Doxycycline reverses cognitive impairment, neuroinflammation and oxidative imbalance induced by D-amphetamine mania model in mice: A promising drug repurposing for bipolar disorder treatment?

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Abstract

Immune-inflammatory mechanisms are involved in the pathophysiology of bipolar disorder. Tetracyclines present neuroprotective actions based on their anti-inflammatory and microglia suppressant effects. Doxycycline (DOXY) is a tetracycline that demonstrates a better usage profile with protective actions against inflammation and CNS injury. Here, we investigated the effects of DOXY against behavioral, neuroinflammatory, and pro-oxidative changes induced by the d-amphetamine mania model. Adult mice were given d-amphetamine 2.0 mg/kg or saline for 14 days. Between days 8 and 14, received lithium, DOXY (25 or 50 mg/kg), or their combination (lithium+DOXY) on both doses. We collected the brain areas prefrontal cortex (PFC), hippocampus, and amygdala to evaluate inflammatory and oxidative alterations. D-amphetamine induced hyperlocomotion and impairment in recognition and working memory. Lithium reversed hyperlocomotion but could not restore cognitive alterations. DOXY alone (at both doses) or combined with lithium reversed d-amphetamine-induced cognitive changes. DOXY, better than lithium, reversed the d-amphetamine-induced rise in TNFα, MPO, and lipid peroxidation. DOXY reduced the hippocampal expression of Iba1 (a marker of microglial activation), inducible nitric oxide synthase (iNOS), and nitrite. Combined with lithium, DOXY increased the phosphorylated (inactivated) form of GSK3β (Ser9). Therefore, DOXY alone or combined with lithium reversed cognitive impairment and neuroinflammation induced by the mice's d-amphetamine model. This study points to DOXY as a promising adjunctive tool for bipolar disorder treatment focused on cognition and neuroimmune changes. Our data provide the first rationale for clinical trials investigating DOXY therapeutic actions in bipolar disorder mania.

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1. Introduction

Bipolar disorder (BD) is a chronic disorder with a prevalence of around 1–5% of the general population for BD types 1 and 2. The cyclic alternation of opposite mood states, namely mania and depression, is the main characteristic of this mental disorder. Mania is the cardinal feature of BD. Manic symptoms are multiple and complex, such as hyperactivity, euphoric, irritable mood, decreased need for sleep, impulsivity, and cognitive changes (Tondo et al., 2017).

Cognition is a core domain of psychopathology in BD. Severe cognitive deficits are present in at least 25% of BD patients. Cognitive impairment is the principal responsible for disability and social burden of the disease independently of acute mood state and a well-described clinical feature of BD progression (Cardoso et al., 2015). Despite the importance of cognitive impairment for BD disability and neuroprogression, current BD drug therapy, including mood stabilizers and antidepressants, does not efficiently restore or reverse the cognitive loss associated with illness progression (Mora et al., 2013).

A close association between neuroinflammation and BD pathophysiology was postulated (Fries et al., 2019). BD patients show elevated serum and cerebrospinal fluid levels of pro-inflammatory cytokines and inflammation-derived metabolites, such as tryptophan catabolites, which correlate positively with symptom severity and psychotic features (Wang and Miller, 2018). In post-mortem studies, it was found an increase in pleiotropic pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and type I cytokines (IFN-γ and IL-12p40) in limbic brain areas of BD subjects (Benedetti et al., 2016). Also, BD patients present changes in glial reactivity markers and gene expression, as observed in brain samples (Giridharan et al., 2019).

Mania-like symptoms and neurobiological alterations induced by psychostimulants have been classically used to predict new antimanic/mood-stabilizing agents. The most studied of these models is the D-amphetamine (AMPH)-induced mania model (Macêdo et al., 2012). The mechanism underpinning AMPH-induced mania-like alterations include: i) AMPH inhibition of dopamine (DA) transporter (DAT) by competition; ii) AMPH stimulation of the vesicular release of DA, and iii) promotion of DAT reverse-transport towards synaptic cleft independently of electrical potential (Calipari and Ferris, 2013). In rodents, hyperactivity is the primary behavioral feature of AMPH-induced manic-like symptoms (Macêdo et al., 2012). However, in the last decades, several authors have reported that AMPH can induce a broader profile of changes, including enhanced sexual behavior, aggressiveness, impulsivity, and cognitive dysfunction, resembling clinical mania (Chaves Filho et al., 2020; van Enkhuizen et al., 2013).

Besides inducing direct changes in DA transmission, AMPH induces a marked brain pro-inflammatory response and microglial activation. Indeed, previous studies have shown AMPH-induced increases in microglial density and reactivity in the limbic striatum, followed by DA nerve terminals damage (Giridharan et al., 2019). In the AMPH mania model, there is a significant rise in pro- and anti-inflammatory cytokines IL-1β, IL-4, IL-10, and TNF-α in mice's serum and brain (Valvassori et al., 2019).

Previous studies have pointed to the neuroprotective effects of tetracyclines against neurodegenerative and neuroinflammatory conditions. In this regard, minocycline (MINO) presents anti-inflammatory, antioxidant, and anti-apoptotic effects (Kim and Suh, 2009), being a promising adjunctive therapy for bipolar depression (Husain et al., 2015). More recently, our research group showed the protective effects of MINO against GBR12909 (a selective DAT

inhibitor)-induced mania-like symptoms and oxidative brain damage in mice (de Queiroz et al., 2018).

Doxycycline (6-Deoxy-5-hydroxytetracycline) is another second-generation tetracycline that demonstrates an impressive ability to suppress microglial activation pro-inflammatory cytokine signaling induced by microbial immunogens and hypoxia (Santa-Cecília et al., 2016). This drug also presented antidepressant-like effects in the LPS animal model (Mello et al., 2013). Compared with MINO, DOXY has a favorable pharmacokinetic and safety profile since it is rapidly and almost entirely absorbed by the gut, presents the least affinity for calcium, and is an inexpensive drug (Kircik, 2010).

Therefore, based on the promising anti-inflammatory and antioxidant effects of DOXY and its more favorable usage profile compared to other tetracycline congeners, in the present study, we sought to test the effects of this drug alone or combined with the standard mood stabilizer lithium (Li) against AMPH-induced hyperlocomotion and cognitive deficits. Based on the involvement of immune disturbances in BD neuropathology, we decided to investigate the effects of DOXY against pro-inflammatory alterations induced by AMPH, such as cytokine expression, microglial activation, nitric oxide (NO) synthesis, and oxidative damage. We hypothesized that DOXY administration, alone or combined with Li, could reverse AMPH-induced behavioral and brain inflammatory changes, representing a new strategy for BD adjunctive therapy.

2. Experimental procedures

2.1. Animals

We used adult male Swiss mice, weighing 25-30 g, provided by the Animal House of the Federal University of Ceara. The animals were housed in open-topped polycarbonate cages (42 × 20.5 × 20 cm), and kept under standard conditions [8 per cage, temperature 22 ± 1°C, humidity 60 ± 5%, 12 h light-dark cycle (lights on at 6 a.m.), and food/water *ad libitum*]. All experimental procedures were performed between 8 a.m. and 2 p.m., according to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services (NIH, 2011) and to the ethical principles adopted by the National Council for Control of Animal Experimentation (CONCEA). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

Doxycycline hydrochloride (DOXY), D-amphetamine (AMPH), and lithium carbonate (Li) were obtained from Sigma (St. Louis, MO, USA). DOXY was dissolved in 2% dimethyl sulfoxide (DMSO) saline. We dissolved the other drugs directly in saline solution (NaCl 0.9%, w/v). The drugs were made up freshly within 1-2 h of dosing and were administered intraperitoneally (IP) in a volume of 0.1 ml/10 g body weight. All other chemicals used were of analytical grade.

2.3. Experimental design

In the present study, we conducted a reversal protocol, aiming to simulate an acute manic episode's treatment, as previously described (Macêdo et al., 2012). To this end, we used a total of 186

animals. Before the beginning of the experiments, mice were habituated for seven days in their home cages. We then divided the animals into nine groups, consisting of 16 randomly allocated animals in two standard cages. After habituation, each animal received for 14 days a daily IP administration of AMPH (2.0 mg/kg) or 0.9 % saline. From the 8th to 14th days of treatment, AMPH or saline-treated animals further received an injection of DOXY (25 and 50 mg/kg, IP), Li (47.5 mg/kg, IP), or saline solution. For drug combination treatment, Li-treated groups received an additional IP injection of DOXY (25 and 50 mg/kg, IP) or saline. In our experiments, all groups received three injections with a 30 min interval between administrations, as shown in Fig. 1.

The selected doses of DOXY were based on previous studies showing the antidepressant-like effects of this drug in mice (Mello et al., 2013). Regarding Li, the dosage regimens were based on previous preclinical reports of this drug's anti-manic effect in the AMPH model (de Souza Gomes et al., 2015). Control animals received three consecutive injections of saline with a 30 min interval between them. The animal weight was measured daily before each drug administration.

Aiming to avoid bias related to the animals' excessive exposure to the behavioral apparatus, each experimental group assigned for behavioral determinations was divided into two cohorts with eight animals each. Accordingly, the first cohort (N = 8/group) was subjected to the open field test and novel object recognition (NOR) task. The second cohort (N = 8/ group) was subjected only to the Y-maze. A 1-hour interval was adopted between behavioral tests. The tests' order was determined based on increasing invasiveness (Wolf et al., 2016). The tests were conducted on the 14th day of treatment, 2 h after the last drug injection, as standardized in this animal model (Macêdo et al., 2012), by two independent trained observers blinded to treatment groups.

We separated the animals into a third cohort (N = 7-8/group) for neurochemical analyses to reduce the influence of stress induced by mice's exposure to behavioral testing in neurochemical parameters, mainly on brain cytokine levels. For the conduction of neurochemical studies, we selected the lowest dose of DOXY, i.e., 25 mg/kg, alone or combined with Li, since no significant difference was observed between the two tested DOXY doses in the behavioral assays. We based the decision to continue the study only with one dose of DOXY on the principle of the 3Rs (Replacement, Reduction, and Refinement). Two-hour after the last drug administration, we sacrificed each mouse by decapitation, cut the brain into 1.0 mm transversal sections using a brain matrix, and arranged it on a frozen glass plate. The brain areas' prefrontal cortex (PFC), hippocampus, and amygdala were carefully dissected using a biopsy punch. Each section's landmarks were based on the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2012). Then, the brain samples were rapidly frozen and stored at -80° C until neurochemical determinations. Fig. 1. presents a graphical view of the experimental design.

2.4. Behavioral tests

2.4.1. Open field test
This test evaluates the influence of drugs on locomotor activity and exploratory behavior. An acrylic apparatus (transparent walls and black background, dimensions 30 × 30 × 15), divided into nine squares, was used (Archer, 1973). The animal was placed in the center of the apparatus and was observed over five minutes. We tested each mouse only once. Between mice, the apparatus was cleaned with a 10% ethanol solution. The number of squares crossed by the mice (considering the four legs in the square) was measured as a direct locomotory activity marker. The test was automatically recorded and analyzed using the Panlab Harvard Apparatus® SMART video tracking version 3.0.03 software.
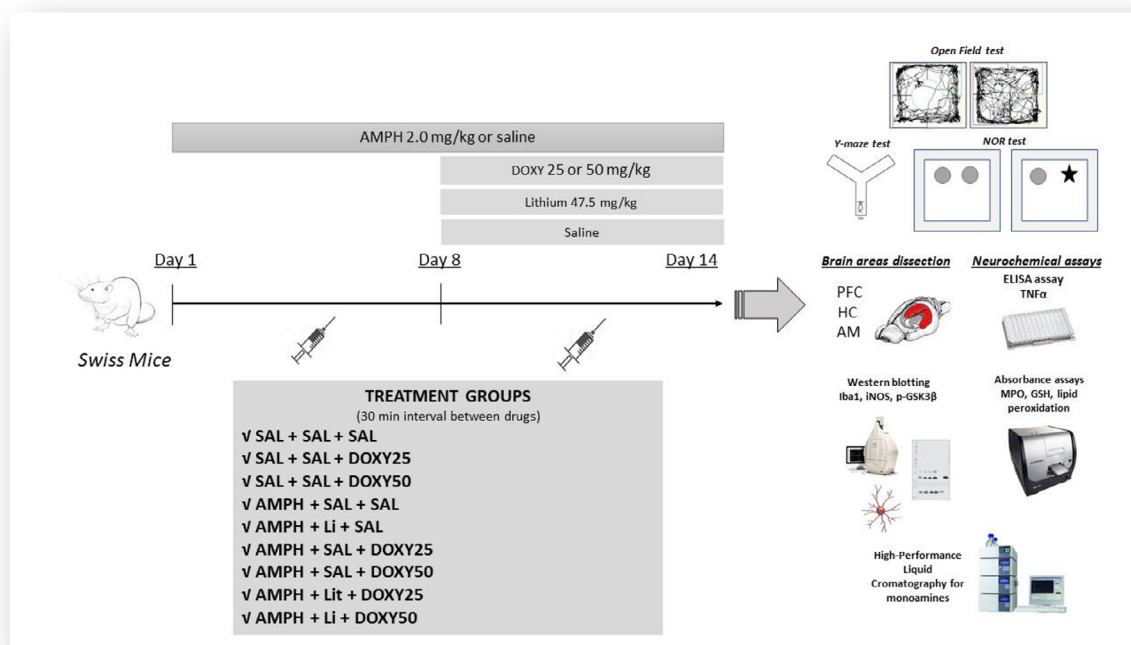
Fig. 1 Experimental design timeline. Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium, PFC: pre-frontal cortex; HC: hippocampus; AM: amygdala; MPO: myeloperoxidase; MDA: malondialdehyde; GSH: glutathione;iba1: ionized calcium-binding adaptor molecule 1; iNOS: inducible nitric oxide synthase; GSK3β: glycogen synthase kinase 3-beta.

2.4.2. Y-maze Test

The apparatus consisted of a Y-shaped maze with three white, opaque plastic arms (length 35, width 5, wall height 10) at a 120 angle from each other arms (labeled A, B and C). Each mouse could freely move through the maze for 8 min. We visually recorded the series of arm entries. A correct alternation was defined as entries in all three arms on consecutive occasions, such as ABC, ACB, BCA, BAC, CBA, CAB. The percentage of correct alternations was calculated as follows: the number of total alternations/(total arm entries x 2), as described in detail elsewhere (Hölter et al., 2015).

2.4.3. Novel object recognition test (NOR)

Novel object recognition is a widely used task to evaluate spatial recognition memory. This test assesses the mouse's ability to discriminate between familiar and novel objects. Firstly, we habituated the mouse to an open field Plexiglas box (30 × 30 × 15 cm size) for 5 min. Twenty-four hours after habituation, the mouse was subjected to a 10 min acquisition trial. In this trial section, the mouse was placed in the same arena with two identical objects situated 15 cm from the arena wall. After 1 h of retention interval, the mice were placed back into the arena and exposed to the familiar object and a novel object (different color and shape) for a further 10 min. As familiar objects, we used two blue lego toys in rectangle shape with 5.0 × 6.5 cm (height x length). We used a green plastic cylinder with 8 cm x 5 cm (height x length) as a novel object. All objects were glue-fixed in the arena floor and carefully cleaned between each animal test. The total time spent exploring each of the two objects (when the animal's snout was directly toward the object at a distance ≤ 2 cm) was recorded. The recognition index was used as a direct measure of recognition memory. It was calculated as follows: (time exploring new object - time exploring familiar object) / (time exploring new object + time exploring familiar object)(Ennaceur and Delacour, 1988).

2.5. Neurochemical assays

2.5.1. Immunoenzymatic assay for TNF-α concentrations

For this test, each brain area was homogenized in 8 volumes of phosphate-buffered saline (PBS) with protease (EMD Biosciences) and phosphatase (Sigma-Aldrich) inhibitors and centrifuged (12,000 g, 5 min). The concentration of the cytokine in 50 µl samples was determined by immunoenzymatic assay ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol and expressed in pg/g of tissue.

2.5.2. Determination of myeloperoxidase (MPO) activity

Myeloperoxidase is a highly oxidative enzyme. This enzyme's extracellular activity gives an estimate of the oxidative stress in inflammatory conditions (Anatoliotakis and Deftereos, 2013). Peroxidase activity with 3,30,5,50-Tetramethylbenzidine (TMB) was measured as described elsewhere (Suzuki et al., 1983). Briefly, brain samples were homogenized in 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB) buffer, pH 6.0 at 4°C at the proportion of 1 ml of buffer for each 50 mg of tissue. After this, samples were centrifuged (12,000 g, 2 min, 4°C), and supernatants collected. 30 µl of the supernatants were mixed with an o-dianisidine solution (0.52 mM o-dianisidine hydrochloride in distilled water) plus 1 ml of hydrogen peroxide 30% (protected from the light). Absorbance was determined at 450 nm in two time-points, 0 and 3 min, to estimate MPO activity (U MPO/min/mg of tissue).

2.5.3. Determination of reduced GSH concentrations

GSH levels were determined to estimate the endogenous antioxidant defenses (Sedlak and Lindsay, 1968). The method was based on Ellman's reagent (DTNB) reaction with free thiol groups. For this end, the samples were homogenized in 0.4 M Tris-HCl buffer, pH

8.9, and 0.01 M DTNB. Reduced GSH levels were determined using a microplate reader set at 412 nm. The results are expressed as ng GSH/mg tissue.

2.5.4. Determination of nitrite levels

Nitrite levels were determined in brain homogenates based on Griess reaction (Green et al., 1981) and expressed as μmol/mg wet tissue.

2.5.5. Measurement of lipid peroxidation

The rate of lipid peroxidation was estimated by determining malondialdehyde (MDA) equivalent concentrations using the thiobarbituric-acid reactive substances (TBARS). The samples were mixed with 100 μl of 35% perchloric acid and were centrifuged at 5000 rpm for 10 min. 150 μl of the supernatants were removed, mixed with 50 μl of 1.2% thiobarbituric acid, and then heated in a boiling water bath for 30 min. After cooling, the MDA levels were determined using a microplate reader set at 535 nm and expressed as μg MDA/mg tissue (Huong et al., 1998).

2.5.6. Western blotting analysis

Hippocampi were homogenized in RIPA lysis buffer [25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 5 mM EDTA; 1% Nonidet-P40 (NP)-40; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS] with protease and phosphatase inhibitors (1 μL of each inhibitor cocktail: 100 μL RIPA). Protein concentrations were determined by Lowry's method according to the manufacturer's protocol to extraction buffers with detergents. SDS polyacrylamide gel electrophoresis (10%) was performed using 50 μg of protein. The proteins were transferred to the PVDF membrane, blocked with BSA 5% for 1 h, and incubated overnight with rabbit polyclonal anti-Iba1 IgG primary antibody (1:1000, Wako Chemicals, USA), mouse polyclonal anti-iNOS IgG (1:500, Sigma-Aldrich, USA), rabbit monoclonal anti-GSK3β IgG (1:1000; Sigma, USA) and rabbit monoclonal anti-phospho-Ser9-GSK3β IgG (1:1000, ThermoFisher Scientific, USA). After washing, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000; Thermo Scientific, USA) or goat anti-mouse IgG secondary antibody (1:3000; Thermo Scientific, USA) for 90 min at room temperature. Signal was detected using the ECL system (Bio-Rad, USA). For each protein investigated, the results were confirmed in three independent experiments and analyzed by a blinded experimenter. After the protein blots' visualization, the protein antibodies were stripped from the membranes that were reprobed with the monoclonal anti-α-tubulin (1: 2000 dilution, Sigma, USA) used as an internal loading control for the above protein blots. The densitometric band analysis was performed in ImageLab software (Bio-Rad, USA). The software automatically determined the bands' background-subtracted density, and the band density quantification in each lane was measured as a total volume of a three-dimensional peek. For each studied protein, the density values were normalized by the internal control α-tubulin ones and expressed as relative protein expression. No significant difference was observed in α-tubulin expression between the tested groups (data not shown).

2.5.7. Determination of monoamines and their main metabolites

The levels of dopamine (DA), noradrenaline (NE), serotonin (5-HT), and their non-conjugated metabolites 3,4-hydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxy-indoleacetic acid (5-HIAA) were assayed by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection. A C18 reverse-phase column (Shim-pack, CLC-ODS 150 × 4.6 mm; Shimadzu, Kyoto, Japan), an amperometric detector (Shimadzu, L-ECD-6A), and a liquid chromatography station were used. The mobile phase used was the following composition: 15.7 g citric acid, 471.5 ml twice-distilled water, NaOH sufficient to bring pH to 3.78

mg octyl sodium sulfate, 20 ml acetonitrile, and 10 ml tetrahydrofuran, and flew at a rate of 0.6 ml/min. The brain tissues were weighed and homogenized in 10% (w/v) of 0.2 M perchloric acid. After centrifugation (12.000 g, 30 min), supernatants were removed and filtered through a membrane (Millipore, Brazil). Each sample (20 μl) was injected into the chromatograph to detect endogenous levels of brain monoamines and their metabolites. The peak areas of the external standards were used to quantify the sample peaks. The values obtained were expressed as ng/g tissue wet weight. DA and 5-HT metabolization rates were calculated (DOPAC+HVA/DA) and (5-HIAA/5-HT), respectively.

2.6. Statistical analyses

All data are present as mean $\pm$ standard error of the mean (SEM). Shapiro-Wilk test was performed to determine the normal distribution of data. Regular one-way analysis of variance (ANOVA) followed by Tukey's or Fisher's LSD (to compare the results obtained with the Western Blotting method) was performed as post hoc tests. The significance level was set at P< 0.05. GraphPad Prism 7.0 Version for Windows, GraphPad Software (San Diego, CA, USA) was used for these analyses.

3. Results

3.1. Doxycycline alone or combined with Li reverses declarative and working memory deficits induced by the AMPH model of mania

Cognitive impairment is frequently observed in BD patients and is related to the neuroprogression of this mental disorder (Cardoso et al., 2015). Manic episodes, in turn, are a hallmark feature of BD, being modeled by AMPH repeated administration (de Souza Gomes et al., 2015). In our results (Fig. 2A), we observed that the repeated administration of AMPH induced a hyperlocomotion (Control vs. AMPH+SAL: 95.00% CI of diff. −114.6 to −19.12, P= 0.0011) that is considered, in a preclinical setting, a mania phenotype. The hyperlocomotion induced by AMPH was significantly reversed by the administration of Li alone or combined with DOXY 25 or 50 (AMPH+SAL vs. AMPH+Li+SAL: 95.00% CI of diff. 19.88 to 118.7, P= 0.0010; AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. 5.429 to 114.3, P= 0.0211; AMPH+SAL vs. AMPH+Li+DOXY50: 95.00% CI of diff. 12.30 to 115.5, P= 0.0056). However, DOXY25 and DOXY50 alone could not reverse AMPH-induced hyperlocomotion (AMPH+SAL vs. AMPH+DOXY25+SAL: 95.00% CI of diff. -6.37 to 96.79, P= 0.1298; AMPH+SAL vs. AMPH+DOXY50+SAL: 95.00% CI of diff. −2.877 to 92.63, P= 0.0811).

Recognition memory is a subcategory of declarative memory related to recognizing previously encountered events or objects. NOR test is a well-established behavioral task to evaluate rodents' recognition memory (Rajagopal et al., 2014). In the NOR test (Fig. 2B), AMPH administration caused a reduction in recognition index (%) compared with control (Control vs. AMPH+SAL: 95.00% CI of diff. 7.329 to 57.85, P= 0.0035). Treatment with DOXY at both doses alone and combined with Li successfully reversed AMPH impairment in this memory domain (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. -63.66 to -11.24, P= 0.0008; AMPH+SAL vs. AMPH+SAL+DOXY50: 95.00%
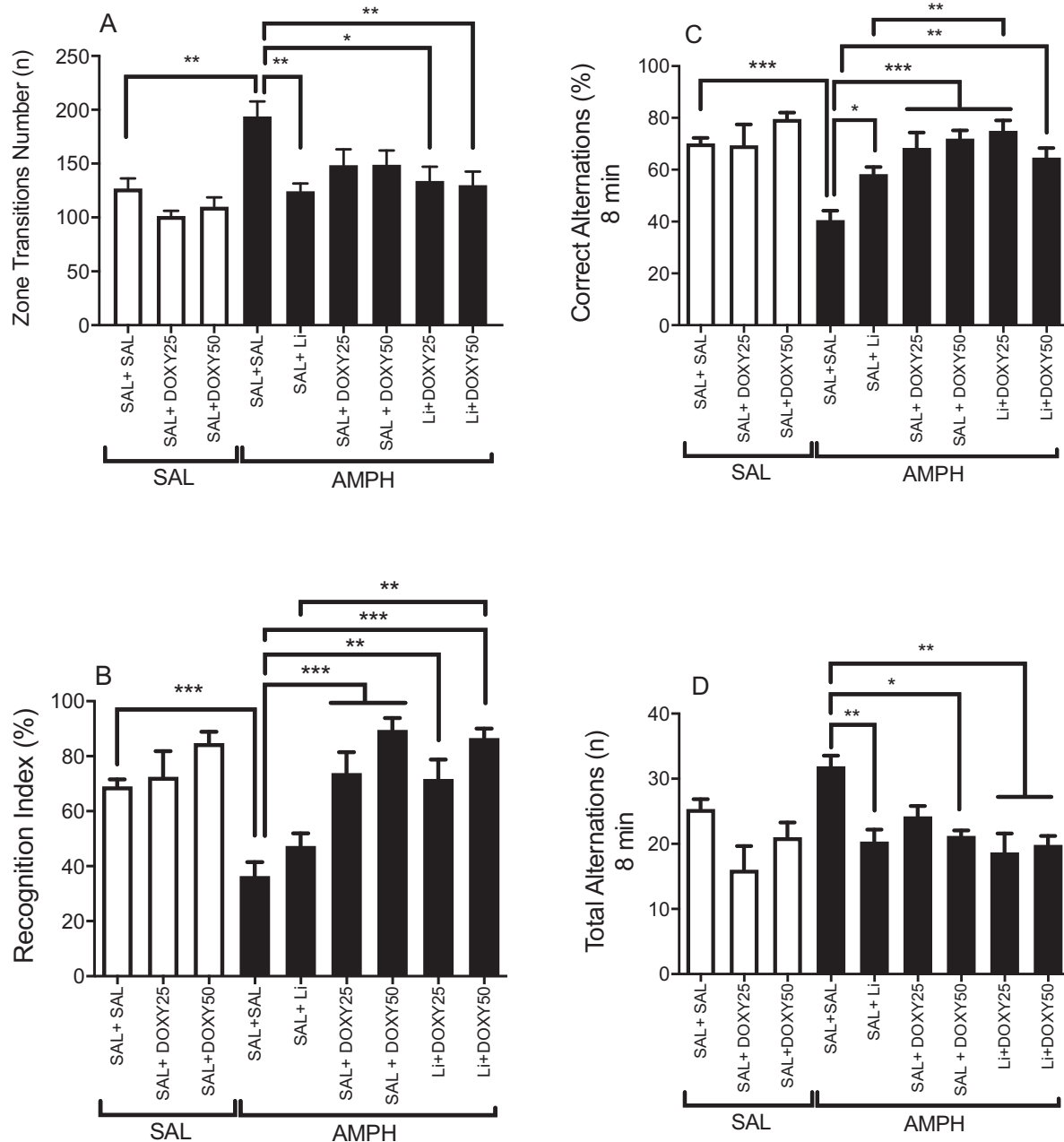
Fig. 2 Effects of doxycycline 25 mg/kg or 50 mg/kg alone and combined with lithium in the reversal of amphetamine-induced changes in open field test - zone transition number (A); novel object recognition test - recognition index (%) (B), and y-maze – correct alternations (%) (C) and total alternations (D). N=8 animals per group. Bars represent mean ± standard error of the mean (SEM). *P<0.05, **P< 0.01, ***P< 0.001 according to one-way ANOVA followed by Tukey′s post-hoc test. Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium.

CI of diff. -79.32 to -26.90, P<0.0001; AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. -61.50 to -9.074, P= 0.0020; AMPH+SAL vs. AMPH+Li+DOXY50: 95.00% CI of diff. -76.43 to -24.00, P< 0.0001). Li alone was not able to reverse the AMPH-induced deficit in recognition memory.

The Y-maze task assesses short-term spatial working memory (Hölter et al., 2015). In our results (Fig. 2C), AMPH repeated administration induced a marked reduction in the percentage of correct alternations in relation to the control group (Control vs. AMPH+SAL: 95.00% CI of diff. 12.81 to 46.40, P< 0.0001). The administration of DOXY, at both doses, alone or combined with Li, significantly reversed AMPH-induced deficits in working memory (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. -45.89 to -9.794, P= 0.0002; AMPH+SAL vs. AMPH+SAL+DOXY50: 95.00%

CI of diff. -49.49 to -13.39, P<0.0001; AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. -53.43 to -15.43, P< 0.0001; AMPH+SAL vs. AMPH+Li+DOXY50: 95.00% CI of diff. -42.13 to -6.028, P= 0.0022). Li alone also reversed AMPH-induced deficits in working memory (AMPH+SAL vs. AMPH+Li+SAL: 95.00% CI of diff. -33.41 to -2.042, P= 0.0160).

Regarding the number of total alternations (Fig. 2D), a parameter correlated with mice motor activity, AMPH repeated administration induced a 1.3-fold, non-significant, increase compared with control. AMPH+Li+SAL, AMPH+Li+DOXY25, or AMPH+Li+DOXY50 (P< 0.01) significantly reduced the number of alternations compared with the AMPH+SAL group. AMPH+SAL+DOXY50 also reduced the number of total entrances in relation to AMPH+SAL-group (P< 0.05).

3.2. Doxycycline alone or combined with Li reverses AMPH-induced neuroinflammatory and pro-oxidative changes

Serum cytokines, such as TNFα, are significantly higher in patients with manic, depressive, and mixed state BD (Luo et al., 2016). In the present study, we observed that AMPH repeated administration increased TNFα levels in all brain areas studied. However, this increase was significant compared with the control group only in the hippocampus (Control vs. AMPH+SAL: 95.00% CI of diff. 3.248 to 31.35, P= 0.0203) and amygdala (Control vs. AMPH+SAL: 95.00% CI of diff. 5.424 to 27.99, P= 0.0100). In the PFC (Fig. 3A), only the group treated with the combination of Li+DOXY25 presented a significant reversal of AMPH-induced alterations in TNFα levels (AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. -34.98 to -5.060, P= 0.0138). On the other hand, in the hippocampus, (Fig. 3B) DOXY25 alone and combined with Li reversed AMPH-induced alterations in TNFα levels (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. 1.394 to 42.01, P= 0.0309; AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. -35.70 to -0.6016, P= 0.0440). In the amygdala (Fig. 3C), only DOXY25 alone significantly reversed this parameter (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. -27.82 to -6.373, P= 0.0070).

The enzyme MPO is a marker and mediator of inflammation and oxidative stress (Ndrepepa, 2019). Bipolar disorder patients present alterations in this enzyme compared with control subjects (Selek et al., 2015). In this study, AMPH treatment induced a significant increase in MPO activity in the PFC (Control vs. AMPH+SAL: 95.00% CI of diff. 15.04 to 81.11, P= 0.0088) and hippocampus (Control vs. AMPH+SAL: 95.00% CI of diff. -117.1 to -45.61, P <0.0001). Only the treatment with Li+DOXY25 significantly reversed AMPH-induced PFC increase in MPO activity, being this effect significant in relation to AMPH+SAL (P= 0.0391) and AMPH+Li+SAL (P= 0.0181) groups (Fig. 3D). In the hippocampus (Fig. 3E), all treatment protocols reversed AMPH-induced increased MPO activity (AMPH+SAL vs. AMPH+Li+SAL: 95.00% CI of diff. 37.29 to 116.5, P <0.0001; AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. 35.15 to 106.6, P <0.0001; AMPH+SAL vs.

AMPH+Li+DOXY25: 95.00% CI of diff. 23.45 to 94.94, P= 0.0003).

An oxidative imbalance is also an important alteration observed in BD. Indeed, regardless of the disorder's phase, BD patients present high oxidative markers and low antioxidant molecules (Andreazza et al., 2007). Here (Fig. 4, A-C), we detected lower levels of the antioxidant GSH in all brain areas dissected from AMPH-treated animals in relation to the control group (PFC - Control vs. AMPH+SAL: 95.00% CI of diff. 17.58 to 578.3, P= 0.0324; Hippocampus - Control vs. AMPH+SAL: 95.00% CI of diff. 61.84 to 646.4, P= 0.0108; Amygdala - Control vs. AMPH+SAL: 95.00% CI of diff. 160.7 to 462.4, P <0.0001). A similar significant reduction in GSH levels was observed in the group that received AMPH+Li when compared with control. In our experimental condition, only the group treated with DOXY25 showed a significant reversal of AMPH-induced GSH deficits in the amygdala (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. -382.6 to -80.86, P= 0.0008), as well as presented a significant increase in the hippocampal levels of this parameter when compared to AMPH group (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. -203.0 to -97.69, P <0.0001).

Regarding lipid peroxidation (Fig. 4 D-F), AMPH caused a significant increase in MDA contents in all brain areas studied (PFC - Control vs. AMPH+SAL: 95.00% CI of diff. -140.2 to -23.28, P= 0.0110; Hippocampus - Control vs. AMPH+SAL: 95.00% CI of diff. -246.9 to -38.85, P= 0.0029; Amygdala - Control vs. AMPH+SAL: 95.00% CI of diff. "-249.8 to -22.64, P= 0.0121). A similar significant increase in lipid peroxidation was observed in the brain areas of animals treated with AMPH+Li in relation to the control group. In the same way, as happened in relation to GSH levels, only the groups that received DOXY25 alone or combined with Li significantly restored hippocampal levels of MDA (AMPH+SAL vs. AMPH+SAL+DOXY25: 95.00% CI of diff. 3.852 to 211.9, P= 0.0386; AMPH+SAL vs. AMPH+Li+DOXY25: 95.00% CI of diff. 33.95 to 242.0, P= 0.0043).

Since the hippocampus presented the main neurochemical changes induced by DOXY treatment, we decided to conduct further evaluations in this brain area. In this regard, AMPH repeated administration caused an increase in iba1 expression compared to the control group (P= 0.0356). Additionally, DOXY25 alone or combined with Li significantly reduced Iba1 expression compared to the AMPH+SAL group (P= 0.0082). Li alone also caused a marked reduction in this marker, but devoid of statistical significance (Fig. 5A).

Regarding iNOS expression (Fig. 5B), AMPH induced a marked increase in this protein expression compared with the control group (P= 0.0253). A similar increase was observed in the AMPH+Li+SAL group compared with the control animals (P= 0.0299). On the other hand, DOXY-treated groups, alone (P= 0.0062) or in combination (P= 0.0330), significantly reversed iNOS expression.

The group treated with AMPH+SAL showed an impressive increase in nitrite's hippocampal levels compared with control (P= 0.0035). The treatment with DOXY25 (P= 0.0013), Li (P= 0.0075) and their combination (P= 0.0005) significantly restored nitrite levels (Fig. 5C).
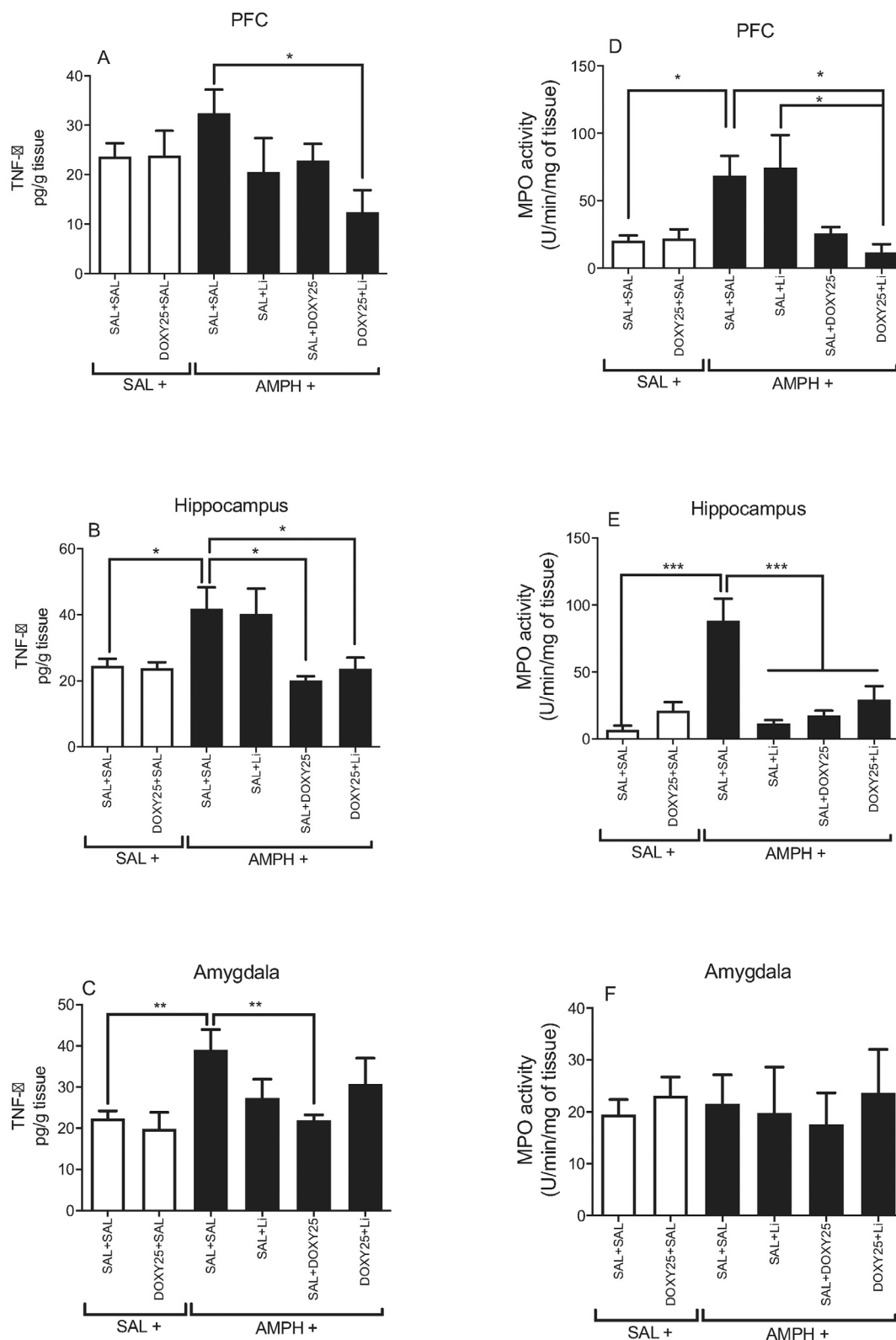
Fig. 3 Effects of doxycycline 25 mg/kg alone and combined with lithium in the reversal of amphetamine-induced changes in TNFα levels and myeloperoxidase (MPO) activity in the mice brain. Graphic plots represent TNFα concentrations in the prefrontal cortex (A), hippocampus (B), and amygdala (C), and MPO activity in the PFC (D), hippocampus (E), and amygdala (F). N=8 animals per group. Bars represent mean ± standard error of the mean (SEM). *P<0.05, **P< 0.01, ***P< 0.001 according to one-way ANOVA followed by Tukey's post-hoc test. Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium.; PFC: prefrontal cortex; MPO: myeloperoxidase.
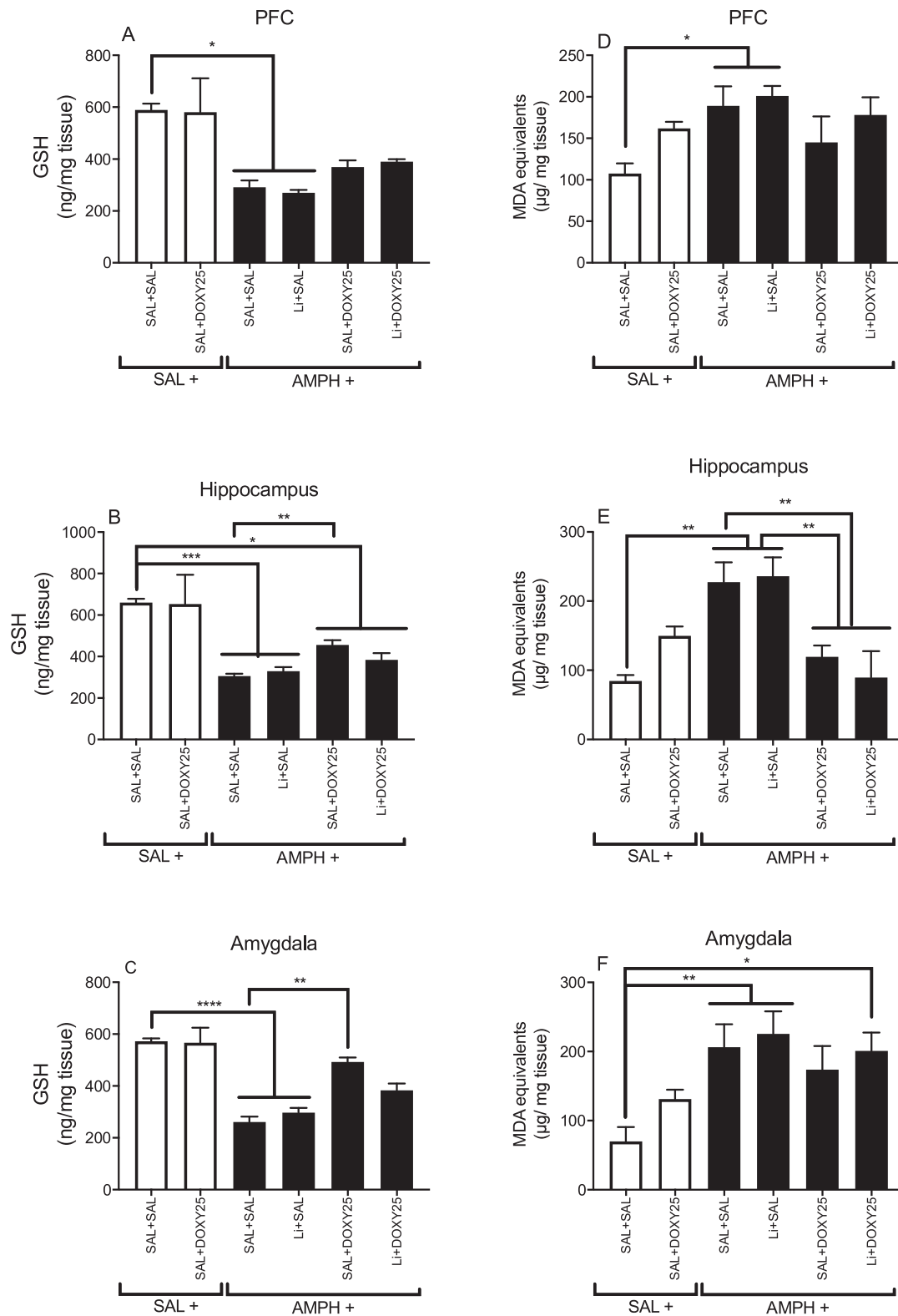
Fig. 4 Effects of doxycycline 25 mg/kg alone and combined with lithium in the reversal of amphetamine-induced changes in oxidative stress markers in the mice brain. Graphic plots represent reduced GSH concentrations in the prefrontal cortex (A), hippocampus (B) and amygdala (C), and malondialdehyde (MDA) levels in the PFC (D), hippocampus (E), and amygdala (F). N=8 animals per group. Bars represent mean ± standard error of the mean (SEM). *P<0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001 according to one-way ANOVA followed by Tukey's post-hoc test. Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium.; PFC: prefrontal cortex; MDA: malondialdehyde; GSH: glutathione.
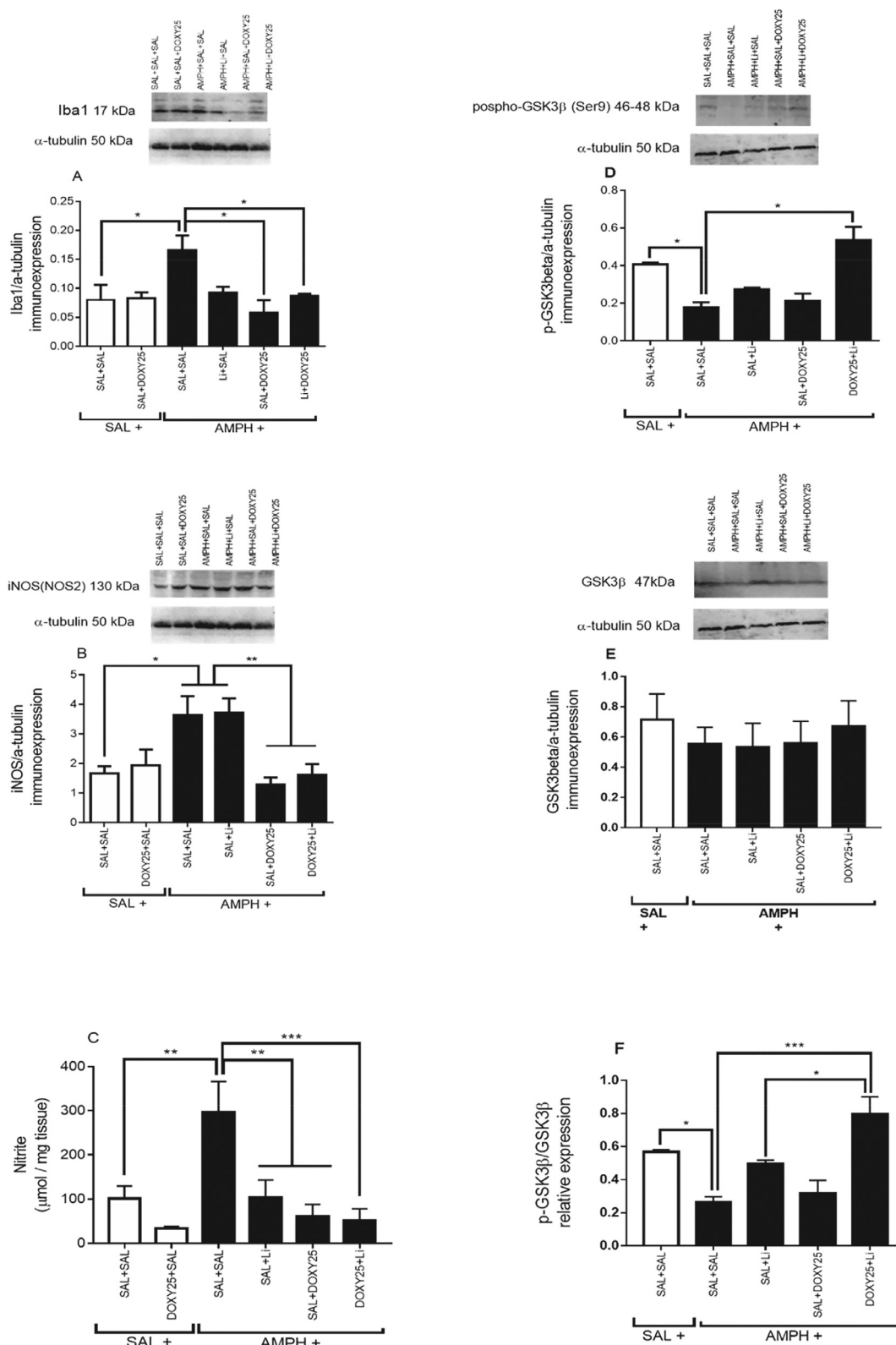
Fig. 5 Effects of doxycycline 25 mg/kg alone and combined with lithium in the reversal of amphetamine-induced changes in microglial activation marker, nitric oxide (NO) synthesis, and GSK3β in mice hippocampus. Graphic plots represent protein expression of ionized calcium-binding adaptor molecule 1 (iba1) (A), inducible nitric oxide synthase (iNOS) (B), nitrite levels (C) phospho-GSK3β (D), GSK3β (E), and phospho-GSK3β/GSK3β (F) in mice hippocampus. N=8 animals per group. Bars represent mean $\pm$ standard error of the mean (SEM). *P<0.05, **P< 0.01, ***P< 0.001, according to one-way ANOVA followed by Tukeys post-hoc test.
Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium; iba1: ionized calcium-binding adaptor molecule 1; iNOS: inducible nitric oxide synthase; GSK3β: glycogen synthase kinase 3-beta.

We found a marked reduction in the phosphorylated form of GSK3β (at Ser9) in the AMPH+SAL group compared with the control group (P= 0.0087) (Fig. 5D). Additionally, only the treatment with Li+DOXY25 significantly reversed this decrease in GSK3β (P= 0.0001). Regarding total GSK3β expression, the tested groups presented no significant differences (Fig. 5E). Finally, for GSK3β phosphorylation rate (p-GSK3β/total-GSK3β), the AMPH+SAL group showed a marked reduction in this parameter compared to the control group (P= 0.0177). The combined treatment (AMPH+Li+DOXY 25) significantly increased this phosphorylation rate compared to both AMPH+SAL (P< 0.0001) and Li groups (P= 0.0128) (Fig. 5F).

3.3. Doxycycline attenuated hippocampal hyperdopaminergic alterations caused by the AMPH mania model

Regarding dopaminergic transmission, as expected, AMPH caused a marked increase in DA amounts (AMPH+SAL vs. control: 95.00% CI of diff. -1.555 to -0.1428, P = 0.0117), followed by a tendency to decrease DOPAC levels in this brain region (AMPH+SAL vs. control, P> 0.05, non-significant) (Fig. 6A-B). AMPH also caused a non-significant trend to reduce DA turnover compared to controls (AMPH+SAL vs. control, P>0.05, non-significant) (Fig. 6D).

DOXY alone significantly reduced DA levels compared to AMPH group (AMPH+SAL+DOXY25 vs. AMPH+SAL: 95.00% CI of diff. −33.41 to −2.042, P= 0.0128) (Fig. 6A). Also, DOXY significantly increased DOPAC (AMPH+SAL+DOXY25 vs. AMPH+SAL: 95.00% CI of diff. 0.3592 to 2.211, P = 0.0182) and HVA levels (AMPH+SAL+DOXY25 vs. AMPH+SAL: 95.00% CI of diff. −1.316 to −.06096, P = 0.0280) compared to AMPH group (Fig. 6B-C). Finally, DOXY restored DA metabolization rate compared to AMPH group (AMPH+SAL+DOXY25 vs AMPH+SAL: 95.00% CI of diff. −0.3093 to 3.771, P = 0.0405) (Fig. 6D).

Regarding 5-HT transmission, AMPH induced a marked increase in 5-HIAA levels compared to the control group (AMPH+SAL vs. control: 95.00% CI of diff. −2.566 to 0.09509, P=0.0313) (Fig. 6F). Also, AMPH induced a significant reduction in total 5-HT contents compared to controls (AMPH+SAL vs. control: 95.00% CI of diff. −0.7493 to 8.025, P= 0.0414) (Fig. 6E). Regarding tested treatments, Li-treated groups showed a significant increase in 5-HT levels compared to AMPH one (AMPH+Li+SAL vs. AMPH+SAL, 95.00% CI of diff. −0.7493 to 8.025, P= 0.0414; AMPH+Li+DOXY25 vs. AMPH+SAL, 95.00% CI of diff. 1.24 to 5.397, P=0.0035) (Fig. 6E).

Finally, AMPH significantly increased NE levels than SAL controls (AMPH+SAL vs. control: 95.00% CI of diff. 2.212 to 4.402, P= 0.001). Li treatment further incremented AMPH-effects in NE levels in the hippocampus compared to controls (AMPH+SAL vs. control: 95.00% CI of diff.−13.91 to −2.434, P= 0.0020) (Fig. 6H). Regarding DOXY effects in control conditions, a tendency to increase HVA levels and total 5-HT amounts were observed in the SAL+DOXY25 group compared to the control group (P> 0.05, non-significant).

4. Discussion

In the present study, we showed for the first time the ability of the second-generation tetracycline DOXY to reverse cognitive deficits and neuroinflammatory changes induced by the AMPH mania model in mice. Of note, DOXY, when compared with Li, showed a superior effect in ameliorating working and recognition memory deficits induced by AMPH. Additionally, DOXY alone, and combined with Li, reversed AMPH-induced increase in brain levels of TNFα, MPO activity, and lipid peroxidation, an effect that was not observed by the treatment with Li alone. DOXY also restored the hippocampal expression of Iba1, a marker of microglial activation, iNOS, and nitrite contents altered by AMPH. The combination of Li+DOXY also caused a marked increase in the phosphorylated (inactivated) form of GSK3β (Ser9). Finally, DOXY partially rescued monoamine changes induced by AMPH in the hippocampus and restored DA metabolization rate. Therefore, the present study points to DOXY as a potential candidate for adjunctive therapy for BD, focusing on the reversal of cognitive and neuroimmune changes observed in this disorder.

Bipolar disorder is mostly recognized as a multifactorial disorder in which a complex interaction between a susceptible genetic heritage and environmental risk factors determine the development of the disease (Berk et al., 2011). In this context, immune dysfunction and chronic low-grade inflammation are contributing mechanisms for BD neurobiology (Fries et al., 2019). Besides that, prefrontal-limbic networks are involved in the pathophysiology of bipolar illness. The first of these networks start in the PFC and modulates amygdala responses to endogenously generated feeling states. Furthermore, several studies have detected alterations in white-matter tracts connecting the subgenual anterior cingulate cortex (ACC) with the amygdala-hippocampal complex, frontal lobe-insula-hippocampus-amygdala-occipital lobe, and frontal lobe-thalamus-cingulate gyrus in bipolar patients relative to healthy controls (Shizukuishi et al., 2013). Based on the evidence of the involvement of PFC, hippocampus, and amygdala in BD pathophysiology, we chose these brain areas for evaluation.

Several studies reported elevated levels of pro-inflammatory cytokines in the serum of BD patients, such as TNF-α, soluble interleukin-2 receptor (sIL-2R), IL-1β, IL-6, and soluble receptor of TNF-type 1 (STNFR1). These cytokines seem to be higher in acute mood episodes, mainly in mania, and to be reduced to basal levels at euthymic states (Sayana et al., 2017). Additionally, some pro-inflammatory markers, particularly IL-1Ra and sTNF-R1, are associated with both general disease severity and psychotic features of BD mania (Hope et al., 2013). Interestingly, these inflammatory changes are not observed in all patients, but only in a subgroup associated with an "inflammatory phenotype". The use of anti-inflammatory and immunomodulatory strategies to this subgroup of patients presenting an inflammatory phenotype may have more significant benefits (Fries et al., 2019).

Cognitive symptoms are the main contributing factors for long-term disability and social burden in BD patients (Cardoso et al., 2015). A significant association between
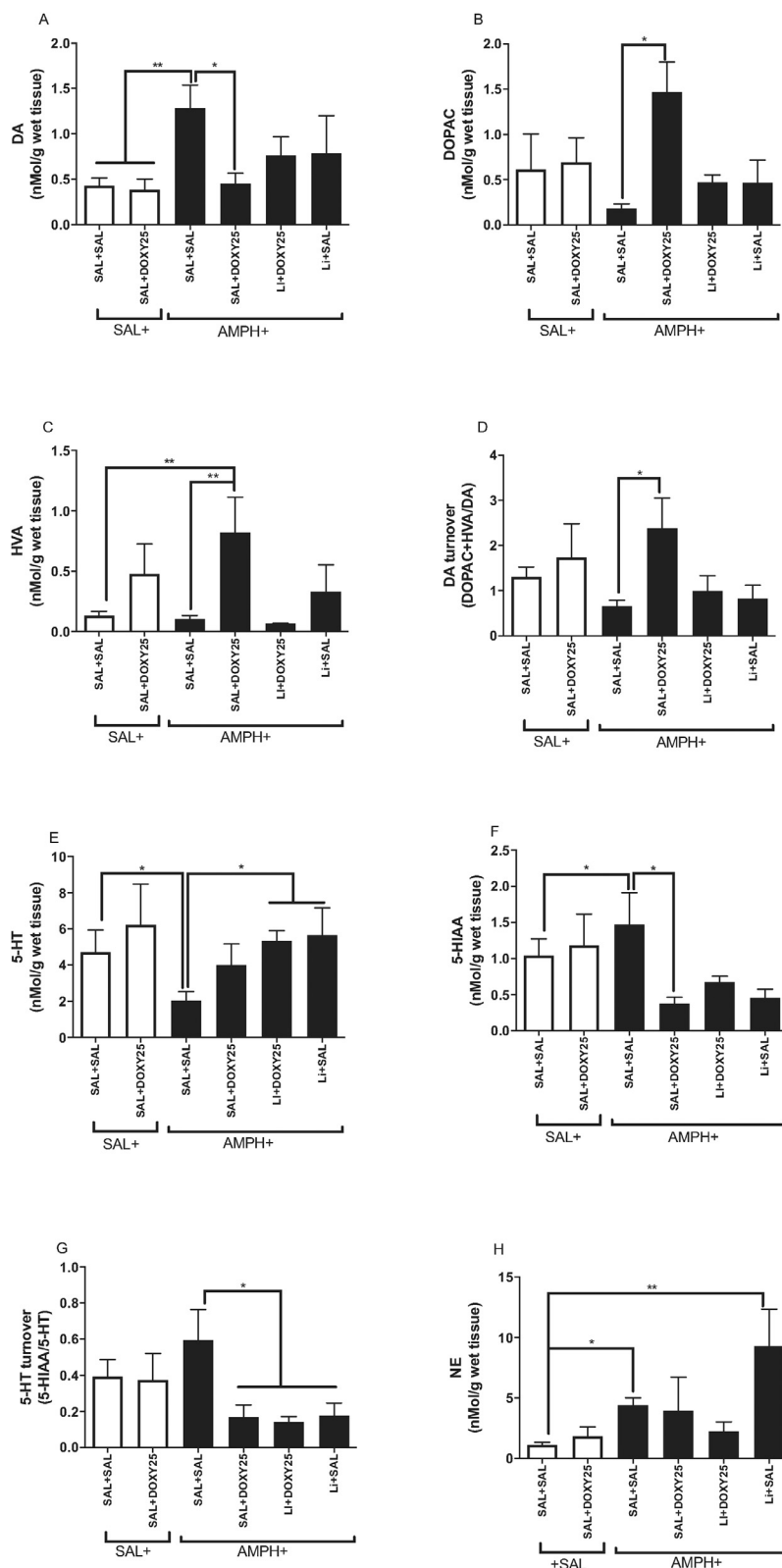
Fig. 6 Effects of doxycycline 25 mg/kg alone and combined with lithium in the reversal of amphetamine-induced changes in monoamines and their metabolites' levels in the mice hippocampus. Graphic plots represent the levels of DA (A), DOPAC (B), HVA (C) DA metabolization/turnover rate (D), 5-HT (E), 5-HIAA (F), 5-HT metabolization/turnover rate, and NE (H). N=7-8 animals per group. Bars represent mean ± standard error of the mean (SEM). *P<0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001 according to one-way ANOVA followed by Tukey's post-hoc test. Abbreviations: SAL: saline; DOXY: doxycycline; AMPH: amphetamine; Li: lithium; DA: dopamine; DOPAC: 3,4-hydroxyphenyl acetic acid; HVA: homovanillic acid; 5-HT: serotonin; 5-HIAA: 5-hydroxy-indoleacetic acid; NE: noradrenaline.

inflammation (i.e., "inflammatory phenotype") and cognitive function has been delineated in BD. In this regard, a negative correlation between overall cognitive function and some specific cognitive domains, such as processing speed and executive function and the serum levels of pro-inflammatory mediators, such as IL-6, TNFα, and IL1Ra was reported (King et al., 2019).

The preclinical model of mania induced by AMPH is a well-known BD mania model (de Souza Gomes et al., 2015; Macêdo et al., 2012). Previous evidence points to a rise in serum and brain pro- and anti-inflammatory cytokines levels (IL-4, IL-6, and TNFα) in animals submitted to this model. These changes in cytokines are partially reduced by the treatment with mood-stabilizing drugs such as Li (Valvassori et al., 2015).

Here, we observed that AMPH induced the typical hyperlocomotion state and caused marked cognitive deficits in working and recognition memory. In our experimental condition, Li treatment successfully reversed the hyperlocomotion behavior but could not restore cognitive changes. Accordingly, previous evidence has described that AMPH induces in mice significant cognitive changes, mainly related to dysfunction in executive and declarative tasks, resembling part of the cognitive profile seen in chronic BD patients (de Souza Gomes et al., 2015). Importantly, we demonstrated, as far as we know for the first time, DOXY's ability alone and combined with Li to fully restore the cognitive performance in AMPH-manic mice.

Second generation tetracyclines present neuroprotective properties (Domercq and Matute, 2004). Regarding DOXY, preclinical evidence points to a notable effect of this drug against neurodegenerative synucleinopathies, such as Parkinson's disease (González-Lizárraga et al., 2017). Also, DOXY demonstrated protective actions against ketamine-induced cognitive impairment (Ben-Azu et al., 2018). Similarly to MINO, anti-inflammatory and microglia suppressant effects of DOXY may justify its behavioral actions (Lazzarini et al., 2013).

In our results, DOXY reversed AMPH-induced increases in the brain levels of the pro-inflammatory cytokine TNFα. This effect was detected in all brain areas studied, i.e., PFC, hippocampus, and amygdala. Furthermore, we observed that only the animals treated with DOXY combined or not with Li, but not Li alone, reversed AMPH-induced alterations. TNFα is a crucial pro-inflammatory mediator produced by glia, mainly microglia, early after an immune challenge. This cytokine is involved in glia initiating and maintaining activation in a positive feedback loop (Lively and Schlichter, 2018). TNFα is also one of the main factors related to microglia-mediated neuronal apoptosis (Guadagno et al., 2013). In this regard, two systematic reviews pointed to TNF-α as the most documented pro-inflammatory factor associated with BD's progressive course, a potential biomarker to discriminate patients in initial and advanced stages (Castaño-Ramírez et al., 2018).

During the inflammatory stimulus, TNF-α works together with reactive oxygen species (ROS) to produce neuronal damage and glial activation. One of the primary mediators of this "harmful relationship" between TNF-α and ROS is the enzyme MPO (Lefkowitz and Lefkowitz, 2008). Indeed, MPO expression is induced by pro-inflammatory cytokines, mainly TNF-α, while MPO also induces TNF-α secretion (Mytar et al., 2004). In the CNS, MPO is produced by activated microglial cells and infiltrated macrophages. When secreted in the extracellular medium, MPO stimulates microglia production of ROS. Therefore, MPO seems to be a significant mediator between inflammation and the associated oxidative damage (Anatoliotakis and Deftereos, 2013).

Here, AMPH increased MPO activity, while DOXY alone or combined with Li efficiently restored MPO activity in the PFC and hippocampus. Based on the relationship between TNF-α and oxidative damage, we also decided to evaluate reduced GSH contents and lipid peroxidation as markers of redox state.

Regarding mania models, the inhibition of DAT functioning and the consequent increase in DA synaptic levels is related to oxidative toxicity (Yamato et al., 2010). This oxidative toxicity is caused by DA metabolization that generates pro-oxidant compounds, such as quinone metabolites (Asanuma et al., 2003). AMPH administration in rodents consistently prompted a rise in brain pro-oxidative markers, such as increased lipid peroxidation, DNA damage, and impairment in endogenous antioxidant enzymes (Macêdo et al., 2012; Macedo et al., 2013).

Our results revealed that DOXY could partially protect the brain areas against AMPH-induced oxidative damage. DOXY (alone or combined with Li) reversed AMPH-induced alterations in GSH levels and lipid peroxidation in the hippocampus. In our experimental conditions, Li was not able to reverse oxidative alterations induced by AMPH.

GSH is the main non-protein thiol in cells, acting as a cofactor for antioxidant and detoxifying reactions. Despite being synthesized exclusively in the cytosol, GSH is distributed in different cell compartments, defending cells against respiration derived ROS (Ribas et al., 2014). We have previously shown MINO protection against oxidative brain damage induced by DAT inhibition by reducing lipid peroxidation and augmenting GSH levels (de Queiroz et al., 2018). This effect of MINO seems to be related to its regulatory effect in the activity of mitochondrial complex I, II, III, and IV in limbic brain areas (Réus et al., 2014) and to its ability to attenuate the increase in striatal DA levels after AMPHs administration (Zhang et al., 2006). Based on the results obtained here, we can infer that DOXY protective effects against AMPH-induced mania-like alterations are potentially associated with its ability to dampen TNFα-MPO inflammatory and oxidant mechanism. Previous studies point to alterations in the WM microstructure as core brain structural features of BD related to the neuroinflammatory condition. A higher concentration of cytokines (i.e., TNF-α, IFN-γ, IL-8, IL-10, IGFBP2, and PDGF-BB) is negatively related to this the integrity of myelin sheaths (Benedetti et al., 2016). Future studies need to address WM alterations in BD patients treated with DOXY to explain further the mechanism of action of this drug in BD patients.

In the present study, DOXY showed significant antioxidant effects in the hippocampus. Hence, we decided to evaluate additional molecular mechanisms for DOXY's protective effect in this brain area. Importantly, compelling evidence implicates the hippocampus as a critical area for BD neuropathology (Chepenik et al., 2012). Findings from both post-mortem and neuroimaging studies have shown hippocampal reduced volume, decreased cell density, de-

creased neuronal plasticity, and altered glial reactivity in BD patients (Savitz et al., 2015).

We observed that the AMPH mania model caused a significant increase in hippocampal Iba1 expression. This Iba1 expression was accompanied by a tremendous increase in iNOS expression and nitrite contents, a final metabolite of NO (Radenovic and Selakovic, 2005). Altogether these results indicate a classical pro-inflammatory activation of regional microglia. One of the most established classifications of microglia (and macrophages) relates to their two main functions, kill/fight or heal/fix, assuming two related phenotypes M1 and M2, respectively (Rath et al., 2014). In this context, iNOS is regarded as a functional marker of the M1 phenotype. Various pro-inflammatory cytokines induce the transcription of iNOS, and this isoform can synthesize large amounts of NO (Chhor et al., 2013). In turn, NO plays important antimicrobial and pro-oxidative functions by generating reactive nitrogen species (RNS), such as peroxynitrite (ONOO−). RNS is highly noxious to cell biomolecules (Brown and Bal-Price, 2003).

DOXY alone or combined with Li demonstrated a remarkable ability to reduce Iba1 expression and iNOS and nitrite levels. Based on these results, we can infer that DOXY can reverse the microglial activation induced by AMPH. These findings follow previous evidence pointing to DOXY's ability to inhibit microglial reactivity in mice models of neurodegenerative diseases and hypoxia (Lazzarini et al., 2013) and prevent microglia production of NO and pro-inflammatory cytokines after LPS challenge (Santa-Cecília et al., 2016). Therefore, these results add new evidence for a better characterization of the AMPH-induced mania model's neurobiological mechanisms and advocate for the use of DOXY as an immunomodulatory drug against neuroinflammatory changes associated with BD.

Previous evidence points to microglia activation in BD patients. For example, in BD brains, HLA-DR-stained microglia displayed thickened processes compared to control tissue, being a marker of classical activation (Rao et al., 2010). Another study reported that microglia cells from BD patients showed decreased DNA methylation in the HCG-9 region, suggesting an increase of inflammation-related gene transcription (Kaminsky et al., 2012). Despite this, other studies showed no differences or reduced activation markers (Giridharan et al., 2019). More clarifying, a significant microgliosis has been noted in suicide BD patients, suggesting that BD's increased microglial reactivity can represent a putative biomarker of suicide (Steiner et al., 2008).

GSK3β is a master kinase involved in the etiology of mood disorders modulated by several signals, among them, the elevation of extracellular DA. Indeed, dopamine can inhibit serine 9 phosphorylation of GSK3 in limbic areas, increasing its kinase activity. The activation of GSK3 involves Akt's inactivation in a protein complex, including the scaffolding protein β-arrestin and GSK3β (Beaulieu et al., 2008). Conversely, inhibition of GSK3 is a common mechanism shared by mood-stabilizing drugs. For example, Li can, directly and indirectly, inhibit GSK3β through phosphorylation at Ser9 (Gould and Manji, 2005). Also, inhibition of GSK3β presents marked anti-inflammatory properties. The mechanism underlying this anti-inflammatory effect seems to be related to the inhibition of GSK3β-induced activation of inflammatory transcription factors, such as NF-κB and signal trans-

ducer and activator of transcription-3 (STAT3) (Jope et al., 2017). Simultaneously, GSK3β inhibits the transcription of anti-inflammatory and reparative mediators, such as CREB signaling (Beurel and Jope, 2010).

As expected, AMPH repeated administration caused a considerable reduction in the GSK3β-Ser9-phosphorylation, which is compatible with AMPH-induced hyperdopaminergic state. Li alone caused a modest increase in the expression of the inactivated form of this kinase. However, its combination with DOXY induced a significant increase in this phosphorylated form. Based on the crucial role of GSK3β in the control of brain inflammation, we can suggest that this kinase's inhibition can represent a possible underlying mechanism for the pro-cognitive and protective effects of Li and DOXY in combination. However, this cannot fully explain the notable anti-inflammatory effects of DOXY since this drug alone could not alter GSK3β phosphorylation levels. We hypothesize that additional mechanisms, such as inhibition of the COX-2 enzyme, regulation of mitochondrial activity, and inhibition of matrix metalloproteases (MMPs), can mediate the immunomodulatory effect of DOXY (Weinberg, 2005).

Based on the modulation of GSK3β by DA, and considering the direct link between DA imbalance and oxidative stress/neuroinflammation in BD (Li and Jope, 2010), we decided to evaluate the monoamines' hippocampal levels (DA, NE, and 5-HT) and their metabolites. We observed that AMPH caused a marked rise in DA and NE levels and decreased 5-HT levels. AMPH slightly suppressed DA metabolism while notably increased 5-HT metabolization rate.

DOXY decreased DA levels, increased DA secondary metabolites (DOPAC and HVA), and normalized DA metabolization rate. This regulatory action of DOXY on DA metabolism can be a potential underlying mechanism for its protection against AMPH-induced oxidative stress. In situations of hyperdopaminergia, DA can suffer metal-catalyzed (Fe^{+3}) auto-oxidation at the catechol moiety generating highly reactive ortho-quinones (Segura-Aguilar et al., 2014). These quinones can interact with several cell components and alter their function and tertiary structure. DA-quinone can further react with ROS in the presence of iron to form the potent pro-oxidative neurotoxin 6-hydroxydopamine (Asanuma et al., 2003). In physiological conditions, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) metabolizes DA, respectively, to DOPAC and HVA, limiting the generation of damaging quinones by DA auto-oxidation (Meiser et al., 2013). Also, an elegant study demonstrated that DA deamination by MAO transfers additional electrons through mitochondrial inter-membrane space contributing to the inner membrane potential without producing ROS (Graves et al., 2020).

Besides that, we observed that the treatment with Li+DOXY25 reversed the decrease in phospho-GSK3β/GSK3β induced by AMPH. The activation of D2 receptors by DA modulates intracellular pathways that lead to GSK3β activation (Li and Gao, 2011). The ability of the combination of Li+DOXY25 in reversing GSK3β activation reflects an important mechanism of these drugs in the reversal of DA-induced oxidative/inflammatory alterations observed in mania. Importantly, GSK3β inhibition attenuates the activation of the pro-inflammatory transcription factor NF-κB, activates the immuno-modulatory transcription factor β-catenin, and in-

duce the secretion of the anti-inflammatory cytokine IL-10 (Klamer et al., 2010).

Here, regarding 5-HT transmission, AMPH decreased hippocampal 5-HT contents and increased the metabolite 5-HIAA and 5-HT metabolization rate. Only recently 5-HT contribution to the neurobiology of BD mania was evidenced. In line with this, Maddaloni et al. (2018) showed that 5-HT depleted *Tph2* mutant mice present an innate behavior resembling mania, which was reversed by mood stabilizer treatment (valproic acid) (Maddaloni et al., 2018). Also, Azechi et al. (2019) reported that Fawn-Hooded (FH) rats that carry dysfunctional gene mutations in the serotonergic system presented hyperactivity and high impulsivity behavior followed by low hippocampal 5-HT levels and increased 5-HT metabolization rate (Azechi et al., 2019). On the other hand, the results obtained with our drug protocols revealed that DOXY had few effects against AMPH-induced 5-HT changes. However, Li normalized both 5-HT contents and metabolization rate. Our result is following previous evidence showing Li's ability to stimulate 5-HT firing release (Treiser et al., 1981) and modulating 5-HT synthesis through tryptophan hydroxylase 2 (TPH2) expression (Scheuch et al., 2010). Therefore, while DOXY effectively counterbalanced the hyperdopaminergic state associated with AMPH, possibly through normalization of DA metabolism, Li partially restored 5-HT contents impaired by this mania model. However, no potential additive effect was observed with these drugs in combination.

Limitations and Perspectives

The present study has some limitations. Firstly, no existing animal model completely mimics the complex phenotypic expression of BD mania. Although the AMPH model resembles the hyperdopaminergic state involved in mania, this BD model lacks complete construct validity. Secondly, we investigated possible targets for DOXY anti-inflammatory/immunomodulatory effect in the AMPH model. Despite the promising results, the primary underlying mechanism for this effect remains not entirely understood. Additional studies need to be conducted to clarify the mechanism of DOXY protective effects. Promising targets for future investigation are energetic mitochondrial complex, cyclooxygenase (COX)-2, and MMPs. Finally, DOXY is an antimicrobial drug that may cause alterations in the gut microbiota. Therefore, studies evaluating gut microbiota alterations in animals submitted to repeated administration of DOXY must be conducted.

5. Conclusions

The present study demonstrates, for the first time, the potential pro-cognitive and anti-inflammatory/immunomodulatory effects of the second-generation tetracycline doxycycline (DOXY) in the AMPH mania model. Notably, DOXY successfully recovered mice's cognitive performance, showing a superior effect in relation to Li. Further, DOXY and its combination with Li protected against brain inflammation and oxidative imbalance induced by AMPH. Since DOXY is a widely used and inexpensive antibiotic with a more favorable safety profile than the related tetracycline MINO, our data provide a rationale for the design of clinical trials based on the re-

purposing of DOXY as an adjunctive treatment to cognitive deficits and inflammatory alterations observed in a subset of BD patients.

Author's disclosures

The authors declare no conflict of interests.

Role of authors

AJMCF, DSM designed the study and wrote the first draft of the manuscript; NLC, AGS, MVRS, PMJ performed the behavioral tests and analyzed the results obtained in the neurochemical tests; PAR, TQ, DCSC performed neurochemical technics and analyzed the results obtained in the behavioral tests; CAP, AJMCF performed Western Blotting tests; MM, CHA performed the statistical analysis and constructed the graphics; All authors contributed to the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2020.11.007.

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