

LITHIUM ATTENUATED THE DEPRESSANT AND ANXIOGENIC EFFECT OF JUVENILE SOCIAL STRESS THROUGH MITIGATING THE NEGATIVE IMPACT OF INTERLUKIN-1 β AND NITRIC OXIDE ON HYPOTHALAMIC–PITUITARY–ADRENAL AXIS FUNCTION

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Abstract—The neuroimmune-endocrine dysfunction has been accepted as one of fundamental mechanisms contributing to the pathophysiology of psychiatric disorders including depression and anxiety. In this study, we aimed to evaluate the involvement of hypothalamic–pituitary–adrenal (HPA) axis, interleukin-1 β , and nitric oxide system in mediating the negative behavioral impacts of juvenile social isolation stress (SIS) in male mice. We also investigated the

possible protective effects of lithium on behavioral and neurochemical changes in socially isolated animals. Results showed that experiencing 4-weeks of juvenile SIS provoked depressive and anxiety-like behaviors that were associated with hyper responsiveness of HPA axis, upregulation of interleukin-1 β , and nitric oxide (NO) overproduction in the pre-frontal cortex and hippocampus. Administration of lithium (10 mg/kg) significantly attenuated the depressant and anxiogenic effects of SIS in behavioral tests. Lithium also restored the negative effects of SIS on cortical and hippocampal interleukin-1 β and NO as well as HPA axis deregulation. Unlike the neutralizing effects of L-arginine (NO precursor), administration of L-NAME (3 mg/kg) and aminoguanidine (20 mg/kg) potentiated the positive effects of lithium on the behavioral and neurochemical profile of isolated mice. In conclusion, our results revealed that juvenile SIS-induced behavioral deficits are associated with abnormalities in HPA-immune function. Also, we suggest that alleviating effects of lithium on behavioral profile of isolated mice may be partly mediated by mitigating the negative impact of NO on HPA-immune function. © 2015 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: juvenile social isolation stress, lithium, nitric oxide, interleukin-1 β , hypothalamic–pituitary–adrenal axis, depressive-like behaviors.

INTRODUCTION

Depression, a condition accounting for enormous social and financial burden, is characterized by despair behaviors, anhedonia, and depressed mood. Adolescence is a pivotal period for brain development accompanied with cortico-limbic maturation (Sisk and Foster, 2004). Ample evidence indicated that experiencing adversity namely by social stressors negatively alters brain function and leads to the occurrence of psychiatric disorders including depression and anxiety (Andersen and Teicher, 2008; Romeo, 2010). Juvenile social isolation stress (SIS) has been shown to produce behavioral and neurochemical changes in social mammals reminiscent of various symptoms observed in depressed patients (Fone and Porkess, 2008; Nestler and Hyman, 2010). A large body of evidence reported that applying SIS to animals provoked behaviors relevant to depression and anxiety (Grippo et al., 2007; Fone and Porkess, 2008; Amiri et al., 2015b).

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Abbreviations: AG, aminoguanidine; FST, forced swimming test; GC, glucocorticoid; HBT, hole-board test; HIPP, hippocampus; HPA, hypothalamic–pituitary–adrenal axis; hprt1, hypoxanthine phosphoribosyl transferase1; i.p., intraperitoneal; iNOS, inducible NOS; IC, isolation condition; IL-1 β , interleukin-1 β ; L-arg, L-arginine; L-NAME, NG-nitro-L-arginine methyl ester; NMDA, N-Methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; OFT, open-field test; PFC, pre-frontal cortex; PND, Postnatal day; SC, social condition; SIS, social isolation stress.

Lithium, a well-known mood stabilizer, has been reported to exert antidepressant properties in preclinical and clinical studies (Austin et al., 1991; Gray and McEwen, 2013). In this context, advantageous effects of lithium on stress-induced psychiatric disorders have been well documented in the literature (Kovacsics and Gould, 2010). Despite several mechanisms of action identified for the therapeutic effects of lithium, the exact mechanisms through which lithium modulates the negative impacts of stress remains elusive. Our previous studies showed that the antidepressant effect of lithium is partly associated with nitric oxide (NO) modulation (Ghasemi et al., 2008, 2009). It has been also demonstrated that lithium exerts anti-inflammatory effects and prevents interleukin-1 β (IL-1 β) overproduction (Albayrak et al., 2013). On the other hand, it has been proposed that lithium is able to modulate the hypothalamic–pituitary–adrenal (HPA) axis dysregulation in the variety of mood disorders (Bschor et al., 2002; Boku et al., 2009). Evidence is accumulating that nitric oxide synthase (NOS) overactivity, IL-1 β overproduction, and HPA-axis dysfunctions are involved in the pathophysiology of psychiatric disorders including depression and anxiety (McEwen, 2005; Dantzer, 2009).

Several lines of research suggested that dysregulation of the neuroimmune-endocrine system is one of the fundamental mechanisms that underlie psychiatric disorders (Pariante and Lightman, 2008). Evidence indicates that experiencing social isolation during adolescence correlates with HPA axis hyper responsiveness and influences brain regions related to mood disorders such as the pre-frontal cortex (PFC) and hippocampus (HIP) (Tsigos and Chrousos, 2002; Serra et al., 2005; Pariante and Lightman, 2008; Hawkey et al., 2012; Gądek-Michalska et al., 2013a). Disruption of the HPA axis activity after chronic stress exposure might be a result of inflammatory cytokines, including IL-1 β and NO overproductions (Gądek-Michalska et al., 2013b). In addition, we have recently shown that inducible NOS (iNOS) activity is associated with co-occurrence of anxiety and depressive-like behaviors following juvenile SIS (Amiri et al., 2015b).

Considering the protective properties of lithium in modulating the negative effects of stress and also the putative role of glucocorticoids (GCs), NO, and IL-1 β in depression and anxiety development, we aimed to investigate the effect of lithium on the depressive and anxiety-like behaviors in animals exposed to juvenile SIS paradigm using behavioral tests. Although the antidepressant and anxiolytic effects of lithium has been established by past studies, the possible effect of this drug on social stressors during adolescence, which is a critical period in developing the brain of animals, has not been illustrated yet. Also, our goal is to evaluate the possible involvement of HPA axis, nitroergic system, and IL-1 β in mediating the protective effect of lithium on mood disorders in socially isolated mice.

EXPERIMENTAL PROCEDURES

Animals

In the current study, we used male NMRI (Naval Medical Research Institute) mice (Pasteur Institute, Tehran, Iran),

weighing 10–14 g and on postnatal day (PND) 21–25. Each experimental group consisted of 6–8 animals. The animals were housed under standard conditions (temperature: 23 \pm 2 °C, 12-h light–dark cycle, and free access to food and water) for four weeks. Animals were housed under two different conditions, social condition (SC), and isolated condition (IC). Socially conditioned mice were housed in groups (six mice per cage) in Plexiglas boxes (25 \times 25 \times 15 cm), while IC mice were housed individually in Plexiglas boxes (24 \times 17 \times 12 cm). Isolated conditioned mice were housed in a separate room and had olfactory and visual contact. The cages of IC mice were cleaned weekly by the same experimenter to avoid minimum handling and social contact. All procedures in our study were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, and we also adhered to institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, TUMS).

Drugs

The following drugs were used in this study: lithium chloride (Merck, Darmstadt, Germany), NG-nitro-L-arginine methyl ester (L-NAME, non-selective NOS inhibitor), aminoguanidine (AG, selective iNOS inhibitor), and L-arginine (L-arg, NO precursor) (Sigma, St Louis, MO, USA). All drugs were dissolved in sterile saline and were administered in a volume of 5 ml/kg mouse weight. All drugs were administered intraperitoneally (i.p.) to animals. Doses and administration time of each drug were chosen according to our pilot treatments and previous published studies (Ghasemi et al., 2008; Amiri et al., 2015b). The efficacy and bioavailability of applied drugs have been documented in the literature suggesting the ability of them to pass through the blood–brain barrier (Gyulai et al., 1991; Sakuma et al., 1992; Cockcroft et al., 1996; Tsuji et al., 2000).

Forced swimming test (FST)

We used FST as a behavioral test in which the prolonged immobility time presents the despair behavior reflecting the depressive-like symptoms (Porsolt et al., 1977). Mice were individually placed in an open glass cylinder (diameter: 10 cm, height: 25 cm) containing 19 cm water (23 \pm 1 °C). Mice were allowed to swim for 6 min, and the immobility time was recorded during the last 4 min of the test. Immobility behavior was considered when the animal remained floating motionless in water and made only those necessary movements to keep its head above water.

Splash test

In rodents, lack of motivation to engage in self-care can be assessed by the splash test. In this test, grooming behavior of mice, which can be considered as an indirect measure of palatable solution intake was

measured. A 10% sucrose solution was squirted on the dorsal coat of animals while they were in their home cages and mice were videotaped for 5 min. Duration of grooming activity behavior including nose/face grooming, head washing, and body grooming were observed by an experimenter who was blind to the treatment conditions (David et al., 2009; Detanico et al., 2009).

Open-field test (OFT)

The OFT was used to elucidate the effects of SIS and treatments on motor function and anxiety-like behaviors (Kuleskaya and Voikar, 2014). The apparatus consisted of a white opaque Plexiglas box measuring (50 cm × 50 cm × 30 cm) which was dimly illuminated. The ground of the box was separated into 16 equal squares. Each mouse was placed gently on the central zone (30 cm × 30 cm) and its behaviors were recorded by a camera for 5 min and were analyzed by Ethovision software version 8 (Noldus, Netherlands). The surface of the apparatus was cleaned with 70% ethanol after each experiment. The distance moved (horizontal activity), the number of rearings (vertical activity), and the time spent in the central zone were evaluated.

Hole-board test (HBT)

The HBT was used to evaluate the anxiety-like behavior of animals (Amiri et al., 2015b). The HBT consisted of a white Plexiglas board (50 cm × 50 cm) with 16 equidistant holes (3 cm in diameter) and was positioned 50 cm above the floor. Animals were placed in the center of the apparatus and the number of head-dips was counted in a 5-min period.

Nitrite assay

To determine the NO levels in the PFC and HIPP, we measured the nitrite level as the NO end-product (Ding et al., 2010; Kordjazy et al., 2015). The SC and IC animals (without any acute stress induction) were decapitated under mild anesthesia using diethyl ether, and then the PFC or HIPP were dissected on the ice-cold surface and immediately immersed in liquid nitrogen. Tissue homogenates were prepared, and then the nitrite level was measured by a colorimetric assay based on the Griess reaction. First, 100 μ L of samples were mixed with 100 μ L Griess reagent. Following 10-min incubation at room temperature, absorbance was measured at 540 nm in an automated plate reader. Concentration of nitrite was determined by reference to a standard curve of sodium nitrite (Sigma, USA) and normalized to the weight of each sample.

Corticosterone assay

To evaluate the HPA axis activity in animals, we measured basal (without any acute stress induction) and post-stress (one hour after an acute stress which is a 6-min forced swimming stress) levels of corticosterone in serum using the previously described method in the SC and IC animals (Veenema et al., 2003; Droste et al.,

2008). Each animal was decapitated under mild anesthesia using diethyl ether and the blood was collected. After that, we centrifuged the obtained blood (3000g, 10 min, at 4 °C) and the serum was collected; then the obtained serum samples were stored at –20 °C until the assay day. Corticosterone levels were measured using a commercial ELISA kit (Biospes, China).

IL-1 β gene expression

The SC and IC animals (without any acute stress induction) were decapitated under mild anesthesia using diethyl ether, and then the PFC or HIPP were dissected on the ice-cold surface and immediately immersed in liquid nitrogen. The tissue homogenates were prepared and the IL-1 β gene expression was evaluated using the described method. Trizol (Invitrogen, Carlsbad, California, USA) was used to isolate total RNA from brain cells. Changes in mRNA levels of desired genes were measured by qRT-PCR after reverse transcription of 1 μ g of RNA from each sample using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). qRT-PCR was performed on a light cycler instrument (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Thermal cycling conditions involved an initial activation step for 30 s at 95 °C followed by 45 cycles including a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was applied to validate whether all primers yielded a single PCR product. The primers used for IL-1 β is: 5'-GAAATGCCACCTTTTGACAGTG-3'(forward), 5'-TG GATGCTCTCATCAGGACAG-3'(reverse). Hypoxanthine phosphoribosyl transferase1 (hprt1) was amplified as normalizer and the fold change in the expression of each target mRNA relative to hprt1 was calculated on the basis of $2^{-\Delta\Delta ct}$ relative expression formulas.

Experiment design and treatments

To determine the effect of SIS on animal behaviors, after 4 weeks exposure to different housing conditions (SC or IC) animals were subjected to the behavioral tests, the FST, splash test, OFT, and HBT. We also examined the effect of SIS on cortical and hippocampal nitrite and IL-1 β levels as well as serum corticosterone concentration.

Then, we evaluated the possible effect of lithium (1, 5, and 10 mg/kg, i.p., 30 min before behavioral tests) on animals' behavior in SC and IC groups. Also, the possible effect of lithium administration on the brain nitrite and IL-1 β levels and serum corticosterone concentration was investigated in this part of the experiment.

In the next step, we investigated whether the nitregic system mediates the effects of lithium on depression and anxiety induced by SIS. In this regard, we treated both SC and IC mice with lithium and either of the following drugs: L-NAME (3 mg/kg, i.p., 45 min before the tests), AG (20 mg/kg, i.p., 45 min before the tests), and L-arg (50 mg/kg, i.p., 45 min before the tests). After co-administration of these drugs with lithium, first, the behavior of each mouse was assessed in open-field or

hole-board paradigm and then the tested animals were evaluated by FST or splash test (each mouse was used in two behavioral tests). To exclude the possible effect of vehicle administration, SC and IC groups were injected with 5 ml/kg sterile physiological saline. We also measured the nitrite levels in PFC and HIPP of all treated groups in order to further investigate the role of nitric system in the observed effects. Serum corticosterone concentration was also measured in experimental groups after acute stress induction. Different groups of SC and IC mice were used for nitrite and corticosterone assays (these animals had not been tested by behavioral tasks), in order to avoid the possible effects of behavioral experiments and manipulations on these parameters. Each experimental animal group consists of 6–8 in behavioral assessments, and 3–8 in molecular evaluations.

Statistical analysis

Comparison between the groups was analyzed using *t*-test and a one-way and a two-way ANOVA followed by multiple comparison tests in the SPSS package software (version 21) and Graph-pad prism software (version 6). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of SIS on behavioral tests, brain IL-1 β and nitrite levels, and serum corticosterone concentration

Applying SIS to mice induced depressive and anxiety-like behaviors and also elevated animals' locomotor activity. Fig. 1a shows that the immobility time increased in the socially isolated animals (IC) compared to the control group (SC) in the FST ($P < 0.001$, Fig. 1a). In the splash test, SIS significantly decreased the grooming activity time in IC mice when compared to SC mice ($P < 0.001$, Fig. 1b). In the OFT, IC mice showed an increase in both total distance moved (horizontal activity) ($P < 0.01$, Fig. 1c) and number of rearings (vertical activity) ($P < 0.001$, Fig. 1d) in comparison with SC animals. Also, IC animals stayed for a shorter period in the central zone compared to the SC group ($P < 0.01$, Fig. 1e). In the HBT, SIS significantly reduced the number of head-dips of mice in comparison with the SC group ($P < 0.001$, Fig. 1f).

In comparison with SC mice, *t*-test analysis showed that IC mice contain higher levels of IL-1 β in both PFC ($P < 0.05$, Fig. 1g) and HIPP ($P < 0.05$, Fig. 1g). Also, socially isolated animals have a higher nitrite level in PFC ($P < 0.001$, Fig. 1h) and HIPP ($P < 0.001$, Fig. 1h) compared to SC mice. Our data have shown that SIS and drug manipulations (lithium) had no effect on the *hprt1* gene expression ($P > 0.05$, data not shown).

Additionally, HPA axis response in IC mice after acute stress was significantly different from post-stress SC animals ($P < 0.001$, Fig. 1i). However, no significant difference was detected in the baseline corticosterone levels between the unstressed IC and SC mice

($P > 0.05$, Fig. 1i). A two-way ANOVA analysis showed significant difference in the serum corticosterone concentrations for the stress induction ($F(1, 16) = 31.44$, $P < 0.001$), housing conditions ($F(1, 16) = 28.34$, $P < 0.001$), and their interaction ($F(1, 16) = 11.13$, $P < 0.01$).

Lithium administration attenuated the depressant and anxiogenic effect of SIS

We intended to evaluate the possible antidepressant effect of lithium in socially isolated animals. Therefore, behaviors of mice after administration of different doses of lithium (1, 5, and 10 mg/kg) were determined in SC and IC groups (Fig. 2a, b). ANOVA analysis revealed that there were significant differences between lithium-treated groups in FST ($F(9, 70) = 5.33$, $P < 0.001$, Fig. 2a) and the splash test ($F(9, 70) = 12.07$, $P < 0.001$, Fig. 2b). Tukey's analysis showed that administration of lithium (1, 5, and 10 mg/kg) had no effect on the immobility time in the FST ($P > 0.05$) or the grooming activity time in the splash test ($P > 0.05$) of SC animals. But, the result showed that administration of lithium at doses 5 and 10 mg/kg significantly reversed the depressant effect of SIS in IC mice in FST ($P < 0.05$ and $P < 0.01$, respectively) and splash test ($P < 0.05$ and $P < 0.01$, respectively). However, administration of the lower dose of lithium (1 mg/kg) did not alter either the immobility or grooming times of IC mice in comparison with saline-treated IC group ($P > 0.05$). Also, our results show that saline treatment did not affect behaviors of IC and SC mice in the tests ($P > 0.05$).

After injection of different doses of lithium, locomotor activity and anxiety-like behaviors were determined in SC and IC mice using behavioral tests including OFT and HBT (Fig. 2c–f). Analysis demonstrated that there were significant differences between lithium-administered groups in distance moved in OFT ($F(9, 70) = 5.894$, $P < 0.001$, Fig. 2c), number of rearings in OFT ($F(9, 70) = 10.15$, $P < 0.001$, Fig. 2d), time spent in the central zone in OFT ($F(9, 50) = 5.156$, $P < 0.001$, Fig. 2e), and number of head-dips in HBT ($F(9, 70) = 7.762$, $P < 0.001$, Fig. 2f). Tukey's analysis showed that lithium (1, 5, and 10 mg/kg) had no effect on SC mice in behavioral tests ($P > 0.05$); while, administration of lithium induced anxiolytic effects in IC animals.

In the OFT, administration of lithium (1, 5, 10 mg/kg) did not alter the number of rearings and total distance moved of IC mice in comparison with saline-treated IC group ($P > 0.05$). However, lithium (10 mg/kg) administration (but not at the lower doses, 1 and 5 mg/kg) improved the time spent in the central zone in IC animals ($P < 0.05$). On the other hand, the results showed that the number of head-dips in IC animals treated with lithium (10 mg/kg, but 1 and 5 mg/kg) was increased compared to the IC saline-injected group ($P < 0.01$). Also, our results showed that saline treatment did not affect the behaviors of both IC and SC mice in the OFT and HBT ($P > 0.05$).

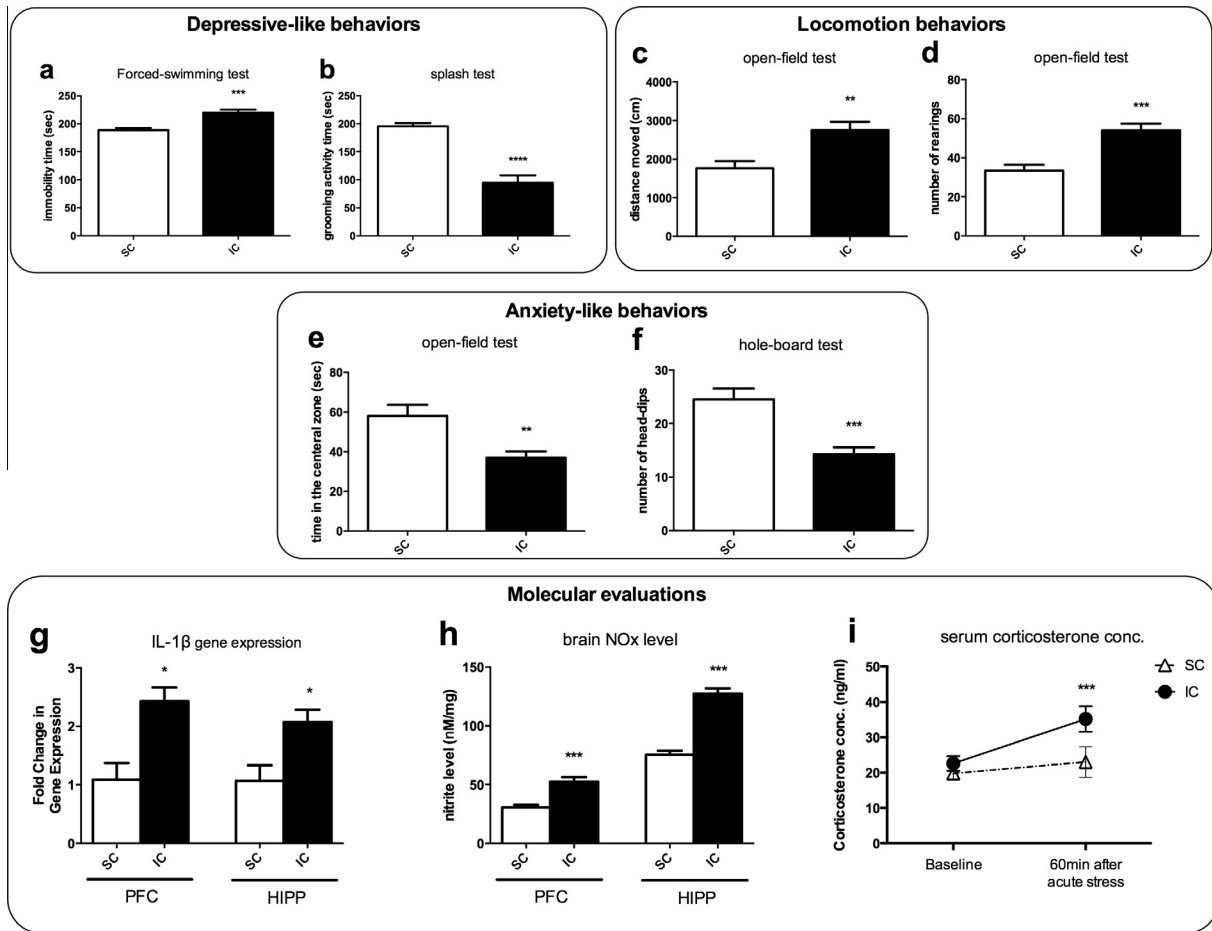


Fig. 1. Effect of housing condition on behaviors related to depression and anxiety and the neuroendocrine system. Effect of different housing conditions, social condition (SC) and isolated condition (IC), on the immobility time in the FST (a), grooming activity time in the splash test (b), total distance moved in the OFT (c), number of rearings in the OFT (d), time spent in the central zone in the OFT (e), number of head dips in the HBT (f), fold changes in the IL-1 β gene expression in the PFC and HIPP (g), cortical and hippocampal nitrite level (h), baseline state and post-stress (60 min after an acute 6 min stress induction) serum corticosterone concentration (i). Values are expressed as the mean \pm S.E.M and were analyzed using *t*-test, a one-way and two-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ compared with the SC control group.

Effects of lithium treatment on brain IL-1 β and nitrite levels, and serum corticosterone concentration

Effects of lithium treatment on cortical and hippocampal IL-1 β and nitrite levels as well as post-stress serum corticosterone concentration in SC and IC animals were determined. A one-way ANOVA revealed significant effect of lithium on IL-1 β levels in the PFC ($F(7, 16) = 5.577$, $p < 0.01$, Fig. 3a) and HIPP ($F(7, 16) = 3.913$, $p < 0.05$, Fig. 3b). Data showed that lithium (10 mg/kg, but not 1 mg/kg) injection considerably reduced the cortical and hippocampal IL-1 β levels in IC mice when compared with the saline-treated IC group ($P < 0.05$). None of these treatments altered the IL-1 β level of PFC and HIPP in SC mice ($P > 0.05$).

We also evaluated the effect of lithium treatment on the nitrite level in PFC ($F(7, 56) = 14.34$, $p < 0.001$, Fig. 3c) and HIPP ($F(7, 56) = 15.04$, $p < 0.001$, Fig. 3d) of SC and IC animals. Results also show that lithium (10 mg/kg) injection reduced the nitrite levels measured in PFC ($P < 0.001$) and HIPP ($P < 0.01$) of IC mice when compared with IC control group. Lithium at the dose of 1 mg/kg did not significantly decrease the

nitrite levels of PFC and HIPP in IC mice ($P < 0.05$). Lithium administration did not affect the nitrite levels of PFC and HIPP in SC mice ($P > 0.05$).

In the next part, we assessed the effects of lithium on post-stress serum corticosterone level in SC and IC animals ($F(7, 32) = 6.510$, $p < 0.001$, Fig. 3e). Our data revealed that treatment with lithium (10 mg/kg, but not 1 mg/kg) in IC mice significantly decreased the corticosterone concentration in serum comparing to saline-treated IC animals ($P < 0.01$). However, lithium (1 and 10 mg/kg) treatment did not change the post-stress serum corticosterone level of SC animals ($P > 0.05$).

Nitroergic system mediates the antidepressant and anxiolytic effect of lithium in socially isolated animals

Fig. 4 shows the effects of subeffective doses of NOS inhibitors alone and in combination with lithium on the behavioral tests of SC and IC animals. ANOVA analysis showed that there were significant differences between all treated groups in FST ($F(11, 84) = 5.722$,

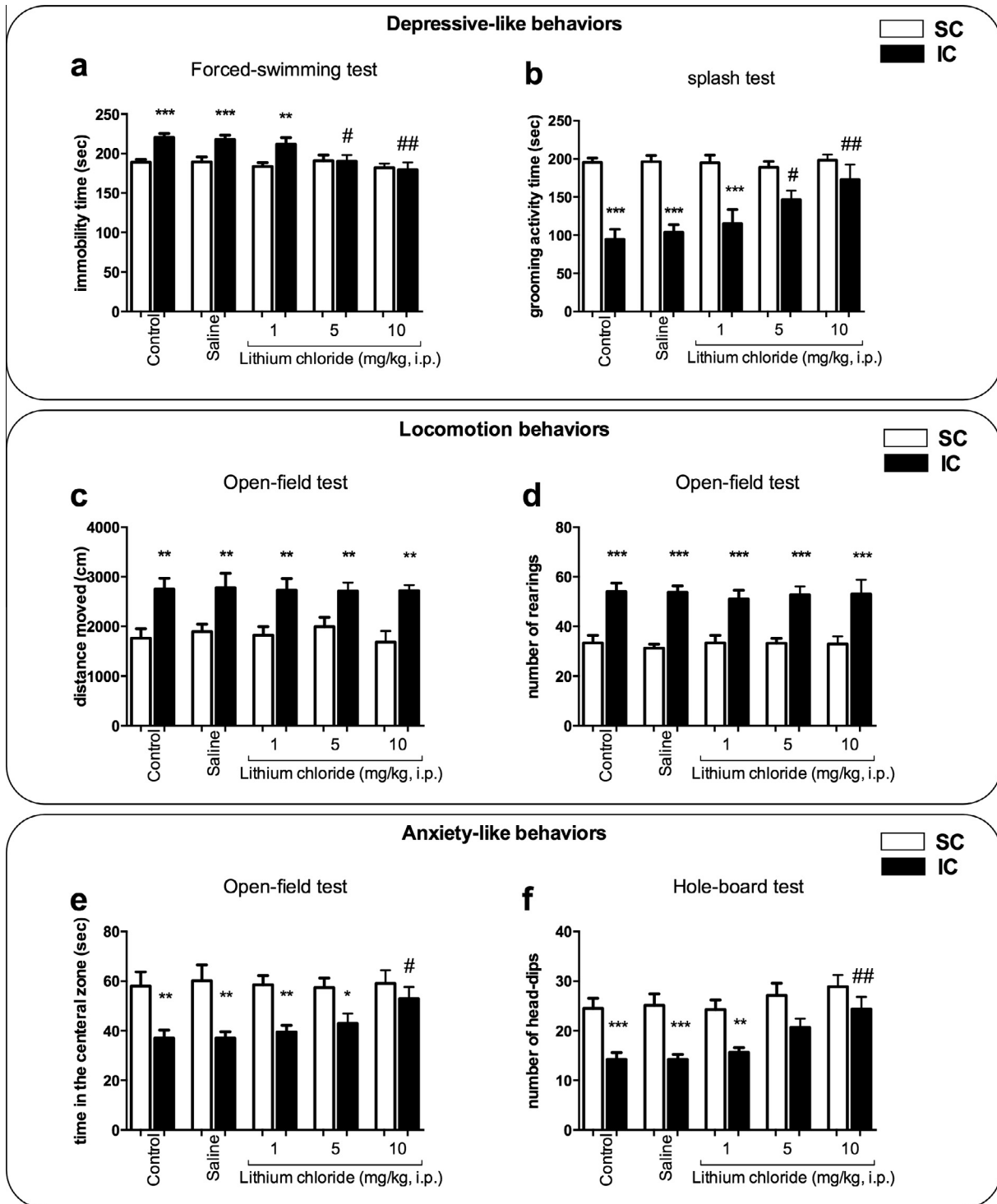


Fig. 2. Effect of lithium chloride treatment on behaviors related to depression and anxiety in different housing conditions. Effects of lithium chloride (1, 5, and 10 mg/kg) on the immobility time in the FST (a), grooming activity time in the splash test (b), total distance moved in the OFT (c), number of rearings in the OFT (d), time spent in the central zone in the OFT (e), number of head dips in the HBT (f). Values are expressed as the mean \pm S. E.M and were analyzed using a one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the SC control group. # $P < 0.05$ and ## $P < 0.01$ compared with the IC saline-treated group.

$P < 0.001$, Fig. 4a) and splash test ($F(11, 84) = 10.88$, $P < 0.001$, Fig. 4b). Tukey's test showed that injection of lithium chloride (1 mg/kg), L-NAME (3 mg/kg), and AG (20 mg/kg) had no effect on SC and IC mice either in FST, or in splash test ($P > 0.05$).

Co-administration of L-NAME (3 mg/kg) and lithium (1 mg/kg) significantly reversed the depressant effect of SIS on IC mice in FST ($P < 0.01$) and splash test ($P < 0.01$); but this treatment did not alter the behavioral tests of SC animals ($P > 0.05$). In addition,

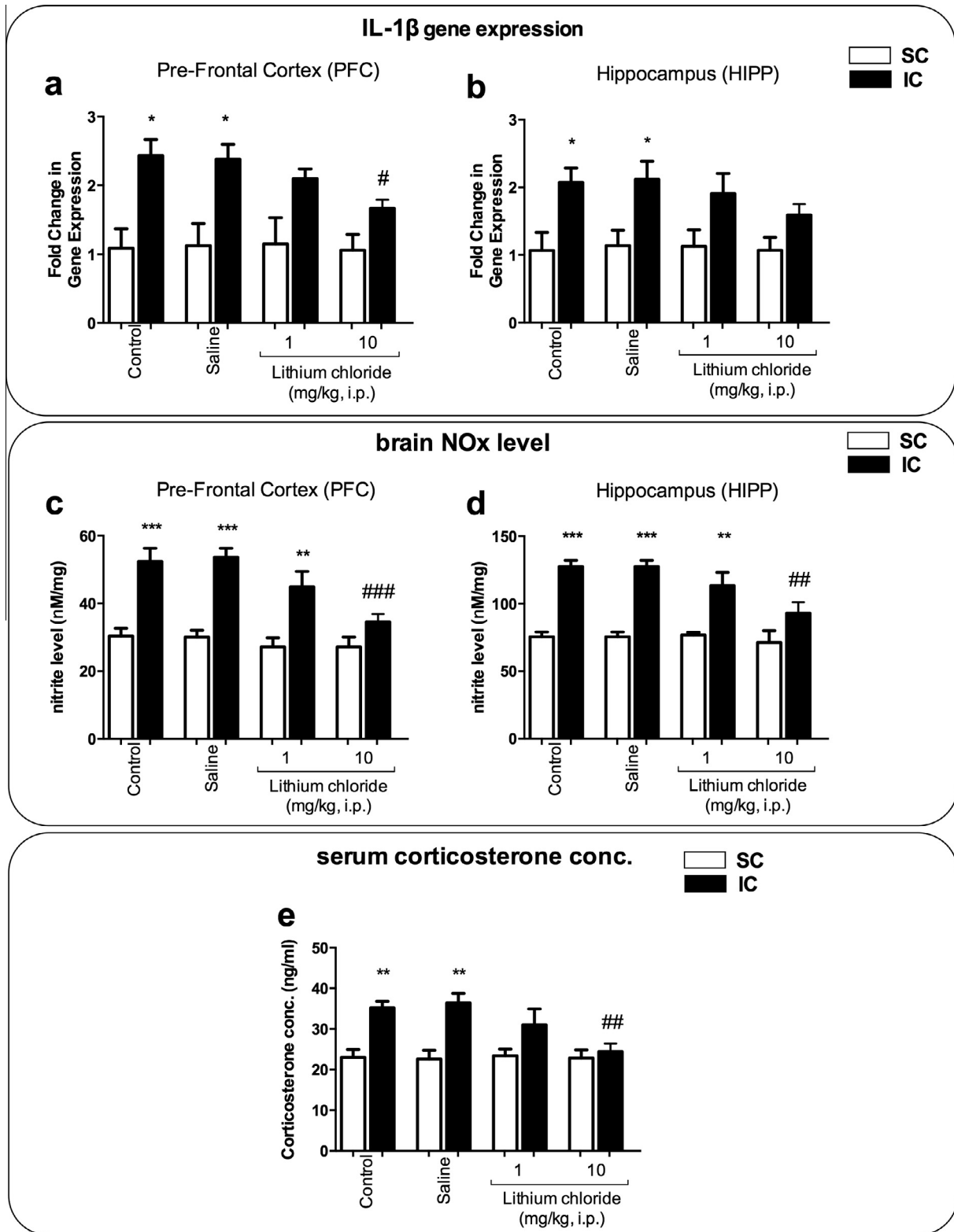


Fig. 3. Effect of lithium chloride treatment on neuroendocrine system in different housing conditions. Effects of lithium chloride (1 and 10 mg/kg) on fold changes in the IL-1 β gene expression in the PFC (a) and HIPP (b), Nitrite levels in the PFC (c) and HIPP (d), Post-stress (one hour after acute 6 min stress induction) serum corticosterone concentration (e). Values are expressed as the mean \pm S.E.M and were analyzed using a one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the IC saline-treated group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with the SC control group.

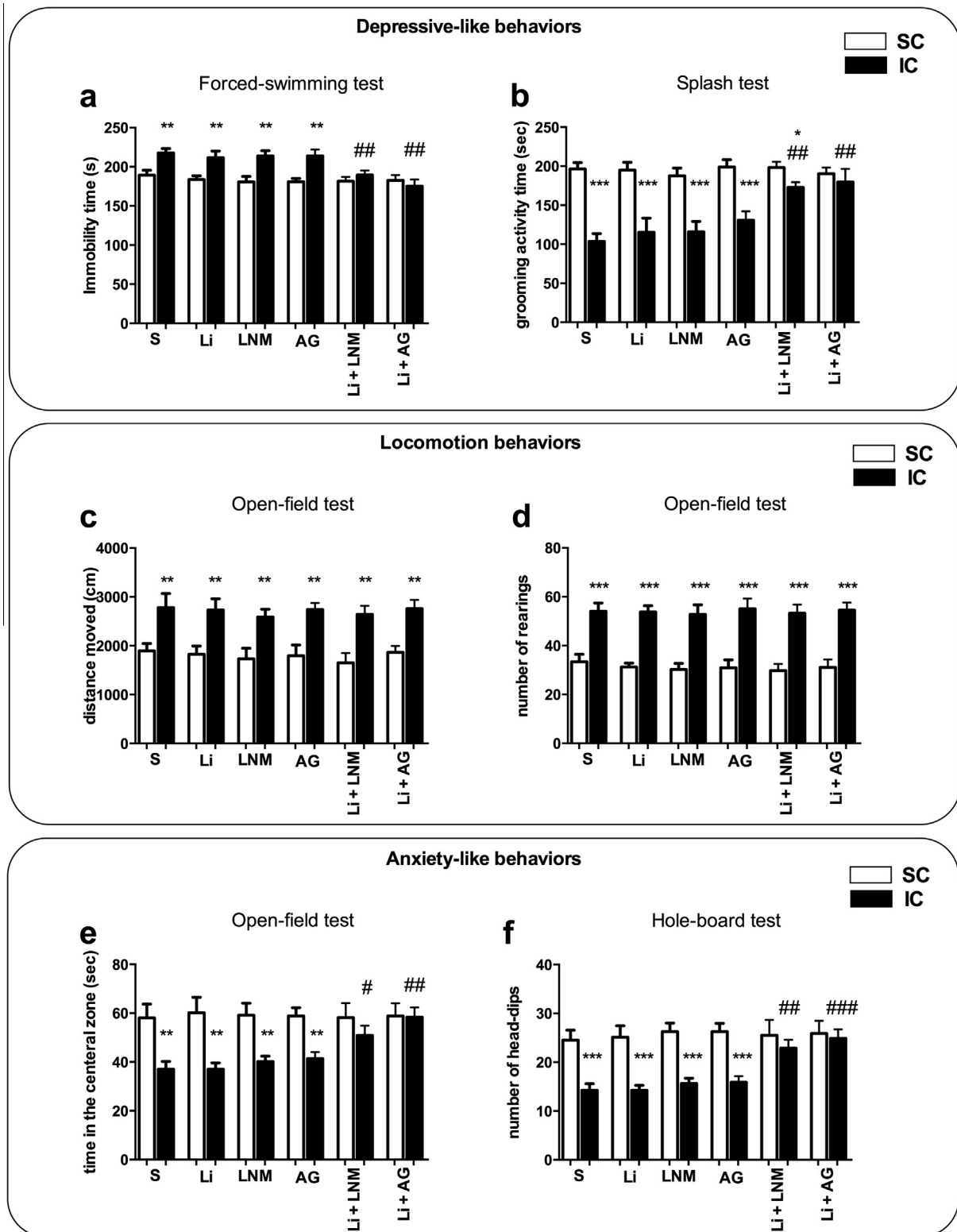


Fig. 4. Effects of lithium chloride and NOS inhibitors co-administration. Effects of lithium chloride (Li) (1 mg/kg) and L-NAME (LNM) (3 mg/kg)/aminoguanidine (AG) (20 mg/kg) co-administration on the immobility time in the FST (a), grooming activity time in the splash test (b), total distance moved in the OFT (c), number of rearings in the OFT (d), time spent in the central zone in the OFT (e), number of head dips in the HBT (f). Values are expressed as the mean \pm S.E.M and were analyzed using a one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the SC control group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with the IC saline-treated (S: saline-treated) groups).

administration of AG (20 mg/kg) significantly augmented the antidepressant effect of lithium (1 mg/kg) in IC animals both in FST ($P < 0.01$) and splash test ($P < 0.01$). However, lithium and AG co-injection did not affect the SC mice in FST and splash test ($P > 0.05$).

In addition, treatment with L-arg (50 mg/kg) abolished the antidepressant effect of lithium 10 mg/kg via decreasing the immobility duration ($F(7, 56) = 6.002$, $P < 0.001$, Fig. 5a) and enhancing the grooming activity time ($F(7, 56) = 13.07$, $P < 0.001$, Fig. 5b) of IC animals when compared to IC saline-treated group. So, there were significant differences between lithium (10 mg/kg) and lithium- (10 mg/kg) + L-arg (50 mg/kg) administered groups in both the FST ($P < 0.05$) and splash test ($P < 0.05$). However, lithium and L-arg injections did not affect the SC mice behaviors either alone or in combination ($P > 0.05$).

In order to evaluate the possible role of nitric oxide system in mediating the anxiolytic effect of lithium, the subeffective doses of NO agents were administered alone and in combination with lithium to SC and IC animals. Analysis showed that there were significant differences between all treated groups in the distance moved in OFT ($F(11, 84) = 6.312$, $P < 0.001$, Fig. 4c), number of rearings in OFT ($F(11, 84) = 14.21$, $P < 0.001$, Fig. 4d), time spent in the central zone in OFT ($F(11, 60) = 4.840$, $P < 0.001$, Fig. 4e), and number of head-dips in HBT ($F(11, 84) = 7.069$, $P < 0.001$, Fig. 4f). Tukey's analysis showed that injection of lithium chloride (1 mg/kg), L-NAME (3 mg/kg), and AG (20 mg/kg) did not alter the SC and IC animals' behaviors in OFT or HBT ($P > 0.05$).

Data have shown that co-administration of L-NAME (3 mg/kg) or AG (20 mg/kg) with lithium (1 mg/kg) did not change the number of rearings and total distance moved of IC animals in the OFT ($P > 0.05$). But L-NAME (3 mg/kg) and lithium (1 mg/kg) co-treatment ($P < 0.05$) as well as AG (20 mg/kg) and lithium (1 mg/kg) co-injection ($P < 0.01$) significantly enhanced the time spent in the central zone of IC mice when compared to IC saline-treated group. In addition, administration of L-NAME (3 mg/kg) and AG (20 mg/kg) significantly augmented the anxiolytic effect of lithium (1 mg/kg) in IC animals in HBT ($P < 0.01$ and $P < 0.001$, respectively). Analysis showed that none of the above-mentioned treatments changed the behaviors of the SC group in the OFT and HBT ($P > 0.05$).

Results showed that L-arg (50 mg/kg) and lithium (10 mg/kg) co-treatment did not change the number of rearings and total distance moved of IC animals in the OFT ($P > 0.05$, Fig. 5c, d). However, treatment with L-arg (50 mg/kg) attenuated the anxiolytic effect of lithium 10 mg/kg in IC mice by decreasing the time spent in the central zone in OFT ($F(7, 40) = 6.371$, $P < 0.001$, Fig. 5e) and head-searching activity in HBT ($F(7, 56) = 10.89$, $P < 0.001$, Fig. 5f). There were significant differences between lithium (10 mg/kg) and lithium (10 mg/kg) + L-arg (50 mg/kg) administered groups in the time in the central zone of OFT ($P < 0.05$) and number of head dips of HBT ($P < 0.01$). Concurrent lithium and L-arg injection did not change the behaviors

of SC mice in the OFT and HBT either alone or in combination ($P > 0.05$).

Pre-frontal cortex and hippocampus nitrite levels in different housing conditions and treatments

The effects of lithium and NO agent administrations on cortical and hippocampal nitrite levels in SC and IC animals were determined (Table 1). A one-way ANOVA revealed significant effect of treatments on the nitrite measured in PFC ($F(9, 70) = 12.59$, $P < 0.001$, Table 1) and HIPP ($F(9, 70) = 8.202$, $P < 0.001$, Table 1). Data showed that lithium (10 mg/kg, but not 1 mg/kg) injection considerably reduced the cortical ($P < 0.001$) and hippocampal ($P < 0.01$) nitrite levels in IC mice when compared with IC control group. We assessed the effects of NOS inhibitors/NO precursor co-administration with lithium on cortical and hippocampal nitrite levels. Our data revealed that co-administration of lithium (1 mg/kg) with L-NAME (3 mg/kg) or AG (20 mg/kg) in IC mice significantly decreased the nitrite levels in PFC ($P < 0.01$ and $P < 0.001$) and HIPP ($P < 0.01$ and $P < 0.01$) comparing to IC animals. Table 1 also shows that L-arg (50 mg/kg) injection abolished the effect of lithium (10 mg/kg) on nitrite levels reduction in the PFC ($P < 0.001$) and HIPP ($P < 0.001$). On the other hand, subeffective doses of NOS inhibitors and NO precursor did not change the nitrite levels in PFC and HIPP when they were applied singly ($P > 0.05$).

Post-stress corticosterone assay in different housing conditions and treatments

The effects of drugs treatment on post-stress (one hour after an acute 6 min forced swimming stress) serum corticosterone concentration in SC and IC animals are shown in Table 1. A one-way ANOVA revealed significant effect of treatments on the serum corticosterone level ($F(9, 40) = 4.081$, $P < 0.001$, Table 1). Tukey's analyses revealed that lithium (10 mg/kg, but not 1 mg/kg) injection significantly reduced the corticosterone level in IC mice when compared with IC control group ($P < 0.01$). Co-administration of lithium (1 mg/kg) with L-NAME (3 mg/kg) or AG (20 mg/kg) in stressed mice significantly decreased the serum corticosterone level after acute stress in IC animals ($P < 0.05$ and $P < 0.01$). We also showed that L-arg (50 mg/kg) injection reversed the normalizing effect of lithium (10 mg/kg) on corticosterone level ($P < 0.05$). None of the applied doses of NOS inhibitors and NO precursor changed the serum corticosterone concentration in IC animals ($P > 0.05$).

DISCUSSION

Results of the current study showed that experiencing 4-weeks of SIS in the adolescence provoked depressive and anxiety-like behaviors in male mice. These behavioral changes were associated with upregulation of IL-1 β , NO overproduction, and HPA axis dysregulation in the socially isolated animals. Our results also revealed that administration of lithium not

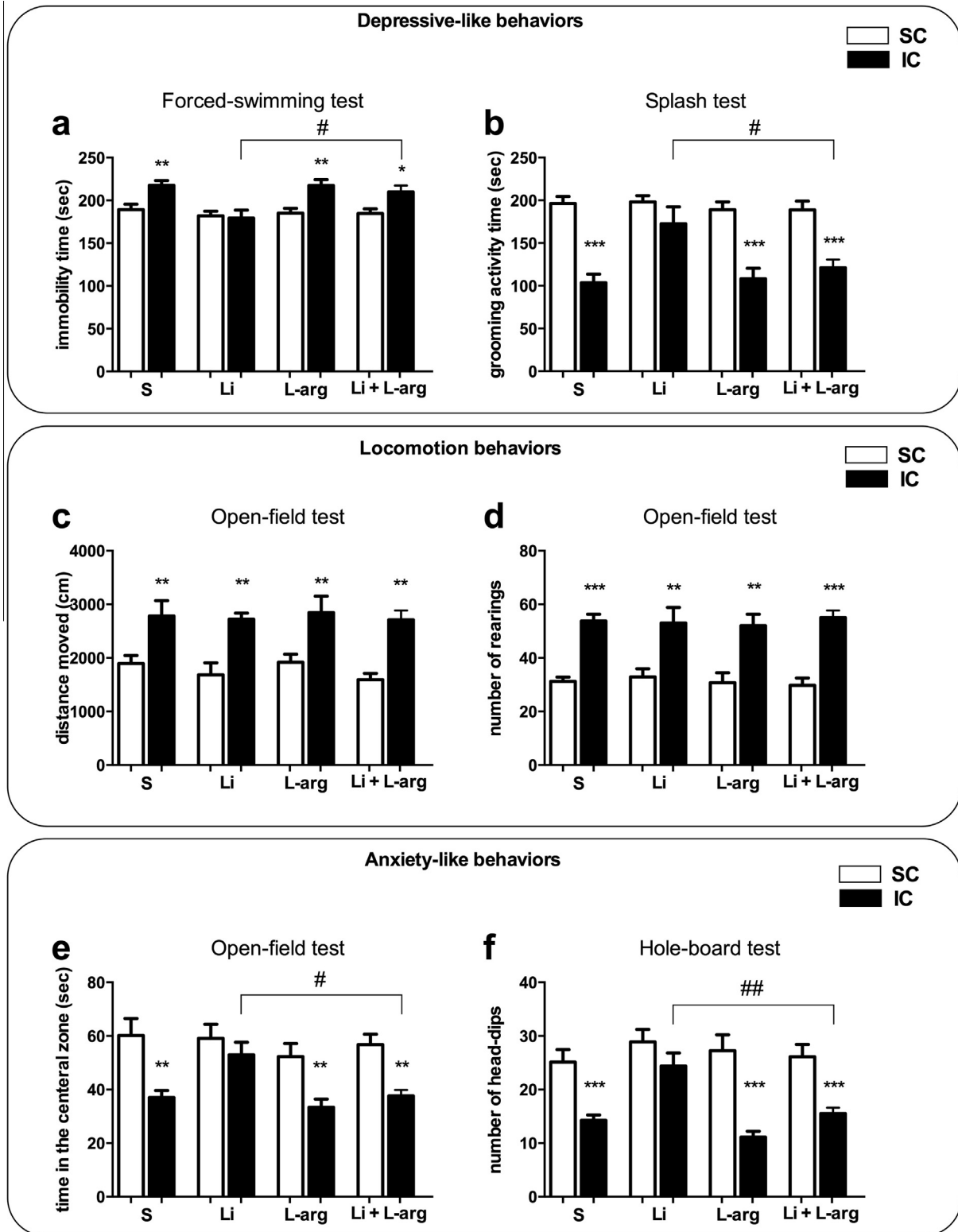


Fig. 5. Effects of lithium chloride and NO precursor co-administration. Effects of lithium chloride (Li) (10 mg/kg) and L-arginine (L-arg) (50 mg/kg) co-administration on the immobility time in the FST (a), grooming activity time in the splash test (b), total distance moved in the OFT (c), number of rearings in the OFT (d), time spent in the central zone in the OFT (e), number of head dips in the HBT (f). Values are expressed as the mean \pm S.E. M and were analyzed using a one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the SC control group. # $P < 0.05$ and ## $P < 0.01$ compared with the IC lithium- (10 mg/kg) treated group (Li group) (S: saline-treated groups).

Table 1. Effect of lithium chloride and NOS inhibitors/NO precursor co-treatments on PFC and HIPP nitrite level and post-stress serum corticosterone concentration

Groups	Nitrite (nM/mg protein)		Serum Corticosterone (ng/ml)
	PFC	HIPP	
Social condition (SC)	30.4 ± 6.4	75.4 ± 10	23 ± 4.3
Isolated condition (IC)	52.4 ± 11 ^{***}	127 ± 13 ^{***}	35.2 ± 3.6 ^{**}
+ Lithium chloride (1 mg/kg)	44.8 ± 12.9 ^{**}	113.1 ± 28 ^{**}	31 ± 8.8
+ Lithium chloride (10 mg/kg)	34.5 ± 6.6 ^{###}	93 ± 23 ^{##}	24.4 ± 4.6 ^{##}
+ L-NAME (3 mg/kg)	51.7 ± 5.2 ^{**}	112 ± 17 ^{**}	33.4 ± 6.1 [*]
+ Aminoguanidine (20 mg/kg)	50.1 ± 4.8 ^{**}	119 ± 14 ^{**}	32.7 ± 5.4 [*]
+ L-arginine (50 mg/kg)	56.2 ± 3.6 ^{**}	139 ± 33 ^{***}	35.8 ± 6.3 ^{**}
+ Lithium chloride (1 mg/kg) + L-NAME (3 mg/kg)	39.2 ± 4.3 ^{##}	94 ± 11 ^{##}	29 ± 2.5 [#]
+ Lithium chloride (1 mg/kg) + Aminoguanidine (20 mg/kg)	32.7 ± 3 ^{###}	89 ± 21 ^{##}	25.4 ± 2.8 ^{##}
+ Lithium (10 mg/kg) + L-arginine (50 mg/kg)	49.8 ± 9.3 ^{***}	121 ± 9 ^{***}	33.7 ± 4 [*]

Effects of lithium chloride (1 mg/kg) and NOS antagonists including L-NAME (3 mg/kg), aminoguanidine (20 mg/kg) co-administrations as well as lithium chloride (10 mg/kg) and L-arginine (50 mg/kg) co-administration on the cortical and hippocampal nitrite level and post-stress (one hour after an acute 6 min stress induction) serum corticosterone concentration in the IC animals. Values are expressed as the mean ± S.D. and were analyzed using a one-way ANOVA followed by Tukey's post hoc test.

* $P < 0.05$ compared with the SC control group.

** $P < 0.01$ compared with the SC control group.

*** $P < 0.001$ compared with the SC control group.

$P < 0.05$ compared with the IC control group.

$P < 0.01$ compared with the IC control group.

$P < 0.001$ compared with the IC control group.

only reversed the depressant and anxiogenic effects of SIS, but also attenuated the expression of IL-1 β and NO overproduction (mostly by iNOS inhibition) in the PFC and HIPP. In addition, we suggest that antidepressant and anxiolytic effects of lithium may be associated with moderating the negative effects of IL-1 β and iNOS on the HPA axis function.

Social isolation stress induces depressive and anxiety-like behaviors through HPA axis dysregulation and IL-1 β /NO overproduction

Experiencing juvenile SIS has been extensively reported to negatively induce long-lasting behavioral, neuroendocrinological, and immunological changes in animals (Sandi and Haller, 2015). Using FST as a valid behavioral test to evaluate the passive behaviors in rodents in response to acute stress, our results showed that exposing juvenile mice to 4-weeks of SIS is able to increase immobility time in the FST reflecting the behavioral despair in IC mice (Cryan and Holmes, 2005). Also, IC mice exhibited a significant decrease in grooming activity time in the splash test indicating that SIS evoked the motivational and self-care difficulties in these animals. Although there are pieces of evidence that has reported SIS do not induce depressive-like behaviors in the FST (Hall et al., 1998; Pisu et al., 2011), there is consensus agreement that SIS, namely during adolescence, is able to provoke depressive-like behaviors not only in the FST but also in other behavioral measures of depression (Grippe et al., 2008; Berry et al., 2012; Evans et al., 2012; Chang et al., 2015). In addition, passive behaviors of stressed animals in our experiments were not related to the changes of locomotor activity. Although the FST is quite tried and trusted, false results might be obtained with sedation and sickness conditions; therefore, we measured the behaviors in OFT relevant to locomotion in order to validate the FST results. This is in agreement with previous studies that have demonstrated SIS

increases the locomotion in rodents. Our results showed that IC mice exhibit an increase in both vertical and horizontal activity in the OFT. In consistence with previous investigations on the anxiogenic effects of SIS, our results revealed that IC mice show anxiety-like behaviors in the OFT (decrease in the time spent in the central zone) and HBT (decrease in number of head-dips) (Fone and Porkess, 2008; Amiri et al., 2015a). We recently demonstrated that juvenile SIS not only accounts for shaping the depressive and anxiety-like behaviors in mice, but also is an animal model for investigation the co-occurrence of anxiety and depressive-like behaviors (Haj-Mirzaian et al., 2015; Amiri et al., 2015b).

Ample evidence indicates the contribution of HPA axis in the pathophysiology of depression. In this regard, both clinical and preclinical studies have shown that depression correlates with elevated serum levels of GCs and the inability of the HPA axis to manage the response to acute stress (Lupien et al., 2009). A comprehensive review by Hawkey and colleagues has investigated the impact of SIS on HPA axis activity and suggests that effects of SIS on HPA axis depend on a variety of factors such as age, species and duration of stress. Furthermore, they concluded that SIS enhances the responsiveness of HPA axis to acute stressors (but not in the basal state) and also, the exaggerated response of HPA axis is accompanied by inflammatory responses in mammals (Hawkey et al., 2012). In response to an acute swim stress, our results showed that while there was no significant difference in basal corticosterone levels between IC and SC mice, IC mice had higher concentrations of corticosterone than their counterparts 60 min after acute stress induction. This result indicates the failure of the HPA axis to restore the corticosterone levels to basal state following exposure to acute stress in socially isolated animals. Also, previous studies on the responsiveness of HPA axis in socially isolated rats have shown that these animals had a greater HPA axis activity following

intracerebroventricular administration of corticotropin-releasing factor (CRF) indicating the impaired negative feedback in the animals (Serra et al., 2005, 2007).

On the other hand, emerging lines of evidence indicates the involvement of inflammatory factors in the pathophysiology of depression (Maes et al., 2009). Role of IL-1 β in modulating the depressive-like behaviors has been well documented in the literature (Goshen et al., 2008). There is a bidirectional relationship between inflammatory factors such as IL-1 β and HPA axis activity. In this regard, it has been shown that chronic exposure to GCs results in activation of IL-1 β and similarly, IL-1 β is able to activate the HPA axis responses (Goshen et al., 2008; Goshen and Yirmiya, 2009; Frank et al., 2014). In agreement with previous research on socially isolated animals, our results demonstrated the upregulation of IL-1 β gene in both HIPP and PFC of IC mice.

Also, results of our laboratory and others suggest that SIS activates the NOS activity in HIPP and PFC that results in increased nitrite levels in these regions of the brain. We recently showed that iNOS plays a role in mediating the co-occurrence of anxiety and depressive-like behaviors in socially isolated mice (Zlatković et al., 2014; Amiri et al., 2015b). Recent studies also have reported the involvement of iNOS in the modulation of depressive and anxiety-like behaviors in animal models of depression (Montezuma et al., 2012; Peng et al., 2012; Tomaz et al., 2014). Considering the immune-HPA axis communications, recent investigations suggest that stimulatory effect of IL-1 β on HPA axis activity may be mediated by the NO generated by iNOS in the PFC and HIPP (Gądek-Michalska and Bugajski, 2005; Gądek-Michalska et al., 2012, 2013b). To support this, it has been demonstrated that application of NOS inhibitors attenuated IL-1 β -induced CRH release and HPA axis responses (Gądek-Michalska et al., 2013b).

Altogether, juvenile SIS caused HPA axis abnormalities, overexpression of IL-1 β and elevated levels of NO in the HIPP and PFC that have already been reported to participate in the development of depression and anxiety-like behaviors.

Lithium attenuated the depressant and anxiogenic effects of juvenile SIS by targeting the negative impact of IL-1 β /NO on HPA axis function

Evidence is accumulating that lithium is not only considered as an effective treatment for mood disorders, but is also able to ameliorate the detrimental outcomes of stress on the brain through different pathways (Gray and McEwen, 2013; Bschor, 2014). Apart from therapeutic effects of lithium that have been attributed to regulation of GSK-3 β and N-methyl-D-aspartate (NMDA) receptors, recent evidence indicates the anti-inflammatory effects of lithium (Ghasemi and Dehpour, 2011; Beurel and Jope, 2014; Nassar and Azab, 2014). Results of our laboratory have previously demonstrated that antidepressant-like effects of both acute and chronic lithium are associated with NO. In this study, our results showed that acute administration of lithium (5 and 10 mg/kg, but not 1 mg/kg) attenuated the negative behavioral effects of SIS on

IC (but not SC) mice. These results were in agreement with similar studies which reported that these doses of lithium do not cause antidepressant and anxiolytic effects in non-stressed animals (Ghasemi et al., 2008; Gould et al., 2008). In consistence with previous reports, we also demonstrated that lithium (10 mg/kg) succeeded to mitigate overexpression of IL-1 β and NO overproduction in PFC and HIPP, as well as moderating the HPA axis hyper responsiveness to acute stress (Boku et al., 2009; Nahman et al., 2012; Nassar and Azab, 2014). As mentioned above, there is evidence suggesting the interactions between inflammatory factors and HPA axis. In this work, we showed that nitric system (mainly iNOS) contributes to positive effects of low-dose lithium against detrimental influences of SIS on animals. In this context, results showed that administration of subeffective dose of lithium along with subeffective doses of L-NAME or AG reversed the depressant and anxiogenic effects of SIS in IC mice. Same treatments with lithium and NOS inhibitors led to a decrease in NO overproduction in HIPP and PFC and, attenuation of HPA axis hyper responsiveness. In addition, lithium failed to produce protective effects in IC mice when animals were pretreated with L-arg indicating that NO (namely iNOS) plays a part in regulatory effects of lithium on HPA axis, inflammatory and behavioral profile of isolated mice.

Nitric oxide plays an important regulatory role in mediating various (patho) physiological processes in the brain. Numbers of studies have indicated that NOS inhibition results in antidepressant-like effects in a variety of animal models of depression (Wang et al., 2008; Mutlu et al., 2009). These reports have speculated that inhibiting NOS in the brain may play enhancing role in the antidepressant effect of many compounds (Harkin et al., 1999; Ghasemi et al., 2008; Krass et al., 2011). Some reports have focused on the involvement of nNOS in the pathophysiology of depression (Joca and Guimarães, 2006); nNOS is a more abundant isoenzyme in the brain in physiological and normal conditions. However, it has been reported that over-expression of iNOS is mostly observed during conditions such as inflammation and chronic stress (Olivenza et al., 2000; Harvey et al., 2004). Under chronic stressful conditions such as SIS, prolonged exposure to GCs results in priming the innate immune system in the brain mostly glial cells. Microglia, the resident macrophages in the brain, is susceptible to GCs-induced metabolic changes and initiates the inflammatory responses through producing inflammatory mediators including IL-1 β and iNOS (Picard et al., 2014; Réus et al., 2015). Inducible NOS not only mediates several inflammatory pathways in psychiatric and neurodegenerative disorders, but is also involved in HPA axis regulations and mediates the effects of IL-1 β on HPA axis activity (Rettori et al., 2009; Gądek-Michalska et al., 2013b; Chen et al., 2015). On the other hand, it has been shown that elevated level of NO has a negative influence on mitochondrial function (Moncada and Erusalimsky, 2002; Doherty, 2011). In order to explain the possible underlying mechanisms account for the effects of lithium, our (un) published findings revealed that juvenile SIS deleteriously change the energy and

antioxidant profile of the HIPP and cortical areas, and inhibition of iNOS not only reversed the mitochondrial dysfunction and energy deficits but also improved the behavioral profile of isolated mice relevant to anxiety and depression (Amiri et al., 2015a). Also, lithium has been well demonstrated to improve the performance of mitochondria and antioxidant system under pathological conditions (Chiu et al., 2015; Silachev et al., 2015; Valvassori et al., 2015). Therefore, it is possible that lithium exerts its effects by reducing the mitochondrial dysfunction and inflammatory responses. Altogether, our data suggests that anxiolytic and antidepressant-like effects of lithium are partly mediated by NO and its attenuating effects on immune-HPA axis function.

On the other hand, in our pilot studies we found that when lithium chloride was injected intraperitoneally at the doses of 1 and 10 mg/kg in SC and IC animals, the serum concentration of Li reached to 0.0345 and 0.281 mM, respectively (30 min after injection). Our results showed that the applied doses of lithium in this study provided therapeutical concentrations of lithium in serum, therefore, it seemed not necessary to investigate the effects of other doses in acute or chronic manner. Also, administration of chronic lithium to IC mice was accompanied by some problems in the study design because there was a concern that any handling or contact with isolated animals for daily oral gavage or intraperitoneal injections could significantly antagonize the effects of SIS. The only way to treat animals with lithium was to dissolve the lithium dose in their drinking water. Considering the different weight gain during the isolation period (4 weeks), it was not possible to treat the mice with the exact dose of lithium because of the variations in mice weight gain. Also, we considered the possible effects of lithium on the developing brain. Since adolescence reflects the age 8–18 years in humans, administration of lithium to juvenile mice at these ages is not clinically relevant. Although we could treat the animals after termination of the isolation process, we rather treated mice with acute lithium in order to investigate the mechanism through which lithium may exert its effects.

CONCLUSION

In conclusion, results of this study revealed that lithium has a protective role against depressant and anxiogenic effects of juvenile SIS. We showed that antidepressant and anxiogenic-like effects of lithium are partially mediated through HPA axis, nitrgergic system, and IL-1 β . Lithium mitigated the HPA axis hyper responsiveness in socially stressed animals through declining the IL-1 β over expression and suppressing NO overproduction (mostly by iNOS inhibition) in the PFC and HIPP of mice.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the article.

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