., 2010). Interestingly, these authors showed that increasing GABAergic activity pharmacologically ameliorates the ongoing paralysis in EAE via inhibition of inflammation. They also demonstrated that the GABAergic agents used act directly on APCs, decreasing MAPK signals and diminishing the subsequent adaptive inflammatory response to myelin proteins.

Beyond the effects of diazepam on the autoimmune, inflammatory response, we report also a positive effect on neurotransmission. Treatment with diazepam inhibited the previously described drop in glutamate release in frontal cortex synaptosomes from EAE animals (Fig. 5). Classically diazepam acts by increasing the frequency of channel opening, thus requiring GABA to be present and classifying them as allosteric agonists (Study and Barker, 1981). Hence, considering we have reported a defective $GABA_A$ function in EAE (Cid et al., 2011), it is possible to speculate that the increased $GABA_A$ activity in the CNS could also contribute to the absence of neurological signs in diazepam-treated rats. Interestingly, alprazolam, other benzodiazepine derivative, also decreased the severity of clinical, histological, and neuroendocrine manifestations of EAE in Lewis rats but only when the animals were exposed to stressful conditions (Núñez-Iglesias et al., 2010). These observations indicate that the anxiolytic effect of benzodiazepines can also account for managing the manifestations of EAE and MS.

CONCLUSION

Our data at present show a beneficial effect of diazepam on the development of EAE. Future experiments using a combination of GABAergic agents specific for central benzodiazepine receptors, intrathecal injections, and the use of benzodiazepines derivatives that do not cross the blood–brain barrier will be conducted in order to dissect the role of GABA in the CNS during the pathogenesis of EAE.

Acknowledgments—The authors wish to thank Dr. M.P. Cid (UNC, Córdoba) for providing the diazepam, and Dr. H. Cejas (Córdoba) for performing routine histology procedures. This work was supported in part by Consejo de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (BID 1201/OC-AR, PICT 31675), Ministerio de Ciencia y Tecnología de la Provincia de Córdoba; and Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SeCyT-UNC), Argentina. M.J.B. and N.C.R. are research fellows, and A.L.D., C.G.M., and G.A.R. are senior career investigators from CONICET.

REFERENCES

Study RE, Barker JL (1981) Diazepam and (-)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of gamma-aminobutyric acid responses in cultured central neurons. Proc Natl Acad Sci USA 78:7180–7184.

Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, Steinman L (2010) Inhibitory role for GABA in autoimmune inflammation. Proc Natl Acad Sci U S A 107:2580–2585.

Bolton C, Paul C (2006) Glutamate receptors in neuroinflammatory demyelinating disease. Mediators Inflamm ID 93684:1–12.

Centonze D, Muzio L, Rossi S, Furlan R, Bernardi G, Martino G (2010) The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. Cell Death Differ 17: 1083–1091.

Cid MP, Vilcaes AA, Rupil LL, Salvatierra NA, Roth GA (2011) Participation of the GABAergic system on the glutamate release of frontal cortex synaptosomes from Wistar rats with experimental autoimmune encephalomyelitis. Neuroscience 189:337–344.

Cua DJ, Hinton DR, Kirkman L, Stohlman SA (1995) Macrophages regulate induction of delayed-type hypersensitivity and experimental allergic encephalomyelitis in SJL mice. Eur J Immunol 25: 2318–2324.

Degano AL, Ditamo Y, Roth GA (2004) Neuronal antigens modulate the immune response associated with experimental autoimmune encephalomyelitis. Immunol Cell Biol 82:17–23.

Degano AL, Roth GA (2000) Passive transfer of experimental autoimmune encephalomyelitis in Wistar rats: dissociation of clinical symptoms and biochemical alterations. J Neurosci Res 59: 283–290.

Demakova EV, Korobov VP, Lemkina LM (2003) Determination of gamma-aminobutyric acid concentration and activity of glutamate decarboxylase in blood serum of patients with multiple sclerosis. Klin Lab Diagn 4:15–17.

Drugan RC, Holmes PV (1991) Central and peripheral benzodiazepine receptors: involvement in an organism's response to physical and psychological stress. Neurosci Biobehav Rev 15:277–298.

Dutta R, Trapp BD (2011) Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. Prog Neurobiol 93:1–12.

Gottesfeld Z, Teitelbaum D, Webb C, Arnon R (1976) Changes in the GABA system in experimental allergic encephalomyelitis-induced paralysis. J Neurochem 27:695–699.

Huang ZJ (2009) Activity-dependent development of inhibitory synapses and innervation pattern: role of GABA signalling and beyond. J Physiol 587:1881–1888.

Ludwin SK (2006) The pathogenesis of multiple sclerosis: relating human pathology to experimental studies. J Neuropathol Exp Neurol 65:305–318.

McFarland HF, Martin R (2007) Multiple sclerosis: a complicated picture of autoimmunity. Nat Immunol 8:913–919.

Mosmann TR, Sad S (1996) The expanding universe of T-cells subsets: Th1, Th2 and more. Immunol Today 17:138–146.

Núñez-Iglesias MJ, Novío S, Almeida-Dias A, Freire-Garabal M (2010) Inhibitory effects of alprazolam on the development of acute experimental autoimmune encephalomyelitis in stressed rats. Pharmacol Biochem Behav 97:350–356.

Pedotti R, De Voss JJ, Steinman L, Galli SJ (2003) Involvement of both allergic and autoimmune mechanisms in EAE, MS and other autoimmune diseases. Trends Immunol 9:479–484.

Rivero VE, Riera CM, Roth GA (1999) Humoral response against myelin antigens in two strains of rats with different susceptibility to experimental allergic encephalomyelitis (EAE). Autoimmunity 29:129–137.

Roth GA, Obata K (1991) Experimental allergic encephalomyelitis: dissociation of immunochemical and clinico-pathological responses in two strains of rats. Neurochem Int 19:213–220.

Scerbo MJ, Rupil LL, Bibolini MJ, Roth GA, Monferran CG (2009) Protective effect of a synapsin peptide genetically fused to the B

subunit of *Escherichia coli* heat-labile enterotoxin in rat autoimmune encephalomyelitis. J Neurosci Res 87:2273–2281.

Sihra TS, Bogonez E, Nicholls DG (1992) Localized Ca^{2+} entry preferentially effects protein dephosphorylation, phosphorylarion, and glutamate release. J Biol Chem 267:1983–1989.

Slavin DA, Bucher AE, Degano AL, Soria NW, Roth GA (1996) Time course of biochemical and immunohistological alterations during experimental allergic encephalomyelitis. Neurochem Int 29: 597–605.

Staykova MA, Cowden WB, Willenborg DO (2002) Macrophages and nitric oxide as the possible cellular and molecular basis for strain and gender differences in susceptibility to autoimmune central nervous system inflammation. Immunol Cell Biol 80:188–197.

Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL (2004) γ-Aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. J Immunol 173:5298–5304.

Tibbs GR, Barrie AP, van Mieghem FJ, McMahon HT, Nicholls DG (1989) Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca^{2+} and glutamate release. J Neurochem 53:1693–1699.

Trapp B, Nave K-A (2008) Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci 31:247–269.

Vercellino M, Merola A, Piacentino C, Votta B, Capello E, Mancardi GL, Mutani R, Giordana MT, Cavalla P (2007) Altered glutamate reuptake in relapsing-remitting and secondary progressive multiple sclerosis cortex: correlation with microglia infiltration, demyelination, and neuronal and synaptic damage. J Neuropathol Exp Neurol 66:732–739.

Vereker E, O'Donnell E, Lynch MA (2000) The inhibitory effect of interleukin-1β on long-term potentiation is coupled with increased activity of stress-activated protein kinases. J Neurosci 20: 6811–6819.

Vilcaes AA, Furlan G, Roth GA (2009) Inhibition of Ca^{2+}-dependent glutamate release in cerebral cortex synaptosomes of rats with experimental autoimmune encephalomyelitis. J Neurochem 108: 881–890.

Zhang N, Wei W, Mody I, Houser CR (2007) Altered localization of $GABA_A$ receptor subunits on dentate granule cell dendrites influences tonic and phasic inhibition in a mouse model of epilepsy. J Neurosci 27:7520–7531.

(Accepted 31 August 2011)
(Available online 17 September 2011)