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Research report

Developmental minocycline treatment reverses the effects of neonatal immune activation on anxiety- and depression-like behaviors, hippocampal inflammation, and HPA axis activity in adult mice

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ABSTRACT

Neonatal infection is associated with increased lifetime risk for neuropsychiatric disorders including anxiety and depression, with evidence showing that dysregulation of the hypothalamic–pituitary–adrenal-(HPA)-axis system may be partly responsible. Preclinical and clinical studies demonstrate that minocycline exhibits antidepressant effects through inhibition of microglial activation and anti-inflammatory actions, and of interest is that recent studies suggest that minocycline alleviates the behavioral abnormalities induced by early-life insults. The current study was designed to determine if developmental minocycline treatment attenuates the neonatal immune activation-induced anxiety- and depression-like symptoms and HPA-axis-dysregulation later in life. To this end, neonatal mice were treated to either lipopolysaccharide or saline on postnatal days (PND) 3–5, then dams during lactation (PND 6–20) and male offspring during adolescence (PND 21–40) received oral administration of minocycline or water via regular drinking bottles. Anxiety- and depression-like behaviors, HPA-axis-reactivity (corticosterone), and hippocampal inflammation (TNF-α and IL-1β) after exposure to stress were evaluated. The results indicated that neonatal immune activation resulted in increased anxiety and depression-like symptoms, HPA-axis-hyperactivity, and elevated the levels of TNF-α and IL-1β in the hippocampus in response to stress in adulthood. Interestingly, developmental minocycline treatment significantly reduced the abnormalities induced by neonatal inflammation in adult mice. In addition, minocycline, regardless of postnatal inflammation, did not have any detrimental effects on the above measured parameters. Considering that minocycline is currently under exploration as an alternative or adjunctive therapy for reducing the symptoms of neurological disorders, our findings suggest that minocycline during development can decrease the behavioral abnormalities induced by early life inflammation in adulthood.

1. Introduction

Extensive research indicates that major depression and anxiety are among the most common psychiatric disorders which frequently cause significant functional impairment in humans (Fried and Nesse, 2015; Löwe et al., 2008; Ravindran and Stein, 2010; Toneatto and Nguyen, 2007). There is significant overlap between these two psychological problems which negatively affect daily activities and quality of life influencing other types of psychiatric disorders (Huppert et al., 2001; Mendlowicz and Stein, 2000). Accumulating evidence suggest that neurological diseases such as autism, schizophrenia, and affective disorders can be programmed by events such as stress and infection in early life (Boksa, 2010; Cottrell and Seckl, 2009; Depino, 2015; Dong et al., 2015; Enayati et al., 2012; Kinney et al., 2008). Although, the etiology of anxiety and depression remains unclear, recent studies by our group have provided evidence that infection/inflammation during the early stages of brain development including prenatal and early postnatal periods may be significantly involved in programming of these disorders in later life (Babri et al., 2014a,b; Doosti et al., 2013; Enayati et al., 2012; Majidi-Zolbanin et al., 2013, 2014, 2015). For

Abbreviations: LPS, lipopolysaccharide; PND, postnatal day; HPA, hypothalamic–pituitary–adrenal axis; IL, interleukin; TNF-α, tumor necrosis factor-alpha; CORT, corticosterone; ANOVA, analysis of variance; SEM, standard error of the mean.

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instance, we and others have developed an animal model of neonatal inflammation through lipopolysaccharide (LPS) administration in postnatal days (PND) 3 and 5 in mice (Doosti et al., 2013; Majidi-Zolbanin et al., 2013) and rats (Sominsky et al., 2012; Walker et al., 2004, 2012) leading to anxiety- and depressive-like behaviors, and persistent abnormalities in hypothalamic–pituitary–adrenal axis (HPA) axis function later in life. Dysregulation of HPA axis has a crucial role in the etiology of depression and anxiety-related disorders, both hypo and hyperactivity of the HPA-axis have indeed been found to be associated with higher risk of depression (Stetler and Miller, 2011; Tsigos and Chrousos, 2002).

Neonatal LPS administration has been shown to activate microglia cells and increase pro-inflammatory cytokines in the brain (Fan et al., 2005a,b). With regard to the relationship between neonatal immune activation and microglial activation, Sominsky et al. (2012) demonstrated that LPS administration in PNDs 3 and 5 results in increased anxiety-like behavior and microglial activation in the hippocampus of adult rats. In support of these findings, Walker et al. (2004, 2012) reported that immune activation with LPS on PND 3 and 5 increases anxiety-like symptoms and levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β in the hippocampus of adult rats in response to stress (Walker et al., 2010). Microglial activation and brain inflammatory cytokines have been implicated in mediating the effects of stress and both of them are known as major triggers for depression (Kreisel et al., 2014; Miller et al., 2009; Raison et al., 2006). In addition, previous studies have shown a relationship between elevated TNF-α and IL-1β and pathophysiology of anxiety and major depression (Bayramgürler et al., 2013; Dowlati et al., 2010; Goshen et al., 2008; Kaster et al., 2012; Krügel et al., 2013; Miller et al., 2009; Raison et al., 2006; Rossi et al., 2012; Simen et al., 2006). Given that hippocampus plays an important role in mediating anxiety- and depression-related behaviors in humans and rodents, this evidence suggests that the neuropathology of anxiety and depression induced by neonatal immune activation might be closely associated with hippocampal inflammation in adulthood.

Emerging evidence from preclinical and clinical studies also shows that the development of the therapeutic strategies against neuropsychiatric disorders with neurodevelopmental origin is an interesting topic for researchers. For example we reported that adolescent fluoxetine treatment can reduce the effects of early postnatal inflammation induced by LPS on anxiety and depression-like behaviors in offspring during adulthood (Doosti et al., 2013; Majidi-Zolbanin et al., 2013). While the majority of studies in this area of research originally tend to address how antidepressant and antipsychotic drugs at different windows of brain development such as prenatal, neonatal and adolescent periods can reduce psychiatric symptoms by regulating different neurotransmitter systems in the brain (Dickerson et al., 2012; Doosti et al., 2013; Ishiwata et al., 2005; Majidi-Zolbanin et al., 2013; Nagano et al., 2012; Rayen et al., 2011; Richtand et al., 2012), a few studies have recently considered the possibilities and ideas about early pharmacological intervention by minocycline, a second-generation tetracycline, for reducing brain inflammatory processes (Fan et al., 2005a,b Zhu et al., 2014a,b). In this context, a series of experiments indicate that minocycline can be a potential therapeutic drug for various neurological disorders, including major depression, anxiety, schizophrenia, Huntington's disease, Parkinson's disease, ischemia (Garrido-Mesa et al., 2013; Soczynska et al., 2012). Findings from animal and human studies support the hypothesis that minocycline can be a potential multi-target agent for the treatment of major depression and anxiety-related disorders through anti-inflammatory and microglial activities, and neuroprotective actions including neurogenesis and anti-glutamate excitotoxicity (Garrido-Mesa et al., 2013; Soczynska et al., 2012). It has been shown that minocycline decreases immobility and enhances the anti-immobility effect of desipramine in the forced swim test in animals showing an antidepressant effect (Molina-Hernández et al., 2008). Moreover, minocycline reduces the expression of pro-inflammatory cytokines and depression-like behavior induced by LPS in mice (O'connor et al., 2009). Minocycline has been used to prevent or ameliorate white matter damage after inflammation and hypoxic-ischemic injury in both adult and neonatal rats (Cai et al., 2006; Fan et al., 2005a). Fan et al. (2005a,b) demonstrated that minocycline treatment attenuates lipopolysaccharide-induced white matter injury in the neonatal rat brain on PND 5, probably through inhibition of microglial activation. In other studies, minocycline treatment during lactation in Fmr1 KO mice, a mouse model of Fragile X Syndrome, alleviated anxiety-like behavior in young mice (Bilousova et al., 2009; Dansie et al., 2013). Overall, the neuroprotective effects of minocycline are assumed to be exerted through antioxidant free-radical scavenging, inhibition of caspase expression and mitogen-activated protein kinases, and the suppression of microglial and astroglial activation and proliferation leading to reduced neuroinflammation and inhibition of apoptotic neuronal loss (Cheng et al., 2015; Soczynska et al., 2012). Minocycline is clinically well tolerated and almost completely absorbed when taken orally and which is also able to pass from the mother to the offspring through the breast milk (Lee et al., 2006; Lin et al., 2005; Luzi et al., 2009; Miyaoka et al., 2012). Since inflammation is thought to be one of the important player in the development of psychiatric diseases, and developmental drug treatment is currently receiving attention as a potential strategy for the treatment of neuropsychiatric disorders, we thought that minocycline could be considered a pharmacological candidate for the prevention of affective-like behaviors induced by neonatal immune activation.

2. Materials and methods

2.1. Subjects and ethics

Adult male and female NMRI mice (10–11 weeks old) were obtained from the animal house of Razi Institute. Animals were maintained under standard laboratory conditions on a 12:12 h light/dark cycle (lights on at 08:00 AM) and controlled temperature (23 ± 1 °C). Food and water were also available ad libitum. All procedures were approved by the Research and Ethics Committee of Tabriz University of Medical Sciences, and conducted in accordance with guidelines from the National Institutes of Health.

2.2. Newborn mice

Following a 2-week period of acclimatization to the new animal housing room, to facilitate the mating, male and female mice were kept together one-by-one in a cage. Female mice were visually monitored daily for confirmation of pregnancy, when it was confirmed the female mice were removed from the breeding cages and housed individually in standard cages. All pregnant animals were allowed to have normal delivery and the first day of birth was considered as PND 0. One day after the birth, all litters were culled to 4 male pups per mother. On the day 21, litters were weaned by removal of the mother and then were housed based on the treatment condition. In order to prevent the possible confounding factors of isolation housing, the offspring were kept in groups of 2 animals in the cages. Only one mouse per litter was used for each of the experiments to avoid the litter-effect.

2.3. Neonatal immune activation

A timeline diagram of the experiments is shown in Fig. 1. The dams were removed from their pups for approximately 5 min and

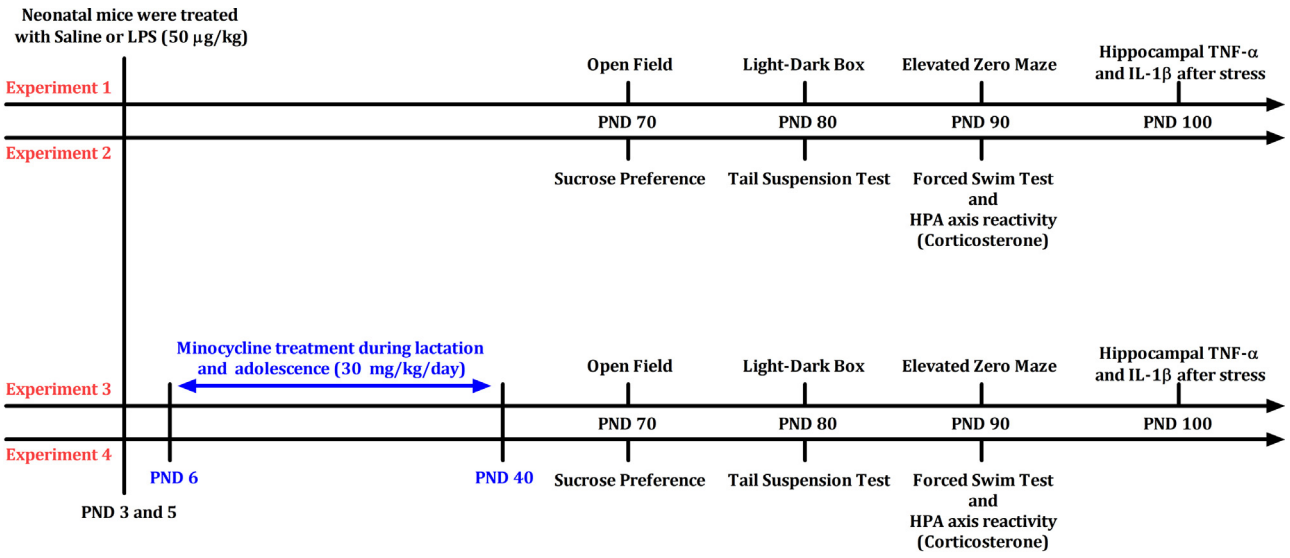

Fig. 1. Experimental design: There were four main experiments in this study, the experiments show the effects of neonatal immune activation alone or in combination with minocycline treatment on anxiety- and depression-like behaviors, HPA axis function and stress-induced inflammatory cytokines (TNF-α and IL-1β) in the hippocampus in male mice in adulthood.

the pups were weighed and received subcutaneously (in the interscapular region) injection of LPS (*Escherichia coli* 0111:B4, Sigma Co., USA; 50 μg/kg) or vehicle solution (1 ml/kg) on the PNDs 3 and 5. Both dose and time of LPS treatment in newborn mice were chosen based on our previous studies, where it has been shown to induce anxiety- and depression-like behaviors in adulthood (Doosti et al., 2013; Majidi-Zolbanin et al., 2013). LPS was dissolved in saline (0.9% NaCl) and injections were performed between 12:00 and 13:00 P.M. Each injection was performed through a needle (30-gauge) connected by polyethylene tubing to a 10-μl Hamilton syringe. Neonate mice were returned to their housing immediately following saline or LPS administration. In order to perform the behavioral tests, mice from both neonatal treatment conditions were divided into 2 experiments (each experiment was only used for three behavioral tests, with a 10-days interval between each test; $N = 9$/group).

2.4. Minocycline treatment

Minocycline (30 mg/kg/day) was administered via drinking water (the bottles were opaque to protect the drug from light) to the nursing mothers (PND 6–20) or adolescent offspring (PND 21–40). We chose this mode of delivery instead of invasive and stressful procedures resulting from repeated injections or oral gavages, to minimize the effect of repeated stress. This method of minocycline administration has been previously reported to result in high (detectable) concentrations of the drug in the blood of adult mice and in the breast milk of lactating dams (Lin et al., 2005; Luzi et al., 2009). Before the experiment began, we recorded the average water consumption according to the body weight per cage per day for each mouse in the laboratory. In addition, minocycline was dissolved in the drinking water and its concentration was calculated at three-day intervals according to the average liquid consumption and body weight per cage. The mice did not receive any source of water except for the drinking water containing minocycline solutions. Thus, they were motivated by thirst to drink the drug solutions. To examine possible effects of the chronic minocycline treatment, the liquid consumption of the control mice was measured every three days which showed no significant change in the liquid intake compared with the drug-receiving mice.

2.5. Behavioral testing

Behavioral assessments began at PND 70. The observers blind to the treatment recorded all parameters for each of the behavioral tests by using a stopwatch. In addition, all behavioral tests were conducted in a quiet room during the light period (between 13:00 and 18:00 h) under illumination of 75 lux and the mice were kept in the room for at least 1 h before the assessment. At the end of each test session, the arena was carefully cleaned with 70% ethanol and after test the cage was transported back to the colony room.

2.5.1. Open field

The open field was conducted as previously described by our group (Amani et al., 2013). The open field apparatus consisted of a white wooden box (40 × 40 × 20 cm) with 16 squares (10 × 10 cm; 12 outer and 4 inner) which was directly illuminated by a 100 W bulb placed 90 cm above the center of the apparatus floor. The test period was initiated when a single mouse was placed in the middle of the apparatus and allowed to move freely for 5 min. The inner zone time in and entries (an entry was defined as all four paws) were recorded as indices of anxiety-like behavior.

2.5.2. Light-dark box

The light–dark box apparatus consisted of a white-black wooden rectangular box (length 46 cm, width 27 cm, and height 30 cm), which was divided into two compartments (light and large: 27 cm × 27 cm, dark and small: 18 cm × 27 cm) by a partition. These areas were connected by a small central open door (7.5 cm × 7.5 cm) located in the center of the partition at floor level. The large compartment was open at the top, illuminated by a 100 W bulb located 90 cm above the apparatus. The small compartment had a removable black lid at the top. To start the test, each mouse was placed in the center of the light compartment, facing away from the door. The animal was allowed to freely explore both compartments for 5 min. The following parameters were recorded: light compartment time, light compartment entries, and latency of entry into the light compartment after the first entry into the dark division. A decrease in the amount of time spent and numbers of entries into the light compartment are indicative of anxiety-like behavior (Amani et al., 2013).

2.5.3. Elevated zero maze

The elevated zero maze was a ring-shaped apparatus, constructed from wood, elevated 40 cm from the floor. This apparatus consisted of a circular platform (outer diameter 46 cm, width 5.5 cm) divided into four quadrants of equal lengths with two open opposite (with 1 cm high curbs to prevent falls), and two equal closed (surrounded by a 20 cm wall from the surface of the maze) quadrants with an open roof. The room was illuminated by a 60-W bulb 1.5 m above the apparatus. Each animal was individually placed into one of the two closed quadrants at the start of their 5 min sessions. The following behavioral parameters were recorded: open quadrant time, open quadrant entries, the number of head dips in the open region; the number of stretch attend postures (defined as the animal having their front two paws out of the closed arm and their hind paws within the closed arm) in the closed quadrants (Salari et al., 2015).

2.5.4. Sucrose preference

The sucrose preference test was performed as described previously by Monteggia et al. (2007), over a 48-h period between PND 70 and 72. No previous food or water deprivation was applied before the test. After three days of acclimation to the two-bottle choice paradigm (two identical water bottles, both containing tap water, were placed on the cages during acclimation, PND 67–70), each offspring was given two bottles, one containing a 2% sucrose solution (A) and the other containing tap water (B). The position of the bottles A and B was changed every 12 h to avoid a "side" bias. The amount of the sucrose solution or water consumed was measured by weighing the bottles immediately before and after the test. The sucrose preference was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed. After the test, offspring were given free access to water. Anhedonia, a core symptom of depression, was defined as a decrease in sucrose preference.

2.5.5. Tail suspension test

The tail suspension test was performed as previously described (Salari et al., 2015). At the beginning of the experiment, each mouse was individually suspended by the tail using a clamp, 2 cm from the end, in a grey wooden enclosure (40 cm high, 30 cm wide and 20 cm deep) such that the head of mouse was about 25 cm above the floor. The total duration of immobility was recorded (in seconds) during the 5 min test period. Any animal that climbed their tails were removed from the experimental group, and were not used in the analysis. Immobility was defined as the lack of motion of the whole body, whereas mobility was defined as movement of the hind legs.

2.5.6. Forced swim test

The forced swim test was performed as described elsewhere (Salari et al., 2015). To apply this behavioral model to mice, the following procedure was adopted: Mice were individually placed into the transparent glass cylinders (Height: 25 cm, Diameter: 10 cm), filled with water to a height of 15 cm and maintained at 25 ± 1 °C. The water was replaced by fresh water between each test. The total duration of immobility was recorded during the last 4 min of the 6 min testing period. At the end of the swimming session, the animals were removed from the cylinder, dried with towels, and placed gently near an electric heater for 15–30 min. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water and making only those movements necessary to keep its head above water. The lack of struggling was considered as indicative of a state of behavioral despair.

2.6. HPA axis reactivity to stress

We assessed HPA axis reactivity in adult male offspring (PND 90) 25 min after the forced swim test. The blood was collected via tail vein puncture and circulating level of corticosterone (CORT; Bio-Medical Assay Company, China) in the serum was measured as previously described by using CORT specific quantitative sandwich ELISA kit according to the manufacturer's instructions. All samples and standards were assayed in duplicate.

2.7. Stress protocol, hippocampal collection and cytokine measurements

We evaluated the impacts of neonatal immune activation and minocycline treatment on IL-1β and TNF-α proteins in the hippocampus following acute restraint stress (mice were individually placed in plastic transparent cylinders, 4 cm diameter and 10 cm long, under a single 60-W light bulb for 60 min) based on a recent study where LPS administration on PNDs 3 and 5 resulted in increased TNF-α and IL-1β in the hippocampus in response of stress (Walker et al., 2010). Immediately after stress exposure, the offspring were deeply anaesthetized by an i.p. injection of ketamine hydrochloride (50 mg/kg; Alfasan, Woerden-Holland) plus Xylazine (5 mg/kg; Alfasan, Woerden-Holland). To prevent contamination of blood cytokine in the brain, mice were perfused with ice-cold pyrogen-free saline before they were killed. Then, whole brains were rapidly removed, placed on an ice-cold surface in a Petri dish filled with saline, and the hippocampus was dissected. The tissues were snap frozen in liquid nitrogen, placed into microcentrifuge tubes and stored at −80 °C until processing. Hippocampal tissue was homogenized in 500 μl buffer (TBS plus 0.2% Triton X-100, 2 mM EDTA, PBS 1 mM PMSF, and protease inhibitor cocktail) and centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatants were collected and total protein was determined by Micro BCA Protein Assay Kit. The levels of IL-1β and TNF-α in the hippocampus was detected using ELISA kits (R&D systems, USA) according to kit instructions. The concentration of cytokine protein is presented as pg per mg protein.

2.8. Statistics

All data were analyzed using the statistical package of SPSS (IBM-Version-21). In order to evaluate the effects of neonatal immune activation in offspring, the data were analyzed using one-way analysis of variance (ANOVA). The interactions between neonatal immune activation and minocycline were analyzed using two-way ANOVA. The data between drug (minocycline) and control (water) subgroups in LPS/saline-treated groups were also analyzed separately by using one-way ANOVA. All data are presented as the mean ± standard error of the mean (S.E.M). A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of minocycline on neonatal immune activation-induced anxiety-like behavior in adult mice

3.1.1. Open field

Male offspring treated with minocycline were tested on PND 70 in the open field. There were no interactions between neonatal immune activation × minocycline treatment in the inner zone time and entries. As shown in Fig. 2, neonatal immune activation with LPS significantly decreased time spent [$F_{1,16} = 6.49$, $P = 0.021$] and number of entries [$F_{1,16} = 6.29$, $P = 0.023$] in the inner zone of the open field, indicating higher levels of anxiety-like behaviors compared to the saline group. Moreover, the results of the open

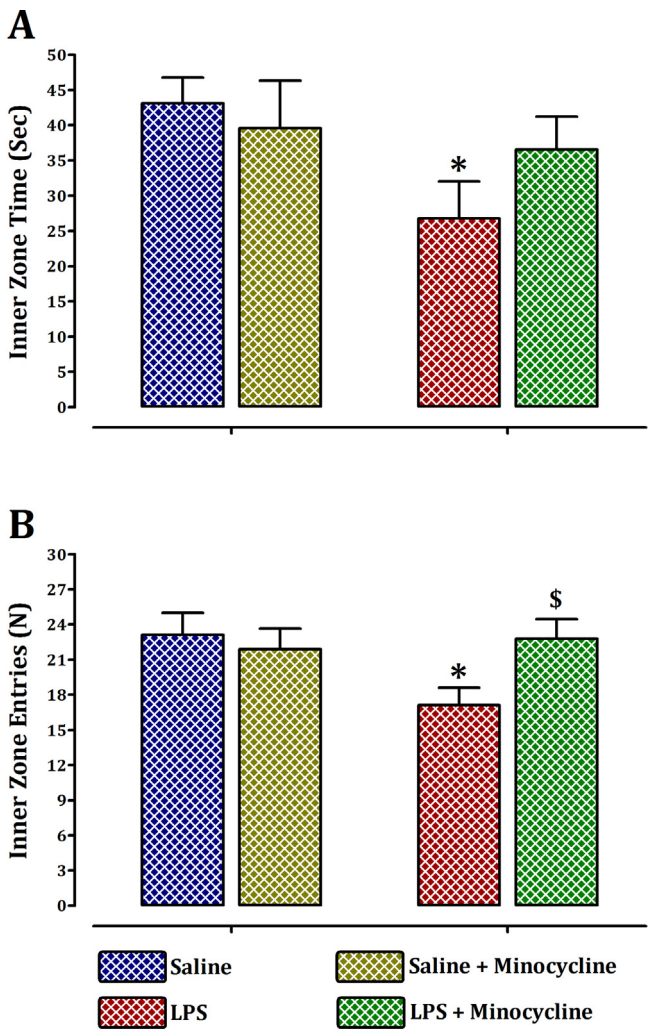

Fig. 2. Effects of neonatal immune activation alone or in combination with minocycline treatment on anxiety-like behavior in the open field in adult male mice, PND 70. Values are presented as mean ± S.E.M. ($N = 9$) inner zone time (A) and inner zone entries (B). Significant differences: $^{*}P < 0.05$, compared to the saline-treated mice; $^{\$}P<0.05$, compared to the LPS-treated mice.

field assessment showed that minocycline treatment during development significantly resulted in decreased the number of entries [$F_{1,16} = 6.41$, $P = 0.022$] into the inner zone in LPS-treated offspring relative to the LPS + water group.

3.1.2. Light–dark box

The light–dark box test was performed ten days after the open field on PND 80 and its results are presented in Fig. 3. Two-way ANOVA revealed a main effect of neonatal immune activation for light compartment time [$F_{1,32} = 5.23$, $P < 0.029$], light compartment entries [$F_{1,32} = 9.53$, $P = 0.004$], and latency [$F_{1},32 = 7.66$, $P = 0.009$], and minocycline treatment for the latency [$F_{1,32} = 6.07$, $P < 0.019$] in the light-dark box test. There was also a significant interaction between neonatal immune activation × minocycline treatment [$F_{1,32} = 8.52$, $P = 0.006$] for the latency. However, there were no significant interactions between neonatal immune activation × minocycline treatment for the other parameters in the test. Additional statistical analysis revealed that neonatal treatment with LPS significantly reduced the light compartment time [$F_{1,16} = 9.7$, $P = 0.007$] and entries [$F_{1,16} = 5.06$, $P = 0.039$], and increased latency [$F_{1,16} = 9.79$, $P = 0.006$]. These findings are indicative for higher levels of anxiety in LPS-treated offspring in comparison with the saline-treated animals. Fig. 3 also shows that minocycline treatment during lactation and adolescence significantly elevated amount of time spent [$F_{1,16} = 5.22$, $P = 0.036$] into the light compartment and reduced the latency [$F_{1,16} = 9.6$, $P = 0.007$] in the LPS offspring relative to LPS/Water offspring. These results confirm the above findings and therefore it can be concluded that minocycline treatment during lactation and adolescence can reverse anxiety-like behavior induced by neonatal immune activation in mice, as a result, we can consider this as a beneficial effect of minocycline for the treatment of anxiety with neurodevelopmental origin in mice.

3.1.3. Elevated zero-maze

After light-dark box paradigm, the offspring were placed in the elevated zero maze on PND 90 and their behavior was recorded for 5 min. Two-way ANOVA revealed a main effect of neonatal immune activation and minocycline treatment on open quadrant time ([$F_{1,32} = 15.48$, $P < 0.001$]; [$F_{1,32} = 7.36$, $P = 0.011$], resp.), open quadrant entries (neonatal immune activation: [$F_{1,32} = 9.74$, $P = 0.004$]), and stretch attend postures ([$F_{1,32} = 10.82$, $P = 0.002$]; [$F_{1,32} = 21.21$, $P < 0.001$], resp.) in closed quadrants. A significant interaction existed between neonatal immune activation and minocycline treatment [$F_{1,32} = 6.25$, $P = 0.018$] for stretch attend postures and there were no interactions for the other parameters. No significant change in the head dips was observed.

In addition, one-way ANOVA revealed that early postnatal exposure to LPS significantly reduced open quadrant time [$F_{1,16} = 14.58$, $P = 0.002$] and open quadrant entries [$F_{1,16} = 11.69$, $P = 0.004$], and increased stretch attend postures [$F_{1,16} = 17.76$, $P = 0.001$], which reflects higher levels of anxiety in LPS-treated offspring relative to the saline-treated group (Fig. 4). On the other hand, as illustrated in Fig. 4, minocycline treatment during development significantly increased open quadrant time [$F_{1,16} = 9.25$, $P = 0.008$] and open quadrant entries [$F_{1,16} = 6.63$, $P = 0.02$], and decreased stretch attend postures [$F_{1,16} = 28.87$, $P < 0.001$], showing lower levels of anxiety-like behavior in LPS offspring in comparison with LPS + water animals. Therefore, these results indicate that minocycline acts as an anxiolytic agent during development and is able to prevent anxiety-like behavior induced by neonatal inflammation in adult mice.

3.2. Effect of minocycline on neonatal immune activation-induced depression-like behavior in adult mice

3.2.1. Sucrose preference

To examine whether minocycline treatment during development exerts a significant effect on depressive-like behavior, we first tested the offspring (PND 70) for sucrose preference to assess offspring's response to a natural reward. It has been suggested that a loss of sensitivity to reward is an indicator of anhedonia-like behavior, one important feature of human major depressive disorder. Two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 8.07$, $P = 0.008$] and minocycline treatment [$F_{1,32} = 4.2$, $P = 0.05$] on sucrose preference test. However, there was no significant interaction between neonatal immune activation × minocycline treatment for sucrose preference. As illustrated in Fig. 5A, mice neonatally treated with LPS showed significantly less preference [$F_{1,16} = 7.4$, $P = 0.015$] for the sucrose solution than saline-treated mice, consistent with an anhedonic response. On the other side, minocycline treatment reversed the decrease in sucrose preference [$F_{1,16} = 4.61$, $P = 0.047$] in LPS offspring, showing higher preference for the sucrose solution and lower depressive-like behavior in LPS offspring in comparison with LPS + water group.

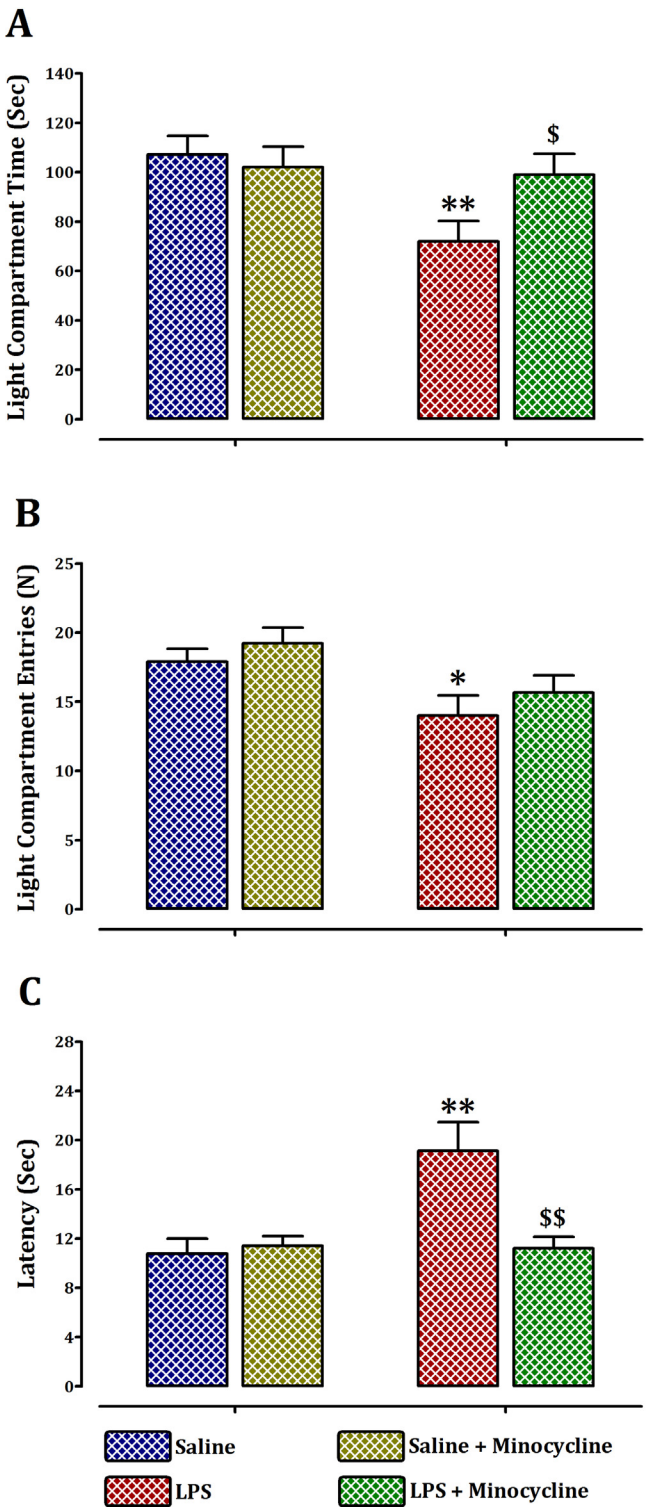

Fig. 3. Effects of neonatal immune activation alone or in combination with minocycline treatment on anxiety-like behavior in the light–dark box in adult male mice, PND80. Values are presented as mean ± S.E.M. ($N = 9$) light compartment time (A), light compartment entries (B), or latency of entry into the light compartment (C). Significant differences: $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the saline-treated mice; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$, compared to the LPS-treated mice.

3.2.2. Tail suspension test

The tail suspension test was performed ten days after sucrose preference test on PND 80 and its data is shown in Fig. 5B. Two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 7.09$, $P = 0.012$] and minocycline treatment [$F_{1,32} = 4.53$, $P = 0.041$] on the immobility time. There was no significant interaction between neonatal immune activation × minocycline treatment in the tail suspension test. Early neonatal exposure to LPS increased the total duration of immobility [$F_{1,16} = 8.74$, $P = 0.009$] in offspring, which is indicative for higher levels of depression in LPS-treated males compared to the saline-treated group. On the other hand, minocycline treatment during lactation and adolescence resulted in decreased the immobility time [$F_{1,16} = 4.53$,

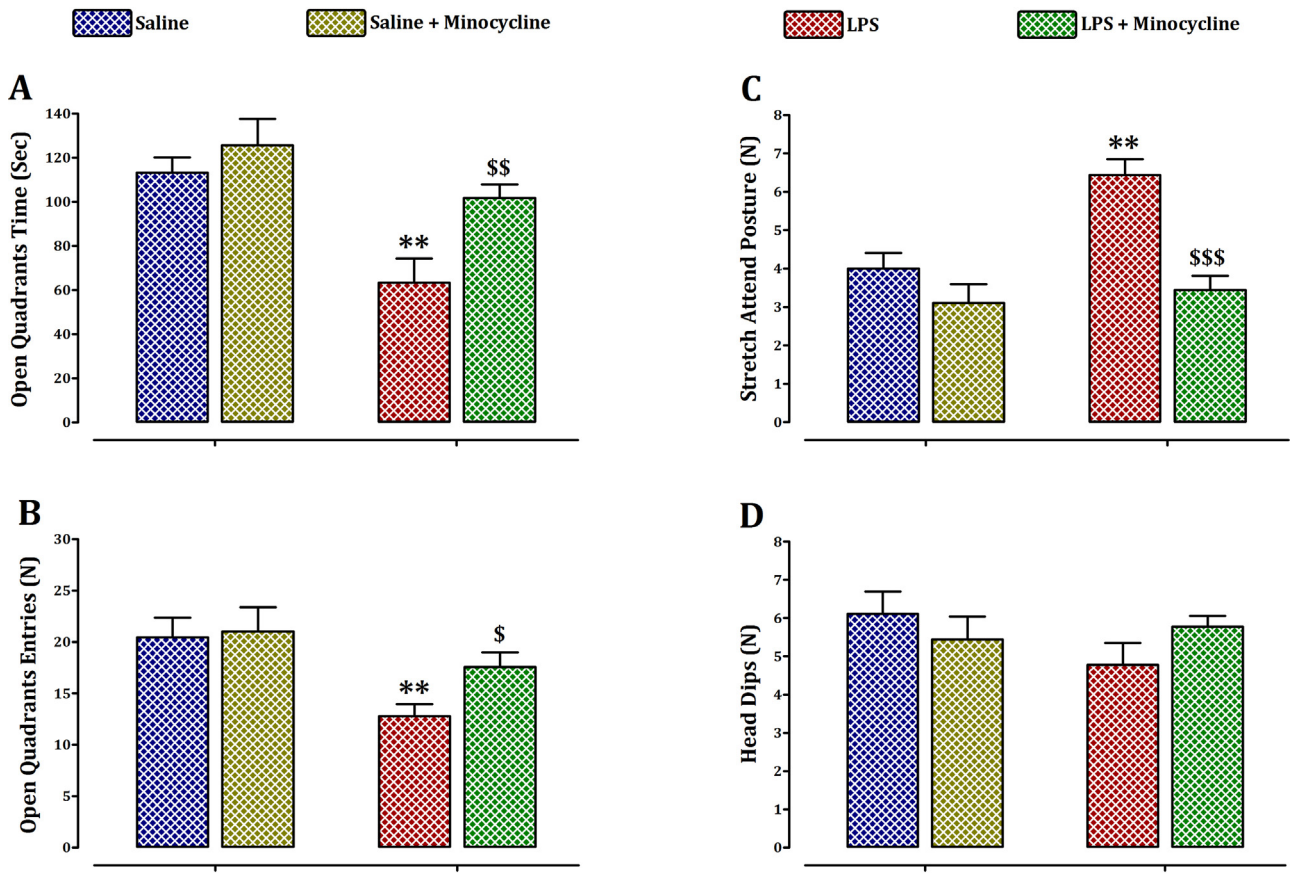

Fig. 4. Effects of neonatal immune activation alone or in combination with minocycline treatment on anxiety-like behavior in the zero maze in adult male mice, PND 90. Values are presented as mean ± S.E.M. ($N = 9$) open quadrant time (A), open quadrant entries (B), the number of the stretch attend postures (C), or the number of head dips (D). Significant differences: $^{**}P < 0.01$, compared to the saline-treated mice; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$, compared to the LPS-treated mice.

$P = 0.049$], which reflects lower levels of depressive-like behavior in LPS offspring relative to the LPS + Water offspring.

3.2.3. Forced swim test

The offspring were tested in forced swim test on PND 90, two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 8.22$, $P = 0.007$] and minocycline treatment [$F_{1,32} = 13.40$, $P = 0.001$] on the immobility time. There was no significant interaction between neonatal immune activation × minocycline treatment in the forced swim test. As shown in Fig. 5C, neonatal immune activation increased the total duration of immobility [$F_{1,16} = 12.21$, $P = 0.003$] in offspring, which reflects higher levels of depression in LPS offspring, as compared to the saline-treated group. On the other side, minocycline treatment during development gave rise to reduced the immobility time [$F_{1,16} = 10.91$, $P = 0.004$], showing lower levels of depressive-like behavior in LPS offspring in comparison with LPS + Water offspring. These findings confirm the above data showing that minocycline treatment during lactation and adolescence can reverse depressive-like behavior induced by neonatal immune activation in mice in adulthood.

3.3. Effect of minocycline following neonatal immune activation on HPA axis reactivity to stress in adult mice

Stress-induced corticosterone levels were measured in response to the elevated plus maze (Fig. 6A) in adult mice. Two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 22$, $P < 0.001$] and minocycline treatment [$F_{1,32} = 7.59$, $P = 0.01$]. However, there was no significant interaction effect for neonatal immune activation × minocycline treatment [$F_{1,32} = 3.75$, $P = 0.62$]. In addition, Fig. 6A shows that neonatal immune activation increased the corticosterone levels [$F_{1,16} = 13.97$, $P = 0.002$] in LPS males relative to the saline-treated group. This finding reflects higher levels of HPA axis reactivity to stress in LPS offspring than saline offspring. One-way ANOVA also indicated that minocycline treatment during development significantly decreased stress-induced corticosterone levels in adult male mice [$F_{1,16} = 9.28$, $P = 0.008$] in response to stress in comparison with the LPS + water group. These findings demonstrate that minocycline exposure during brain development can normalize HPA axis reactivity to stress following neonatal immune activation.

3.4. Effect of minocycline following neonatal immune activation on stress-induced hippocampal IL-1β and TNF-α in adult mice

3.4.1. IL-1β in the hippocampus

As illustrated in Fig. 6B, the levels of IL-1β protein in the hippocampus were measured after stress exposure in adult mice. Two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 44.68$, $P < 0.001$] and minocycline treatment [$F_{1,32} = 23.8$, $P < 0.001$]. A significant interaction existed between neonatal immune activation and minocycline treatment [$F_{1,32} = 6.7$, $P = 0.014$]. One way ANOVA indicated that postnatal immune activation elevated the hippocampal IL-1β levels [$F_{1,16} = 30.26$, $P < 0.001$] in LPS-treated mice, as compared to the saline-treated mice. The levels of IL-1β in the hippocampus of the saline-minocycline-treated mice were significantly lower [$F_{1,16} = 4.93$, $P = 0.041$] than those saline-water-treated mice. Furthermore, Fig. 6B shows that developmental minocycline exposure markedly reduced the levels of IL-1β protein in the hippocampus of the

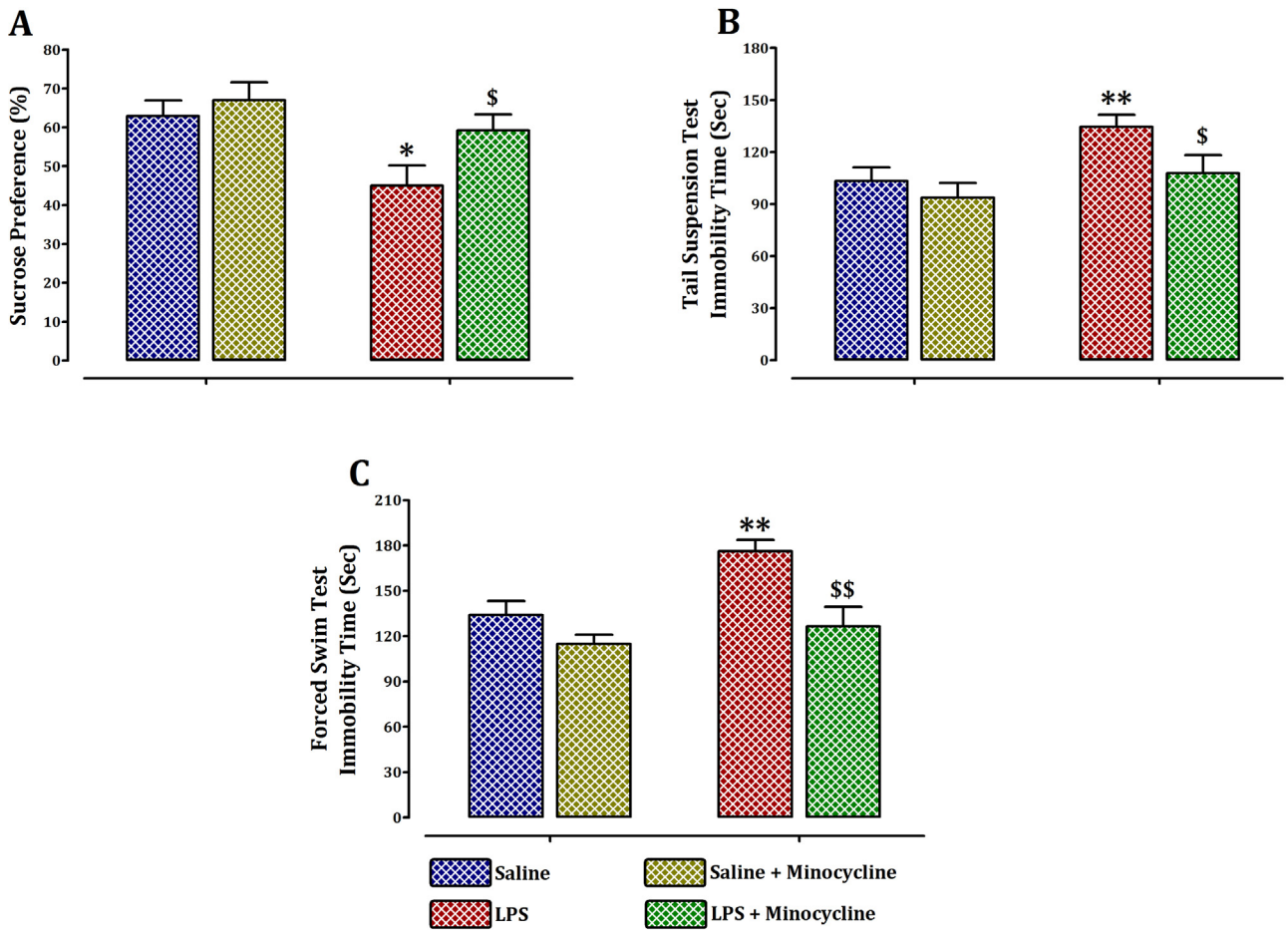

Fig. 5. Effects of neonatal immune activation alone or in combination with minocycline treatment on depression-like behaviors (sucrose preference, PND 70: A; tail suspension test, PND80: B; forced swim test, PND90: C) in adult male mice. Values are presented as mean ± S.E.M. ($N = 9$) percentage of sucrose preference, or of total duration of immobility. Significant differences: $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the saline-treated mice; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$, compared to the LPS-treated mice.

mice that neonatally were exposed to LPS [$F_{1,16} = 18.97$, $P < 0.001$]. These data clearly demonstrate that minocycline treatment following neonatal immune activation during perinatal and adolescent periods can decrease stress-induced IL-1β in the hippocampus.

3.4.2. *TNF-α in the hippocampus*

We also measured the levels of TNF-α protein in the hippocampus after stress exposure in adult mice. Two-way ANOVA revealed a main effect of neonatal immune activation [$F_{1,32} = 44.1$, $P < 0.001$] and minocycline treatment [$F_{1,32} = 20.97$, $P < 0.001$]. However, there was no significant interaction for neonatal immune activation and minocycline treatment. As shown in Fig. 6C, the data analysis revealed that neonatal immune activation resulted in an increase in TNF-α protein in the hippocampus [$F_{1,16} = 27.31$, $P < 0.001$] in mice. We also found a significant lower level of TNF-α protein [$F_{1,16} = 10.44$, $P = 0.005$] in the hippocampus of the saline-minocycline-treated mice in comparison with the saline-water-treated mice. Moreover, minocycline treatment following neonatal immune activation significantly decreased the hippocampal TNF-α levels [$F_{1,16} = 11.31$, $P = 0.004$] in adult mice after stress exposure. These findings indicate developmental minocycline treatment following neonatal immune activation can reduce stress-induced TNF-α in the hippocampus.

4. Discussion

Our results (Fig. 7) confirm and extend the earlier findings, suggesting that neonatal immune activation can be a risk factor for the development of anxiety- and depression-like behaviors, HPA axis hyperactivity and hippocampal inflammation in adulthood (Doosti et al., 2013; Majidi-Zolbanin et al., 2013; Walker et al., 2004, 2010, 2012). Given that we have previously discussed in detail the influence of neonatal immune activation on HPA axis development and its relevance to anxiety- and depression-like behaviors elsewhere (Doosti et al., 2013; Majidi-Zolbanin et al., 2013), here we further focus on the role of hippocampal inflammation and minocycline effects on these affective disorders. Consistent with our findings it has been shown that neonatal infection increases the expression of glial cell marker and IL-1β-related genes in the hippocampus of neonate and adult rats in response to LPS administration (Bilbo et al., 2005a). Besides, Walker et al. (2004, 2012) reported that early neonatal LPS exposure in rats significantly elevates anxiety-like behavior and hippocampal IL-1β and TNF-α levels after exposure to stress in adulthood (Walker et al., 2010). Interestingly, this group later showed that postnatal LPS administration results in increased inflammation and microglial activation in the hippocampus in adult rats that were co-incident with an elevation in levels of anxiety-like behavior (Sominsky et al., 2012). In light of this evidence, a wide spectrum of studies suggest that dysregulation of inflammatory processes need to be carefully considered as a major pathophysiological mechanism of major depression (Dowlati et al., 2010; Miller et al., 2009; Raison et al., 2006). From a neuroimmunological point of view, pro-inflammatory agents such as IL-1β and TNF-α are thought to be among the most important players in the pathogenesis of major depression and anxiety-related disorders, through their direct effects on neural cells within CNS and alterations in

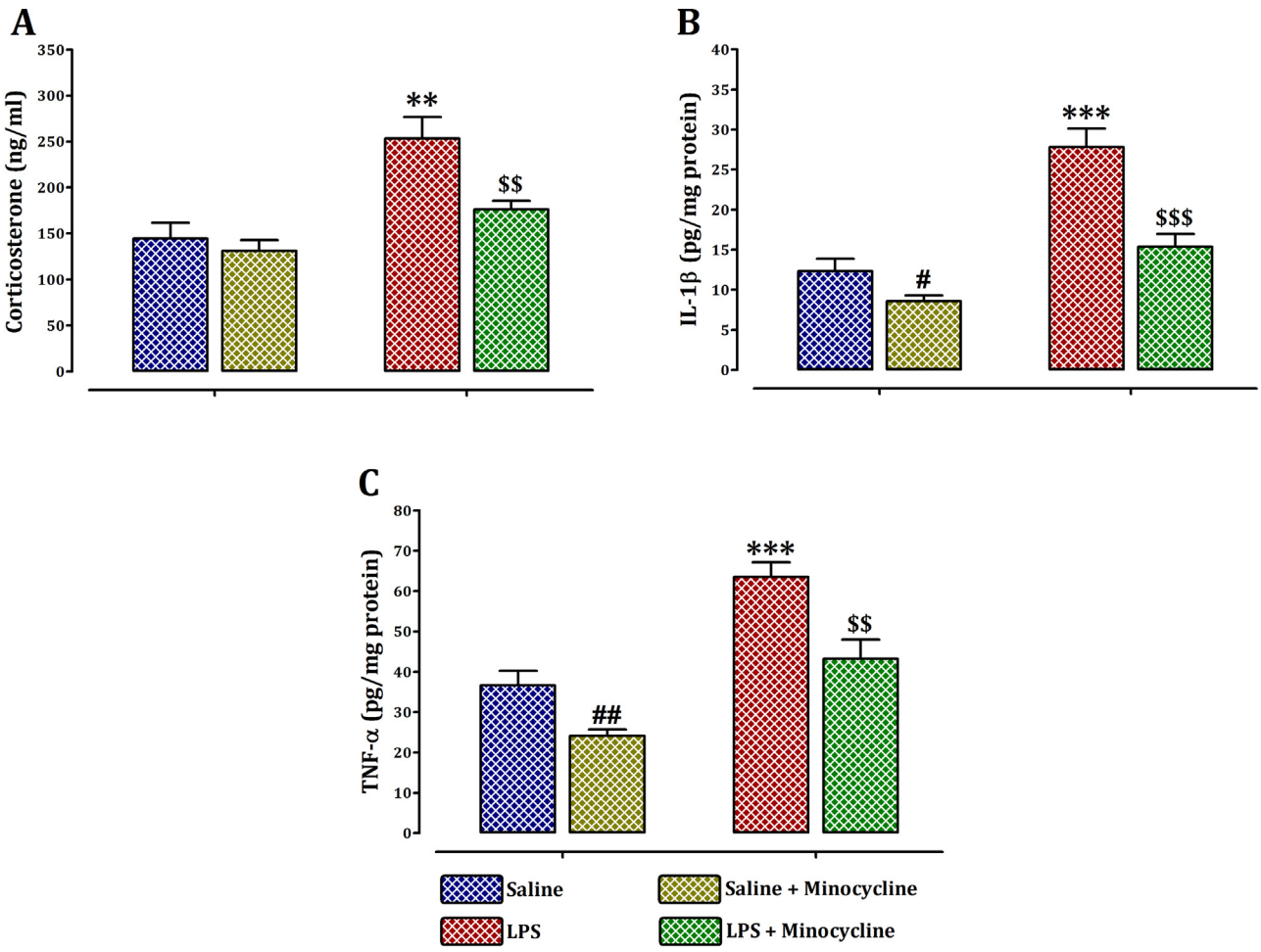

Fig. 6. Effects of neonatal immune activation alone or in combination with minocycline treatment on HPA axis reactivity to stress (corticosterone levels, PND90) and stress-induced inflammatory cytokines (IL-1β: B; and TNF-α: C; PND 100) in the hippocampus in adult male mice. Values are presented as mean ± S.E.M. ($N = 9$) corticosterone concentration, or cytokine protein levels. Significant differences: $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to the saline-treated mice; $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to the saline-treated mice; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$, compared to the LPS-treated mice.

neurotransmitters and neuropeptides levels (Dowlati et al., 2010; Loftis et al., 2010; Miller et al., 2009; Raison et al., 2006; Salim et al., 2012). This view, in agreement to our findings, is supported by clinical studies where IL-1β and TNF-α appear to be significantly higher in patients with major depression, and of interest is that antidepressant drugs can reverse these altered levels (Dowlati et al., 2010; Hannestad et al., 2011; Owen et al., 2001; Song et al., 2009; Tuglu et al., 2003). Preclinical studies also support these findings, in recent years, it has repeatedly been reported that these two cytokines have a direct role in the regulation of depression and anxiety-like symptoms in animal models (Babri et al., 2014b; Chen et al., 2013; Goshen et al., 2008; Kaster et al., 2012; Rossi et al., 2012). Further confirmations come from previous literature where treatment with anti-TNF and -IL-1β agents or the deletion of related genes was associated with decreased depressive and anxiety symptoms (Bayramgürler et al., 2013; Goshen et al., 2008; Karson et al., 2013; Koo and Duman, 2009; Krügel et al., 2013; Murray et al., 2013; Simen et al., 2006). Considering that the major source of TNF and IL-1β in the CNS is activated microglia and astrocytes, and the fact these glia cells and cytokines are involved in inflammation, neuronal death and neurogenesis (Ben-Hur et al., 2003; Ekdahl et al., 2009; Goshen et al., 2008; Iosif et al., 2006; Kohman and Rhodes, 2013; Zunszain et al., 2012), in this study, their potential effects on the structure and function of hippocampal neurons might have influenced the behavior in mice. The point which needs to be taken into consideration is that hippocampus is involved in the regulation of depression (Bremner et al., 2000) and anxiety (Engin and Treit, 2007) behaviors and HPA axis activity (Herman et al., 2005). While there is a strong relationship between inflammation in the brain and the etiology and pathophysiology of major depression and anxiety-related disorders, little attention has been paid to where depression is characterized by increased inflammation in the context of HPA axis hyperactivity and glucocorticoid resistance (Pariante and Lightman, 2008). According to the fact that increased CORT level during inflammation may result from stimulation of the HPA axis by pro-inflammatory cytokines such as IL-1β and TNF-α (Munck et al., 1984), it can be concluded that elevated levels of IL-1β and TNF-α in the hippocampus and HPA axis dysregulation as a result of neonatal immune activation in mice likely contribute to alterations in anxiety- and depression-like phenotypes in adulthood.

In the second part of this study, we show that the adverse consequences of neonatal immune activation on anxiety- and depression-like behaviors, and hippocampal inflammation were reversed by developmental minocycline treatment in adult male mice. In addition, developmental minocycline exposure, regardless of postnatal inflammation, did not have any detrimental effects on the above measured parameters. Recent studies have demonstrated the neuroprotective effects of minocycline on anxiety and depression-related disorders in humans and animals (Soczynska et al., 2012). For instance, minocycline has shown to be beneficial in reducing the immobility time, an indicator of antidepressant effect,

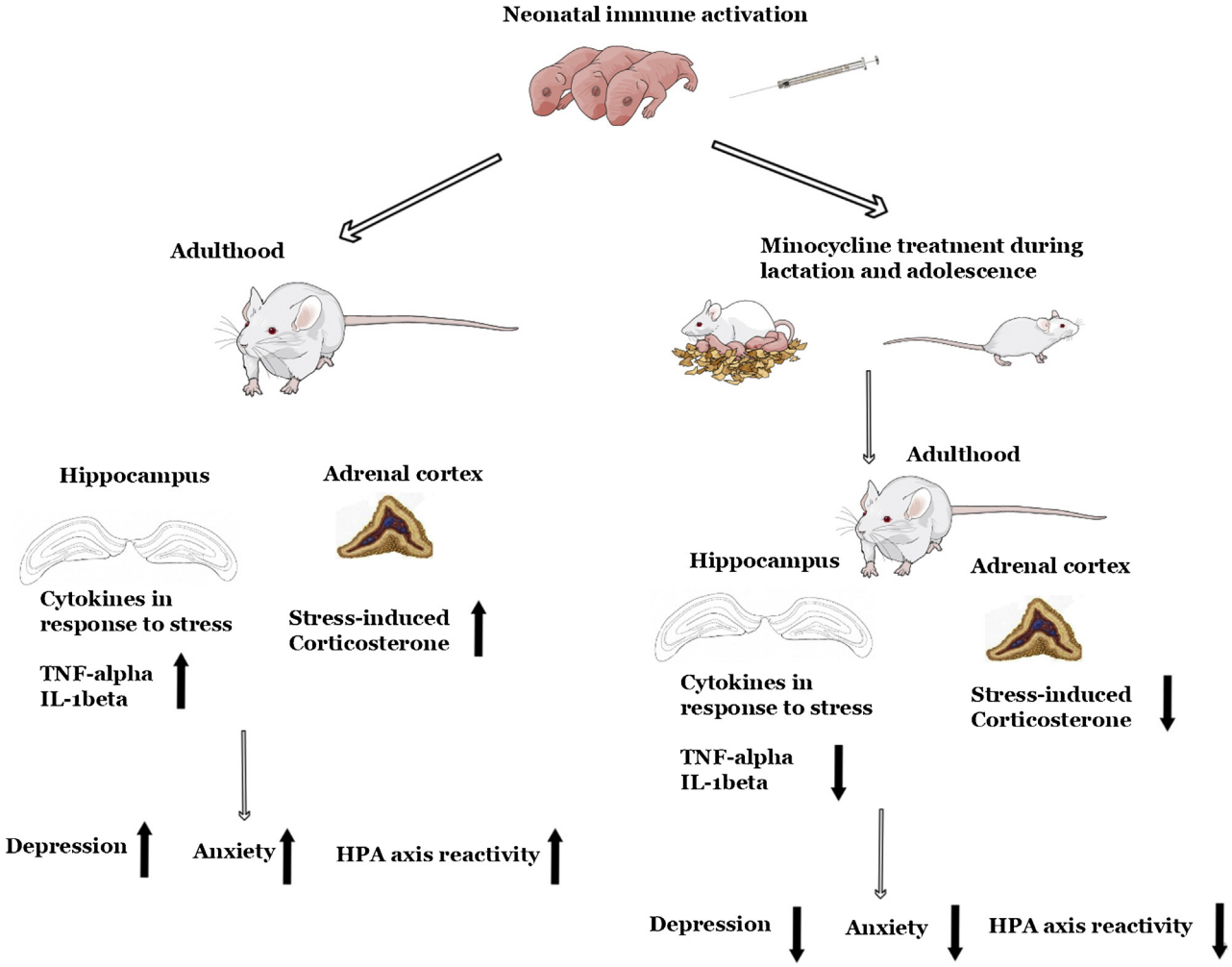

Fig. 7. This figure indicates the summary of results. ↑ = Increase and ↓ = Decrease.

in the forced swim test (Molina-Hernández et al., 2008). Furthermore, minocycline was able to attenuate LPS-induced expression of pro-inflammatory cytokines and prevent LPS- and interferon-alpha-induced depressive-like behavior in mice (O'connor et al., 2009; Zheng et al., 2015). In line with this, it has been shown that minocycline attenuates LPS-induced neuroinflammation, sickness behavior, and anhedonia in adult mice through reduced microglial activation and decreased mRNA levels of IL-1β in the hippocampus (Henry et al., 2008). Another study found that minocycline affects the maturation of dendritic spines in the developing hippocampal neurons, resulting in anxiolytic behavior in young Fmr1 KO mice (Bilousova et al., 2009). Interestingly, in agreement with our findings, a recent study demonstrated that adolescent minocycline treatment attenuated the behavioral deficits and inhibited the activated microglia in the brain of offspring that prenatally exposed to inflammation (Zhu et al., 2014b). Further evidence comes from the experiments where minocycline has been used to prevent or ameliorate white matter damage induced by neonatal exposure to LPS, the protective effect of minocycline was associated with suppressed microglial activation and decreased the levels of IL-1β and TNF-α proteins in the neonatal rat brain (Fan et al., 2005a). Along with these finding, Zhu et al. (2014a,b) reported that neonatal intra-hippocampal injection of LPS in rats leads to behavioral alterations such as deficits in social interaction, novel object recognition and prepulse inhibition, and increased the number of activated microglial cells in the hippocampus, which application of either minocycline, risperidone or both of them significantly rescued these abnormalities (Zhu et al., 2014a). Consistent with these findings, previous literature supports the idea that impaired neuroprotection like decreased neuronal survival and neurogenesis in the hippocampus is strongly involved in the pathogenesis of major depression disorder, thus these actions can be considered as potential pathophysiological factors and therapeutic targets for patients with major depression (Pae et al., 2008). On the other side, it has been shown that neonatal *E. coli* administration reduces both the survival and neurogenic potential of hippocampal progenitors and impairs adult performance in hippocampus-dependent memory tasks, which is attributed to decreased hippocampal neurogenesis (Bilbo et al., 2005b; Bland et al., 2010). Moreover, neonatal exposure to LPS has been shown to induce an acute expansion of the hippocampal microglia population, resulting in reduced neurogenesis in the hippocampus of rats (Smith et al., 2014). Although the findings from the present study do not provide direct evidence about the effect of neonatal immune activation on the neuronal survival and neurogenesis, our results may reflect the ability of inflammation to modulate depression and anxiety-like behaviors through inhibition of neurogenesis in the hippocampus. This conclusion comes from previous studies in which LPS and TNF-α have shown to be the known agents impairing newborn and adult neurogenesis in different brain regions like hippocampus (Borsini et al., 2015; Cacci et al., 2005; Das and Basu, 2008; Järlestedt et al., 2013; Monje et al., 2003). In support of this argument we recently demonstrated that early neonatal TNF-α treatment in mice results in increased anxiety and depression-like behavior in adulthood (Babri et al., 2014b).

The question now is how minocycline treatment can reverse the effects of neonatal immune activation in adulthood, in other words, what are the potential mechanisms? Given that neuroinflammation is mediated by microglia and astrocytes in the CNS, we think that the neuroprotective mechanism of minocycline appears to be directly related to its anti-inflammatory effects. It has clearly been documented that minocycline suppresses microglial and astroglial activation, inhibits the increased production of pro-inflammatory cytokines, like TNF-α and IL-1β, enhance neuroplasticity and neurogenesis in the hippocampus (Cheng et al., 2015; Garrido-Mesa et al., 2013; Jiang et al., 2015; Soczynska et al., 2012). Since brain is very vulnerable and sensitive to inflammation during early neonatal development, an age when murine developmental neurogenesis is highly active (Smith et al., 2014), it is very likely that the negative effect of LPS on structure and function of hippocampal neurons might have been enhanced by increased levels of TNF-α and IL-1β in adulthood. It is obvious that the hippocampus is an important limbic structure which plays a crucial role in anxiety and depression, and HPA axis system (Bremner et al., 2000; Engin and Treit, 2007; Herman et al., 2005). Previous literature has unequivocally linked the existence of structural and functional disorders in the hippocampus, such as a reduction in the total number of neurons, to major depression (Bremner et al., 2000) which the effectiveness of antidepressants such as fluoxetine appear to be dependent on induction of hippocampal neurogenesis (Santarelli et al., 2003). Similarly, administration of minocycline markedly increases hippocampal neurogenesis and reduces the levels of pro-inflammatory cytokines in the brain (Garrido-Mesa et al., 2013; Jiang et al., 2015; Soczynska et al., 2012). From a therapeutic point of view, based on the above evidence from experimental and clinical studies with minocycline, we postulate that minocycline may treat neonatal immune activation-induced anxiety and depression-like behaviors, and HPA axis dysregulation through the inhibition of microglial and/or astroglial activation, anti-inflammatory activity and upregulating hippocampal neurogenesis. Considering that neonatal infection can have profound effects on human's long-term health during adulthood. In this study, we used the neonatal immune activation model for inducing anxiety- and depression-related behaviors in mice, which is based on the well-documented association between early life exposure to infection and increased risk of neuropsychiatric disorders including depression and anxiety in adulthood. Using animal models like this one can be of great value, to help understand and uncover the etiologies of affective disorders which are multifactorial, resulting from a complex interplay between genetic and environmental factors. Given that the neuroprotective effects of minocycline are currently under exploration as an alternative or adjunctive therapy for alleviating the symptoms of neuropsychiatric disorders, finding potential therapeutic efficacy of minocycline in neurodevelopmental disorders makes it an attractive drug for further investigation in humans. Much more work is also needed to understand the benefits and risks of developmental minocycline treatment against psychiatric disorders induced by common early-life insults including infection and stress.

Conflict of interest

None.

Authors' contributions

Ali-Akbar Salari conceived and designed the study; Jafar Majidi, Morteza Kosari-Nasab and Ali-Akbar Salari performed research, Ali-Akbar Salari analyzed and interpreted the data, and wrote the paper. All authors read and approved the final version of the manuscript.

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