Psychopharmacology
DOI 10.1007/s00213-014-3791-z

ORIGINAL INVESTIGATION

Involvement of nitric oxide in improving stress-induced behavioural alteration by glatiramer acetate treatment in female BALB/c mice

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Received: 25 September 2013 / Accepted: 25 October 2014

Abstract
Rationale Oxidative stress and neurotrophins are among the most important factors involved in several pathophysiological brain processes. In addition, long-term exposure to stressful situations has deleterious effects on behaviour. We have previously shown that stressed female BALB/c mice show poor learning performance and that this behaviour is reversed by glatiramer acetate (GA) treatment.
Objectives We investigated the involvement of the hippocampal oxidative status and neurotrophin levels in cognitive deficit and the improvement of this deficit by GA treatment in chronic stressed BALB/c mice.
Methods Female BALB/c mice were exposed to a chronic mild stress (CMS) model for 9 weeks. During the last 3 weeks of the stress exposure, one group of mice was subcutaneously injected four times with 100 μg GA/mouse. Following this period, behavioural studies were performed. The mice were then sacrificed, and biochemical studies were performed on the hippocampus.
Results The stressed mice exhibited a significant decline in their performance in the open-field and in object-in-place tasks. This decline was accompanied by an increase in reactive oxygen species (ROS) and a decrease in nitric oxide (NO) production by neuronal nitric oxide synthase (nNOS). Neither antioxidant defences nor neurotrophin protein levels were involved in this process. Interestingly, the administration of GA re-established the normal levels of ROS, restored nNOS activity and improved learning performance.
Conclusions The GA treatment improved learning and memory in female BALB/c mice under chronic stress through a mechanism that involves the regulation of NO production, which in turn modulates the ROS levels.

Keywords Chronic stress · Neuronal nitric oxide synthase · Reactive oxygen species · Neurotrophins · Hippocampus · Glatiramer acetate

Introduction

Stress is defined as any situation that disturbs physiological or psychological homeostasis. In general, the short-run response to stress is proposed to be beneficial for the adaptation of the organism to a dangerous situation, but long-term exposure to the stressor ultimately results in harmful outcomes. A previous study described that chronic stress exposure has several consequences on behavioural, endocrine and immunological response (McEwen 2008).

Oxidative stress has been associated with the pathogenesis of several neurodegenerative and neurological disorders. However, in unfavourable conditions, antioxidant defences may be insufficient, leading to increased vulnerability and eventually cell death (Ischiropoulos and Beckman 2003). Oxidative stress results from the generation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide and peroxynitrite (Oliver et al. 1990). Accumulated evidence indicates that excessive stress leads to brain oxidative stress, a key mechanism in the pathology of brain disorders (Madrigal et al. 2006; Schiavone et al. 2013).

Nitric oxide (NO) is involved in several physiological and pathological brain processes, including hippocampal responses to stress (Reagan et al. 1999). NO production might result in either toxicity or neuroprotection, depending on the NO level, the location of NO production, the extent of oxidative stress and the type of neurodegenerative process (Lipton

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Published online: 07 November 2014

1999). In general, controlled NO production is necessary for the normal repair of damaged tissue. However, a high production of NO can interact with the superoxide anion generated by mitochondria or through other mechanisms, leading to the formation of the powerful oxidant species peroxynitrite (Miranda et al. 2000). Most studies have focused on the biological effects of NO. The interactions between NO and ROS have demonstrated that ROS and NO mutually regulate each other's signalling mechanisms (Thomas et al. 2008). The fusion of NO with O_2 to give peroxynitrite is physiologically significant (Hill et al. 2010). This radical–radical reaction has a high rate constant and is favoured instead of O_2 dismutation to hydrogen peroxyde. As a result, highly cytotoxic and long-lived ROS are replaced by peroxynitrite, which is short-lived in the cellular environment (Pryor et al. 2006).

In this context, we have shown that female 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 behaviour (Palumbo et al. 2007, 2010) related to a diminished NO production by neuronal NO synthase (nNOS), an increase in ROS production (Palumbo et al. 2007) and a decrease in adult neurogenesis (Palumbo et al. 2012).

Neurotrophins are another class of factors that modulate behaviour. They are growth factors that play crucial roles in the formation and plasticity of neuronal networks and have been involved in the pathophysiology of suicide (Dwivedi 2012) and depression (Jiang and Salton 2013). The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). In particular, BDNF plays an important role in the processes of synaptic plasticity and learning. An increased expression of BDNF can positively affect the generation of long-term potentiation and memory (Pang et al. 2004). Some studies have shown that NO regulates BDNF expression, which affects neural progenitor cell proliferation and differentiation in the mammalian brain (Canossa et al. 2002; Cheng et al. 2003).

Glatiramer acetate (GA) is an approved drug for treating multiple sclerosis, which consists of acetate salts with synthetic polypeptides containing L-alanine, L-glutamate, L-lysine and L-tyrosine (Teitelbaum et al. 1971). Schwartz's group demonstrated that GA could be used to stimulate an immune-mediated neuroprotective response in various models of acute central nervous system (CNS) injury and neurodegenerative disease (Schwartz et al. 2009; Kipnis et al. 2000). The authors proposed that this beneficial effect is mediated by a well-controlled inflammatory reaction because GA can act as a "universal antigen" that weakly activates a broad range of T cells. A brief treatment with GA induced a weak Th1 response that may decrease neurodegeneration, whereas a prolonged treatment resulted in a Th2 response that may decrease autoimmune disease (Kipnis and Schwartz 2002). Additionally, treatment with CNS-related peptides that weakly activate self-reactive T cells can ameliorate the depressive behaviour induced by CMS in rats (Lewitus et al. 2009). In this model, the treatments were preventive and were applied before exposure to stress. We previously demonstrated that GA was able to reverse the deleterious effects of chronic stress, such as impairment of learning and memory and diminished adult neurogenesis (Palumbo et al. 2012).

This work aimed to analyse the involvement of the hippocampal oxidative status and neurotrophin levels in cognitive deficit and its improvement by GA treatment in BALB/c mice under chronic stress.

The CMS scheme used both previously and in the present study includes a weekly 17-h period of paired housing. Male mice fight when they are together in a cage, particularly if they are under stress. Therefore, this first attempt to investigate the relation among oxidative status, the neurotrophin levels and cognitive deficit under chronic stress and GA effect was performed only in female BALB/c mice. However, we considered it important to extend this study using male and female mice and adjust our CMS scheme to determine whether gender differences exist in the mechanisms underlying the cognitive deficit under CMS and its reversion by GA treatment.

Materials and methods

Animals

Inbred female BALB/c mice were obtained from the Veterinary School of the University of Buenos Aires (Buenos Aires, Argentina). Sixty-day-old mice weighing between 23 and 25 g at the beginning of the experiments were used. The mice were maintained on a 12:12-h light/dark cycle under controlled temperatures (18–22 °C). Unless indicated otherwise, food and water were freely available. The animals were maintained 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 beginning the experiments, the phases of the oestrous cycle were monitored daily to verify that all mice had a synchronised oestrous cycle.

Experimental design

Figure 1 presents an experimental scheme used in the present work. The mice were randomly assigned to two groups: one that was housed in normal conditions (control mice) and another that was subjected to CMS (CMS mice). Six weeks later, the control and CMS mice were injected with either the vehicle or the GA. The chronic stress protocol (see below) was continued upon the GA treatment to rule out any effect due to the lack of stress. Mice of each group were used to evaluate behavioural performance, hippocampal oxidative state and

neurotrophin protein levels. After behavioural testing, mice were left undisturbed in their home cages 48 h prior to sacrifice. The experimental protocols were approved by the Institutional Committee for the Use and Care of Laboratory Animal (CICUAL, School of Medicine, University of Buenos Aires, Argentine).

CMS model

The stress scheme was slightly modified from those previously used in rats (Willner et al. 1992) and mice (Monleon et al. 1995). The animals were housed singly and exposed to one 8-h period of food deprivation, one 16-h period of water deprivation, two periods (7 and 17 h) of 45° cage tilt, one 17-h period in a soiled cage (100 ml water in sawdust bedding), two periods of continuous overnight illumination and one 17-h period of paired housing (animals were always housed in the same pairs, but the host cages were alternated between each member of the pair). All individual stressors used were classified as "mild" according to the Animals Scientific Procedures Act of 1986 (UK legislation). The individual stressors were dispersed throughout 9 weeks in a manner similar to that previously described (Palumbo et al. 2007).

GA treatment

GA consists of acetate salts of synthetic polypeptides containing L-alanine, L-glutamate, L-lysine and L-tyrosine (Teitelbaum et al. 1971). After 6 weeks of stress exposure, the animals were injected with GA for 3 weeks. Each mouse received four subcutaneous injections of 100 μg of GA dissolved in phosphate-buffered saline (PBS) at a final volume of 150 μl, according to the procedure described by Butovsky et al. (2006). During the first week of the GA treatment, the animals were injected twice at a 24-h interval and once per week for the following 2 weeks. GA (Copaxone® 323K253890604 batch no. 538768, Teva Pharmaceutical Industries, Petah Tiqva, Israel) was kindly provided by Teva-Tuteur, Argentina. As indicated above, the stress exposure was continued after the GA injection to avoid any effect due to the lack of stress (Palumbo et al. 2012). PBS was used as the control vehicle. The mice were tested 7 days after the last GA injection.

Behavioural tests

Open-field habituation

An open-field test was performed between 5:00 and 7:00 pm using a rectangular chamber (l×w×h, 42×35×15 cm) that was divided into 30 squares of 7×7 cm and made of grey polyvinylchloride (PVC), as previously described (Frisch et al. 2005; Dillon et al. 2008). On the first day, the animals were placed in the open field and their locomotor activity was assessed. The behavioural parameters registered during 5-min sessions were (1) crossings (horizontal activity): the number of horizontal lines crossed, (2) rearing (vertical activity): the number of times a mouse stood on its hind legs and (3) corner time: the time spent in any corner of the open field. After 24 h, the mice were re-exposed to the open field to evaluate any changes in the behavioural parameters. The sessions were recorded using a video camera (Sony DCB-DVD810). The habituation was estimated as the relative decrease in activity between the first and second exposures to the open field.

Object-in-place task

The object-in-place task is a procedure that was developed to test rodent memory, which combines the elements of traditional measures of object recognition and object placement. Prior to testing object memory, the mice were habituated to the open field for 10 min each day for three or four consecutive days. This procedure comprised a sample phase and a test phase separated by a 1-h delay. In the sample phase, each mouse was placed in the centre of the open field and was allowed 5 min to investigate four distinct objects, each located 5 cm from the corner walls. During the delay period, all objects were cleaned with alcohol to remove olfactory cues and any sawdust stuck to the object. For the test phase, two of the original four objects, those on either the right or the left of the open field, exchanged positions, whereas the two remaining objects remained in their original positions. The subjects were allowed to explore the objects for 3 min. The results are expressed as the discrimination ratio (DR), defined as the difference between the times spent with the moved objects (Mt) and the unmoved objects (Ut) divided by the total time spent with the moved and unmoved objects [DR=(Mt−Ut)/(Mt+Ut)] (Cost et al. 2014).

Determination of the oxidation state in hippocampus

NOS activity

The NO synthase (NOS) activity was evaluated through the conversion of [^{14}C]-L-arginine (300 mCi/mmol, PerkinElmer Inc., Waltham, MA, USA) to [^{14}C]-L-citrulline, as described previously (Palumbo et al. 2007). The hippocampi were homogenised by sonication in 1 mL of 20 mM HEPES buffer (pH 7.4) with 1 mM dithiothreitol (DTT), 1 μM leupeptin, 0.45 mM $CaCl_2$ and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). The homogenates were incubated at 37 °C for 30 min and under 5 % CO_2 in O_2 in the presence of [^{14}C]-L-arginine (0.5 μCi). The reaction was terminated by quick cooling using ice, and the samples were centrifuged at 20,000×g for 10 min at 4 °C. The supernatants were passed through 2-mL Dowex AG 50 WX-8 (sodium form) (Bio-Rad,

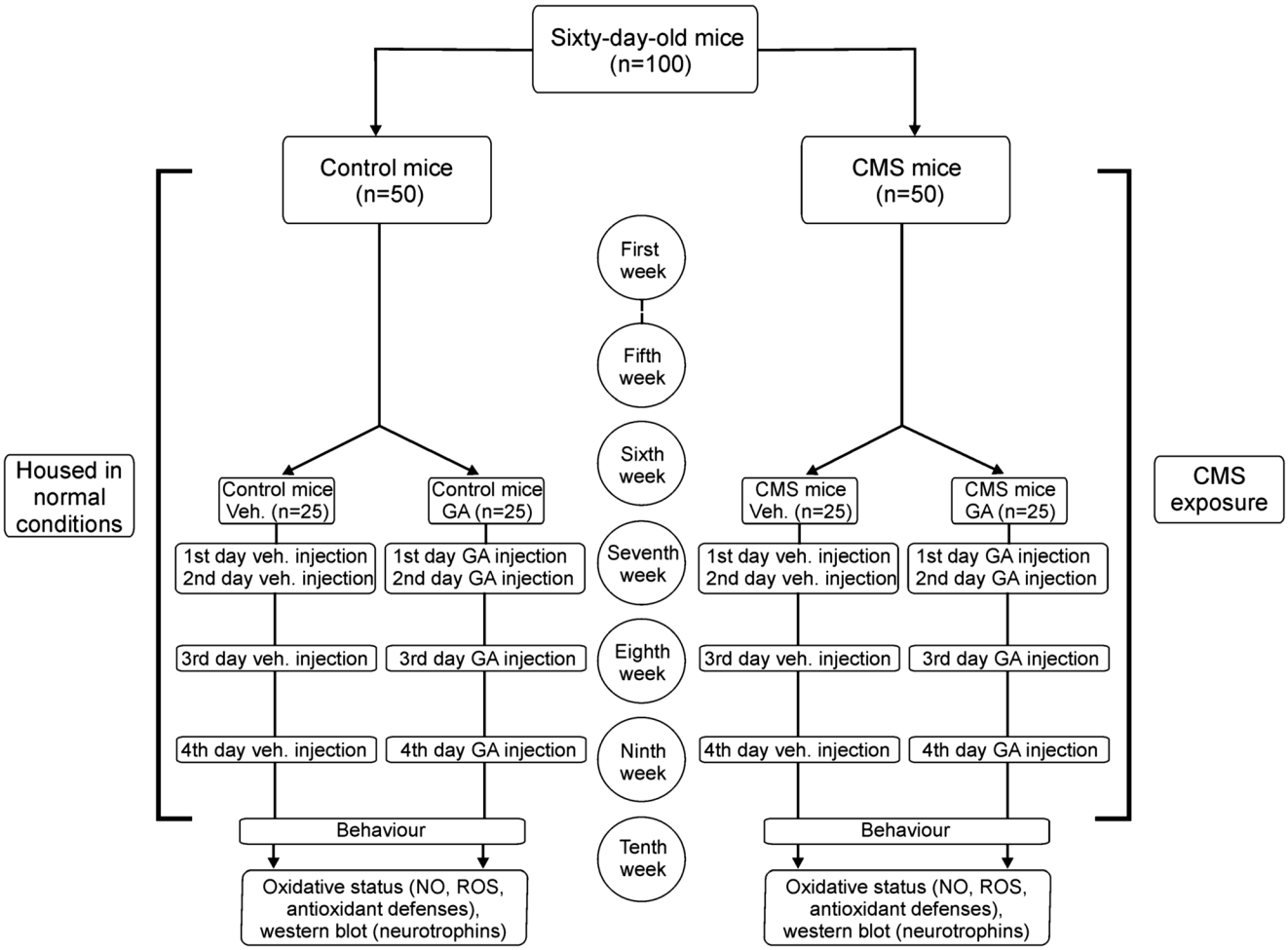

Fig. 1 Scheme of the experimental design used in the present work. The mice were divided into two groups: one housed in normal conditions (control, $n=50$) and the other subjected to *chronic mild stress* (CMS, $n=50$). Each group was then divided into two subgroups: two control subgroups and two CMS subgroups ($n=25$ in each group). After 6 weeks of exposure, one subgroup of control mice and one subgroup of CMS mice were injected with the vehicle ($n=25$) or glatiramer acetate (*GA*) ($n=25$). These mice were evaluated for behaviour, oxidative state and neurotrophin levels. The chronic stress protocol was continued after the GA treatment to rule out any effect due to the lack of stress

Hercules, CA, USA) columns. [^{14}C]-L-citrulline was eluted with 2 mL water and quantified by liquid scintillation counting. The results are expressed as picomoles of [^{14}C]-L-citrulline produced per gram of tissue in 30 min. In some experiments, the eNOS inhibitor L-N5-1-iminoethyl)ornithine hydrochloride (L-NIO, 0.8 μmol/L) or the nNOS inhibitor 7-NI (10 μmol/L) (Sigma-Aldrich, St. Louis, MO, USA) was added. The nNOS activity was calculated by considering the values obtained in the presence of the eNOS inhibitor (L-NIO), whereas the eNOS activity was calculated by considering the values obtained in the presence of the nNOS inhibitor (7-NI).

Analysis of ROS

The ROS levels were determined based on a previously described method (Keston and Brand 1965; Palumbo et al. 2007). The hippocampi were dissected on ice and homogenised in ice-cold Locke's buffer (NaCl 154 mmol/L, KCl 5.6 mmol/L, $NaHCO_3$ 3.6 mmol/L, $CaCl_2$ 2.3 mmol/L, glucose 5.6 mmol/L and HEPES 5 mmol/L). Aliquots of the homogenates (0.5-mg tissue/mL) were incubated with 2,7-dichlorofluorescin diacetate (final concentration 5 μmol/L) at 37 °C for 15 min. The results are expressed as picomoles per milligram of tissue per minute. In some experiments, the ROS levels were determined at different times (0.5, 2 and 24 h) after adding 7-NI (10 μmol/L).

Catalase activity measurement

The catalase (CAT) activity was assayed using the method described by Beers and Sizer (1952). Briefly, the tissues were homogenised at 10 %*w*/*v* in 50 mM phosphate buffer and centrifuged at 42,000×*g* for 15 min. A supernatant aliquot was incubated with 0.036 % (*w*/*w*) hydrogen peroxide solution (H_2O_2). The time required to decrease the absorbance at

240 nm from 0.45 to 0.40 absorbance units was recorded. One absorbance unit is defined as the amount of CAT that decomposes 1.0 μmol of H_2O_2/min at pH 7 at 25 °C. The results were calculated as CAT units per milligram of tissue and expressed as the mean±SEM.

Superoxide dismutase activity measurement

The superoxide dismutase (SOD) activity was determined using the procedure described by McCord and Fridovich (1969). The tissues were homogenised at 10 %*w*/*v* in a 216 mM, pH 7.8 phosphate buffer solution and centrifuged at 900×*g* at 4 °C for 10 min. A supernatant aliquot was mixed with 216 mM, pH 7.8 phosphate buffer, 10.7 mM EDTA, 1.1 mM cytochrome c and 0.108 mM xanthine at 25 °C. The reaction was started by adding 0.1 mL of xanthine oxidase enzyme solution (2 U/mL) (Sigma-Aldrich, St. Louis, MO, USA). The increase in the absorbance at 550 nm was recorded for 5 min. One unit of SOD is defined as the amount of SOD that inhibits the rate of reduction of cytochrome c by 50 % in a coupled system, using xanthine and xanthine oxidase at pH 7.8 and 25 °C in a 3-mL reaction volume. The results were calculated as SOD units per milligram of tissue and expressed as the mean±SEM.

Determination of glutathione

The levels of reduced glutathione (GSH) were determined using the GSH reductase recycling assay described by Tietze (1969). The hippocampi were sonicated in 100 mM sodium phosphate buffer, pH 7.5, with 1 mM EDTA. Trichloroacetic acid (TCA, 5 %) was added to precipitate proteins, and the homogenates were centrifuged at 3700 rpm and 4 °C for 10 min. The total and oxidised glutathione (GSSG) were determined for each sample. The homogenates were incubated for 1 h with 1 M 3-vinyl pyridine (Sigma-Aldrich, St. Louis, MO, USA), a scavenger of GSH, to quantify GSSG. Next, the reaction mixture (1 mM DTNB, 200 U/mL GSH reductase, 1 mM NADPH and 100 mM sodium phosphate buffer, pH 7.5, with 1 mM EDTA) was added. The absorbance was measured at 405 nm in an ELISA plate reader (Bio-Rad, Hercules, CA, USA). The GSH levels were calculated as the difference between total GSH and GSSG.

Western blot analysis

Neurotrophins from the homogenates of the hippocampi were analysed by immunoblot analysis. The hippocampi were homogenised in 3× sodium dodecyl sulphate (SDS) sample buffer [2 % SDS, 10 % (*v*/*v*) glycerol, 62.5 mM Tris–HCl, pH 6.8, 0.2 % bromophenol blue and 10 mM 2-mercaptoethanol]. The proteins were separated using SDS-PAGE on 15 % polyacrylamide gels and transferred to PVDF membranes. Non-specific binding sites were blocked with blocking buffer (5 % non-fat dried milk, containing 0.1 % Tween 20 in 100 mM Tris–HCl, pH 7.5 and 0.9 % NaCl) for 1 h. The membranes were subsequently incubated with anti-BDNF 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NT-3 1:100 (Abcam Inc., Cambridge, MA, USA) and anti-NGF 1:500 (Abcam Inc., Cambridge, MA, USA) for 18 h. Anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control for equal loading and transfer efficiency. Next, the membranes were incubated for 1 h with HSP-conjugated anti-rabbit immunoglobulin (Ig) G 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as secondary antibodies. The membranes were analysed by enhanced chemiluminescence (Amersham Biosciences, UK). The optical density (OD) of the bands on the films was determined by quantitative densitometry with a computerised image processing system (ImageQuant TL 7.0, GE Healthcare, Uppsala, Sweden). The densitometric analysis was performed using Gel-Pro Analyzer (version 3.1.00.00, Media Cybernetics, Inc. Rockville, MD, USA). The OD of β-actin bands was used as an internal control for differences in sample loading. For each blot, normalised values were expressed as the percentage of relative normalised controls and used for the statistical analyses.

Statistical analysis

The data were analysed using two-way ANOVA to examine the significance of the main effects and interactions. When the interaction was significant, simple effects analysis (*F*) was used, but when the interaction was not significant, the Bonferroni's (*t*) post hoc test was applied. Open-field habituation data and ROS production in the presence of the NOS inhibitor were analysed by two-way ANOVA for repeated measures (*F*), followed by the Bonferroni (*t*) test. Student's *t* test (*T*) was used to evaluate the open-field exploratory activity of the two groups during the first exposure. The differences between the means were considered significant if $p<0.05$.

Results

GA treatment reduces the effects of CMS on learning and memory

To evaluate the effect of stress and GA treatment on learning and memory performance, we used two different tasks: the open-field task to determine the ability to habituate to a novel environment and the object-in-place task to study short-term memory. As behavioural assessments can influence the animals' behaviour in subsequent tests, different mice were tested in each task. In agreement with our previous results (Palumbo

et al. 2012), the stressed mice had a poor habituation in the open-field test, which was reversed by the GA treatment (Fig. 2). The two-way ANOVA for repeated measures indicated changes in behavioural parameters, depending on the condition (control or CMS) and time (0 or 24 h) [interaction: condition × time; crossings: $F(1, 18)=29.400$, $p<0.001$; rearing: $F(1, 18)=12.976$, $p<0.01$; corner time: $F(1, 18)=12.684$, $p<0.01$]. In the control mice, the second exposure showed a decrease in the number of crossings (59 %, post hoc Bonferroni test; $t(18)=8.775$, $p<0.001$)) and rearing (44 %, $t(18)=6.806$, $p<0.001$) and an increase in the time spent in a corner (71 %, $t(18)=6.789$, $p<0.001$), compared with the first exposure. The CMS mice showed a reduced habituation capacity. No differences were observed in the number of crossings ($t(18)=1.106$, not significant (NS)), rearing ($t(18)=1.712$, NS) and the corner times ($t(18)=1.753$, NS) at 24 h compared with those at 0 h. However, the CMS mice injected with GA could habituate to the novel environment, similar to the control BALB/c mice. The two-way ANOVA for repeated measures indicated changes in behavioural parameters, depending on the condition (vehicle or GA) and/or time (0 or 24 h) [interaction: condition × time; crossings: $F(1, 18)=6.463$, $p<0.02$; rearing: $F(1,18)=17.478$, $p<0.001$; corner time: $F(1,18)=0.179$, NS]. For the GA-treated CMS mice, Bonferroni's post hoc test showed a decrease in the number of crossings ($t(18)=4.461$, $p<0.001$) and rearing ($t(18)=7.313$, $p<0.001$) and an increase in the corner time ($t(18)=3.478$, $p<0.01$) at 24 h. However, the GA treatment did not affect the performance of the control mice, with respect to the habituation [two way ANOVA for repeated measures, interaction: condition × time; crossings: $F(1, 18)=0.01$, NS; rearing: $F(1, 18)=2.327$, NS; corner time: $F(1, 18)=0.347$, NS]. For the GA-treated control mice, the post hoc Bonferroni test showed a significant difference in the parameters determined 24 h after the first exposure [crossings ($t(18)=12.920$, $p<0.001$); rearing ($t(18)=11.419$, $p<0.001$); corner time ($t(18)=7.511$, $p<0.001$)].

The short-term memory of the stressed and control mice, either injected or not injected with GA, was examined by the object-in-place task. Figure 3 shows the discrimination performance of the different experimental groups. The two-way ANOVA revealed significant changes, depending on the condition and treatment [interaction: condition × treatment; $F(3, 20)=98.260$, $p<0.0001$]. The simple effects analysis showed that the stressed mice failed to discriminate between the

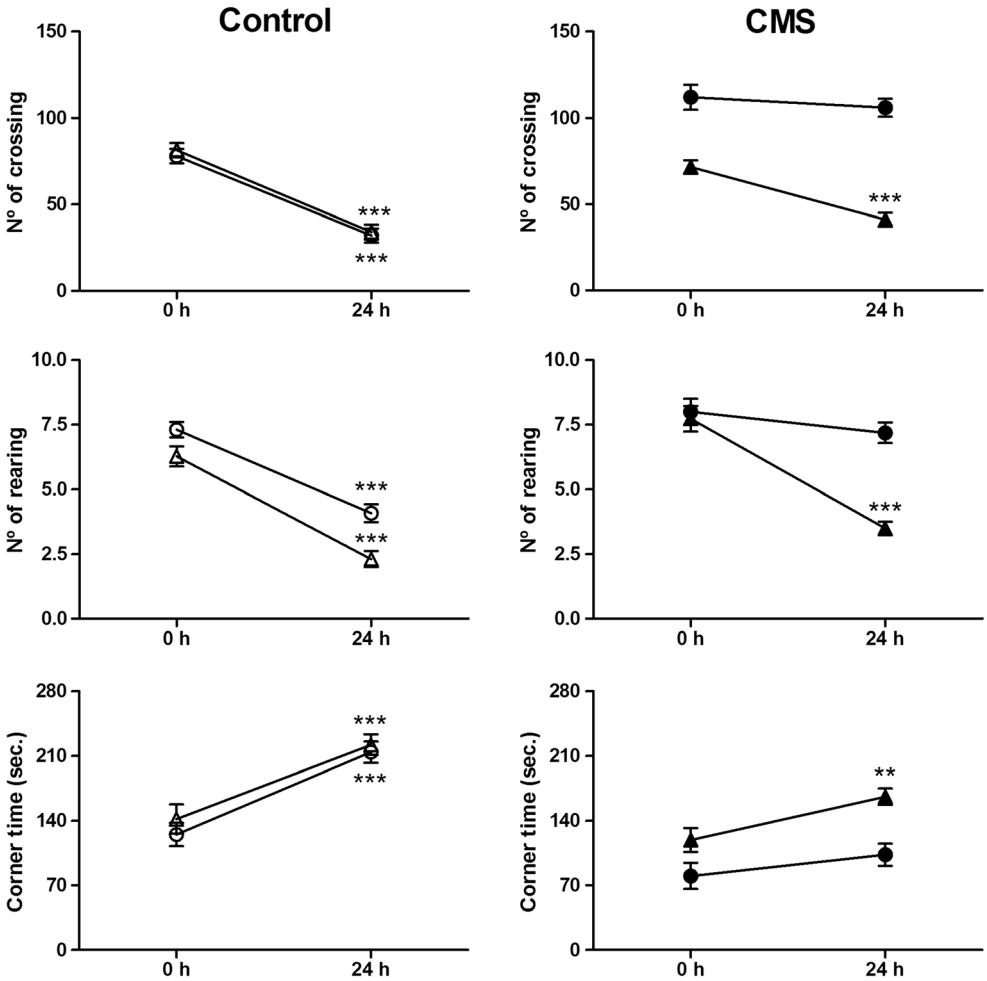

Fig. 2 Performance in open field in control, CMS and GA-treated BALB/c mice. The figure shows the number of crossings, the corner time and the number of rearing measured in the control (*white*) and CMS (*black*) BALB/c mice injected with either the vehicle (*circle*) or the GA (*triangle*) during the first exposure (0 h) and after 24 h. The *bars* denote the mean±SEM of ten mice for each group. *$p<0.05$; ***$p<0.001$ compared with the first exposure

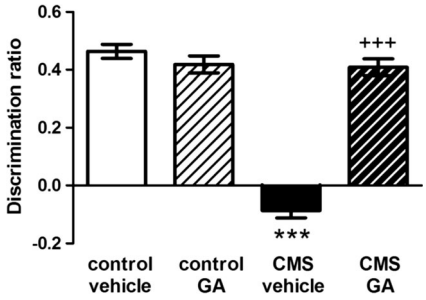

Fig. 3 Object-in-place task. The bar chart shows the discrimination ratio in the control (*white bars*) and CMS (*black bars*) BALB/c mice injected with either the vehicle (*plain bars*) or the GA (*striped bars*). The data represent the mean±SEM of six animals for each group. ***$p<0.001$ with respect to the vehicle-treated control mice; +++$p<0.001$ compared with vehicle-treated CMS mice

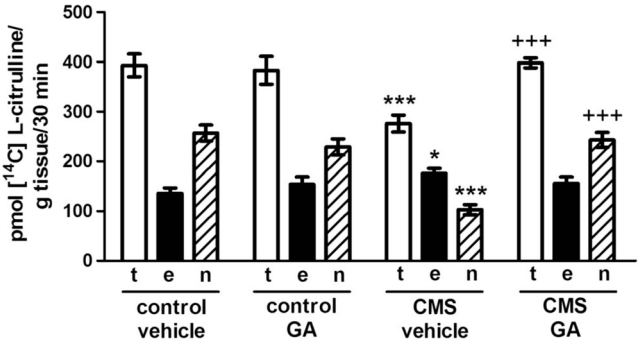

Fig. 4 Hippocampal nitric oxide synthase (NOS) activity. The graph presents the NOS activity expressed in picomoles [^{14}C]-L-citrulline per gram of tissue in 30 min. Total NOS (*t*, *white bars*), eNOS (*e*, *black bars*) and nNOS (*n*, *striped bars*) activity was determined in hippocampal homogenates from the BALB/c control and CMS mice injected with either the vehicle or the GA. The results represent the mean±SEM for seven animals per each group. *$p<0.05$, ***$p<0.001$ with respect to vehicle-treated control mice; +++$p<0.001$ with respect to vehicle-treated CMS mice

moved and unmoved objects, whereas the control group preferentially explored the moved objects ($F(1, 20)=202.222$, $p<0.0001$). The discrimination deficit observed in the CMS mice was completely reversed by the GA administration ($F(1, 20)=164.444$, $p<0.0001$). However, the GA treatment did not affect the performance of the control mice in any of the three tests performed ($F(1, 29)=2.222$, NS). These results demonstrate that GA treatment can improve the memory impairment induced by chronic stress.

Effect of GA treatment on the hippocampal oxidative status of stressed mice

To evaluate whether chronic stress affects the oxidative status in the hippocampi of BALB/C mice and whether GA treatment reverses the effects induced by chronic stress, we studied the activity of NOS, the levels of ROS and antioxidant defences such as the CAT, SOD and GSH levels.

NOS activity

Previously, we demonstrated that the total NOS activity was significantly lower in hippocampal homogenates from the CMS mice than in those from the control BALB/c mice (Palumbo et al. 2007). As presented in Fig. 4, the total NOS activity was lower in the CMS mice than in the control mice. This decrease was due to a decrease in the nNOS activity. Further, the eNOS activity was higher in the CMS mice than in the control mice. The GA treatment in the CMS mice restored the NOS and nNOS activity levels to normal. The two-way ANOVA showed significant changes in the total NOS and nNOS activity, depending on the condition and treatment [interaction: condition × treatment; total NOS: $F(3, 24)=10.251$, $p<0.01$ and nNOS: $F(3, 24)=32.800$, $p<0.001$]. The simple effects analysis showed a significant decrease in the total NOS activity ($F(1, 24)=16.171$, $p<0.001$) and nNOS activity ($F(1, 24)=55.540$, $p<0.001$) in the stressed mice compared with the control mice. This decrease was reversed by the GA treatment (CMS vehicle versus CMS GA, total NOS: $F(1, 24)=17.606$, $p<0.001$; nNOS: $F(1, 24)=45.728$, $p<0.001$). No significant difference was observed in the eNOS activity, depending on the condition and treatment [interaction: condition × treatment; $F(3, 24)=2.290$, NS]. Bonferroni analysis showed a significant increase in the eNOS activity ($t(12)=2.394$, $p<0.05$) in the stressed mice compared with the control mice. However, the control mice injected with GA showed non-significant differences in the total NOS, nNOS and eNOS activity compared with the control mice injected with the vehicle (Fig. 3).

ROS production

The two-way ANOVA revealed a significant relation between the ROS levels and the condition and treatment of the mice [interaction: condition × treatment; $F(3, 20)=4.50$, $p<0.05$]. The simple effects analysis showed significant differences in ROS production in the hippocampus from the CMS mice compared with those from the control mice [$F(1, 20)=28.444$, $p<0.001$]. The GA treatment in the stressed mice reversed the ROS levels [$F(1, 20)=18.778$, $p<0.001$] (Fig. 5a).

To study the involvement of NOS in ROS production, the ROS levels were determined in the presence of 7-NI, a selective nNOS inhibitor, in the mice hippocampi. As presented in Fig. 5b, the nNOS inhibition produced a time-dependent increase in the ROS levels in the four experimental groups. The two-way ANOVA for repeated measures showed that there was a non-significant interaction between the groups [interaction: condition × treatment $F(3, 40)=0.077$, NS]. The Bonferroni post hoc test showed a significant increase in the ROS levels after 24 h of nNOS inhibition (24 h vs basal values, control vehicle $p<0.001$, CMS vehicle $p<0.001$,

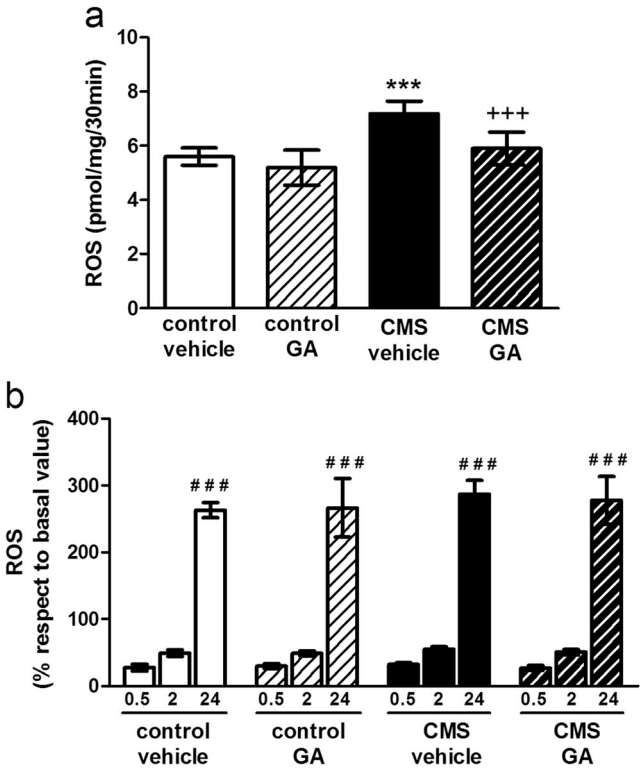

Fig. 5 ROS levels. The ROS production was determined in the hippocampi from the control (*white bars*) and CMS (*black bars*) mice injected with either the vehicle (*plain bars*) or the GA (*striped bars*). The results represent the mean±SEM for six mice from each group, and two independent experiments were performed in duplicate with three mice in each group. **a** ROS production in the absence of the NOS inhibitor. ***$p<0.001$ with respect to control mice injected with vehicle; +++$p<0.001$ with respect to CMS mice injected with vehicle. **b** ROS production in the presence of 7-NI (10 μmol/L) at different times after adding 7-NI (0.5, 2 and 24 h). ###$p<0.001$ with respect to the corresponding basal value

control GA $p<0.001$, CMS GA $p<0.001$). These results indicated that GA affects the ROS levels through a mechanism that involves NOS activity. However, the participation of other mechanisms cannot be disregarded.

Antioxidant defences

Chronic stress caused no significant changes in CAT, SOD or GSH levels. However, the GA treatment induced an increase in CAT activity in the control mice. The two-way ANOVA showed a non-significant interaction between the condition and treatment for the CAT, SOD and GSH levels [interaction: condition × treatment; CAT: $F(1, 20)=0.73$, NS; SOD: $F(1, 20)=1.37$, NS; GSH: $F(1, 20)=0.55$, NS]. The Bonferroni test indicated that the GA treatment only increased the CAT activity in control mice ($p<0.05$) (Fig. 6).

Western blot of neurotrophins

We evaluated whether neurotrophin-3 (NT-3), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) protein levels were different between the hippocampi from the control and the CMS mice, using Western blot analysis. Additionally, we studied whether the GA treatment affected the expression levels in both groups. The two-way ANOVA revealed that non-significant differences depended on the condition [control versus CMS, NT-3: $F(1, 20)=0.12$, NS; NGF: $F(1, 20)=0.24$, NS; BDNF: $F(1, 20)=0.003$, NS] and treatment [vehicle versus GA, NT-3: $F(1, 20)=0.003$, NS; NGF: $F(1, 20)=3.69$, NS; BDNF: $F(1, 20)=0.01$, NS] (Fig. 7).

Discussion

In the present work, we showed that GA treatment improved learning and memory in BALB/c mice under chronic stress through a mechanism that involves the regulation of NO production, which modulates ROS levels. We also analysed the neurotrophin expression levels and antioxidant defences but did not find any alteration in either stressed or GA-treated mice.

Several studies support the notion that a deregulated redox signalling can be crucial in the pathogenesis of various neurodegenerative and neurological disorders, including stress. Oxidative species are produced during normal physiologic processes and perform numerous roles in regulating cell function. Excess oxidative species are neutralised by intrinsic antioxidant mechanisms. However, under adverse situations, the antioxidant defences can be insufficient, resulting in structural and functional changes that induce cellular injury (Ischiropoulos and Beckman 2003). In BALB/c mice, chronic stress induced an increase in ROS production, which was related not to decreased antioxidant defences but to a diminution in NO production. This decrease was primarily due to nNOS activity and could not be compensated by increased eNOS activity. This increase in eNOS activity can be a compensatory mechanism to balance NO production in the hippocampus to minimise stress injury, as previously suggested (Zorrilla Zubilete et al. 2005; Hernandez et al. 2004; Palumbo et al. 2009). GA treatment restored the NO levels in the CMS mice and simultaneously decreased the ROS levels. In addition, the in vitro inhibition of NO production by nNOS in both control and GA-treated mice resulted in an increase in the ROS levels. Based on these results, we postulate that GA acts through a mechanism that involves NO production and consequently regulates ROS levels.

However, the precise mechanism through which GA restores NOS activity must be further examined. It has been previously demonstrated that immune treatment with GA induces a weak activation of self-reactive T cells (Kipnis and Schwartz 2002). Moreover, we have previously found that GA treatment reverses the deleterious effects of stress on neurogenesis and memory and increases interferon (IFN)-γ

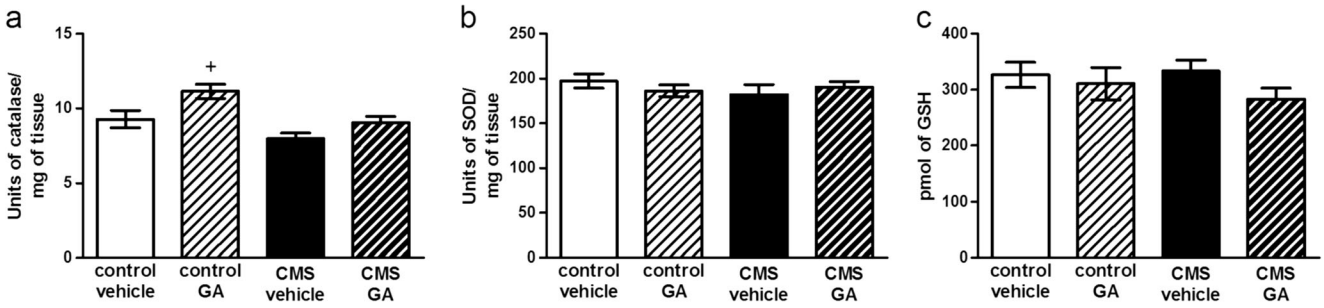

Fig. 6 Antioxidant defences. Catalase (CAT, **a**), superoxide dismutase (*SOD*, **b**) and reduced glutathione (*GSH*, **c**) levels were determined in the hippocampi from the control (*white bars*) and CMS (*black bars*) mice injected with either the vehicle (*plain bars*) or the GA (*striped bars*). The results represent the mean±SEM of six mice of each group, and two independent experiments were performed in duplicate with three mice in each group. $+p<0.05$ with respect to the corresponding control mice injected with vehicle

production (Palumbo et al. 2012). Possibly, IFN-γ could induce the expression of nNOS in the hippocampus. Further studies are necessary to confirm this possibility.

Conversely, previous studies have suggested an interaction between the NO and BDNF pathways in regulating neuronal function. Xiong et al. (1999) showed that BDNF and NO production are co-regulated in rodent neocortical neurons. In addition, Cheng et al. (2003) postulated a positive feedback loop between NO and BDNF, regulating neural progenitor cell proliferation and the differentiation of mammalian brains. However, we found that CMS exposure induced no changes in the NT-3, NGF or BDNF protein levels in the hippocampus. In addition, GA treatment did not affect the neurotrophin levels. In general, chronic stress is considered to decrease neurotrophin levels, particularly BDNF, in the hippocampus. However, the effects of stress on BDNF levels in the hippocampus appear to depend on several factors such as the type, intensity, duration and frequency of the stressors and the number of exposures (Neto et al. 2011). Several studies have reported no effects or even an increase in the BDNF levels in the hippocampus, following CMS exposure (Neto et al. 2011).

It is noteworthy that the GA treatment had no effect on the control mice in terms of the effects on behaviour, neurotrophin levels, NOS activity or ROS production. The lack of effect or an opposite effect in control animals is commonly observed in pharmacological treatments (Mitic et al. 2013; Nishiyori and Ueda 2008; Lewitus et al. 2009). However, the GA treatment induced an increase in CAT activity. Considering that this increase in CAT activity is not observed in GA-treated CMS mice, it is reasonable to hypothesise that GA has different mechanisms of action in normal conditions and in stress conditions. Thus, stress triggers responses involving mechanisms (such as a decrease in NO production) that can be modulated by GA. These mechanisms are absent under normal conditions; therefore, GA has no significant effects on the control mice.

Finally, it should be noted that we used female mice for our investigation. These female mice were in the same phase of the cycle at the beginning of the experiment, and we assumed that they were in the same phase of the cycle in all experiments. It is important to consider gender differences in cognition reported under both basal conditions and following chronic stress. Usually, the results of various studies in rodent species indicated that males perform better than females in tasks requiring the use of spatial learning and memory (Gresack and Frick 2003; Perrot-Sinal et al. 1996). However, under chronic stress exposure, the females are more resistant to stress-mediated cognitive impairments than males are (Bowman 2005; Conrad et al. 2003; Kitraki et al. 2004). However, the results strongly depended on the strain used

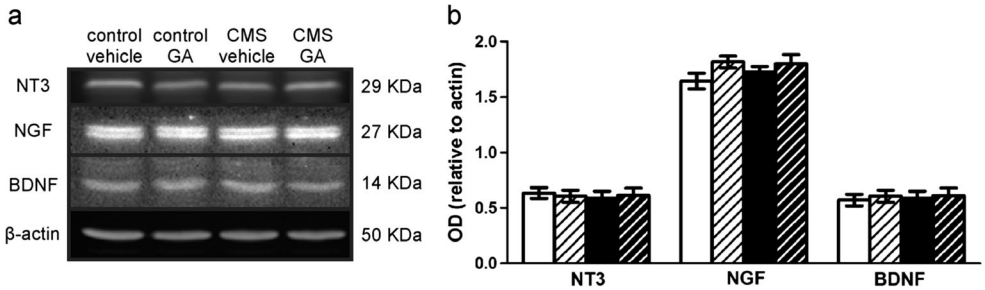

Fig. 7 Neurotrophin expression. **a** Representative Western blot for neurotrophin-3 (*NT-3*), nerve growth factor (*NGF*) and brain-derived neurotrophic factor (*BDNF*) in the hippocampi from the control (*white bars*) and CMS (*black bars*) mice injected with the vehicle (*plain bars*) or GA (*striped bars*). **b** Densitometric analysis of NT-3, NGF and BDNF expressed in arbitrary units of optical density (*OD*) relative to actin. The results represent the mean±SEM of three independent experiments with two mice in each group

(Franceschelli et al. 2014). Considering these findings, future studies are needed to establish whether gender differences exist with respect to the effect of CMS and GA administration.

Conclusions

The present findings highlight a critical role of nNOS in the effect of GA treatment on the cognitive decline induced by chronic stress. A decrease in NO production results in an increase in ROS levels, which in turn induces tissue toxicity, thereby impairing learning and memory. GA treatment attenuates the cognitive deficit induced by chronic stress exposure by regulating NO production. Finally, the present results may be useful in generating new strategies for treating the consequences of stress- and age-related memory impairments.

Acknowledgments The authors thank María Rosa Gonzalez Murano for her technical assistance, Daniel Gonzalez for his valuable help in the animal stress model and Noemí Cappano and Patricia Fernandez for their secretarial assistance. This work was supported by grants from CONICET (PIP 00281) and from the University of Buenos Aires (UBACyT 2002010010633) to AMG.

Conflict of interest The authors declare that there are no conflicts of interest.

References

Beers RF Jr, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195:133–140

Bowman RE (2005) Stress-induced changes in spatial memory are sexually differentiated and vary across the lifespan. J Neuroendocrinol 17:526–535

Butovsky O, Koronyo-Hamaoui M, Kunis G, Ophir E, Landa G, Cohen H, Schwartz M (2006) Glatiramer acetate fights against Alzheimer's disease by inducing dendritic-like microglia expressing insulin-like growth factor 1. Proc Natl Acad Sci U S A 103:11784–11789

Canossa M, Giordano E, Cappello S, Guarnieri C, Ferri S (2002) Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. Proc Natl Acad Sci U S A 99:3282–3287

Cheng A, Wang S, Cai J, Rao MS, Mattson MP (2003) Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. Dev Biol 258:319–333

Conrad CD, Grote KA, Hobbs RJ, Ferayorni A (2003) Sex differences in spatial and non-spatial Y-maze performance after chronic stress. Neurobiol Learn Mem 79:32–40

Cost KT, Lobell TD, Williams-Yee ZN, Henderson S, Dohanich G (2014) The effects of pregnancy, lactation, and primiparity on object-in-place memory of female rats. Horm Behav 65:32–39

Dillon GM, Qu X, Marcus JN, Dodart JC (2008) Excitotoxic lesions restricted to the dorsal CA1 field of the hippocampus impair spatial memory and extinction learning in C57BL/6 mice. Neurobiol Learn Mem 90:426–433

Dwivedi Y (2012) Brain-derived neurotrophic factor in suicide pathophysiology. In: Dwivedi Y (ed) The neurobiological basis of suicide. CRC Press, Boca Raton, Chapter 8. Available from: http://www.ncbi.nlm.nih.gov/books/NBK107216/

Franceschelli A, Herchick S, Thelen C, Papadopoulou-Daifoti Z, Pitychoutis PM (2014) Sex differences in the chronic mild stress model of depression. Behav Pharmacol 25:372–383

Frisch C, De Souza-Silva M, Söhl G, Güldenagel M, Willecke K, Huston JP, Dere E (2005) Stimulus complexity dependent memory impairment and changes in motor performance after deletion of the neuronal gap junction protein connexin36 in mice. Behav Brain Res 157:177–185

Gresack JE, Frick KM (2003) Male mice exhibit better spatial working and reference memory than females in a water-escape radial arm maze task. Brain Res 982:98–107

Hernandez R, Martinez-Lara E, Del Moral ML, Blanco S, Cañuelo A, Siles E, Esteban FJ, Pedrosa JA, Peinado MA (2004) Upregulation of endothelial nitric oxide synthase maintains nitric oxide production in the cerebellum of thioacetamide cirrhotic rats. Neuroscience 126:879–887

Hill BG, Dranka BP, Bailey SM, Lancaster JR Jr, Darley Usmar VM (2010) What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. J Biol Chem 285:19699–19704

Ischiropoulos H, Beckman JS (2003) Oxidative stress and nitration in neurodegeneration: cause, effect, or association? J Clin Invest 111:163–169

Jiang C, Salton SR (2013) The role of neurotrophins in major depressive disorder. Transl Neurosci 4:46–58

Keston AS, Brand TR (1965) The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal Biochem 11:1–5

Kipnis J, Schwartz M (2002) Dual action of glatiramer acetate (Cop-1) in the treatment of CNS autoimmune and neurodegenerative disorders. Trends Mol Med 8:319–323

Kipnis J, Yoles E, Porat Z, Cohen A, Mor F, Sela M, Cohen IR, Schwartz M (2000) T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. Proc Natl Acad Sci U S A 97:7446–7451

Kitraki E, Kremmyda O, Youlatos D, Alexis MN, Kittas C (2004) Gender-dependent alterations in corticosteroid receptor status and spatial performance following 21 days of restraint stress. Neuroscience 125:47–55

Lewitus GM, Wilf-Yarkoni A, Ziv Y, Shabat-Simon M, Gersner R, Zangen A, Schwartz M (2009) Vaccination as a novel approach for treating depressive behavior. Biol Psychiatry 65:283–288

Lipton SA (1999) Neuronal protection and destruction by NO. Cell Death Differ 6:943–951

Madrigal JL, Garcia-Bueno B, Caso JR, Perez-Nievas BG, Leza JC (2006) Stress-induced oxidative changes in brain CNS. Neurol Disord Drug Targets 5:561–568

McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055

McEwen BS (2008) Central effects of stress hormones in health and disease: understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol 583:174–185

Miranda KM, Espey MG, Wink DA (2000) A discussion of the chemistry of oxidative and nitrosative stress in cytotoxicity. J Inorg Biochem 79:237–240

Mitic M, Simic I, Djordjevic J, Radojcic MB, Adzic M (2013) Gender-specific effects of fluoxetine on hippocampal glucocorticoid receptor phosphorylation and behavior in chronically stressed rats. Neuropharmacology 70:100–111

Monleon S, Aquila P, Parra A, Simon VM, Brain PF, Willner P (1995) Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. Psychopharmacology 117:453–457

Neto FL, Borgesa G, Torres-Sanchezb S, Micob JA, Berrocosob E (2011) Neurotrophins role in depression neurobiology: a review of basic and clinical evidence. Curr Neuropharmacol 9:530–552

Nishiyori M, Ueda H (2008) Prolonged gabapentin analgesia in an experimental mouse model of fibromyalgia. Mol Pain 4:52. doi: 10.1186/1744-8069-4-52

Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA (1990) Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci U S A 87:5144–5147

Palumbo ML, Fosser NS, Ríos H, Zorrilla Zubilete MA, Guelman LR, Cremaschi GA, Genaro AM (2007) Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory. J Neurochem 102:261–274

Palumbo ML, Zorrilla-Zubilete MA, Cremaschi GA, Genaro AM (2009) Different effect of chronic stress on learning and memory in BALB/c and C57BL/6 inbred mice: involvement of hippocampal NO production and PKC activity. Stress 4: 350–361

Palumbo ML, Canzobre MC, Pascuan CG, Ríos H, Wald M, Genaro AM (2010) Stress induced cognitive deficit is differentially modulated in BALB/c and C57Bl/ 6 mice: correlation with Th1/Th2 balance after stress exposure. J Neuroimmunol 218:12–20

Palumbo ML, Trinchero MF, Zorrilla-Zubilete MA, Schinder AF, Genaro AM (2012) Glatiramer acetate reverts stress-induced alterations on adult neurogenesis and behavior. Involvement of Th1/Th2 balance. Brain Behav Immun 26:429–438

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487–491

Perrot-Sinal TS, Kostenuik AM, Ossenkopp KP, Kavaliers M (1996) Sex differences in performance in the Morris water maze and the effects of initial nonstationary hidden platform training. Behav Neurosci 110:1309–1320

Pryor WA, Houk KN, Foote CS, Fukuto JM, Ignarro LJ, Squadrito GL, Davies KJ (2006) Free radical biology and medicine: it's a gas, man! Am J Physiol Regul Integr Comp Physiol 291:R491–R511

Reagan LP, McKittrick CR, McEwen BS (1999) Corticosterone and phenytoin reduce neuronal nitric oxide synthase messenger RNA expression in rat hippocampus. Neuroscience 91:211–219

Schiavone S, Jaquet V, Trabace L, Krause KH (2013) Severe life stress and oxidative stress in the brain: from animal models to human pathology. Antioxid Redox Signal 18:1475–1490

Schwartz M, London A, Shechter R (2009) Boosting T-cell immunity as a therapeutic approach for neurodegenerative conditions: the role of innate immunity. Neuroscience 158:1133–1142

Teitelbaum D, Meshorer A, Hirshfeld T, Arnon R, Sela M (1971) Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. Eur J Immunol 1:242–248

Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA (2008) The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med 45:18–31

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal Biochem 27: 502–522

Willner P, Muscat R, Papp M (1992) Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev 16:525–534

Xiong H, Yamada K, Han D, Nabeshima T, Enikolopov G, Carnahan J, Nawa H (1999) Mutual regulation between the intercellular messengers nitric oxide and brain-derived neurotrophic factor in rodent neocortical neurons. Eur J Neurosci 11:1567–1576

Zorrilla Zubilete MA, Ríos H, Silberman DM, Guelman LR, Ricatti JM, Genaro AM, Zieher LM (2005) Altered nitric oxide synthase and PKC activities in cerebellum of gamma-irradiated neonatal rats. Brain Res 1051:8–16