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Glatiramer acetate reverts stress-induced alterations on adult neurogenesis and behavior. Involvement of Th1/Th2 balance

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ABSTRACT

Long-term exposure to stressful situations has deleterious effects on adult neurogenesis, behavior, and the immune system. We have previously shown that stressed BALB/c mice show poor learning performance, which correlates with an increase in the T helper 1/T helper 2 (Th1/Th2) cytokine balance. Glatiramer acetate (GA) can stimulate autoreactive T cells. In this work we investigated the effects of GA treatment on BALB/c mice exposed to chronic mild stress (CMS). Stressed mice exhibited a significant decline in their performance in the open field and Y-maze tasks, which was accompanied by a reduction in dentate gyrus neurogenesis and an altered Th1/Th2 balance. Interestingly, after 6 weeks of CMS exposure administration of GA reestablished normal levels of adult neurogenesis, restored the Th1/Th2 balance, and improved learning performance. These results demonstrate that GA treatment can reverse the learning impairment induced by stress through a mechanism that likely involves the regulation of the cytokine balance and adult neurogenesis.

1. Introduction

Stress is defined as any situation capable of perturbing the physiological or psychological homeostasis. While response to stress is a necessary survival mechanism, prolonged stress can produce severe consequences affecting behavioral, endocrine and immunological parameters (McEwen, 2008). Among them, the hippocampus, a limbic area involved in learning and memory, is particularly sensitive to stress (Kim and Yoon, 1998). In particular, structural alterations in the hippocampal formation and reduction of neurogenesis in the adult dentate gyrus have been observed in different animal models of chronic stress (McEwen, 2001). The main mediators in the adaptive response to stressors involve glucocorticoids, catecholamines and the balance between T helper 1 (Th1) versus T helper 2 (Th2) cytokines (McEwen, 2008). In previous reports (Palumbo et al., 2007, 2010) we have shown that BALB/c mice exposed to chronic mild stress (CMS) display poor learning performance in both open field and passive avoidance inhibitory tasks and a decrease in spontaneous alternation behavior. Moreover, animals under stress showed a morphological alteration in hippocampus related to diminished nitric oxide production by neural nitric oxide synthase and an increment of oxidative stress (Palumbo et al., 2007). We have also found that chronic stress induces a decrease in T-cell proliferative response *in vitro* and a lower T-cell dependent antibody production *in vivo*, with changes in stress hormone regulation of T-lymphocyte reactivity (Silberman et al., 2004). Recently, we found a correlation between poor memory performance and the increase of the Th2/Th1 balance (Palumbo et al., 2010).

Immune activity in the central nervous system (CNS) has long been considered detrimental and, in general, it is associated to inflammatory processes. However, recent studies have shown that immune cells may play an essential role in protecting the injured CNS. Schwartz and their group have formulated the concept of "protective autoimmunity". They demonstrated in numerous very well designed experimental works that the physiologic autoreactive T cells response plays a crucial role in neuroprotection following CNS injury (for review see Schwartz et al., 2008, 2009). Autoreactive T cell responses were shown to play a crucial role in neuroprotection following CNS injury or neurodegenerative pathology (Hauben et al., 2000; Yoles et al., 2001; Graber and Dhib-Jalbut, 2009; Benner et al., 2004; Beers et al., 2008). Furthermore, CNS-specific T cells that confer neuroprotection might also induce autoimmune diseases such as multiple sclerosis or experimental autoimmune encephalomyelitis (Kipnis et al., 2002). The

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efficacy of the neuroprotective autoimmune response may depend upon regulatory mechanisms whose activity is genetically determined (Kipnis et al., 2001).

Glatiramer acetate (GA) consists of acetate salts of synthetic polypeptides containing L-alanine, L-glutamate, L-lysine, and L-tyrosine (Teitelbaum et al., 1971). Schwarzt's group demonstrated that GA, an approved drug for the treatment of multiple sclerosis, could be used to promote an immune-mediated neuroprotective response in various models of acute CNS injury (Kipnis et al., 2000). They propose that this beneficial effect is obtained through a well-controlled inflammatory reaction, and that the activity of GA in driving this reaction derives from its ability to serve as a "universal antigen" by weakly activating a wide spectrum of self-reactive T cells.

In this context, the aim of the present work was to investigate the GA effects in CMS BALB/c mice. Particularly, we studied learning and memory in open field and in a spatial memory test and the Th1/Th2 cytokine balance. In addition, since spatial learning has been tightly linked to neurogenesis in the dentate gyrus and both processes are affected by chronic stress (Koehl and Abrous, 2011; McEwen, 2010), we also investigated whether adult hippocampal neurogenesis is modulated by GA treatment.

2. Materials and methods

2.1. Animals

Inbred female BALB/c mice were purchased from Veterinary School of the University of Buenos Aires. Sixty-day-old mice weighing between 23 and 25 g at the beginning of the experiments were used. Mice were housed and maintained on an 8:00 AM to 8:00 PM light/dark cycle under controlled temperatures (18–22 °C). Except as indicated below, food and water were freely available. Animal care was in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996. Two weeks before the beginning of the experiments, phases of the estrous cycle were monitored daily in order to verify that all mice have a synchronized estrous cycle.

2.2. Experimental design

Fig. 1 shows a scheme of the experimental design used in the present work. Mice were distributed in two groups, one group was housed in normal conditions (control mice) and the other group was subjected to CMS (CMS mice). Six weeks later, control and CMS mice were injected with vehicle or GA. The chronic stress protocol (see below) was continued upon the GA treatment to rule out any effect due to the lack of stress. All animals were behavior tested before their sacrifice. Mice of each group were used to evaluate cytokine balance or adult neurogenesis. Subgroups used for neurogenesis analysis were injected four weeks before their sacrifice with multiple injection of BrdU. Experimental protocols were approved by the Internal Ethics Committee of the School of Medicine of the University of Buenos Aires, the Institutional Animal Care and Use Committee.

2.3. Chronic mild stress model

The stress scheme was slightly modified from those previously used in rats (Willner et al., 1992) and mice (Monleon et al., 1995). Animals were housed singly and exposed to: one 16-h period of water deprivation; two periods of continuous overnight illumination; two periods (7 and 17 h) of 45° cage tilt; one 17-h period in a soiled cage (100 ml water in sawdust bedding); one period (8 h) of food deprivation; one 17-h period of paired housing (animals were always housed in the same pairs, but host cages were alternated between each member of the pair). All individual stressors used had been classified as "mild" according to the Animals Scientific Procedures Act of 1986 (UK legislation). The stressors were scheduled throughout nine weeks in a similar manner to that previously described (see CMS scheme in Palumbo et al., 2007). Animals were left undisturbed in their home cages 24 h prior to sacrifice.

2.4. GA treatment

After six weeks of stress exposure animals were injected for three weeks with GA. Each mouse was subcutaneously (s.c.) injected four times with 100 μg per injection of GA dissolved in phosphate buffered saline (PBS) at a final volume of 150 μl, according Butovsky et al. (2006b). GA (Copaxone® 323K253890604 batch No. 538655, Teva Pharmaceutical Industries, Petah Tiqva, Israel) was kindly provided by Teva-Tuteur, Argentina. During the first week of GA treatment animals received two injections with a 24-h interval between them and once per week for the following two weeks. As indicated above, stress exposure was continued upon the GA injection to rule out any effect due to the lack of stress. In Fig. 1 there are one scheme showing how GA was administered. PBS was used as control vehicle. Mice were tested seven days after the last GA injection.

2.5. Behavioral tests

2.5.1. Open field

Open-field habituation was performed using a rectangular chamber (42 cm × 35 cm × 15 cm) made of gray polyvinylchloride (PVC) (Frisch et al., 2005). The floor of the open field was uniformly divided into 30 squares of 7 × 7 cm. A low-level loudspeaker provided a broad spectrum masking noise. The apparatus was cleaned with water containing 0.1% acetic acid after each trial. Mice were acclimated to the testing room for at least 20 min prior to testing. On the first day animals were placed in the open field (first exposure) and locomotor activity was evaluated. Behavioral parameters recorded during 5-min sessions were: (1) crossings (horizontal activity): the number of horizontal lines crossed; (2) rearings (vertical activity): the number of times a mouse stood on its hind legs with forelegs in the air or against the wall; and (3) corner time: the time spent in any corner. After 24 h mice were re-exposed to the open field to evaluate changes in behavioral parameters. The open field test was performed between 5:00 and 7:00 pm. Sessions were recorded using a video camera (Sony DCB-DVD810). Because mice demonstrate less exploratory activity in a familiar environment, this simple test assesses the ability of the mouse to learn and remember the open-field chamber. Habituation was estimated as the relative decrease in activity between the first exposure and re-exposures to the open field (Frisch et al., 2005).

2.5.2. Y-maze spontaneous alternation

Spontaneous alternation behavior in a Y-maze task was recorded and evaluated as a spatial memory task. The apparatus consisted of three identical black plexiglass arms (l × w × h, 28 × 10 × 20 cm). Mice were acclimated to the testing room for at least 20 min prior to testing. At the beginning of the session, mice were placed at the end of one fixed arm of the Y-maze and allowed to explore freely for 6 min. The sequence of arm entries was recorded using a video camera (Sony DCB-DVD810). An alternation refers to three successive visits to the three separate arms of the maze. The percent alternation was calculated as the number of alternations divided by the total arm entries minus 2, multiplied by 100 (Dillon et al., 2008; Kim et al., 2008).

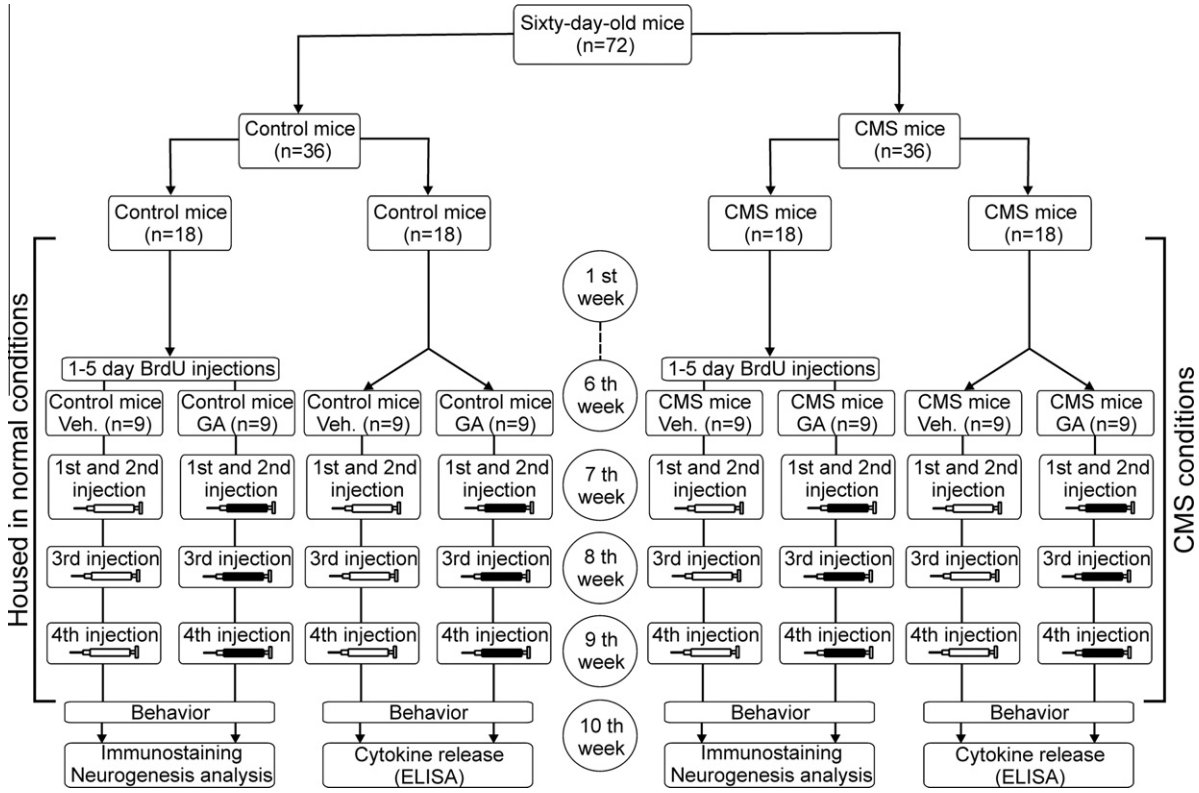


Fig. 1. Scheme of the experimental design used in the present work. Mice were distributed in two groups, one group was housed in normal conditions (control, $n = 36$) and the other group was subjected to chronic mild stress (CMS, $n = 36$). In turn, each group was divided in four subgroups, two control subgroups and two subgroups CMS ($n = 18$ each group). After six weeks exposure, one subgroup of control and CMS animals were injected with vehicle (white syringe) ($n = 9$) or GA (black syringe) ($n = 9$). These mice were used to evaluate behavior and cytokine release. The other subgroups of mice were injected four weeks before their sacrifice with BrdU. The half of animals of these subgroups were injected with vehicle ($n = 9$) or GA ($n = 9$). These mice were used to evaluate behavior and adult neurogenesis. The chronic stress protocol was continued upon the GA treatment to rule out any effect due to the lack of stress.

2.6. Evaluation of adult neurogenesis

2.6.1. Administration of 5-bromo-2′-deoxyuridine and tissue preparation

Mice received intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU) 50 mg/kg every 12 h for 5 consecutive days. Four weeks after the last BrdU injection, mice were anesthetized (100 μg ketamine + 10 μg xylazine g^{-1}, i.m.) and perfused intracardially with PBS and then with 4% paraformaldehyde (PFA). Housing, treatments, surgery and euthanasia were carried out according to NIH guidelines, and all experiments were carried out following guidelines laid by the Leloir Institute animal welfare committee. Brains were removed and sectioned (40 μm) using a sliding microtome (Leica, Wetzlar, Germany).

2.6.2. Immunohistochemistry

For BrdU immunostaining, free-floating coronal sections (40-μm thick) were washed with Tris-buffered saline (TBS) and incubated for 2 h in 50% formamide solution at 65 °C and then in 2 N HCl at 37 °C. Sections were washed with TBS, blocked for 1 h with TBS containing 3% donkey serum and .25% Triton X-100, and incubated for 72 h with the primary antibodies in blocking solution. Primary antibodies were: rat anti-BrdU (1:200; Boehringer Manheim, Roche, Basel, Switzerland) and mouse anti-neuron-specific nuclear protein (anti-NeuN; 1:50, kindly provided by F.H. Gage), included in some sections to corroborate the neuronal phenotype. Secondary antibodies were donkey anti-rat Cy-3 and donkey anti-mouse Cy-5 (1:250; Jackson ImmunoResearch, West Grove, Pennsylvania, United States).

2.6.3. Cell counting

For immunohistochemical analysis of adult hippocampal neurogenesis, 1 every 12 coronal sections spanning the entire dentate gyrus were taken for each mouse. Neurogenesis was assessed counting $BrdU^+$ cells using fluorescence microscopy (Zeiss Axiovert 135 M) with a 10× objective and a DDC camera (Hamamatsu ORCA C474295). Only $BrdU^+$ cells located in the granule cell layer and subgranular zone of the dentate gyrus (DG) were counted. The total number of labeled cells per dentate gyrus was then obtained for each mouse by multiplying the number of $BrdU^+$ cells × 12. It is well known that, four weeks after of the last BrdU injection the majority of $BrdU^+$ cells in the dentate gyrus are mature neurons (Kempermann et al., 2003). The phenotype $BrdU^+$ cell was corroborated by NeuN labeling. Confocal images were taken using a Zeiss Pascal confocal microscope (Zeiss, Jena, Germany) with a 1-μm pinhole. Co-localization was analyzed in single optical planes taken through the entire *z*-axis of each cell using Zeiss LSM Image Browser Software.

2.7. Determination of cytokine production

2.7.1. Cell suspensions and culture conditions

Lymphoid cell suspensions from control and CMS mice were obtained as previously described (Edgar et al., 2002). Briefly, mice were sacrificed by decapitation and lymph nodes (axilary, inguinal and mesenteric) were removed and disrupted through a 1 mm metal mesh, and the cell suspension was filtered through a 10 μm nylon mesh. The suspension was depleted of red bloods and dead cells by centrifugation over Ficoll/Hypaque (density 1.084 from Sigma–Aldrich). After three washes in Roswell Park Memorial

Institute (RPMI)-1640 medium, cells were re-suspended in RPMI 1640 supplemented with 10% of batched-tested non-stimulatory fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 50 μM beta-mercaptoethanol. Cell viability was estimated according to the Trypan blue exclusion criteria and was higher than 90%.

2.7.2. Cytokine release

To stimulate cytokines production lymphoid cells (1×10^6/ml) were incubated with concanavaline A (1 μg/ml) for 24 h at 37 °C in a 5% CO_2 atmosphere in a Falcon 24-well plate, as previously described (Palumbo et al., 2010; Takeno et al., 2004). After incubation, culture supernatants were harvested and their IFN-γ, IL-2, IL-6, IL-10 and IL-4 levels were determined by ELISA kits (Amersham Biosciences, Little Chalfont, Buckinghamshire). It is important to note that 1 μg/ml of Concanavaline A is the optimal concentration that stimulated T cells proliferation given a peak of proliferation at the third day of culture.

2.7.3. Cell viability and proliferation

Cell viability was estimated according to the Trypan Blue (Sigma–Aldrich) exclusion criteria. Proliferation was determined by addition 5 μCi [^{3}H]thymidine per well for the last 18-h period of culture. The thymidine incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters (Whatman) of the acid insoluble macromolecular fraction.

2.8. Statistical analysis

Data were analyzed using two-way ANOVA to examine significance of main effects and interactions. When interaction was significant, simple effects analysis (F) was made. When interaction was not significant, the Bonferroni's (t) post hoc test was applied. Open field habituation was analyzed by two-way ANOVA for repeated measures (F) followed by Bonferroni (t). Student́s *t*-test (T) was used to evaluate open field exploratory activity during the first or second exposure between two groups. Differences between means were considered significant if $p < .05$.

3. Results

3.1. GA treatment reduces the effects of CMS on learning and memory

The interactions between CMS, learning, and GA were investigated using the open field and the Y-maze tasks, which evaluate non-associative memory and spatial learning, respectively. As described above (see Section 2.2) mice were treated with GA after six weeks of stress exposure. Learning and memory were evaluated one week after the last GA injection (see Figs. 2A and 3A).

We utilized the open field task to determine the ability to habituate to a novel environment. As expected from our previous report (Palumbo et al., 2007), stressed mice displayed a more intense exploratory activity during the first exposure (the first time that mouse was put into the open field). As can be seen in Fig. 2, CMS mice injected with vehicle showed an increase in crossings ($T(18) = 3.38$, $p < .01$) and rearings ($T(18) = 2.14$, $p < .05$) and a parallel decrease in the corner time ($T(18) = 2.11$, $p < .05$) (Fig. 2B–D, respectively) with respect to control mice.

Concerning to habituation for vehicle injected mice, two-way ANOVA for repeated measures indicated changes in behavioral parameters depending on condition (control or CMS) and time (0 or 24 h) [interaction: condition × time; crossings: $F(1,18) = 20.200$, $p < .001$; rearings: $F(1,18) = 6.661$, $p < .02$; corner time: $F(1,18) = 13.861$, $p < .002$]. Control BALB/c mice displayed lower levels of exploratory activity when were re-exposure to open field (24 h after the first exposure). Habituation was reflected in the 48% decrease in number of crossings in the x–y plane (post hoc Bonferroni test; $t(18) = 9.780$, $p < .001$; Fig. 2B), 57% reduction in the number of rearings ($t(18) = 8.986$, $p < .001$; Fig. 2C), and 59% increase in corner time ($t(18) = 7.970$, $p < .001$; Fig. 2D). However, CMS showed a reduced habituation capacity. No differences were observed in the number of crossings ($t(18) = .279$, NS), rearings ($t(18) = 2.265$, NS) and corner time ($t(18) = 1.275$, NS) at 24 h when compared to 0 h (Fig. 2B–D, respectively).

GA treatment improves the altered performance caused by CMS. GA treated mice showed a smaller number of crossings during the first exposure to the open field ($T(18) = 3.76$; $p < .01$; Fig. 2B), although no significant differences were found for rearings ($T(18) = .53$; NS; Fig. 2C) and corner time ($T(18) = 1.61$; NS; Fig. 2D). Interestingly, CMS mice injected with GA were able to habituate to the novel environment similarly to control BALB/c mice. Two way ANOVA for repeated measures indicated changes in behavioral parameters depending on condition (vehicle or GA) and time (0 or 24 h) [interaction: condition × time; crossings: $F(1,18) = 4.151$, $p < .05$; rearings: $F(1,18) = 8.043$, $p < .02$; corner time: $F(1,18) = 13.095$, $p < .002$]. Bonferroni's post hoc test showed for GA-treated CMS mice, a decrease in the number of crossings ($t(18) = 3.140$; $p < .05$; Fig. 2B) and rearings ($t(18) = 6.275$; $p < .001$; Fig. 2C), as well as an increase in corner time ($t(18) = 6.393$; $p < .001$; Fig. 2D) at 24 h. In addition, a significant difference in exploratory activity was observed in the second exposure to open field between CMS mice treated with GA and CMS mice injected with vehicle. A significant lower number of crossings ($T(18) = 7.56$, $p < .0001$), number of rearings ($T(18) = 5.62$, $p < .0001$) and higher corner time ($T(18) = 6.39$, $p < .0001$) was recorded for GA-treated CMS mice.

However, GA treatment did not affect the performance of control mice with respect to the habituation [two way ANOVA for repeated measures, interaction: condition × time; crossings: $F(1,18) = 1.577$, NS; rearings: $F(1,18) = .004$, NS; corner time: $F(1,18) = .153$, NS]. Post-hoc Bonferroni showed for GA-treated control mice a significant difference in the parameters determined 24 h after the first exposure [crossings ($t(18) = 11.56$, $p < .001$); rearings ($t(18) = 8.896$, $p < .001$); corner time ($t(18) = 7.418$, $p < .001$)] (Fig. 2B–D, respectively).

We used the Y-maze to evaluate spatial memory by measuring the rate of spontaneous alternation among arms. Stressed mice showed a reduced alternation that indicates poor memory performance that was reverted after GA treatment (Fig. 3B). Two-way ANOVA indicated significant changes in the percentage of spontaneous alternations depending on condition (control or CMS) and treatment (vehicle or GA) [interaction: condition × treatment; $F(3,31) = 16.39$, $p < .001$]. Simple effects analysis showed a significant decrease in CMS mice compared to control both injected with vehicle [$F(1,28) = 24.09$, $p < .001$]. This impaired behavior was fully reverted by GA administration [$F(1,28) = 19.36$, $p < .001$]. Simple effects analysis showed no differences between control mice injected with vehicle or GA [$F(1,28) = 2.42$, NS]. These results demonstrate that GA treatment can reverse the memory impairment induced by chronic stress.

3.2. GA treatment restores adult hippocampal neurogenesis

A decrease in the rate of adult hippocampal neurogenesis has been described after CMS (Lee et al., 2006; Guo et al., 2009; Dagytė et al., 2011), which could be one of the mechanisms underlying the memory deficits described above. To address this question we monitored the correlation of adult neurogenesis with the impaired memory after CMS exposure and their reversion with GA treatment. Control and stressed mice received two daily injections of BrdU 50 mg/kg during five consecutive days, four weeks before

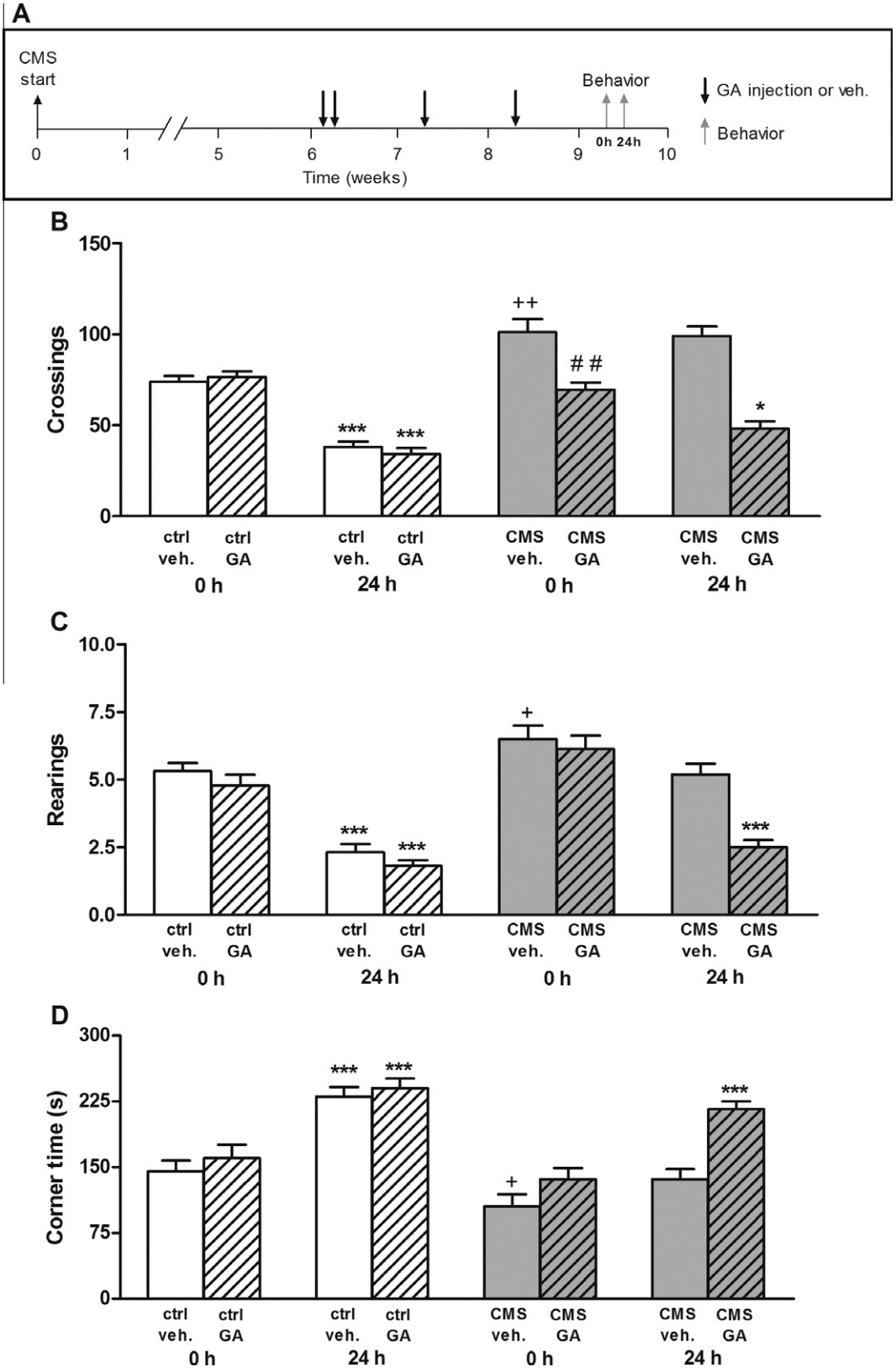


Fig. 2. Habituation in open field task. A, Schematic representation of the experiment timeline. Number of crossings (panel B), rearings (panel C) and corner time (panel D) were measured in control (ctrl) and CMS mice injected with vehicle (veh.) or GA during the first exposure (0 h) and after 24 h. Bars denote mean ± SEM of 10 mice for each group. (∗) and (∗∗∗) denote $p < .05$ and $p < .001$ compared to the first exposure; (+ and ++) denote $p < .05$ and $p < .01$ compared to non-stressed mice; (##) indicates $p < .01$ with respect to the corresponding vehicle-treated mice.

sacrifice. GA or vehicle treatment was administered according to the scheme shown in Fig. 4A. Adult neurogenesis was assessed by counting $BrdU^+$ cells in the dentate gyrus of the hippocampus. Four weeks after labeling, most newborn cells within the granule

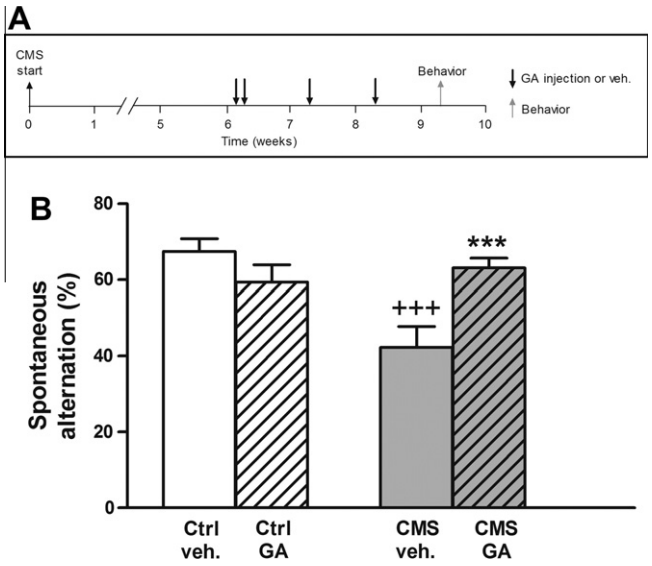


Fig. 3. Mice behavior in the Y-maze. (A) Schematic representation of the experiment timeline. (B) The bar chart shows the spontaneous alternation behavior in a Y-maze in control (ctrl) and CMS mice injected with vehicle (veh.) or GA. Data represents mean ± SEM of eight animals for each group. (+++) denotes $p < .001$ with respect to ctrl + veh.; (***) indicates $p < .001$ compared to CMS + veh.

cell layer were neurons (Kempermann et al., 2003) (Fig. 4D and E). Two-way ANOVA indicated significant changes in the number of $BrdU^+$ cells depending on condition (control or CMS) and treatment (vehicle or GA) [interaction: condition × treatment; $F(3,20) = 4.630$, $p < .05$]. As can be seen in Fig. 4B, CMS mice exhibited a significant decrease (about 30%) in the number of $BrdU^+$ cells respect to control mice (simple effects analysis; $F(1,20) = 12.602$; $p < .01$). Moreover, GA treatment did not affect adult neurogenesis in control mice ($F(1,20) = .870$; NS), but it did restore the original levels of adult neurogenesis, increasing a 27% respect to CMS mice injected with vehicle ($F(1,20) = 4.415$; $p < .05$) (Fig. 4B). Fig. 4C depicts representative images of $BrdU^+$ cells in the dentate gyrus using fluorescence microscopy.

3.3. Modulation of cytokine levels by GA treatment

We have previously shown that CMS decreases the level of Th1 and increases Th2-type cytokines, altering the Th1/Th2 balance (Palumbo et al., 2010). To evaluate if the effect of GA treatment on behavior and neurogenesis are also reflected in the cytokine levels, the production of IFN-γ, IL-2 (Th1 cytokines), IL-4, IL-10 and IL-6 (Th2 cytokines) in stimulated T cells were determined. Two-way ANOVA revealed that significant changes in IFN-γ, IL-10, IL-4 and IL-6 production were observed depending on the condition (control or CMS) and the treatment (vehicle or GA); but not for IL-2 [interaction: condition × treatment, IFN-γ: $F(3,23) = 17.53$, $p < .001$; IL-4: $F(3,23) = 12.07$, $p < .01$; IL-10: $F(3,23) = 23.43$, $p < .001$; IL-6: $F(3,23) = 81.96$, $p < .001$ and IL-2: $F(3,23) = .07$, NS]. As shown in Fig. 5, cytokine release indicates that CMS animals injected with vehicle showed a lower production of IFN-γ [simple effects analysis; $F(1,20) = 52.143$, $p < .001$] and a higher release of IL-4 [$F(1,20) = 26.889$, $p < .001$], IL-10 [$F(1,20) = 15.667$, $p < .001$] and IL-6 [$F(1,20) = 454.00$, $p < .001$] than control animals. No changes were found for IL-2 levels between control and CMS animals (Bonferroni's post hoc test; $t(20) = 2.038$, NS).

Most CMS-induced changes in cytokine levels were reverted by *in vivo* GA administration. CMS mice treated with GA displayed fully restored levels for IFN-γ [simple effects analysis; $F(1,20) = 23.327$, $p < .001$; IL-4: $F(1,20) = 49.333$, $p < .001$; IL-10: $F(1,20) = 31.667$, $p < .001$ and IL-6: $F(1,20) = 143.00$, $p < .001$] when compared to CMS mice injected with vehicle. No changes were observed in IL-2 (Bonferroni's post hoc test; $t(20) = .975$, NS). These findings indicate that stress induces a decrease in the Th1/Th2 balance, which it is reverted by GA treatment.

It is important to note that GA administration did not modify cytokine levels in control mice. Simple effect analysis showed no differences for the analyzed cytokines between control mice injected with vehicle or GA [IFN-γ: $F(1,20) = 1.211$, NS; IL-10: $F(1,20) = .333$, NS; IL-4: $F(1,20) = 4.278$, NS; IL-6: $F(1,20) = 1.00$, NS and IL-2: $t(20) = .541$, NS].

To rule out the possibility that changes in cytokines levels were due to different number of cells present in each well after 24 h of incubation; we determined the cell viability and the proliferation under these culture conditions. As can be seem in the table inserted in Fig. 5, no significant differences were found in these parameters between the different experimental groups.

4. Discussion

In this study we show that chronic stress exposure in BALB/c mice induces cognitive deficits and a decrease in adult neurogenesis, which are paralleled by changes in the Th1/Th2 balance. These alterations are reverted by GA treatment.

It was described that the immune system can signal the central nervous system through the action of cytokines (Eskandari et al., 2003). Moreover, deregulation of cytokines (Th1 and Th2) has been involved in the pathogenesis of many human illnesses such as autoimmune diseases, sleep disturbance, major depression and other disorders (Kaufmann et al., 2007; Schwarz et al., 2001). It has been proposed that immunity to self might play an important role in maintenance, protection, and repair of the healthy and diseased CNS (see Schwartz et al., 2008, 2009; Graber and Dhib-Jalbut, 2009).

On the other hand, the notion that adult hippocampal neurogenesis is functionally relevant and it plays a key role in learning and memory has been extensively documented during the last decade (Deng et al., 2010; Koehl and Abrous, 2011). In general, behavioral evaluation of rodents with reduced adult neurogenesis has consistently suggested an involvement of adult-born neurons in learning and memory. In this context, our results show an impairment in learning and memory that could be related to a decrease in adult neurogenesis observed in mice under chronic stress. The immune system plays a key role in the proliferation, migration and survival of neural stem/progenitor cells (NPCs) of the adult brain. When activated by IL-4, microglia can induce a bias towards oligodendrogenesis, whereas IFN-γ-activated microglia promotes neuronal differentiation (Butovsky et al., 2006a). IFN-γ can promote neuronal differentiation and neurite outgrowth of murine adult NPCs (Johansson et al., 2008; Wong et al., 2004). We here found that stress exposure decreases IFN-γ while it increases IL-4, IL-10 and IL-6 which correlate with a reduced adult neurogenesis and a poor memory performance in mice. GA treatment was able to reverse the deleterious effects on neurogenesis and memory in parallel with an increase of IFN-γ production. These results could be indicating a possible proneurogenic effect for this cytokine. A recent study found that adult neurogenesis in transgenic mice, in which IFN-γ is selectively expressed under an oligodendrocyte-specific promoter, is elevated relative to naïve wild-type controls. This result suggests that the mere presence of IFN-γ within the brain parenchyma is sufficient to promote adult neurogenesis (Baron et al., 2008). Further studies to know cytokine expression variations in hippocampus after CMS as well as after GA treatment are necessary to understand the interplay between GA treatment, peripheral immune system, neurogenesis and behavior.

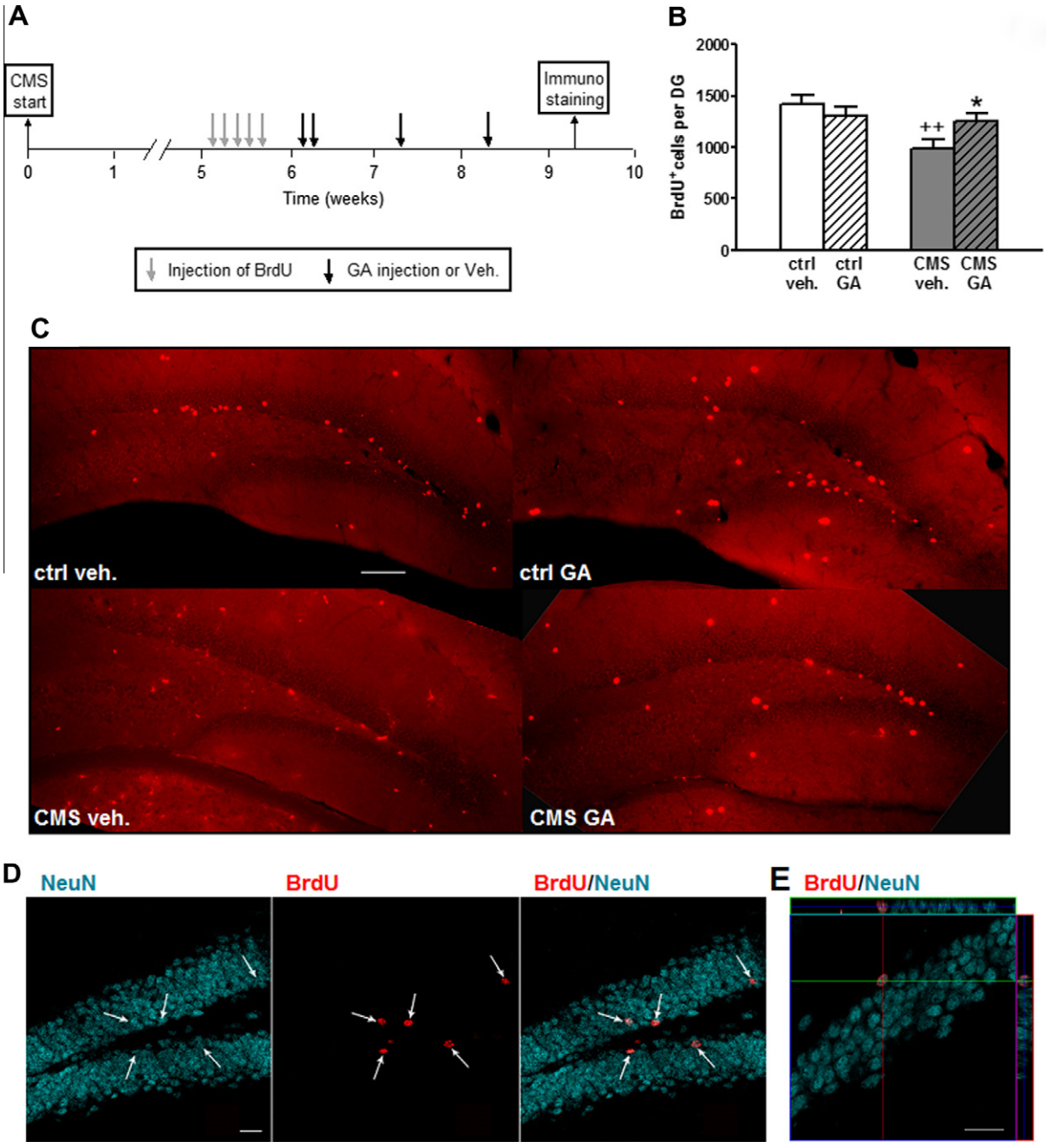
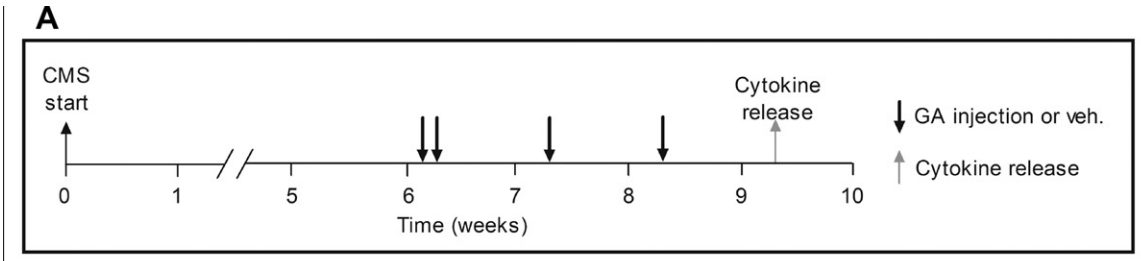
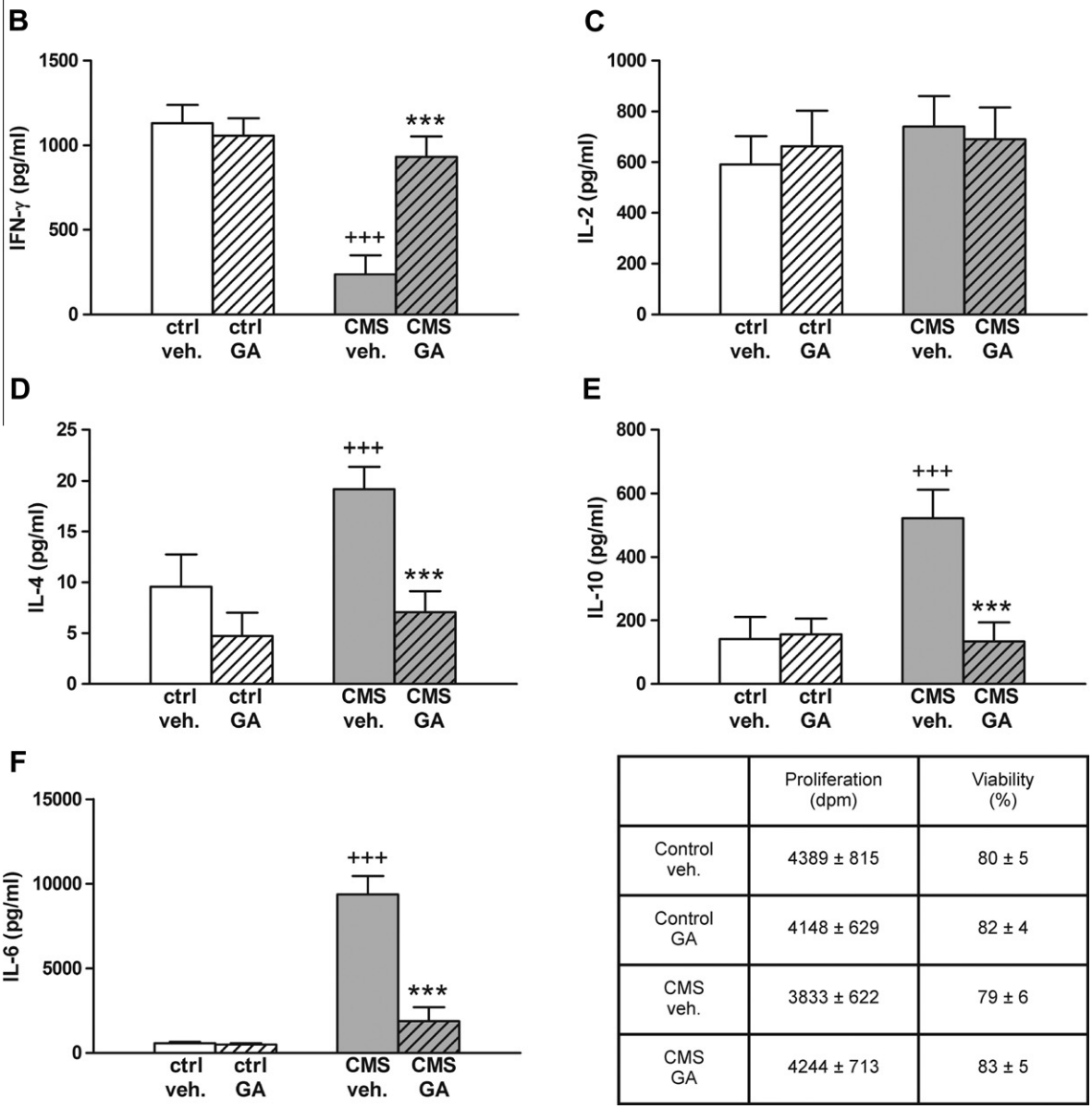


Fig. 4. Adult hippocampal neurogenesis. (A) Experimental timeline depicting BrdU (gray arrows) and GA (black arrows) administration. (B) quantification of $BrdU^+$ cells in the dentate gyrus (DG) monitored 28 days after the last BrdU injection in control (ctrl) and CMS mice injected with vehicle (veh.) or GA. Data are mean ± SEM for 6 mice in each group. (++) and (∗) denote $p < .01$ with respect to ctrl + veh., and $p < .05$ compared to CMS + veh. (C) representative images of the dentate gyrus in mice belonging to each of the groups as indicated. BrdU immunofluorescence is shown in red. Calibration bar: 100 μm. (D) representative single-plane confocal micrographs of the dentate gyrus of control + GA mice double-stained for BrdU (red) and NeuN (blue; calibration bar: 20 μm). E, representative confocal micrograph depicting a $BrdU^+$ cell located in the subgranular zone stained for NeuN in CMS + veh. mice. The image shows the overlay of the blue and red channel in single optical sections together with the orthogonal projections onto the x–z (top) and y–z (right) planes. Calibration bar: 20 μm.

GA was originally synthesized to mimic the activity of myelin basic protein (MBP) by inducing experimental autoimmune encephalomyelitis (EAE) in laboratory animals but was found to be non-encephalitogenic and even to suppress MBP-induced EAE (Teitelbaum et al., 1971). In humans, daily administration of GA resulted in the development of a T helper 2 (Th2)/Th3-type response over time (Arnon and Aharoni, 2009). On the other hand, it was demonstrated that GA could be to promote an immune-mediated neuroprotective response in various models of acute CNS injury and neurodegenerative disease (Schwartz et al., 2009). It is striking how the same compound (GA) provide both properly regulated immune suppression with a shift to Th2 (in the case of the autoimmune disease) and properly regulated immune activation with Th1 activation (in the case of the neurodegenerative disease). Reports indicate that MS patients treated with GA initially show a Th1-type response, which later switches towards Th2 (Farina et al., 2001). Thus, it was propose that a short pretreatment leads to a weak Th1 response that may attenuate neurodegeneration, whereas prolonged pretreatment leads to a Th2 response that may lessen autoimmune disease (Kipnis and Schwartz, 2002). In





	Proliferation (dpm)	Viability (%)
Control veh.	4389 ± 815	80 ± 5
Control GA	4148 ± 629	82 ± 4
CMS veh.	3833 ± 622	79 ± 6
CMS GA	4244 ± 713	83 ± 5

Fig. 5. Cytokine levels. (A) Schematic representation of the experiment timeline. The concentration of INF-γ (panel (B), IL-2 (panel C), IL-4 (panel D), IL-10 (panel E) and IL-6 (panel F) was determined in supernatants of lymphocytes from lymph nodes stimulated with Con A for 24 h from control (ctrl) and CMS mice, injected with GA or vehicle. Table insert in figure show the cell viability and proliferation after 24 h of culture. ^{3}H-tymidine uptake (dpm) for non-stimulated cells was: control + veh.: 2345 ± 544, control + GA: 2622 ± 428, CMS + veh.: 2529 ± 632, CMS + GA: 2735 ± 602. Data show mean ± SEM of three independent experiments with two animals for each group. (+++) and (***) denote $p < .001$ with respect to ctrl + veh. and $p < .001$ with respect to CMS + veh.

this context, it was demonstrated that immune treatment with CNS-related peptide that activate weakly self-reactive T cells can ameliorate depressive behavior induced by CMS in rats. The behavioral outcome was also accompanied by restoration of hippocampal BDNF levels and neurogenesis (Lewitus et al., 2009). It is important to note that in this model, treatments were always applied as a preventive treatment before exposure to stress.

Our present results show that GA treatment after stress exposure is able to revert the stress-induced memory impairment. We postulate that the decrease in IFN-γ along with the IL-6 and IL-4 increased production could constitute a mechanism that may contribute to a decrease in neurogenesis and to the memory impairment induced by CMS exposure. In this scenario, reestablishing the Th1/Th2 balance by GA treatment restored adult neurogenesis

and memory performance. It is important to note that GA treatment did not have any effect on control mice in terms of effects on behavior or neurogenesis. Taking into account that a short treatment with GA weakly activates self-reactive T cells, this response could be not able to alter physiological conditions. It should be noted that we use females for our investigation and two weeks before the beginning of the experiments, phases of the estrous cycle were monitored daily in order to verify that all mice have a synchronized estrous cycle. Although we assume that all females are in the same phase of the cycle for each of the major stages of our study, we did not check whether this assumption is fulfilled. In the last years, it has been considered that estrous cycles decrease the homogeneity of study populations and confound effects of experimental manipulations. Most studies analyzing the role of sex hormones on different parameters use the classic endocrine paradigm of ovariectomy and estradiol replacement and pregnant female. But, there are few studies studying the influence of hormonal variations related to the phase of the estrous cycle in behavior and cytokine balance. Concerning behavior, the most consistent finding is that during proesturs and estrus rats and mice display less anxiety-like behavior than during diestrus (Meziane et al., 2007). Concerning to cytokine production, it has suggested that the increased P4 and E2 concentrations during the luteal phase play a role in the deviation of the immune response towards a Th2-type response (Dosiou et al., 2004; De León-Nava et al., 2009). In a recent and interesting review Beery and Zucker (2011) analyze the sex bias in biomedical research. They emphasize that many studies over the past 90 years established that replicable results are just as likely to emerge from investigation of female as male mammals, including scores that did not track female reproductive cycles (for review see Beery and Zucker, 2011). Taking into account these considerations and the low variability of our results within each experimental group, we think that our results are not due to a not synchronized estrous cycle. However, further studies are necessary to establish sex steroids influence on GA effects.

Finally, the present findings highlight a critical role of the peripheral immune system in the cognitive decline induced by chronic stress. Thus, T-cell trafficking to the CNS would enable to contribute to maintain appropriate levels of neurogenesis, as well as, additional aspects of adult brain plasticity. In this context, GA may become a very useful tool to understand the cellular and molecular mechanisms that mediate the deleterious actions of chronic stress.

5. Conflicts of interest

The authors declare that there are no conflicts of interest.

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