Pharmacology, Biochemistry and Behavior 97 (2010) 350–356

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Inhibitory effects of alprazolam on the development of acute experimental autoimmune encephalomyelitis in stressed rats

María J. Núñez-Iglesias, Silvia Novío, Antonio Almeida-Dias, Manuel Freire-Garabal *

Lennart Levi Stress and Neuroimmunology Laboratory, Department of Pharmacology, School of Medicine and Nursing, University of Santiago de Compostela, C/San Francisco, s/n.15782 Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 15 May 2010
Received in revised form 27 August 2010
Accepted 6 September 2010
Available online 15 September 2010

Keywords:
Anxiety
Anxiolitic benzodiazepine agonist
Autoimmmune nervous system disease
Corticosterone
Experimental autoimmune encephalomyelitis

ABSTRACT

The progression and development of multiple sclerosis (MS) has long been hypothesized to be associated with stress. Benzodiazepines have been observed to reduce negative consequences of stress on the immune system in experimental and clinical models, but there are no data on their effects on MS, or experimental autoimmune encephalomyelitis (EAE), a model for human MS. We designed experiments conducted to ascertain whether alprazolam could modify the clinical, histological and neuroendocrine manifestations of acute EAE in Lewis rats exposed to a chronic auditory stressor. EAE was induced by injection of an emulsion of MBP and complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37Ra. Stress application and treatment with drugs (placebo or alprazolam) were initiated 5 days before inoculation and continued daily for the duration of the experiment (days 14 or 34 postinoculation).Our results show significant increases in the severity of neurological signs, the histological lesions of the spinal cord (inflammation), and the corticosterone plasmatic levels in stressed rats compared to those non-stressed ones. Treatment with alprazolam reversed the adverse effects of stress. These findings could have clinical implications in patients suffering from MS treated with benzodiazepines, so besides the psychopharmacological properties of alprazolam against stress, it has beneficial consequences on EAE.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE), a heterogeneous inflammatory, autoimmune, demyelinating, and neurodegenerative disease of the central nervous system (CNS) mediated by autoreactive T lymphocytes directed against the neuroantigen, myelin basic protein (MBP), is widely considered as an animal model for human multiple sclerosis (Dowdell et al., 1999; Liu et al., 2007). EAE is extremely well characterized in terms of clinical and histopathologic abnormalities and it is most often studied in rat and mouse models. So, EAE is inducible in the Lewis rat, which exhibits an acute monophasic disease, and in selected mouse strains, which show a remitting–relapsing or chronic course of paralysis (Whitacre et al., 1998).

Current research evidence suggests that interactions between genetic and environmental factors contribute to modulate the susceptibility to degenerative disorders, including inflammatory and autoimmune diseases of the CNS (Marchetti et al., 2001). In this context, it has been hypothesized that disease onset (Welsh et al., 2009) and progression (Gold et al., 2005) and clinical exacerbation (Mohr et al., 2004; Welsh et al., 2009) in multiple sclerosis (MS), are associated with stressful life events, being more important than the severity of stressors in relation to MS relapse risk (Brown et al., 2006). However, a controversial issue in MS research concerns the extent to which psychological stress contributes to the development of the disorder (Ackerman et al., 2002). Several studies have suggested that stress is a potent trigger for MS disease activity (Ackerman et al., 2003; Golan et al., 2008; Grant et al., 1989; Mohr et al., 2002; Sibley, 1997; Stip and Truelle, 1994; Warren et al., 1982), but other studies either have failed to confirm this relationship (Pratt, 1951) or have shown a protective role for stress (Nisipeanu and Korczyn, 1993; Sibley et al., 1991).

The prescription of benzodiazepines to manage the symptoms of MS, such as the spasticity associated with anxiety (Rode et al., 2003) and catatonic features (Hung and Huang, 2007), is common. Nevertheless, to date, there are no data on the effects of these compounds on the development of EAE. In order to further elucidate this relationship, we conducted pharmacological studies in vivo examining the effects of alprazolam, a benzodiazepine anxiolytic drug, on the development of EAE in Lewis rats exposed to noxious stimulation. The aim of this work is to ascertain whether alprazolam could modify the clinical, pathological and neuroendocrine manifestations of acute EAE in stressed Lewis rats.

* Corresponding author. Department of Pharmacology, School of Medicine, University of Santiago de Compostela, C/San Francisco, s/n. 15782 Santiago de Compostela, Spain. Tel.: +34 677 910 037; fax: +34 981 573 191.
E-mail addresses: snl@usc.es (M.J. Núñez-Iglesias), snl@usc.es (S. Novío), snl@usc.es (A. Almeida-Dias), snl@usc.es, manuel.freire-garabal@usc.es (M. Freire-Garabal).

0091-3057/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2010.09.002

2. Methods

2.1. Animals

Male Lewis rats (250 to 300 g; Charles River S.A., Barcelona, Spain) were housed in a standard animal facility, allowed free access to food and water. All the experimental protocols were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by Institutional Animals Ethics Committee. In addition, all efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Procedure

Rats were divided in two series: SERIES A: animals receiving complete Freund's adjuvant (CFA; no EAE), SERIES B: rats with EAE. In each of both series, rats were divided into five groups according to the psychopharmacological treatment they were submitted to: GROUP 1: unstressed controls ($n=4$); GROUP 2: unstressed rats injected with placebo ($n=4$); GROUP 3: unstressed rats injected with alprazolam ($n=4$); GROUP 4: stressed rats injected with placebo ($n=4$); and GROUP 5: stressed rats injected with alprazolam ($n=4$).

2.3. Induction of EAE

Rats were immunized in each hind foot with a mixture of purified MBP (final concentration 1 mg/ml) isolated from guinea pig brain (Sigma Chemical Company, St. Louis, MO, USA) emulsified in complete Freund's adjuvant (MBP-CFA) containing *Mycobacterium tuberculosis* H37Ra (final concentration 5 mg/ml; Difco Laboratories, Detroit, Michigan) in a volume of 50 µl (Ahmed et al., 2001).

2.4. Assessment of clinical EAE

Animals were monitored daily for clinical signs of EAE and scored blind by the same observer using the following criteria: 0 = normal; 0.5 = loss of tonicity in distal half of tail; 1 = piloerection; 2 = loss in tail tonicity; 3 = hind leg paralysis; 4 = paraplegia; and 5 = moribund (Stanislaus et al., 2001).

2.5. Assessment of histological EAE

Animals were sacrificed by anesthetic overdose on days 14 or 34 post-immunization (PI; 4 animals per group) and their cervical spinal cords were examined by light microscopy. Spinal tissue was selected for analysis because a heavy lesion load can be guaranteed in this CNS area during the onset of acute EAE (Bolton and Paul, 1997). The upper 1.5 cm of tissue was dissected and snap frozen. Cervical cord sections were cut at 5-µm thickness, at a standard depth and stained with haematoxylin and eosin or with anti-MBP polyclonal antibodies (Sigma). For MBP immunostaining, the peroxidase/anti-peroxidase method was applied. Histological severity of inflammation was scored in a blind fashion from 0 to 4: 0, no inflammatory cells; 1, leptomeningeal and adjacent subpial cell infiltration; 2, mild perivascular cuffing; 3, extensive perivascular cuffing; and 4, extensive perivascular cuffing plus severe parenchymal cell infiltration. Demyelination in the spinal cord was also scored according to the following grading system: 0, no demyelination; 0.5, traces of perivascular or subpial demyelination; 1, confluent perivascular or subpial demyelination; 2, massive perivascular or subpial demyelination (e.g. one-half the cross-section of spinal cord); and 3, extensive demyelination (transverse myelitis) (Tanuma et al., 2000).

2.6. Corticosterone assay

Circulating corticosterone levels in rats were also determined to exclude the possibility that treatment regimens enhanced endogenous steroid levels which are known to influence the course of EAE (MacPhee et al., 1989). Blood was collected from rats sacrificed on day 34 PI and assayed for corticosterone as described by Zenker and Bernstein (1958). The standard curve is plotted and the amount, A, in sample is read from that graph. The concentration, C, was obtained by the formula: $C = A \times 100/V$, where V is the number of ml of plasma used. The results were expressed as µg/100 ml (mean ± S.E.M.).

2.7. Stress procedure

Noise was produced by a loud speaker (15 W), installed at a distance of 30 cm above the cage, and driven by a white noise generator emitting all the frequencies in the range 0–20 kHz. A precision sound level meter was used to set the intensity of sound to 100 dB uniformly in the cage. The rats were subjected to a broad band noise at 100 dB daily for 5 s every minute during (at random) either a 1- or 3-hour period around midnight, at the height of the diurnal activity cycle (Monjan and Collector, 1977), starting 5 days prior to the inoculation and continuing throughout the experiment. Unstressed rats were exposed only to the normal activity of the animal room.

2.8. Drug treatments

Alprazolam (1 mg/Kg of body weight) (Pharmacia & Upjohn, MI, USA) was intraperitoneally (i.p.) injected in a volume of 1 ml/kg of 1% aqueous solution of carboxymethylcellulose as vehicle. The same volume of diluent was used as placebo. Drugs were daily administered at 9.30 p.m. Treatment was initiated 5 days before immunization and continuing throughout the experiment.

2.9. Statistical analysis

Statistical analysis was performed using an ANOVA with grouping of means by Student Newman–Keuls multiple range tests (SPSS 15.0). Differences were considered to be significant when the probability (P) value was <0.05.

3. Results

3.1. Clinical evaluation

Clinical EAE signs appeared on day 6 PI being more severe in injected rats with placebo or alprazolam than in control ones. All control rats exhibited neurological signs of EAE between days 13 and 17 after immunization, by which time control rats had almost completely recovered from those. The general sequence of events following the sensitization, is characterized 13 days later by a monophasic ascending paralysis, followed by recovery within 3 days. As illustrated in Fig. 1a, acoustic stress increased the severity of neurological signs and alprazolam partially reversed that adverse effect. Besides, the onset of severe neurological signs (grade 5) was significantly delayed by treatment of stressed rats with alprazolam ($p<0.05$) and disappeared significantly earlier ($p<0.01$) compared to the stressed animals injected with placebo.

The results in Fig. 1b showed that the daily injection placebo or alprazolam exerted a stressor effect in unstressed rats. This observation is consistent with the observation that all unstressed rats injected with alprazolam showed no detectable neurological signs from day 34 PI whereas not unstressed rats injected with placebo.

Given that, chronic stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, which includes an increased visceral adiposity and decreased lean body (bone and muscle mass) mediated by glucocorticoids, not only has profound inhibitory effects on growth hormone and sex steroid production, but also antagonizes the actions of these hormones on fat tissue catabolism (lipolysis), and muscle and

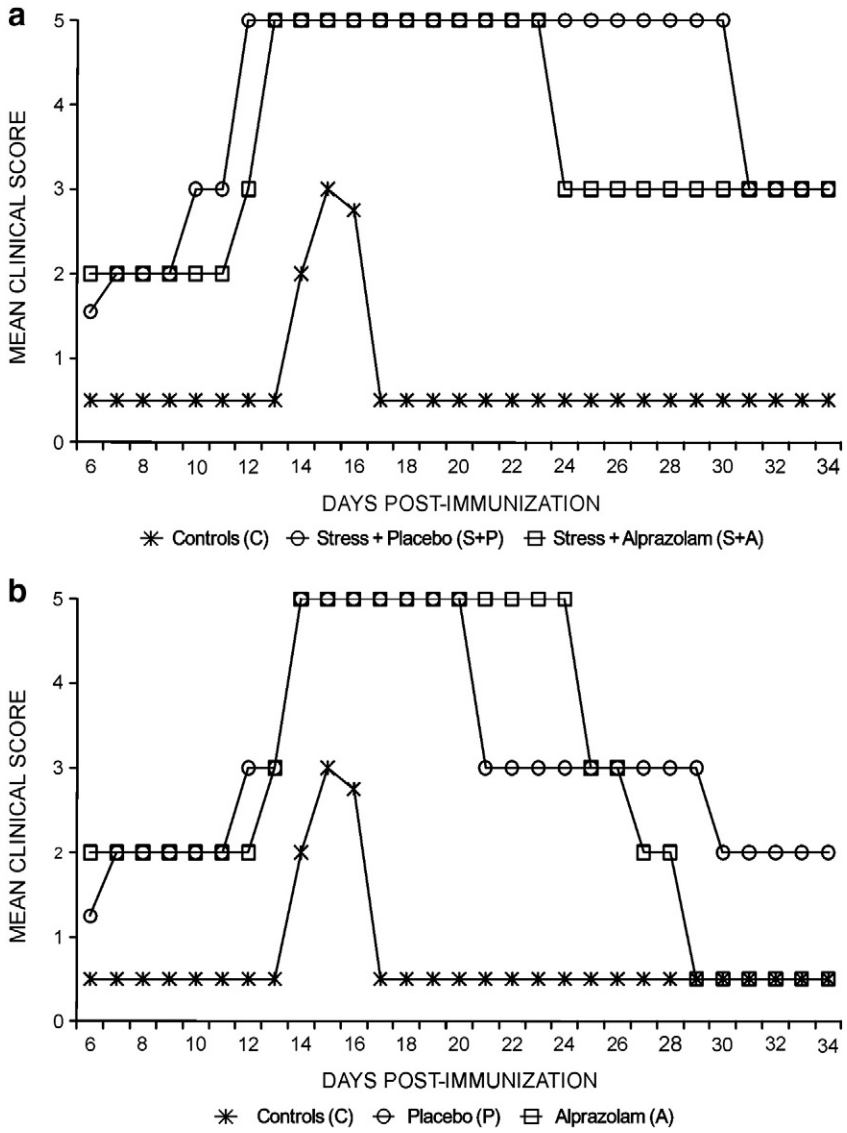

Fig. 1. Effects of alprazolam on neurological status of stressed (a) and non-stressed (b) EAE-induced Lewis rats. Animals were monitored daily for clinical signs of EAE using the following criteria: 0 = normal; 0.5 = loss of tonicity in distal half of tail; 1 = piloerection; 2 = loss in tail tonicity; 3 = hind leg paralysis; 4 = paraplegia; and 5 = moribund. Differences between P and S + P were significant on days 12–13 and 21–30. Differences between A and S + A were significant on days 13, 24 and 29–34. Differences between S + P and S + A were significant on days 12 and 24–30. Differences between P and A were significant on days 21–24 and 29–34.

bone anabolism (Chrousos, 2000), we have assessed body weight from the beginning of the study (5 days before inoculation) (Fig. 2). It can be seen from Fig. 2a, at the onset phase acoustic stress, that the body weight decreased significantly ($p<0.01$) in comparison to control rats. Alprazolam treatment in stressed rats reduced significantly ($p<0.05$) the body weight loss compared to stressed rats injected with placebo. No significant difference was observed neither between control rats and non-stressed animals injected with placebo nor between non-stressed rats injected with placebo or alprazolam (Fig. 2b).

3.2. Pathological examination

The results are summarized in Figs. 3 and 4. Inflammatory lesions characterized by perivascular infiltration of inflammatory cells were observed in the spinal cord of all animals. The perivascular inflammatory infiltrate was significantly higher in the stressed animals injected with placebo as compared to the control group and the stressed rats injected with alprazolam. None of the groups immunized with MBP-CFA developed significant demyelinating lesions (data not shown), as we have previously reported (Nuñez et al., 2007).

3.3. Corticosterone assay

Stressed rats injected with placebo had significantly ($p<0.01$) higher levels of corticosterone than controls. Corticosterone levels appeared significantly decreased ($p<0.01$) in stressed rats injected with alprazolam compared with those of stressed rats injected with placebo, but still remained significantly ($p<0.01$) elevated compared to controls (Table 1). Since exposure to stress activates the HPA axis, which is associated with corticosterone increase and EAE severe clinical signs (Bernard et al., 1992), and treatment with anxiolytic benzodiazepine agonist reduces corticosterone in stressed rats (Bizzi et al., 1984; Owens et al., 1989), studies were carried out to determine if corticosterone plasmatic levels could be correlated with clinical and histological scores and whether the attenuation of stress-induced corticosterone by alprazolam could be correlated with reduced clinical and histological scores. Pearson's correlation coefficients

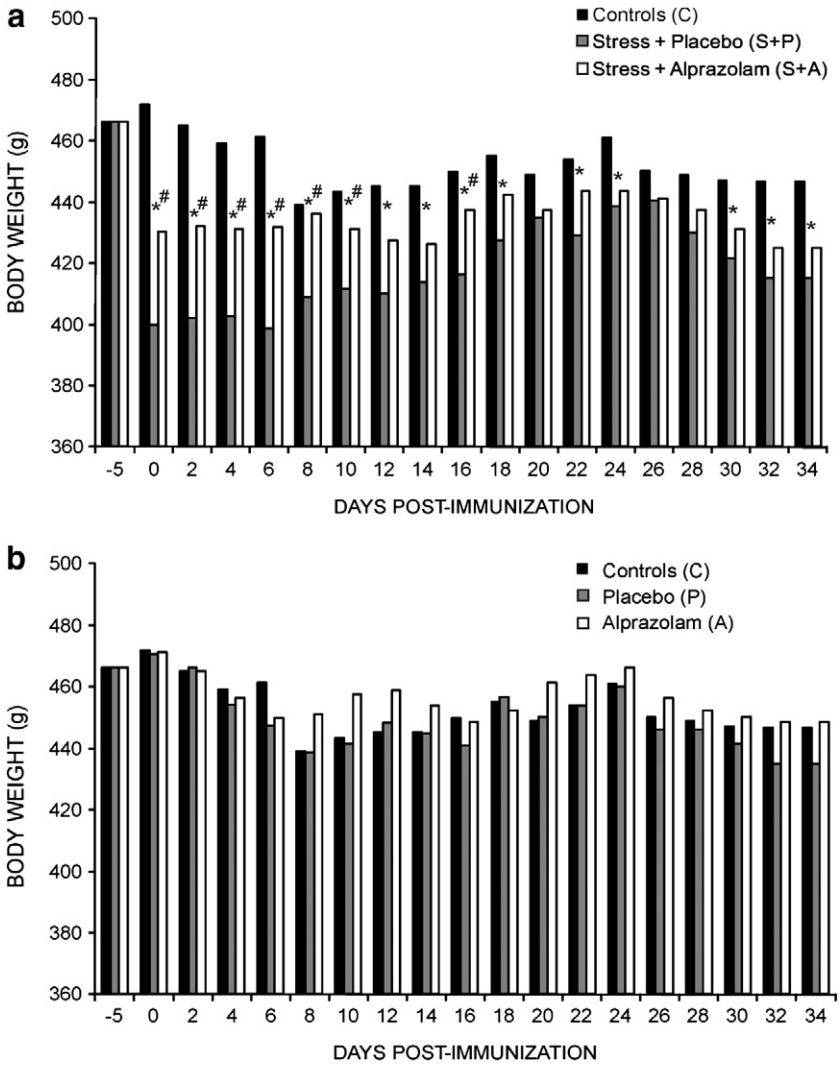

Fig. 2. Effects of stress on body weight of EAE-induced Lewis rats throughout the 34 days of treatment. Values are mean score of 4 animals. Significant differences with respect to the body weight were noted between control rats and animals injected with placebo (*$p<0.05$). Significant differences with respect to the body weight were noted between rats injected with placebo and alprazolam (#$p<0.05$).

among the parameters mentioned previously showed a statistically positive correlation (Table 2).

4. Discussion

The presented data show that sound stress increased EAE severity in Lewis rats. We observed an enhancement of the clinical signs and the perivascular inflammatory infiltrate of EAE besides increased corticosterone levels in stressed rats relative to unstressed animals. These adverse effects were partially reversed by alprazolam, which reduced the latent period and histological lesions of the spinal cord and proportionally suppressed the stress-induced increase in corticosterone plasmatic levels.

Preclinical research in EAE is merely just exploratory and, currently, none of the described animal models of MS has been able to reproduce the whole spectrum found in human disease. Nevertheless, the suitably adapted applications of the EAE model have contributed to: 1) a better understanding of the pathology of MS; 2) creating better biomarkers for its diagnosis and prognosis; and 3) developing improved and safe therapies for this disease (Steinman and Zamvil, 2006). New therapies for treatment of MS have been approved from the US Food and Drug Administration (glatiramer acetate, mitoxantrone and natalizumab) (Steinman and Zamvil, 2006), and experiments are constantly demonstrating new targets for potential treatments of this disease (Lock et al., 2002). Since MS is a complicated disease with many symptoms (anxiety, pain, depression, etc.), combinations of therapies might be tried in the EAE models to search for potential synergies between drugs and unexpected adverse drug interactions, which should highlight the importance of multidisciplinary care interventions that target disease symptoms control.

Numerous triggers of MS have been proposed, including bacterial or viral infections, bacterial superantigens, physical injury, or stressful life events (Mohr et al., 2004). Of these, the role of psychological stress had been by far the most controversial (Ackerman et al., 2003; Golan et al., 2008; Gold et al., 2005; Grant et al., 1989; Mohr et al., 2002; Nisipeanu and Korczyn, 1993; Pratt, 1951; Sibley, 1997; Sibley et al., 1991; Stip and Truelle, 1994; Warren et al., 1982). This discrepancy may had been the result of a number of research design problems, including infrequent monitoring of patients, subjective reporting bias, different patient groups, lack of adequate controls and different outcome parameters. However, there is also evidence to show that the relationship between MS and stressful life events is complex and may rely on one or more factors such as stressor chronicity, frequency, severity and type (Brown et al., 2005). So, whereas traumatic stressors reduce the risk of exacerbations (Nisipeanu and Korczyn, 1993), more moderate stressors have been shown to trigger the disease activity (Ackerman et al., 2002). The findings of Nisipeanu and Korczyn's

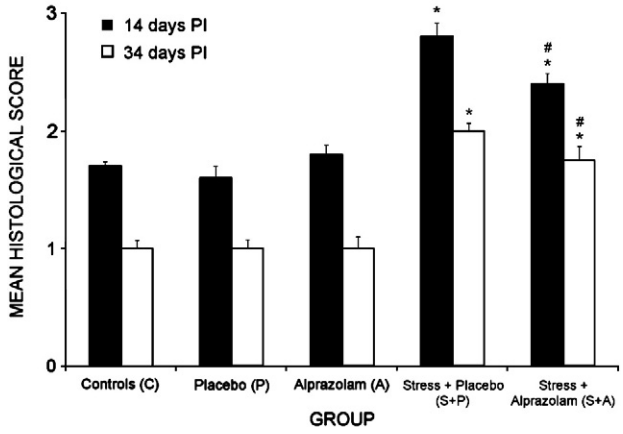

Fig. 3. Histopathological analysis of EAE on days 14 and 34 PI (4 animals per group). Histological severity of inflammation was scored in a blind fashion from 0 to 4: 0, no inflammatory cells; 1, leptomeningeal and adjacent subpial cell infiltration; 2, mild perivascular cuffing; 3, extensive perivascular cuffing; and 4, extensive perivascular cuffing plus severe parenchymal cell infiltration. None of the mean scores for unstressed rats injected with alprazolam or placebo were statistically different ($p>0.05$) from those of controls. Stressed rats injected with placebo showed the mean histological score significantly higher ($^{*}p<0.01$) in comparison to the control rats. In contrast, the mean histological score for stressed rats injected with alprazolam was significantly ($\#p<0.01$) lower than those of the stressed animals injected with placebo, but still remained significantly ($^{*}p<0.01$) elevated compared to controls.

Table 1
Effects of alprazolam chronic treatment on corticosterone plasmatic levels 34 days after immunization in stressed Lewis rats.

Groups	Corticosterone levels
Controls	38.66 ± 1.86
Placebo	40.38 ± 1.06
Alprazolam	40.08 ± 1.08
Stress + Placebo	48.28 ± 1.30[a]
Stress + Alprazolam	43.42 ± 0.56[a,b]

Corticosterone was assayed as described in Methods section. The results are expressed as the mean of triplicate determinations (μg/100 ml ± S.E.M.).
[a] Significant difference from control rats ($p<0.01$).
[b] Differences between placebo and alprazolam significant at $p<0.01$.

study (Nisipeanu and Korczyn, 1993) had a huge repercussion in that moment, because they contradicted most of studies which supported the contribution of psychological stress to the activity of MS. Nevertheless, in the present date, several authors agree that this discrepancy might be due to research design problems (Golan et al., 2008; Goodin, 2008). Therefore, the small number of patients studied, the lack of a measure of subjective stress, the type of statistical analysis used (simple regression to the mean), etc. have limited the reliability of the findings in that study.

Regarding EAE models, there is also a controversy on the effects of stress on EAE in rodents, depending on the type of the stressor and time of administration. Other experimental variables such as sex, strain, time of day, and the kinetics of immune response development are also important on the effect of stress on EAE (Griffin et al., 1993). The immersion of rats for 5 min in a water bath (44 °C) for 10 or 13 days after the immunization diminished the severity of clinical EAE and delayed the onset of disease. Nevertheless, if stress was administered prior to immunization, this resulted in the marginal suppression of clinical EAE (Owhashi et al., 1997). In the same way, restraint stress administered prior to neuroantigen immunization suppressed murine relapsing EAE (Dowdell et al., 1999) and electric stress, administered during 19 days after immunization, but not during 19 days before, suppressed the appearance and development of EAE (Bukilica et al., 1991). Curiously, investigation of the mechanism underlying the stress-induced suppression of EAE (Griffin et al., 1993) revealed that restraint stress did not alter the clinical course of EAE in rats challenged with MBP 68–88 encephalitogenic peptide, suggesting that restraint stress may affect processing and/or presentation of the MBP molecule. In relation to sound stress, it is known that it can delay the onset of the disease (Bukilica et al., 1991) or increase the severity of EAE when administered neonatally (Dimitrijevic et al., 1995).

Activation of the inflammatory immune system might affect many neuroendocrine and central neurotransmitter processes provoked by stressors and vice versa (Anisman, 2009). Studies in MS and its animal models have recently shown disruptions in the communication between the immune system and the two major stress–response systems, the HPA axis and the autonomic nervous system, playing the functional status of the HPA axis a relevant role in the control of EAE (Gold et al., 2005). During the experimentally induced disease in Lewis rats, the endogenous levels of glucocorticoids are elevated being the recovery from the disease clearly dependent on this endocrine change (MacPhee et al., 1989). This endocrine response is immunologically mediated so, it is mainly the result of the stimulation of the HPA axis by cytokines (such as IL-1) produced during the immune response that induces the autoimmune disease (Del Rey et al., 1998). In EAE models, the negative feedback system mediated via the glucocorticoid receptors seems to be disturbed (Gold et al., 2005), favoring the stressors the perpetuation of this dysregulation, as it was shown by increased corticosterone levels in stressed rats relative to unstressed animals. The clinical relevance of an increased HPA axis activity is supported by the observation that this phenomenon is related to the clinical disease course (Then Bergh et al., 1999). Since benzodiazepines, especially alprazolam, possess a clear inhibitory influence on the activity of the HPA axis (Arvat et al., 2002; Bizzi et al., 1984; Owens et al., 1989), we evaluated the efficacy of alprazolam to modify the manifestations of acute EAE in stressed

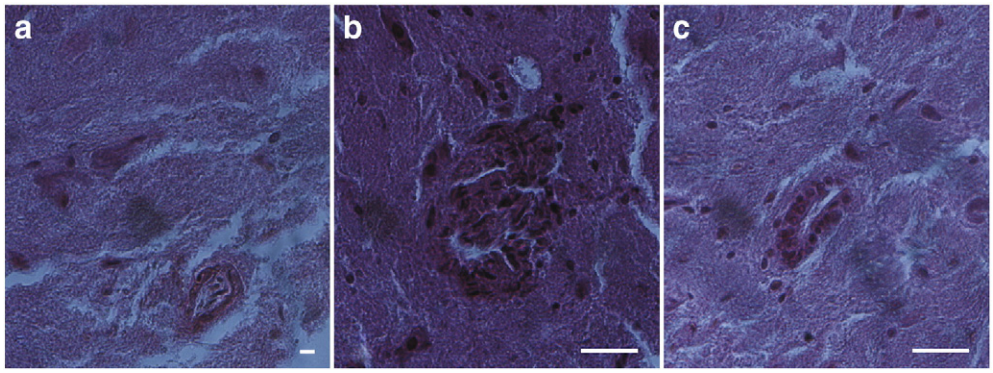

Fig. 4. Histopathological analysis of EAE. (a) Control rats, (b) stressed rats injected with placebo, and (c) stressed rats injected with alprazolam. Stressed rats injected with alprazolam showed less pronounced inflammatory infiltrate in comparison with placebo rats (data in Fig. 3). Bar = 100 μm.

Table 2
Pearson's correlation coefficient among diagnostic parameters in the EAE.

	Corticosterone	Inflammatory infiltrate	Neurological signs	Weight
Corticosterone		0.8707[a]	0.7141[a]	0.8463[a]
Inflammatory infiltrate	0.8707[a]		0.8346[b]	−0.7901[b]
Neurological signs	0.7141[a]	0.8346[b]		−0.9524[a]
Weight	0.8463[a]	−0.7901[b]	−0.9524[a]	

Using the data obtained on 34 PI day, the clinical signs to corticosterone plasmatic levels correlation and the histological signs to corticosterone plasmatic levels correlation were calculated using the Pearson's correlation coefficient, r.
[a] Significant correlation ($p<0.01$).
[b] Significant correlation ($p<0.05$).

Lewis rats. Several mechanisms could explain the effect of alprazolam observed in the present study. First, both in vivo and in vitro studies have shown that the GABA-benzodiazepine-chloride ionophore receptor complex is modulated by corticosterone levels (Acuña et al., 1990) and conversely that HPA axis hormones, including CRF, ACTH and corticosterone, are modulated by GABA and benzodiazepines (Arvat et al., 2002). It has been reported that anxiolytics reduce the response of CRF, ACTH and corticosterone to stress (Arvat et al., 2002; Bizzi et al., 1984; Owens et al., 1989). It could be due to the suppression of the stress-induced increase in plasma ACTH levels which were attributed to activation of GABA-linked benzodiazepine receptors in the CNS. Central pharmacological effects related to central type benzodiazepine receptors acting by facilitating inhibitory GABA neurotransmission in the CNS, may regulate the release of neuroendocrine hormones involved in the immune response to stress. A second mechanism for the conferred EAE attenuation in stressed rats by alprazolam could be its potent platelet activating factor (PAF) antagonist properties. Some findings indicate that activation and control of the coagulation cascade, modulated by antigen-specific mediators of cellular immunity, appear to be of prime importance in the EAE (Inoue et al., 1996). Susceptibility and resistance to EAE in rodents correlate with the induction of procoagulant and anticoagulant activities. Anticoagulants produced by cells from non-susceptible EAE rodents suppress the common coagulation pathway by inhibiting trombin and factor Xa activities (Geczy et al., 1984). It was found that in washed human platelets the alprazolam potently inhibited PAF-induced changes in shape, aggregation, and secretion, being the effects specific for PAF activation (Kornecki et al., 1984). Ng and Wong (1988) also showed that alprazolam could inhibit the [^{3}H]PAF binding to the human peripheral blood mononuclear leukocytes. An interesting possibility raised by Bernardini et al. (1989) suggests that PAF plays a role in the activation of the HPA axis and glucocorticoid secretion and can serve as a mediator in the interactions of the immune system with the CNS. PAF is a stimulator of the HPA axis in the rat which causes significant stimulation of hypothalamic CRH, pituitary ACTH and adrenal corticosterone secretion, which is inhibited by alprazolam. In addition, the PAF stimulates ACTH secretion by dispersed rat pituicytes which are also inhibited by the alprazolam (Bernardini et al., 1989). The specific antagonism of PAF action by psychotropic drugs suggests that PAF or PAF-like phospholipids may play a role in neuronal function (Kornecki et al., 1987).

Besides the mechanisms previously described, downstream effects of the alprazolam on immunological and inflammatory parameters important for EAE must be underscored. Antigen-specific T cells constitute only a small proportion of infiltrating leukocytes in EAE or MS lesions (Cross et al., 1990). Secondarily recruited inflammatory cells account for the vast majority of infiltrating cells and play a pivotal role in CNS tissue damage (Ransohoff, 1999). Although the detailed mechanisms by which inflammatory cells enter the CNS compartment are not completely understood, increasing evidence suggests that cytokines are essential for this process (Karpus and Ransohoff, 1998). Enhanced expression of proinflammatory cytokines in the CNS, such as the monocyte chemoattractant protein 1 (MCP-1), has been demonstrated both in EAE models (Juedes et al., 2000) and in human case series (Simpson et al., 1998), having showed that the severity of relapsing EAE is reduced by anti-MCP-1 antibodies (Karpus and Kennedy, 1997). Additionally, mice that lack C–C chemokine receptor 2 (CCR2), the major receptor on monocytes for MCP-1, fail to develop EAE after active immunization (Fife et al., 2000) and are resistant to the induction of EAE by the adoptive transfer of primed T cells from syngenic wild-type mice (Izikson et al., 2000). The effect of alprazolam on the expression levels of cytokines has been scantily studied (Chang et al., 1992; Oda et al., 2002). Oda et al. (2002) have noted a potent inhibitory activity of this benzodiazepine on IL-1alpha-elicited MCP-1 production in T98G cells. Likewise, alprazolam inhibits the production of cytokines IL-1beta and JE in LPS-stimulated mouse macrophage cells (Oda et al., 2002) and reduces the production of IL-2 by murine splenic T cells (Chang et al., 1992). These findings suggest that alprazolam might prevent the infiltration of determinate regions by an excess of proinflammatory cytokines, i.e. to inhibit c-Rel-associated immunity and inflammation-related substances. Since the excess production of proinflammatory cytokines exacerbates MS or EAE (Karpus and Ransohoff, 1998), the previously described action of alprazolam might explain the improvement of manifestations associated with EAE in rats treated with alprazolam, compared to animals injected with placebo.

Our data at present show the beneficial effects of alprazolam on the development of EAE. However, dissecting the molecular mechanisms underlying these observations is extraordinarily difficult because they are intermixed with variable contributions of environmental, genetic, age, gender and reproductive factors. EAE is a good tool for studying basic mechanisms of brain inflammation and immune-mediated CNS tissue injury, and for obtaining proof of principle. Whether a certain drug has the potential to block or enhance these pathways, and they are relevant for MS patients in general, the subpopulation of patients has to be determined in respective clinical studies (Steinman and Zamvil, 2006). Nevertheless, our data about the effects of alprazolam on the development of EAE might have important clinical implications in MS patients because benzodiazepines are used for the treatment of catatonic features and spasticity associated with anxiety in these patients.

Acknowledgement

We thank Isabel Tarrío for the technical help.

References

Ackerman KD, Heyman R, Rabin BS, Anderson BP, Houck PR, Frank E, et al. Stressful life events precede exacerbations of multiple sclerosis. Psychosom Med 2002;64:916–20.
Ackerman KD, Stover A, Heyman R, Anderson BP, Houck PR, Frank E, et al. Relationship of cardiovascular reactivity, stressful life events, and multiple sclerosis disease activity. Brain Behav Immun 2003;17:141–51.
Acuña D, Fernandez B, Gomar MD, del Aguila CM, Castillo JL. Influence of the pituitary-adrenal axis on benzodiazepine receptor binding to rat cerebral cortex. Neuroendocrinology 1990;51:97-103.
Ahmed Z, Gveric D, Pryce G, Baker D, Leonard JP, Cuzner ML, et al. Myelin/axonal pathology in interleukin-12 induced serial relapses of experimentalallergic encephalomyelitis in the Lewis rat. Am J Pathol 2001;158:2127–38.
Anisman H. Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. J Psychiatry Neurosci 2009;34:4-20.
Arvat E, Giordano R, Grottoli S, Ghigo E. Benzodiazepines and anterior pituitary function. J Endocrinol Invest 2002;25:735–47.
Bernard CCA, Mandel TE, Mackay IR. Experimental models of human autoimmune disease: overview and prototypes. In: Rose N, Mackay IR, editors. The Autoimmune Diseases II. London: Academic Press; 1992. p. 47-106.
Bernardini R, Calogero AE, Ehrlich YH, Brucke T, Chrousos GP, Gold PW. The alkyl-ether phospholipid platelet-activating factor is a stimulator of the hypothalamic-pituitary-adrenal axis in the rat. Endocrinology 1989;125:1067–73.
Bizzi A, Ricci MR, Veneroni E, Amato M, Garattini S. Benzodiazepine receptor antagonists reverse the effect of diazepam on plasma corticosterone in stressed rats. J Pharm Pharmacol 1984;36:134–5.
Bolton C, Paul C. MK-801 limits neurovascular dysfunction during experimental allergic encephalomyelitis. J Pharmacol Exp Ther 1997;282:397–402.

Brown RF, Tennant CC, Dunn SM, Pollard JD. A review of stress–relapse interactions in multiple sclerosis: important features and stress-mediating and -moderating variables. Mult Scler 2005;11:477–84.
Brown RF, Tennant CC, Sharrock M, Hodgkinson S, Dunn SM, Pollard JD. Relationship between stress and relapse in multiple sclerosis: part II. Direct and indirect relationships. Mult Scler 2006;12:465–75.
Bukilica M, Djordjevic S, Maric I, Dimitrijevic M, Markovic BM, Jankovic BD. Stress-induced suppression of experimental allergic encephalomyelitis in the rat. Int J Neurosci 1991;59:167–75.
Chang MP, Castle SC, Norman DC. Mechanism of immunosuppressive effect of alprazolam: alprazolam suppresses T-cell proliferation by selectively inhibiting the production of IL2 but not acquisition of IL2 receptor. Int J Immunopharmacol 1992;14:227–37.
Chrousos GP. The role of stress and the hypothalamic-pituitary-adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes. Int J Obes Relat Metab Disord 2000;24:S50–5.
Cross AH, Cannella B, Brosnan CF, Raine CS. Homing to central nervous system vasculature by antigen-specific lymphocytes. I. Localization of 14C-labeled cells during acute, chronic, and relapsing experimental allergic encephalomyelitis. Lab Invest 1990;63:162–70.
Del Rey A, Klusman I, Besedovsky HO. Cytokines mediate protective stimulation of glucocorticoid output during autoimmunity: involvement of IL-1. Am J Physiol 1998;275:R1146–51.
Dimitrijevic M, Markovic BM, Laban O, Jankovic BD. Suppression of experimental allergic encephalomyelitis in offspring of DA and Wistar rats following immunization of mother with encephalitogen. J Neuroimmunol 1995;58:43–50.
Dowdell KC, Gienapp IE, Stuckman S, Wardrop RM, Whitacre CC. Neuroendocrine modulation of chronic relapsing experimental autoimmune encephalomyelitis: a critical role for the hypothalamic-pituitary-adrenal axis. J Neuroimmunol 1999;100:243–51.
Fife BT, Huffnagle GB, Kuziel WA, Karpus WJ. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. J Exp Med 2000;192: 899–905.
Geczy CL, Roberts IM, Meyer P, Bernard CC. Susceptibility and resistance to experimental autoimmune encephalomyelitis and neuritis in the guinea pig correlate with the induction of procoagulant and anticoagulant activities. J Immunol 1984;133:3026–36.
Golan D, Somer E, Dishon S, Cuzin-Disegni L, Miller A. Impact of exposure to war stress on exacerbations of multiple sclerosis. Ann Neurol 2008;64:143–8.
Gold SM, Mohr DC, Huitinga I, Flachenecker P, Sternberg EM, Heesen C. The role of stress–response systems for the pathogenesis and progression of MS. Trends Immunol 2005;26:644–52.
Goodin DS. The impact of war-stress on MS exacerbations. Ann Neurol 2008;64:114–5.
Grant I, Brown GW, Harris T, McDonald WI, Patterson T, Trimble MR. Severely threatening events and marked life difficulties preceding onset or exacerbation of multiple sclerosis. J Neurol Neurosurg Psychiatry 1989;52:8-13.
Griffin AC, Lo WD, Wolny AC, Whitacre CC. Suppression of experimental autoimmune encephalomyelitis by restraint stress: sex differences. J Neuroimmunol 1993;44: 103–16.
Hung YY, Huang TL. Lorazepam and diazepam for relieving catatonic features in multiple sclerosis. Prog Neuropsychopharmacol Biol Psychiatry 2007;31:1537–8.
Inoue A, Koh CS, Shimada K, Yanagisawa N, Yoshimura K. Suppression of cell-transferred experimental autoimmune encephalomyelitis in defibrinated Lewis rats. J Neuroimmunol 1996;71:131–7.
Izikson L, Klein RS, Charo IF, Weiner HL, Luster AD. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. J Exp Med 2000;192:1075–80.
Juedes AE, Hjelmström P, Bergman CM, Neild AL, Ruddle NH. Kinetics and cellular origin of cytokines in the central nervous system: insight into mechanisms of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. J Immunol 2000;164:419–26.
Karpus WJ, Kennedy KJ. MIP-1alpha and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. J Leukoc Biol 1997;62:681–7.
Karpus WJ, Ransohoff RM. Chemokine regulation of experimental autoimmune encephalomyelitis: temporal and spatial expression patterns govern disease pathogenesis. J Immunol 1998;161:2667–71.
Kornecki E, Ehrlich YH, Lenox RH. Platelet-activating factor-induced aggregation of human platelets specifically inhibited by triazolobenzodiazepines. Science 1984;226:1454–6.
Kornecki E, Lenox RH, Hardwick DH, Bergdahl JA, Ehrlich YH. Interactions of the alkyl-ether-phospholipid, platelet activating factor (PAF) with platelets, neural cells, and the psychotropic drugs triazolobenzodiazepines. Adv Exp Med Biol 1987;221:477–88.
Liu T, Donahue KC, Hu J, Kurnellas MP, Grant JE, Li H. Identification of differentially expressed proteins in experimental autoimmune encephalomyelitis (EAE) by proteomic analysis of the spinal cord. J Proteome Res 2007;6:2565–75.
Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat Med 2002;8:500–8.
MacPhee IA, Antoni FA, Mason DW. Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J Exp Med 1989;169:431–45.
Marchetti B, Morale MC, Testa N, Tirolo C, Caniglia S, Amor S, et al. Stress, the immune system and vulnerability to degenerative disorders of the central nervous system in transgenic mice expressing glucocorticoid receptor antisense RNA. Brain Res Brain Res Rev 2001;37:259–72.
Mohr DC, Goodkin DE, Nelson S, Cox D, Weiner M. Moderating effects of coping on the relationship between stress and the development of new brain lesions in multiple sclerosis. Psychosom Med 2002;64:803–9.
Mohr DC, Hart SL, Julian L, Cox D, Pelletier D. Association between stressful life events and exacerbation in multiple sclerosis: a meta-analysis. BMJ 2004;328:731, doi: 10.1136/bmj.38041.724421.55.
Monjan AA, Collector MI. Stress-induced modulation of the immune response. Science 1977;196:307–8.
Ng DS, Wong K. Specific binding of platelet-activating factor (PAF) by human peripheral blood mononuclear leukocytes. Biochem Biophys Res Commun 1988;155:311–6.
Nisipeanu P, Korczyn AD. Psychological stress as risk factor for exacerbations in multiple sclerosis. Neurology 1993;43:1311–2.
Nuñez MJ, Balboa J, Rey-Méndez M, Brenlla J, González-Peteiro M, Rodrigo E, et al. Effects of amphetamine and cocaine on the development of acute experimental allergic encephalomyelitis in Lewis rats. Hum Exp Toxicol 2007;26:637–43.
Oda T, Ueda A, Shimizu N, Handa H, Kasahara T. Suppression of monocyte chemoattractant protein 1, but not IL-8, by alprazolam: effect of alprazolam on c-Rel/p65 and c-Rel/p50 binding to the monocyte chemoattractant protein 1 promoter region. J Immunol 2002;169:3329–35.
Owens MJ, Bissette G, Nemeroff CB. Acute effects of alprazolam and adinazolam on the concentrations of corticotropin-releasing factor in the rat brain. Synapse 1989;4: 196–202.
Owhashi M, Shouzui Y, Arita H. Stress down-regulates experimental allergic encephalomyelitis (EAE) but permits activation and localization of autoreactive V beta 8.2+ T cells. Int J Neurosci 1997;89:177–88.
Pratt RTC. An investigation of the psychiatric aspects of disseminated sclerosis. J Neurol Neurosurg Psychiatry 1951;14:326–36.
Ransohoff RM. Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines. J Neuroimmunol 1999;98:57–68.
Rode G, Maupas E, Luaute J, Courtois-Jacquin S, Boisson D. Medical treatment of spasticity. Neurochirurgie 2003;49:247–55.
Sibley WA. Risk factors in multiple sclerosis. In: Raine CS, McFarland HF, Tourtellotte WW, editors. Multiple Sclerosis: Clinical and Pathogenetic Basis. London: Chapman and Hall; 1997. p. 141–8.
Sibley WA, Bamford CR, Clark K, Smith MS, Laguna JF. A prospective study of physical trauma and multiple sclerosis. J Neurol Neurosurg Psychiatry 1991;54:584–9.
Simpson JE, Newcombe J, Cuzner ML, Woodroofe MN. Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. J Neuroimmunol 1998;84:238–49.
Stanislaus R, Singh AK, Singh I. Lovastatin treatment decreases mononuclear cell infiltration into the CNS of Lewis rats with experimental allergic encephalomyelitis. J Neurosci Res 2001;66:155–62.
Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol 2006;60: 12–21.
Stip E, Truelle JL. Organic personality syndrome in multiple sclerosis and effect of stress on recurrent attacks. Can J Psychiatry 1994;39:27–33.
Tanuma N, Shin T, Matsumoto Y. Characterization of acute versus chronic relapsing autoimmune encephalomyelitis in DA rats. J Neuroimmunol 2000;108:171–80.
Then Bergh F, Kümpfel T, Trenkwalder C, Rupprecht R, Holsboer F. Dysregulation of the hypothalamo-pituitary-adrenal axis is related to the clinical course of MS. Neurology 1999;53:772–7.
Warren S, Greenhill S, Warren KG. Emotional stress and the development of multiple sclerosis: case-control evidence of a relationship. J Chron Dis 1982;35:821–31.
Welsh CJ, Steelman AJ, Mi W, Young CR, Storts R, Welsh Jr TH, et al. Neuroimmune interactions in a model of multiple sclerosis. Ann NY Acad Sci 2009;1153:209–19.
Whitacre CC, Dowdell K, Griffin AC. Neuroendocrine influences on experimental autoimmune encephalomyelitis. Ann NY Acad Sci 1998;840:705–16.
Zenker N, Bernstein DE. The estimation of small amounts of corticosterone in rat plasma. J Biol Chem 1958;231:695–701.