Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice

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Article Info
Article history:
Received 17 May 2012
Revised 17 October 2012
Accepted 24 October 2012
Available online 8 November 2012

Keywords:
Folic acid
Antidepressant
Major depression
Acute restraint stress
Oxidative stress
Antioxidant

Abstract
Experimental and epidemiological studies have shown the close relationship between stressful events, depression, and cognitive impairment. Folic acid has been reported to present antidepressant-like effects in both experimental and clinical approaches. However, the mechanisms mediating such effects are not understood. In the present study, we evaluated if folic acid administration to mice could protect against restraint stress-induced depressive-like behavior and cognitive deficit. Considering that oxidative stress has been pointed as a key event involved with depressive disorders, cerebrocortical and hippocampal oxidative stress-related parameters, such as the activities of antioxidant enzymes (mainly those related to the hydroperoxide-detoxifying system) and markers of lipid peroxidation, were also investigated. Restraint stress induced depressive-like behavior in the forced swimming test and memory impairment in the object recognition test, without altering locomotor activity of mice. Folic acid (50 mg/kg, p.o.) was able to prevent the stress-induced increase on immobility time in the forced swimming test, but did not prevent memory impairment. Moreover, restraint stress increased thiobarbituric acid reactive substance levels, and catalase, glutathione peroxidase and glutathione reductase activities in the cerebral cortex and hippocampus, and superoxide dismutase activity in the hippocampus. Folic acid treatment restored the activity of the antioxidant enzymes and reduced lipid peroxidation in the hippocampus. Glutathione, a non-enzymatic antioxidant, was not altered by stress and/or folic acid administration. Together, the results of the present work reinforce the notion that folic acid displays a specific antidepressant profile in the restraint stress paradigm that may be at least partly due to its antioxidant role.

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Introduction
Stress may be defined as a state of threatened homeostasis that can produce adaptive physiologic and behavioral responses depending on severity, type and duration of stressful events in an attempt to re-establish body homeostasis (Chrousos, 2009; Jaggi et al., 2011; Munhoz et al., 2008). These physiological, psychological, and cognitive alterations induced by stress affect different organs and systems, including the central nervous system (CNS) (Linthorst and Reul, 2008; Munhoz et al., 2008). A consequence of stressful events is the increased susceptibility to different psychiatric diseases, including depression (Calabrese et al., 2011), which frequently is accompanied by cognitive deficits (Marazziti et al., 2010; Murrough et al., 2011). Stressful life events have a considerable causal association with the pathophysiology of this disorder, especially in genetically predisposed individuals (Charney and Manji, 2004; Lanumey et al., 2008).

Many animal models of stress-induced depression are widely used to investigate potential biochemical parameters affected in depression, as well as screen antidepressant drugs (Kaluff et al., 2007). Using stress to induce a feeling of loss of control might result in a behavioral state analogous to depression (Calabrese et al., 2011; Kubera...
et al., 2011). Restraint stress is frequently employed to induce a depressive behavioral state in rodents. In particular, it has been widely used in acute and chronic stress studies (Capra et al., 2010; Christiansen et al., 2011; Huynh et al., 2011; Poleszak et al., 2006; Sekiguchi et al., 2006).

Stress exerts detrimental effects on several cellular functions, as evidenced by defective plasma antioxidant defenses in conjunction with enhanced lipid peroxidation in depressive patients (Bilici et al., 2001; Khanzode et al., 2003; Ozcan et al., 2004), indicating that oxidative damage is an important mechanism in the pathophysiology of depression in humans (Maes et al., 2011). Similarly, restraint stress in rodents precipitates many neurochemical and hormonal abnormalities that are often associated with an imbalance in the brain’s intracellular redox state. Several studies have reported that restraint stress enhances lipid peroxidation and the activity of antioxidant enzymes in brain regions (Balk et al., 2010; García-Fernández et al., 2012), including the cerebral cortex and hippocampus, two structures closely related to the pathophysiology of depression (Duman and Voleti, 2012; Yu and Chen, 2011). On the other hand, decreased antioxidant enzyme activities have also been reported in rodents submitted to restraint stress (Balk et al., 2010; Kumari et al., 2010).

It is well known that aversive stimuli (like stress), especially in the brain, may result in the production of reactive oxygen species (ROS), such as superoxide anion radical (O$_2^-$), hydroxyl radical (HO$^.$), and hydrogen peroxide (H$_2$O$_2$). When ROS production exceeds the antioxidant capacity, they could lead to lipid peroxidation, especially in membranes, which plays an important role in tissue injury (Halliwell, 1992). Important endogenous antioxidant enzymes, which inhibit the formation of ROS or promote the removal of free radicals and their precursors, include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) (McCord and Fridovich, 1988). SOD is the first line of defense against ROS and catalyzes the dismutation of superoxide anion radical (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) (McCord and Fridovich, 1988). This molecule, H$_2$O$_2$, can be reduced to water and molecular oxygen by either CAT (Chelkani et al., 2004) or GPx (Flohe, 1971). Besides detoxifying H$_2$O$_2$, GPx can also reduce lipid and non-lipid hydroperoxides at the expense of reduced glutathione (GSH), which is in turn oxidized, forming glutathione disulfide (GSSG) (Flohe, 1971). GSH is the most important non-enzymatic endogenous antioxidant and can be regenerated by GSR with the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) (Krohne-Ehrich et al., 1977). This non-enzymatic antioxidant plays a role in detoxifying a variety of electrophilic xenobiotics, producing less toxic compounds (Jakoby, 1978).

Although the underlying pathophysiological mechanisms of stress-induced depression are not completely established, novel targets have been identified for the development of novel pharmacological treatments affording higher efficacy, lower side effects, and faster action (Lee et al., 2010). There is evidence that folic acid is an important micro-nutrient that may contribute to depressive disorders and its treatment (Coppens and Bolander-Gouaille, 2005; Morris et al., 2008; Sarris et al., 2009). Besides its essential role to the synthesis of DNA, RNA, and proteins for neurological function (Fenech, 2010; Kronenberg et al., 2009), folic acid has been postulated as a putative antidepressant agent, based on preclinical (Brockard et al., 2008a; Budni et al., 2012a, b) and clinical (Astorg et al., 2008; Papakostas et al., 2004) studies. In addition, antimanic-like (Brockard et al., 2010), cognitive-enhancing (Matté et al., 2009a; Singh et al., 2011) and neuroprotective (Budni et al., 2011; Matté et al., 2009b; Yu et al., 2009) properties have been reported for this vitamin. Although the aforementioned studies point to antidepressant effects of folic acid in both experimental and clinical approaches, the potential involvement of anti- and pro-oxidative effects in such effects is not clear.

Using the above considerations, the aim of our present work was to study if folic acid administration to mice could protect against acute restraint stress (ARS)-induced changes in parameters related to depressive, locomotor/exploratory and cognitive behaviors, as well as if cerebrocortical and hippocampal oxidative stress could be involved in such events. Based on the potential antioxidant effects of folic acid, we hypothesized that it could prevent ARS-induced depressive behavior and cognitive decline by modulating oxidative stress-related events.

Materials and methods

Animals

Male Swiss mice weighing 40–45 g (4-month old) were maintained at 21–23 °C with free access to water and food, under a 12:12 h light:dark cycle (lights on at 7:00 a.m.). All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval by the Ethics Committee of the institution and all efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Drugs and treatment

Folic acid (Sigma Chemical Co., St. Louis, U.S.A) was dissolved in distilled water and administered orally (p.o.) by gavage at a dose of 50 mg/kg 1 h before the ARS procedure. Folic acid solution was freshly prepared before administration and was administered in a volume of 1 ml/kg. Prior to the experiments, the animals were maintained at free access to water and food (Nuvilab CR-1®, Brazil, containing 1.0 mg/kg of folic acid). Thus, the control animals were not folic acid deficient and folic acid treated animals received an extra (supplementation) amount of this vitamin, exceeding the daily dietary intake. To develop this study mice were divided into four groups, as follows: (1) vehicle + non-stressed; (2) folic acid + non-stressed; (3) vehicle + stressed; (4) folic acid + stressed. The dose of folic acid was chosen based on previous studies from our group (Brockard et al., 2008a,b, 2009; Budni et al., 2012a,b).

Acute restraint stress (ARS) procedure

ARS protocol was adapted from the previous procedure (Kumar and Goyal, 2008; Poleszak et al., 2006; Zafri et al., 2009). The animals were divided into four groups as mentioned above. Stressed groups were administered with vehicle or folic acid and 1 h after the treatment they were submitted to stress. In the period (1 h) that elapsed between the folic acid treatment and stress procedure, the animals were maintained in their home cages with free access to water and food. The immobilization was applied for a period of 7 h using an individual rodent restraint device made of Plexiglas fenestrated. This restrained all physical movement without causing pain. The animals were deprived of food and water during the entire period of exposure to stress. After 7 h, independent groups of animals were released from their enclosure and 40 min post-release the animals were submitted to the FST (n=8–10 per group), or ORT (n=8–10 per group), or sacrificed for the biochemical studies (n=8–10 per group). Non-stressed groups were treated with vehicle or folic acid and were kept without food and water during the entire period of exposure to stress.

Behavioral tests

Forced swimming test (FST)

Briefly, mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C, the total duration of immobility was measured during a 6-min period as described previously (Brockard et al., 2008b; Budni et al., 2007). Each mouse was judged to be immobile...
when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect and an increase of immobility time, when compared to the control group, is considered a depressive-like effect (Kaster et al., 2012; Porsolt et al., 1977).

Open-field test
To assess the possible effects of folic acid on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Budni et al., 2007; Rodrigues et al., 1996). Animals were individually placed in a wooden box (40 × 60 × 50 cm) with the floor divided into 12 rectangles. The number of squares crossed with all paws (crossing) was counted in a 6 min session. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

Object recognition test (ORT)
The object recognition was performed as previously described (Réus et al., 2008). The task took place in a 40 × 60 × 50 cm open-field surrounded by 50 cm high walls made of plywood with a frontal glass wall. The floor of the open-field was divided into 12 equal rectangles by black lines. The training session was conducted by placing individual mouse for 5 min in the apparatus, in which two identical objects (objects A1 and A2; both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a long-term recognition memory test given 24 h after training, the mice explored the open-field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150–200 g), but distinctive shapes. A recognition index calculated for each animal was calculated in the test session, and it reports the ratio TB/(TA + TB) (TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). Between trials, the objects were washed with 10% ethanol solution. Exploration was defined as sniffing (exploring the object 3–5 cm away from it) or touching the object with the nose and/or forepaws.

Biochemical analysis

Tissue preparation
Forty minutes after the ARS procedure, the animals were killed by decapitation and the cerebral cortices and hippocampi were removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 × g, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities and for the quantification of the levels of GSH and thiobarbituric acid reactive substances (TBARS).

Activity of antioxidant enzymes
Glutathione reductase (GSR) activity was determined based on the protocol developed by Carlberg and Mannervik (1985). Briefly, GSR reduces GSSG to GSH at the expense of NADPH, the disappearance of which can be followed at 340 nm. Glutathione peroxidase (GPx) activity was determined based on the protocol developed by (Wendel, 1981) by indirectly measuring the consumption of NADPH at 340 nm. The GPx uses GSH to reduce the tert-butyl hydroperoxide, producing GSSG, which is readily reduced to GSH by GSR using NADPH as a reducing equivalent donor. Therefore, GSR activity was determined by indirectly measuring the consumption of NADPH. Both GSR and GPx activities were expressed as nmol NADPH oxidized/min/mg protein. Catalase activity was measured by the method of Aebi (1984). The reaction was started by the addition of freshly prepared 30 mM H2O2. The rate of H2O2 decomposition by catalase was measured spectrophotometrically at 240 nm and the enzyme activity was expressed as μmol H2O2 consumed/min/mg protein. Superoxide dismutase activity was assayed spectrophotometrically as described by Misra and Fridovich (1972). The spectrophotometer used for these assays was a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). This method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. The SOD enzymatic activity was expressed as units (U)/mg protein.

Glutathione levels
Glutathione (GSH) levels were measured as non-protein thiols (NPSH), based on the protocol developed by Ellman (1955). This methodology quantifies only the reduced form of glutathione, which is responsible for the antioxidant properties of this peptide. In addition, a small percentage (around 5%) of other low-molecular-weight thiols is also measured. Briefly, hippocampal and cerebrocortical homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at 5000 × g for 10 min, and the supernatant was incubated with DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) in a 1 M phosphate buffer, pH 7.0. Absorbances were measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels, which were expressed as nmol NPSH/mg protein.

Thiobarbituric acid reactive species (TBARS) formation
TBARS levels, a measurement of lipid peroxidation, were determined in the hippocampal and cerebrocortical homogenates according to the method described by Okawa et al. (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. This method is not specific because aldehydes other than MDA also react with TBA (Halliwell and Gutteridge, 1999). However, the method provides a general view of the peroxidative state in biological systems. The samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as a standard.

Determination of protein
The protein content was quantified according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

Statistical analysis
All data are presented as mean ± SEM. Data were analyzed by two-way ANOVA or two-way repeated measures ANOVA, followed by Duncan’s post hoc test when the F value was significant. Differences were considered statistically significant if p ≤ 0.05.

Results

Behavioral tests

Forced swimming test (FST)
Fig. 1A shows the effect of treatment of mice with folic acid or vehicle on the depressive-like behavior elicited by ARS. The two-way ANOVA revealed significant differences for folic acid treatment [F(1, 26) = 33.11, p < 0.01], restraint stress [F(1, 26) = 13.28, p < 0.01], and ARS vs. folic acid treatment interaction [F(1, 26) = 4.38, p < 0.05]. Post-hoc analyses indicated that stressful stimuli significantly (p < 0.01) reversed the increase in immobility time in stressed mice. Folic acid administration significantly (p < 0.01) reversed the increase in immobility time in stressed mice. Folic acid administration, in non-stressed mice, also significantly (p < 0.05) decreased the immobility time in the FST as compared to non-stressed mice treated with vehicle. There are no significant differences (p = 0.28) between non-stressed folic acid-treated...
mice (mean of immobility time: 245.71±7.93 s) and stressed folic acid-treated mice (mean of immobility time: 256.43±5.77 s) in the FST.

**Locomotor activity**

The result depicted in Fig. 1B shows that locomotor activity of mice submitted to 7 h of ARS treated with folic acid or vehicle was not altered in the open-field test. The two-way ANOVA revealed no significant differences for folic acid treatment [F(1,28)=0.05, p=0.82], restraint stress [F(1,28)=0.06, p=0.80], and treatment vs. restraint stress interaction [F(1,28)=0.05, p=0.82].

**Object recognition test (ORT)**

In order to evaluate if restraint stress induces impairment of cognitive function the ORT was carried out. Repeated-measures ANOVA showed significant differences for the stress group [F(1,23)=99.51, p<0.01] and folic acid vs. stress interaction [F(1,23)=5.56, p<0.05], but not for R1 vs. folic acid group [F(1,23)=0.099, p=0.76]. There were significant differences for repetition 1 (R1) vs. stress (R1) [F(1,23)=123.27, p<0.01] and R1 vs. folic acid vs stress group interaction [F(1,23)=5.10, p<0.05], but not for R1 vs. folic acid group [F(1,23)=0.49, p=0.49]. In the training session, all groups (non-stressed or stressed mice) showed no differences in the exploration between the two objects (means±SEM of recognition index: vehicle/non-stressed=0.494±0.010; folic acid/non-stressed=0.497±0.005; vehicle/stressed=0.491±0.015; folic acid/stressed=0.481±0.015). If mice remember an object, they prefer to explore and sniff the novel object when the original object is replaced by a new one. In the test session (24 h after the training session), mice from the vehicle/non-stressed group (0.685±0.023) and folic acid/non-stressed group (0.473±0.003) preferred to explore the novel object, while the stressed mice group (0.474±0.003) explored the novel and original objects similarly, which is indicative of a cognitive impairment induced by restraint stress (p<0.01). Folic acid treatment (folic acid/stressed=0.426±0.027) was not capable of preventing the decline in the recognition of a novel object induced by restraint stress, since we observed a similar frequency of exploring and sniffing the novel object or the original one in the folic acid/stressed group. Also, it is important to mention that post hoc analysis indicated a significant difference between the vehicle/non-stressed and folic acid/non-stressed groups in the test session (p<0.01), since folic acid induced an increase in the recognition index (11%) when compared with animals treated with vehicle in the test session (Fig. 2).

**Measurement of biochemical parameters**

**Lipid peroxidation**

TBARS levels were measured because malondialdehyde (MDA) is a late product of lipid peroxidation. The presence of MDA may be an indicative of a more evident oxidative damage to the membranes, compromising their functions and overall cellular integrity (Hernández-Martínez et al., 2011). Fig. 3 shows that TBARS levels were significantly increased in the cerebral cortex and hippocampus of stressed mice when compared to non-stressed mice. Treatment with folic acid (50 mg/kg, p.o.) significantly prevented the increase of TBARS levels in the hippocampus (Fig. 3B), but not in the cerebral cortex (Fig. 3A), when compared to stressed animals. The two-way ANOVA showed significant differences for folic acid treatment [F(1,17)=8.79, p<0.01] and folic acid treatment vs. ARS interaction [F(1,17)=5.51, p<0.05], but not for ARS [F(1,16)=6.36, p=0.092]. Folic acid administration to non-stressed mice did not produce any significant effect on TBARS levels.

**GSH levels**

Fig. 4 shows that no significant statistical difference was observed in the GSH level in the cerebral cortex (Fig. 4A) and hippocampus

Fig. 1. Effect of treatment with folic acid on immobility time in the FST (panel A) and on locomotor activity (panel B) in mice submitted to restraint stress. Values are expressed as mean±SEM (n=7–8). **p<0.01 and *p<0.05 compared with the non-stressed group treated with vehicle. #p<0.01 compared with the stressed group treated with vehicle, according to two-way ANOVA followed by Duncan’s post hoc test.

Fig. 2. Effect of treatment with folic acid in the object recognition test (ORT) in mice submitted to restraint stress. Recognition index for the objects in the training and test sessions for non-stressed and stressed groups of mice. Results are presented as mean±SEM of the recognition index (n=6–8). The test session was performed 24 h after the training session. **p<0.001 compared with the same group of training session. ##p<0.01 indicates difference from the recognition index between the vehicle/non-stressed and folic acid/non-stressed groups of test session, according to two-way repeated measures ANOVA followed by Duncan’s post hoc test.
Antioxidant enzyme activities

Restraint stress significantly increased CAT activity in the cerebral cortex (Fig. 5A) and hippocampus (Fig. 5B) of stressed mice as compared to non-stressed mice. This increase induced by ARS was significantly blunted by the treatment with folic acid, but this effect was observed only in the hippocampus. In the non-stressed mice group, the treatment with folic acid did not alter CAT activity in both cerebral structures. Two-way ANOVA revealed a significant folic acid vs. ARS interaction [F(1,17) = 8.91, p < 0.01] for hippocampal CAT activity, although no significant effects were observed for folic acid treatment [F(1,17) = 4.23, p = 0.055] and ARS [F(1,17) = 0.44, p = 0.51] alone. In the cerebral cortex, two-way ANOVA revealed a significant main effect of folic acid treatment [F(1,18) = 26.74, p < 0.01], but not of ARS [F(1,18) = 1.92, p = 0.18], as well as a non significant folic acid treatment vs. ARS interaction [F(1,18) = 0.04, p = 0.85].

As depicted in Fig. 5D, ARS caused an increase on SOD activity in the hippocampus and this effect was reversed by folic acid treatment (folic acid treatment [F(1,19) = 6.90, p < 0.05], ARS [F(1,19) = 10.30, p < 0.01] and folic acid treatment vs. ARS interaction [F(1,19) = 8.32, p < 0.01]). Fig. 5C shows that no significant statistical alteration was observed in SOD activity in the cerebral cortex of stressed mice compared to non-stressed animals (folic acid treatment [F(1,19) = 7.57, p < 0.05], ARS [F(1,19) = 0.004, p = 0.95], and folic acid treatment vs. ARS interaction [F(1,19) = 5.94, p < 0.05]). The treatment with folic acid alone did not alter CAT and SOD activities in the evaluated structures, independent on stress condition.

As can be observed in Figs. 6A (cerebral cortex) and B (hippocampus), stressed mice displayed increased GPx activity when compared with the non-stressed control group. This effect was significantly reversed by folic acid treatment in the hippocampus (folic acid treatment [F(1,17) = 7.03, p < 0.05], ARS [F(1,17) = 0.29, p = 0.59] and folic acid treatment vs. ARS interaction [F(1,17) = 13.18, p < 0.01]), but not in the cerebral cortex (folic acid treatment [F(1,18) = 21.47, p < 0.01], ARS [F(1,18) = 1.06, p = 0.31], and folic acid treatment vs. ARS interaction [F(1,18) = 1.41, p = 0.25]). The administration of folic acid alone did not affect the GPx activity in the non-stressed group in both cerebral structures.

Finally, the results illustrated in the Figs. 6C (cerebral cortex) and D (hippocampus) show that the exposure of mice to ARS also resulted in a significant increase of activity in the antioxidant enzyme GSR in the hippocampus and cerebral cortex. This increase was significantly abolished by folic acid treatment in stressed mice in both brain regions. The administration of folic acid alone to non-stressed mice caused no significant alteration on GSR activity. Two-way ANOVA revealed a significant difference for folic acid treatment [F(1,19) = 19.28, p < 0.01], ARS [F(1,19) = 4.78, p < 0.05], and folic acid treatment vs. ARS interaction [F(1,19) = 5.14, p < 0.05] in the cerebral cortex. In the hippocampus, the two-way ANOVA revealed significant differences for folic acid treatment [F(1,19) = 4.82, p < 0.05], ARS [F(1,19) = 6.03, p < 0.05], and folic acid treatment vs. ARS interaction [F(1,19) = 12.76, p < 0.01].

Discussion

The present study shows that ARS induced depressive-like behavior, cognitive deficits, and oxidative imbalance (increased hippocampal and cerebrocortical TBARS levels, SOD, CAT, GPx, and GSR

(Fig. 4B) of stressed mice compared to non-stressed animals treated with vehicle or folic acid.

![Fig. 3. Effect of treatment with folic acid on thiobarbituric acid reactive substances (TBARS) in the cerebral cortex (panel A) and hippocampus (panel B) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n = 5–6). **p < 0.01 compared with the non-stressed group treated with vehicle, ##p < 0.01 compared with the stressed group treated with vehicle, according to two-way ANOVA followed by Duncan's post hoc test.](image)

![Fig. 4. Effect of treatment with folic acid on glutathione levels in the cerebral cortex (panel A) and hippocampus (panel B) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n = 6). Two-way ANOVA showed no significant effect.](image)
Fig. 5. Effect of treatment with folic acid on SOD activity (panel A: cerebral cortex; panel B: hippocampus) and on CAT activity (panel C: cerebral cortex; panel D: hippocampus) of mice submitted to restraint stress. Values are expressed as mean±SEM (n=5–6). **p < 0.01 compared with the non-stressed group treated with vehicle, ##p < 0.01 and #p < 0.05 compared with the stressed group treated with vehicle, according to two-way ANOVA followed by Duncan’s post hoc test.

Fig. 6. Effect of treatment with folic acid on GPx activity (panel A: cerebral cortex; panel B: hippocampus) and on GSR activity (panel C: cerebral cortex; panel D: hippocampus) of mice submitted to restraint stress. Values are expressed as mean±SEM (n=5–6). **p < 0.01 and *p < 0.05 compared with the non-stressed group treated with vehicle, ##p < 0.01 and #p < 0.05 compared with the stressed group treated with vehicle, according to two-way ANOVA followed by Duncan’s post hoc test.
activities). The depressive-like behavior and all the neurochemical changes observed in the hippocampus were restored by folic acid treatment. On the other hand, folic acid was not able to protect against cognitive impairment induced by restraint stress. Glutathione, a non-enzymatic antioxidant, was not altered by stress and folic acid administration.

Taking into account that stressful life events have been reported to facilitate the evolution of depressive disorders (Calabrese et al., 2011), chronic or ARS in rodents has been widely used as a model of depression (Capra et al., 2010; Christiansen et al., 2011; Huyhn et al., 2011; Poleszak et al., 2006; Sevgi et al., 2006). Because ARS represents the most severe type of stress which causes emotional stress in rodents and has a comparative effect in humans (Calabrese et al., 2011; Kubera et al., 2011), it was used in the present study in an attempt to induce depressive-like behavior. Moreover, the major advantage of using restraint stress as a stress-induced model of depression is that it produces an inescapable physical and mental stress to which adaptation is seldom exhibited (Jaggi et al., 2011).

Numerous studies have reported that rodents (mice and rats) exposed to emotional stress, such as restraint stress, in different durations of stressful events, exhibit depressive-like behavior, evidenced by increased immobility time, particularly in the FST (Capra et al., 2010; Park et al., 2010; Poleszak et al., 2006; Zafir et al., 2009) and tail suspension test (Hayase, 2011; Park et al., 2010). This behavioral alteration in mice is comparable to the depressed mood in humans (Wong and Licinio, 2004). Corroborating these studies, our results indicate that ARS for 7 h induced an increase of immobility time in the FST in mice, without causing changes in the locomotor activity. Further corroborating several recent studies that have suggested an antidepressant potential of folic acid (Brocardo et al., 2008a,b, 2009; Budni et al., 2012a,b), the present study clearly shows that this depressive-like behavior was reversed by folic acid treatment. Moreover, folic acid in non-stressed mice induced antidepressant-like effects in the FST, 8 h after its administration, indicating a prolonged effect of this vitamin. Folic acid alone did not affect the locomotion of non-stressed or stressed mice in the OFT. Experimental evidence shows that folate blood half-life in mice is 7 days (McKay et al., 2011). Taking into account that brain folate availability essentially depends on blood folate levels (Ramaekers, 2004), it is reasonable to infer that folic acid levels attained in serum and brain are sufficient to produce the observed effects in the experimental protocol used.

It has been shown that folic acid (administered by oral, intracerebroventricular, or intraperitoneal routes) displays an antidepressant-like effect in mice submitted to the FST and the TST (Brocardo et al., 2008b), two behavioral tests predictive of antidepressant activity (Porsolt et al., 1977; Steru et al., 1985). Several mechanisms may be involved in the antidepressant-like effect of this vitamin. Brocardo et al. (2008b) showed that serotonergic (5-HT1A and 5-HT2A/2C receptors) and noradrenergic (α1- and α2-adrenoceptors) systems are implicated in the antidepressant-like effect of folic acid. Furthermore, the antidepressant-like effects of this vitamin were shown to be dependent on the inhibition of either N-methyl-D-aspartate (NMDA) receptors, nitric oxide (NO), or cyclic guanosine monophosphate (cGMP) synthesis (Brocardo et al., 2008a), as well as through an interaction with the opioid system (μ and δ receptors) (Brocardo et al., 2009). More recently, we showed that the antidepressant effects of folic acid might also be dependent, at least in part, on the inhibition of glycogen synthase kinase-3 (GSK-3β), activation of peroxisome proliferator-activated receptor-γ (PPARγ) (Budni et al., 2012b), and inhibition of different types of potassium channels (Budni et al., 2012a). However, there were no studies in the literature investigating the potential relationship between the antidepressant-like effects of folic acid and its properties to modulate the anti-/pro-oxidant status in the central nervous system.

Because of its crucial role in the metabolism of homocysteine to methionine, catalyzed by methionine synthase, folic acid deficiency leads to the accumulation of homocysteine, which has been associated with depression (Khanna et al., 2011). Moreover, it has been shown that folic acid deficiency can result in low levels of monoamines (serotonin, norepinephrine, and dopamine), which could contribute to the development of depressive symptoms (Morris et al., 2008). Fava (2007) showed that folic acid augmentation may be used to enhance the efficacy of antidepressants in non-responder patients to antidepressant treatment and improve residual symptoms during antidepressant treatment. It is also important to mention that genetic factors can also affect folic acid levels. One example is the inherited deficiency in the methylene tetrahydrofolate reductase (MTHFR) gene, which is caused by a C677T-polymorphism and limits the conversion of folic acid to its biologically active form, l-methylfolate. Noteworthy, this polymorphism has been suggested to be related with depression (Fathy et al., 2011).

Taking into account that drugs which induce hyperlocomotion may give a “false” positive effect in the FST, and drugs decreasing locomotion may give a “false” negative result (Borsini and Meli, 1988), in the present study the effects of folic acid or vehicle in non-stressed mice as well as in mice submitted to ARS were tested in the OFT. Folic acid administered to stressed or non-stressed mice did not significantly alter locomotor activity in the OFT, a result that indicates that the depressive-like effect induced by ARS and the reversal of this effect elicited by folic acid are not due to any locomotor effect.

The restraint stress applied in this study induced considerable cognitive impairment in the ORT, a result that is in agreement with several literature data (Baker and Kim, 2002; Nagata et al., 2009). Moreover, a study by Li et al. (2012) showed that restraint stress can affect different memory components since it impaired the memory retrieval and interrupted the consolidation of short-term memory into long-term memory in the ORT. Interestingly, our results show that folic acid treatment was not able to abolish the restraint stress-induced cognitive deficits in the ORT, but this vitamin produced an antidepressant-like effect in this animal model. However, it is interesting to note that mice treated with folic acid (non-stressed group), in the session test, preferred to explore the novel object more than the original object, as compared with the vehicle/non-stressed group, suggesting a potential cognitive enhancer role of folic acid. Indeed, several studies have indicated that folic acid has a cognitive improvement property, since it caused an improvement on memory status in elderly rats (Singh et al., 2011) and in cognitive deficits induced by hyperhomocysteine (Matté et al., 2005a). Additionally, a deficiency of this vitamin is associated with cognitive impairment in rodents (Troen et al., 2008), and many clinical studies have indicated that folic acid is important in cognitive performance, contributing to the notion that low folic acid itself might be a risk factor for cognitive impairment (De Lau et al., 2007; Durga et al., 2006; Kado et al., 2005; Ramos et al., 2005). However, in spite of all this literature evidence, in the present study, although folic acid treatment caused a cognitive improvement in non-stressed mice, it was not able to protect against restraint stress-induced cognitive deficits. However, it cannot be ruled out that a higher dose of folic acid or a repeated treatment with this vitamin would be able to reverse the cognitive deficit induced by restraint stress.

The results of the present work also indicate oxidative imbalance, alteration of markers of oxidative damage to lipids, and antioxidant defense in the hippocampus and cerebral cortex of mice submitted to restraint stress for 7 h. The results show that depressive-like behavior and the cognitive impairment induced by restraint stress were accompanied by a significant lipid peroxidation, as evidenced by increased amount of TBARS levels in the cerebral cortex and hippocampus of mice. Our results are in accordance with previous findings, which show that acute immobilization stress significantly increased MDA in the brain of rodents (Kumar and Goyal, 2008; Kumar et al., 2010; Zafir et al., 2009). Also supporting our results, clinical studies indicate raised levels of MDA in depressed patients (Bilici et al., 2001; Khanzode et al., 2003). Taking into account that lipid
peroxidation is one of the major consequences of free-radical-mediated injury to the brain (Dotan et al., 2004), the present study suggests that behavioral alterations caused by restraint stress may be associated with oxidative damage in the cerebral cortex and hippocampus.

The ARS-induced raise in lipid peroxidation was reversed by folic acid treatment in the hippocampus, but not in the cerebral cortex. This finding is somewhat similar to the one reported by Brocardo et al. (2010), which showed that folic acid treatment prevented ouabain-induced increase of lipid peroxidation in the hippocampus of rats. Moreover, Singh et al. (2011) showed that long-term folic acid treatment (8 weeks) significantly decreased lipid peroxidation in the rat cerebral cortex, midbrain, and cerebellar regions. In line with this, folate deprivation led to increased hippocampal lipid peroxidation in rats (Chen et al., 2011). Furthermore, a clinical study performed by Racek et al. (2005) indicated that folic acid supplementation in patients with hyperhomocysteinemia induced partial prevention of plasmatic lipid peroxidation (Racek et al., 2005).

The present study found that the restraint stress caused an increase in activity of the antioxidant enzymes directly involved in the neutralization of ROS, namely SOD (hippocampus), CAT, GSR and GPx (cerebral cortex and hippocampus). These findings are in agreement with the results of several clinical studies. Bilici et al. (2001) showed that depressed patients, especially melancholic patients, had higher activities on plasma GSR, erythrocyte GPx, and SOD than those of healthy controls. Moreover, a recent study reported that patients, during acute depressive episodes, had significantly higher activity of SOD and CAT on erythrocytes, as compared to healthy controls (Galecki et al., 2009). Other human studies show increased SOD activity in the prefrontal cortex of postmortem patients (Michel et al., 2007) and in erythrocytes of patients with depressive disorder (Kotan et al., 2011). Similarly, pre-clinical studies have also reported changes in the activities of antioxidant enzymes in response to experimental stress; however, data are not always in agreement. In fact, both increased (Balk et al., 2010; Fontella et al., 2005; Kim et al., 2005) and decreased antioxidant enzyme activities (Balk et al., 2010; Kumar and Goyal, 2008; Kumar et al., 2010) have been observed after experimental stress. These differences may be a consequence of a number of variations of the procedures or animals used, including strain, age and gender of animals, as well as intensity, duration, frequency and type of stressor (Buyultekin and Mostofsky, 2009).

With respect to the results presented herein, increased SOD, CAT, GPx and GSR activities (especially in the hippocampus) might be due to a compensatory response to increased free radical formation induced by ARS in mice. In this regard, some lines of evidence have reported that the occurrence of pro-oxidative stimulus is necessary to trigger an increase in the levels (and consequently, activities) of antioxidant enzymes (Bea et al., 2009; Suzuki et al., 2008).

Our results show no alterations in glutathione (GSH) levels in the cerebral cortex and hippocampus of mice, independent on stress condition or folic acid treatment. This is in agreement with a recent study, which observed no changes in GSH level in the brain of rats submitted to restraint stress for 24 h (Méndez-Cuesta et al., 2011). The absence of changes in GSH levels as a result of stress and folic acid treatment suggests that this non-enzymatic antioxidant does not play a significant role in this model upon the conditions employed in this study.

Our results are in line with evidence that oxidative stress has been implicated in the pathology of depression and that antioxidants may offer resistance against oxidative stress (Maes et al., 2011). Folic acid was able to protect against restraint stress-induced oxidative-like behavior and oxidative stress, particularly in the hippocampus. Similar to folic acid, classical antidepressant treatment also attenuates the increase of antioxidant enzyme activities in depressed patients (Kotan et al., 2011). A study performed by Bilici et al. (2001) showed that depressive patients, especially melancholic patients, had higher MDA levels, plasma GSR, erythrocyte GPx, and SOD activities than those of healthy controls. After treatment for 3 months with fluoxetine, sertraline, fluvoxamine, and citalopram, the enzymatic activities and MDA levels of the patients were significantly decreased to normal levels. Although the presented results for mice are in line with those for depressed patients (Bilici et al., 2001; Kotan et al., 2011), they do not necessarily indicate that folic acid acts similarly to these antidepressants.

Because the activities of hippocampal and/or cortico-cerebral antioxidant enzymes were changed after 7 h of restraint stress, one could speculate that these effects represent a consequence of immobilization (resting) in the stressed animals. Although this possibility could not be definitively ruled out, evidence shows that the activities of glutathione-related antioxidant enzymes are not significantly affected by the circadian cycles (Onchovaroa et al., 2006, 2008). Based on such evidence and on the fact that animals are immobile while sleeping, it is reasonable to suppose that the “psychophysiological stress” (but not just immobilization) was the major factor affecting antioxidant enzyme activities. This idea is reinforced by previous studies indicating that different types of stressing agents (i.e., unpredictable stress, post-traumatic stress disorders, among others) affect the activity of brain antioxidant enzymes, such as SOD, GPx and GSR (Diehl et al., 2012; Filipović and Pajović, 2009; Moretti et al., 2012).

Conclusions

This investigation revealed that the antidepressant action of folic acid might be associated with its capability of maintaining pro-/anti-oxidative homeostasis in the hippocampus of mice subjected to ARS. Taking into account that (i) the behavioral and biochemical outcomes observed in animals subjected to ARS are similar to those found in human depression and that (ii) many depressive patients do not tolerate or respond adequately to the available classical anti-depressants, the present findings warrant further studies to evaluate the therapeutic relevance of folic acid for the treatment of depression and as a co-adjutant treatment with antidepressants.

Acknowledgments

This study was supported by the FINEP research grant “Rede Instituto Brasileiro de Neurociência” (IBN-Net/CNPq), CAPES, CNPq and Núcleo de Excelência em Neurociências Aplicadas de Santa Catarina (NENASC) Project/PRONEX Program of CNPq/FAEPSC (Brazil).

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