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What is This?

Prevention and reversal of ketamine-induced schizophrenia related behavior by minocycline in mice: Possible involvement of antioxidant and nitrergic pathways

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Abstract

It has been hypothesized that oxidative imbalance and alterations in nitrergic signaling play a role in the neurobiology of schizophrenia. Preliminary evidence suggests that adjunctive minocycline treatment is efficacious for cognitive and negative symptoms of schizophrenia. This study investigated the effects of minocycline in the prevention and reversal of ketamine-induced schizophrenia-like behaviors in mice. In the reversal protocol, animals received ketamine (20 mg/kg per day intraperitoneally or saline for 14 days, and minocycline (25 or 50 mg/kg daily), risperidone or vehicle treatment from days 8 to 14. In the prevention protocol, mice were pretreated with minocycline, risperidone or vehicle prior to ketamine. Behaviors related to positive (locomotor activity and prepulse inhibition of startle), negative (social interaction) and cognitive (Y maze) symptoms of schizophrenia were also assessed. Glutathione (GSH), thiobarbituric acid-reactive substances (TBARS) and nitrite levels were measured in the prefrontal cortex, hippocampus and striatum. Minocycline and risperidone prevented and reversed ketamine-induced alterations in behavioral paradigms, oxidative markers (i.e. ketamine-induced decrease and increase in GSH levels and TBARS content, respectively) as well as nitrite levels in the striatum. These data provide a rationale for evaluating minocycline as a novel psychotropic agent and suggest that its mechanism of action includes antioxidant and nitrergic systems.

Keywords

Minocycline, schizophrenia, ketamine-induced model, social interaction, prepulse inhibition, cognition

Introduction

Schizophrenia is a severe, chronic and debilitating mental disorder characterized by positive (e.g. hallucinations), negative (e.g. blunted affect and social isolation), and cognitive symptoms (e.g. executive and memory dysfunction) (Larson et al., 2010). Although first-generation antipsychotics are effective in reducing positive symptoms, these agents have been relatively less effective in mitigating the severity of negative symptoms and cognitive deficits. The pertinence of the latter observation is underscored by several lines of evidence indicating that negative symptoms and cognitive dysfunction are the principal cause of functional impairment in individuals with schizophrenia (Shamsi et al., 2011; Tandon et al., 2009; Wyatt et al., 1988).

Treatment with first-generation antipsychotics can be associated with treatment-emergent extrapyramidal symptoms as evidenced by acute events (e.g. akathisia) and/or tardive dyskinesia (Jones et al., 2006; Tandon et al., 2010). Although the atypical antipsychotics were once thought to be more effective and safe than the first-generation drugs (Tandon et al., 2010), the results of large-scale trials failed to demonstrate a superior efficacy for the atypical drugs in cognitive or social outcomes (Jones et al., 2006; Keefe et al., 2007; Lieberman, 2007). Relatively little progress

has been made in developing new therapeutic targets for negative and cognitive symptoms (Buckley and Stahl, 2007). The relative absence of genuinely novel psychotropic agents, for most severe and persisting psychiatric disorders, is largely a consequence of

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the unavailability of sufficient disease models (Chatterjee et al., 2012; Yee and Singer, 2013).

A widely used animal model of schizophrenia involves the acute or repeated administration of ketamine (Becker and Grecksch, 2004; Bubenikova-Valesova et al., 2008). Ketamine has been used clinically as a dissociative anesthetic with multimodal mechanisms of action, which include a noncompetitive antagonism to the glutamate N-methyl-d-aspartic acid receptor (NMDAR) and a D2-dopamine receptor agonist with a slightly lower affinity for 5-HT2 receptors (Kapur and Seeman, 2002). In rodents, NMDAR blockade induces deficits in prepulse inhibition (Jentsch and Roth, 1999; Kamiyama et al., 2011), memory and social interaction (Becker and Grecksch, 2004; Chindo et al., 2012; Duan et al., 2013), which models the positive, cognitive and negative symptoms of schizophrenia, respectively (Javitt et al., 2012).

Minocycline is a second-generation tetracycline with neuroprotective effects (Garrido-Mesa et al., 2013) as evaluated in several neurodegenerative conditions like Parkinson's (Du et al., 2001), Huntington's (Chen et al., 2000) and Alzheimer's disease (Choi et al., 2007). These neuroprotective effects of this substance reside in its anti-inflammatory, neurotrophic, antioxidant, direct radical-scavenging and anti-apoptotic properties (Garrido-Mesa et al., 2013; Kraus et al., 2005). Minocycline may also protect neurons against glutamate-induced excitotoxicity, which has been implicated in the pathophysiology of several neuropsychiatric conditions (Dean et al., 2012).

In the last decade, the neurotherapeutic potential of minocycline for the treatment of mental disorders has been explored (Dean et al., 2012), mainly for mitigating depressive symptoms (Soczynska et al., 2012) and psychopathology associated with schizophrenia (Zhang et al., 2007). Importantly, there is a lack of preclinical studies investigating the putative mechanisms of action for minocycline as a therapeutic target for schizophrenia (Garrido-Mesa et al., 2013). Presently, preliminary clinical studies point to a possible benefit of adjunctive minocycline for the treatment of negative and cognitive symptoms of schizophrenia (Chaudhry et al., 2012; Levkovitz et al., 2010; Miyamoto et al., 2013).

Taken together, the pharmacological and therapeutic profile of minocycline suggests that it may have clinical application in schizophrenia with the possibility of not only mitigating psychopathology, but also modifying hypothesized disease processes (Dean et al., 2012, Torrey and Davis, 2012). This study herein aimed to extend knowledge regarding minocycline's putative psychotropic properties by (1) investigating the possible effects of minocycline in the prevention and/or reversal of ketamine-induced schizophrenia-like behavior; (2) determining the effects of minocycline against ketamine-induced oxidative imbalance through the determinations of lipid peroxidation and reduced glutathione (GSH) levels and (3) evaluating the alterations in nitrite levels in mouse brain areas related to schizophrenia pathophysiology, namely the prefrontal cortex (PFC), hippocampus (HC) and striatum (ST).

Methods

Animals

Male Swiss mice (25–30 g) were used. The animals were maintained in a controlled temperature (23±1ºC) with a 12h dark/light cycle (lights on at 07:00) and free access to water and food.

Procedures were conducted in accordance with the Brazilian College of Animal Experimentation (COBEA) guidelines for the care and use of laboratory animals, as well as the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. This study was approved by the Ethical and Animal Research Committee of Federal University of Ceara.

Drugs

The animals received intraperitoneal (i.p.) injections of minocycline hydrochloride (25 or 50 mg/kg, Sigma-Aldrich, St Louis, USA) dissolved in 2% dimethyl sulfoxide (DMSO). Ketamine hydrochloride (20 mg/kg, König, Brazil) was diluted in distilled water. Risperidone (0.5 mg/kg, Risperdal® Jassen-Cilag, Brazil) was dissolved in distilled water. Controls were administered 2% DMSO (vehicle). All drugs were administered in a volume of 0.1 ml/10g body weight.

Experimental procedure

An overview of the experimental design is depicted in Figure 1. Briefly, in the prevention paradigm the maintenance treatment phase of schizophrenia was simulated (Leucht et al., 2003). Different groups of animals (*n* = 10–15) were treated daily with vehicle, minocycline (25 or 50mg/kg, i.p.) or risperidone 0.5 mg/kg, i.p. for 14 days. Between the eighth and 14th days, these animals additionally received a daily dose of ketamine (20mg/kg, i.p.) or vehicle 30 min after minocycline administration. In the reversal model, we simulated the acute treatment of psychotic episodes (Hatta et al., 2009). Briefly, each animal group (*n* = 10–15) received one daily i.p. injection of ketamine 20 mg/kg or vehicle for 14 days. From the eighth day of treatment onwards, these animals additionally received a daily i.p. administration of vehicle, minocycline (25 or 50 mg/kg) or risperidone 0.5 mg/kg, with a 30 min interval between treatments. Risperidone was used as the standard antipsychotic because a ketamine-induced model of schizophrenia has been demonstrated to be more responsive to atypical antipsychotics (Becker and Grecksch, 2004).

Behavioral determinations of prepulse inhibition of the startle reflex (PPI), locomotor activity evaluated by the open field test (OFT), spatial recognition memory evaluated by the Y-maze task (YMT) and social interaction test (SIT) were registered at the 14th day of treatment 30 min after the last drug administration. Following the behavioral determinations, mice were sacrificed by decapitation and the PFC, HC and ST were dissected, rapidly frozen and stored at −70°C until assayed.

Behavioral tests

PPI. The test session commenced by placing a subject in the stabilimeter cage for a 5-min exposure to the background noise. After this acclimatization period, mice were presented with a series of 10 stimuli (pulse alone – 120 dB, 50 ms duration), with an inter-trial interval of 20 s. The purpose of this phase was to allow within-session habituation to the startle stimulus. Thereafter, the PPI modulation of the acoustic startle was tested. The protocol consisted of 74 trials pseudo-randomly divided into seven different categories presented with an inter-trial interval of 20 s: 20 presentations of pulse alone (120 dB, 50 ms duration), eight presentations of each prepulse intensity alone (70, 75 and 80 dB, 3000 Hz
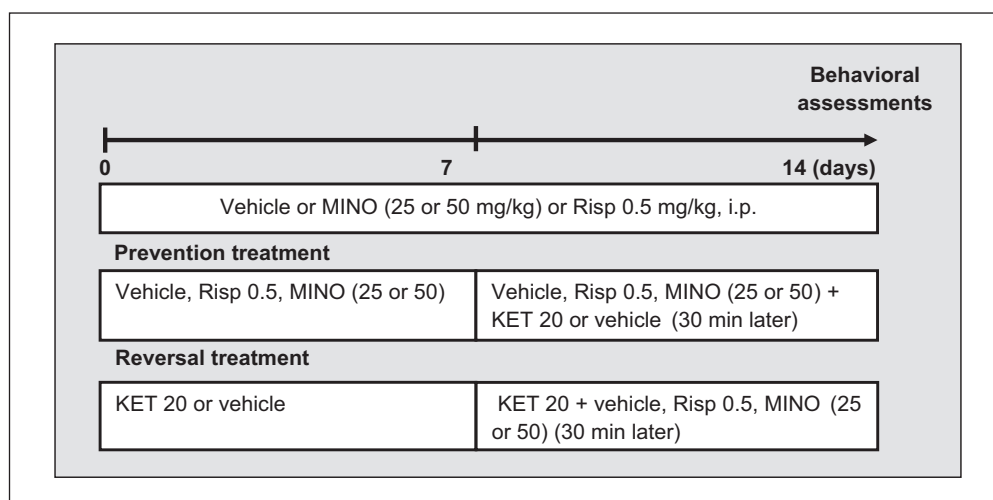
Figure 1. Schematic diagram of the experimental protocol.
i.p.: intraperitoneal; KET: ketamine; MINO: minocycline, RISP: risperidone.

frequency, 20 ms duration) and 10 presentations of each prepulse intensity + pulse (with 50 ms interval) (Levin et al., 2011).

OFT. The open-field (Archer, 1973) area was made of acrylic (transparent walls and black floor, 30 cm × 30 cm × 15 cm) divided into nine squares of equal area. This apparatus was used to evaluate the exploratory activity of the animal. The observed parameter was the number of squares crossed (with all four paws).

YMT. Spontaneous alternation performance was assessed using a YMT, which allows the evaluation of cognitive searching behavior. Each arm of the maze was 40 cm long, 25 cm high and 6 cm wide and converged to an equal angle. Each mouse was placed at the end of one arm and allowed to freely move through the maze during 8 min. The series of arm entries was recorded visually. An alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as total of alternations/(total arm entries − 2), as previously described (Dall'igna et al., 2007; Yamada et al., 1996).

SIT. The testing apparatus consisted of a 60 × 40 cm Plexiglas box divided into three chambers. Mice were able to move between chambers through a small opening (6 × 6 cm) in the dividers. An iron cage in each of the two side chambers contained the probe mice. Test mice were placed in the center chamber. Mice were allowed 5 min of exploration time in the box, after which an unfamiliar, same-sex probe mouse from the same experimental group was placed in one of two restraining cages (Radyushkin et al., 2009). The time spent in each of the three chambers was measured, and social preference was defined as follows: (% time spent in the social chamber) − (% time spent in the opposite chamber).

Determination of oxidative stress parameters

Determination of GSH levels. GSH levels were evaluated to estimate endogenous defenses against oxidative stress. The method was based on Ellman's reagent (DTNB) reaction with free thiol groups. Homogenates 10% (w/v) in EDTA 0.02M were added to a 50% trichloroacetic acid solution. After centrifugation (3000 r/min for 15 min), the homogenate supernatant was collected and the production levels of GSH were determined as described elsewhere (Sedlak and Lindsay, 1968). Briefly, the samples were mixed with 0.4MTris-HCl buffer, pH 8.9 and 0.01M DTNB. GSH level was determined by the absorbance at 412nm and was expressed as ng of GSH/g wet tissue.

Measurements of lipid peroxidation. Lipid peroxide formation was analyzed by measuring the thiobarbituric acid reacting substances (TBARSs) in the homogenates. The samples were briefly mixed with 50mM potassium phosphate monobasic buffer pH 7.4, 63μL of the homogenate was mixed with 100μL of 35% perchloric acid, then the samples were centrifuged (5000 rpm/10 min) and 150μL of the supernatant was retrieved and mixed with 50μL of thiobarbituric acid 1.2% then heated in a boiling water bath for 30 min. After cooling, the lipid peroxidation was determined by the absorbance at 535nm and was expressed as μmol of malondialdehyde (MDA)/mg of protein (Ohkawa et al., 1979).

Nitrite determination. In order to assess the effects of treatments with respective drugs on nitric oxide (NO) production, nitrite levels were determined in the mouse brain homogenates immediately after decapitation in all groups. After centrifugation (10,000 rpm for 10 min), the homogenate supernatant was collected and the production of NO was determined based on Griess reaction (Green et al., 1981; Radenovic and Selakovic, 2005). Briefly, 100μL of supernatant was incubated with 100μL of Griess reagent (sulfanilamine in 1% H_3PO_4/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/1%H3PO4/distilled water, 1:1:1:1) at room temperature for 10 min. The absorbance was measured at 550nm via a microplate reader. The standard curve was prepared with several concentrations of $NaNO_2$ (ranging from 0.75 to 100μM) and was expressed as μmol/g of protein.

Statistical analysis

Mean amplitude of startle response to pulse-alone (P) and prepulse + pulse (PP+P) trials were calculated for each subject. The level of PPI in each rat was determined by expressing the prepulse
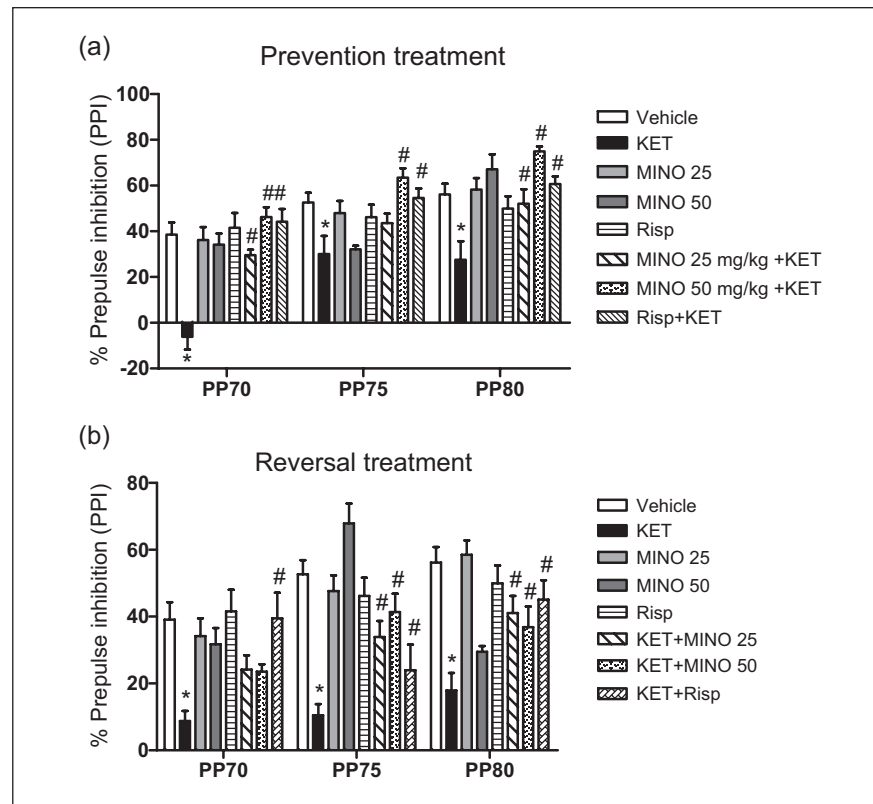
Figure 2. Percentage of prepulse inhibition of startle (PPI) of animals submitted to the ketamine(KET)-induced model of schizophrenia and subjected to the prevention treatment (a) or reversal treatment (b) with minocycline (MINO) or risperidone (Risp). Bars represent mean ± standard error of the mean of the percentage of PPI (n = 8–10 animals/group).
*p < 0.05 versus control.
#p < 0.05 versus ketamine according to two-way ANOVA followed by Bonferroni's post hoc test.

+ pulse startle amplitude as a percentage decrease from pulse-alone startle amplitude, according to the following formula: %PPI=100−[100×(PP/P)]. Using this formula, a 0% value denotes no difference between amplitude of startle response to pulse alone and to the prepulse + pulse and, consequently, to PPI. The behavioral results of prepulse inhibition were analyzed by two-way ANOVA followed by a Bonferroni post hoc test. The results of the other behavioral determinations and oxidative stress parameters were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Previously, the normal distribution of the data was evaluated. Significance level was set at $p \leq 0.05$. Data analyses were performed using GraphPad Prism software, version 5.0 for Windows (copyright 1992–2007; GraphPad Software, San Diego, California, USA).

Results

Prevention and reversal of ketamine-induced PPI deficits by minocycline

In the present study, the analysis of PPI data by repeated measures two-way ANOVA revealed a significant interaction between 'prepulse intensities' and 'experimental groups' in both prevention (df = 14, F = 2.799, p = 0.0012) and reversal (df = 14, F = 5.13, p < 0.0001) protocols (Figure 2(a) and (b)). In the prevention protocol, Bonferroni post hoc test showed a significant decrease in PPI

levels following ketamine administration in the prepulse intensities of 70 (p < 0.001), 75 (p < 0.01) and 80 dB (p < 0.001) when compared with vehicle-treated animals. Pretreatment with minocycline (at both doses) and risperidone significantly prevented the impairment in PPI levels induced by ketamine in the prepulse intensities of 70 and 80 dB (p < 0.001). In the prepulse intensity of 75 dB this effect was observed only in the groups treated with minocycline at the higher dose (i.e. 50 mg/kg) and in the risperidone-treated group (p < 0.001) (Figure 2(a)).

In the reversal protocol (Figure 2(b)) ketamine administration promoted a significant decrease in PPI levels in all prepulse intensities evaluated (p < 0.001) as compared with vehicle-treated animals. In the same pattern observed in the prevention protocol, the administration of minocycline (at both doses) and risperidone reversed ketamine-induced PPI deficits in the prepulse intensities of 75 and 80 dB (p < 0.01). In the prepulse intensity of 70 dB only risperidone was able to reverse ketamine-induced PPI impairment (p < 0.001). The administration of minocycline (25 and 50 mg/kg) and risperidone alone caused no alterations in PPI levels in any of the studied protocols.

Prevention and reversal of ketamine-induced locomotor alterations by minocycline

In the prevention ($F(7,71)$ = 23.23, p < 0.001) (Figure 3(a)) and reversal ($F(7,80)$ = 16.05, p < 0.001) (Figure 3(b)) protocols,
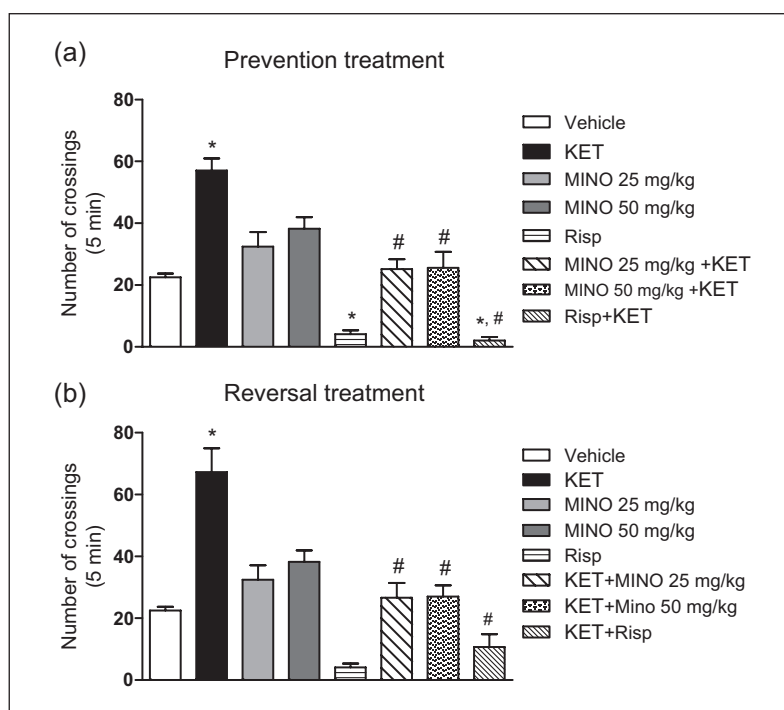
Figure 3. Number of crossings in the prevention treatment. Bars represent mean ± standard error of the mean of the number of crossings ($n = 8$–10 animals/group).

*$p < 0.05$ versus control.

#$p < 0.05$ versus KET according to one-way ANOVA followed by Tukey's post hoc test. MINO: minocycline; Ket: ketamine; Risp = risperidone.

ketamine administration significantly increased the number of crossings in the OFT when compared with vehicle-treated animals. In both protocols the administration of minocycline (25 and 50 mg/kg) and risperidone prevented ($p < 0.001$) and reversed ($p < 0.001$) ketamine-induced hyperlocomotion. Risperidone when given alone caused a significant decrease in locomotor activity as compared with vehicle-treated animals in the prevention ($p < 0.05$) paradigm, while the administration of minocycline alone did not promote significant changes on this parameter on both protocols.

Prevention and reversal of the ketamine-induced alterations in the social preference and working memory by minocycline

Our data showed that ketamine administration significantly decreased the percentage for social preference in the prevention ($F(7,67) = 4.405$, $p < 0.01$) and reversal protocols ($F(7,71) = 6.861$, $p < 0.001$) when compared with vehicle-treated animals. Minocycline 50 mg/kg ($p < 0.01$) and risperidone ($p < 0.05$) prevented the decrease for social preference, with both active and vehicle-treated animals performing at comparable levels. Furthermore, minocycline (at both doses, $p < 0.001$) and risperidone ($p < 0.01$) reversed ketamine-induced decrements in the percentage of social preference (Figure 4(a) and (c)).

In the evaluation of working memory performance (Figure 4(b) and (d)), the animals treated with ketamine in the prevention ($F(7,82) = 13.95$, $p < 0.05$) and reversal ($F(7,84) = 5.067$, $p < 0.001$) protocols exhibited a significant decrease in the percentage of correct alternations in the YMT as compared to vehicle-treated animals. The administration of minocycline (25 and 50 mg/kg)

significantly prevented ($p < 0.05$) and reversed ($p < 0.001$) the alterations caused by ketamine administration. Conversely, risperidone decreased the percentage of correct alternations when compared with vehicle- and ketamine-treated animals in the prevention protocol ($p < 0.01$), but reversed ketamine-induced alterations ($p < 0.05$).

Minocycline (25 and 50 mg/kg) and risperidone when given alone caused no alterations in the percentage of social contacts and correct alternations in the Y maze.

Prevention and reversal of oxidative and nitrite alterations by minocycline

The evaluation of oxidative stress parameters in the PFC, HC and ST of animals submitted to the prevention protocol is presented in Table 1. Ketamine administration significantly decreased GSH levels by 58% in the PFC ($F(7,77) = 253.8$, $p < 0.001$) and by 65% in the ST ($F(7,62) = 243.1$, $p < 0.001$) when compared with vehicle-treated animals. Pretreatment with minocycline (25 and 50 mg/kg) significantly prevented this decrease in the PFC ($p < 0.01$) and ST ($p < 0.05$). Risperidone, in turn, when given alone increased the levels of GSH in the PFC 10-fold and six-fold in the HC and ST as compared with vehicle-treated animals. Almost the same pattern of increase was observed in the PFC ($p < 0.01$), HC ($p < 0.001$) and ST ($p < 0.001$) of animals pre-treated with risperidone prior to ketamine administration. The administration of minocycline alone caused no alterations in GSH levels in the brain areas studied. There was also no alteration in the HC of animals pre-treated with minocycline prior to ketamine administration.
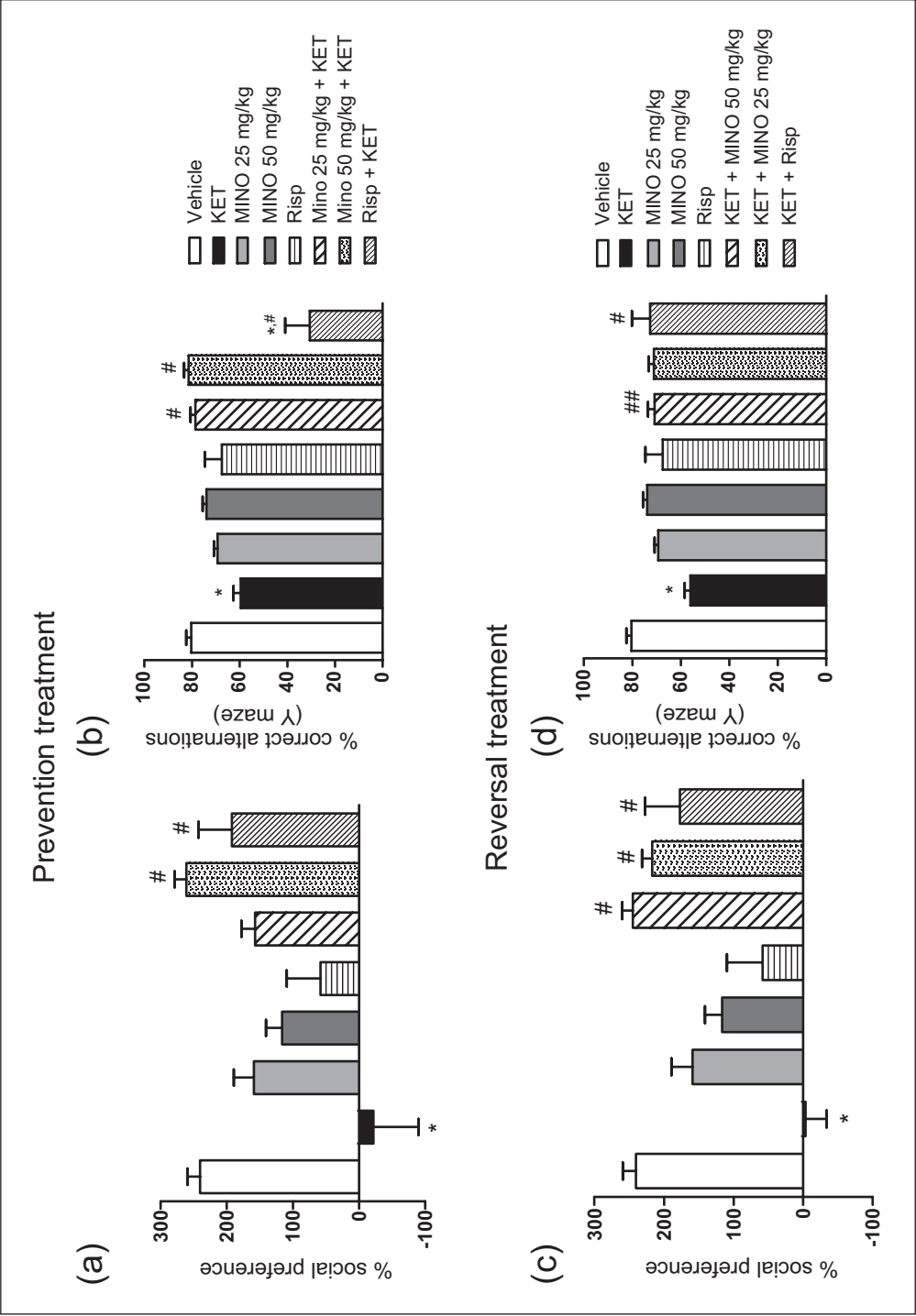
Figure 4. Percentage of social contacts (a) and percentage of correct alternations in the Y maze (b) of animals submitted to the prevention treatment. Percentage of social contacts (c) and percentage of correct alternations in the Y maze (d) of animals submitted to the reversal treatment with minocycline (MINO) or risperidone (Risp). Bars represent mean ± standard error of the mean of the number of crossings ($n = 8$–10 animals/group).
*$p < 0.05$ versus control.
#$p < 0.05$ versus KET according to one-way ANOVA followed by Tukey's post hoc test. Ket: ketamine.

Table 1. Neurochemical alterations (oxidative stress parameters and nitrite levels) in brain areas of animals submitted to the *prevention* treatment.

	Prefrontal	Hippocampus	Striatum
GSH (ng/g wet tissue)			
Vehicle	839.3 ± 31.5	1122 ± 113.3	1308 ± 115.9
KET	345.1 ± 23.0*	878.9 ± 79.03	463.4 ± 77.2*
MINO 25 mg/kg	550.7 ± 45.1	607.9 ± 67.7	895.2 ± 64.0
MINO 50 mg/kg	502.5 ± 57.8	607.3 ± 69.1	896.4 ± 121.1
Risp	8041 ± 108.9*	8116.0 ± 90.2*	7899 ± 162.9*
MINO 25 mg/kg + KET	882.0 ± 60.7#	993.9 ± 47.6	733.8 ± 123#
MINO 50 mg/kg + KET	795.6 ± 49.6#	956.6 ± 48.0	846.9 ± 71.1#
Risp + KET	6682 ± 407.8*,#	5629 ± 608.1*,#	6425 ± 213.1*,#
TBARS (µmol of MDA/mg protein)			
Vehicle	290.6 ± 22.2	401.9 ± 27.3	340.9 ± 17.6
KET	480.7 ± 22.1*	529.3 ± 22*	474.3 ± 20.2*
MINO 25 mg/kg	398.9 ± 21.8	304.7 ± 32.9	374.4 ± 26.3
MINO 50 mg/kg	398.9 ± 21.8	378.9 ± 18	381.1 ± 14.5
Risp	199.6 ± 30.9	116.6 ± 26.4*	135.6 ± 14.3*
MINO 25 mg/kg + KET	457.5 ± 26.2	457.9 ± 43.8	344.3 ± 34.2#
MINO 50 mg/kg + KET	372.0 ± 13.4#	328.5 ± 29.5#	329.9 ± 21.1#
Risp + KET	82.8 ± 8.1*,#	124.9 ± 18.4*,#	99.5 ± 6.3*,#
Nitrite (µmol/mg protein)			
Vehicle	1.36 ± 0.06	2.06 ± 0.65	1.31 ± 0.06
KET	1.2 ± 0.08	1.50 ± 0.08	2.82 ± 0.55*
MINO 25 mg/kg	1.58 ± 0.17	1.41 ± 0.06	1.48 ± 0.07
MINO 50 mg/kg	1.50 ± 0.09	0.96 ± 0.09	1.42 ± 0.06
Risp	1.08 ± 0.16	2.24 ± 0.32	0.91 ± 0.08
MINO 25 mg/kg + KET	2.25 ± 0.5	1.68 ± 0.09	2.07 ± 0.17
MINO 50 mg/kg + KET	1.44 ± 0.06	1.31 ± 0.08	1.27 ± 0.08#
Risp + KET	1.10 ± 0.13	0.91 ± 0.10	1.26 ± 0.35#

Mice (8–10/group) were administered with vehicle, MINO (25 or 50mg/kg, intraperitoneally (i.p.)) or RISP 0.5 mg/kg for 14 days once a day. Between the eighth and 14th days, these animals additionally received one dose of KET 20mg/kg or vehicle, i.p., daily. Results are expressed as means ± SEM and analyzed by ANOVA and Tukey's test as a post hoc.

*#$p < 0.05$ *vs*. vehicle and KET groups, respectively.

GSH: glutathione; MDA: malondialdehyde; KET: ketamine; MINO: minocycline; Risp: risperidone; TBARS: thiobarbituric acid reactive substances.

Ketamine administration caused an increase in TBARS levels in the PFC ($F(7,94) = 23.94$, $p < 0.001$), HC ($F(7,92) = 29.74$, $p < 0.05$) and ST ($F(7,96) = 31.46$, $p < 0.001$) as compared with vehicle-treated animals. The administration of minocycline 50 mg/kg prior to ketamine prevented these alterations induced by ketamine in all brain areas studied ($p < 0.001$), while pre-treatment with minocycline (25 mg/kg) prevented this alteration in only the ST ($p < 0.01$). Pretreatment with risperidone significantly decreased TBARS levels as compared with vehicle- and ketamine-treated animals in all brain areas studied ($p < 0.001$). In relation to the administration of minocycline and risperidone alone, minocycline (25 and 50 mg/kg) caused no alterations in TBARS levels, while risperidone in the HC ($p < 0.001$) and ST ($p < 0.001$) decreased this parameter.

In the prevention protocol, ketamine promoted an increase in nitrite levels only in the ST of vehicle-treated mice. Pretreatment with minocycline (50 mg/kg) and risperidone was able to significantly prevent this increase ($p < 0.05$). No alterations in this parameter were observed in the other brain areas studied.

Regarding the alterations in oxidative parameters and nitrite levels in animals subjected to the reversal treatment as shown in Table 2, ketamine administration significantly decreased GSH levels in the PFC ($F(7,76) = 987.3$, $p < 0.001$), HC ($F(7,80) =$ 377.5, $P < 0.001$) and ST ($F(7,90) = 108.3$, $p < 0.001$) when compared with vehicle-treated animals. This decrease was reversed only by minocycline (50 mg/kg) and risperidone in all brain areas studied ($p < 0.001$). Risperidone, in the same way as observed in the prevention protocol, caused a significant increase in GSH levels, both when used alone ($p < 0.001$) and when administered before ketamine in all brain areas studied ($p < 0.001$). The administration of minocycline (25 and 50 mg/kg) alone caused no alterations in GSH levels.

The levels of lipid peroxidation evaluated here by TBARS measures were increased by ketamine administration in the PFC ($F(7,95) = 16.18$, $p < 0.001$), HC ($F(7,105) = 24.62$, $p < 0.001$) and ST ($F(7,112) = 36.68$, $p < 0.001$) when compared with vehicle-treated animals. The administration of minocycline (25 and 50 mg/kg) and risperidone reversed ketamine-induced alterations in TBARS levels in the HC ($p < 0.05$) and ST ($p < 0.05$). In the PFC this reversal was observed only in the animals treated with minocycline 25 mg/kg (Table 2).

Nitrite levels in the same pattern as observed in the prevention protocol significantly increased only in the ST of ketamine-treated animals ($F(7,126) = 4.190$, $p < 0.001$). Only minocycline 50 mg/kg and risperidone significantly reversed this alteration ($p < 0.001$). The administration of minocycline (25 and 50 mg/kg) and

Table 2. Neurochemical alterations (oxidative stress parameters and nitrite levels) in brain areas of animals submitted to the *reversal* treatment.

	Prefrontal	Hippocampus	Striatum
GSH (ng/g wet tissue)			
Vehicle	808.0 ± 31.4	1260 ± 123.7	1250 ± 115.0
KET	341.1 ± 22.0*	399.7 ± 13.6*	388.7 ± 15.2*
MINO 25 mg/kg	550.7 ± 45.1	504.5 ± 43.0	659 ± 52.6
MINO 50 mg/kg	677 ± 112.7	713.2 ± 96.8	1001 ± 158.9
Risp	8041 ± 108.9*	8116.0 ± 90.2*	7899 ± 162.9*
KET + MINO 25 mg/kg	230.4 ± 11.2*	221.1 ± 6.3*	364.5 ± 49.6
KET + MINO 50 mg/kg	1781 ± 221.3#	3549 ± 536.9*,#	2418 ± 317.7#
KET + Risp	7499 ± 170.6*,#	7657 ± 184*,#	6692 ± 778.4*,#
TBARS (µmol of MDA/mg protein)			
Vehicle	293.4 ± 16.6	393.2 ± 28.4	341.1 ± 13.7
KET	490.5 ± 26.8*	498.9 ± 13.4*	469.5 ± 11.8*
MINO 25 mg/kg	285.0 ± 25.9	304.7 ± 32.9	374.4 ± 26.3
MINO 50 mg/kg	379.3 ± 21.2	374.3 ± 18.2	380.4 ± 13.4
Risp	156.3 ± 30.0*	117.0 ± 27.0*	121.4 ± 14.4*
KET + MINO 25 mg/kg	303.9 ± 30.7#	348.2 ± 38.2#	371.6 ± 24.7#
KET + MINO 50 mg/kg	414.1 ± 40.7	348.6 ± 18.4#	343.4 ± 17.4#
KET + Risp	213.4 ± 24.3#	186.2 ± 22.5*,#	181.7 ± 26.3*,#
Nitrite (µmol/mg protein)			
Vehicle	1.31 ± 0.05	1.32 ± 0.08	1.28 ± 0.05
KET + vehicle	1.37 ± 0.11	1.57 ± 0.13	2.60 ± 0.41*
MINO 25 mg/kg	1.58 ± 0.17	1.68 ± 0.14	1.65 ± 0.10
MINO 50 mg/kg	1.5 ± 0.09	1.61 ± 0.10	1.58 ± 0.11
Risp	1.08 ± 0.17	0.96 ± 0.09	0.91 ± 0.08
KET + MINO 25 mg/kg	1.79 ± 0.17	1.99 ± 0.23	1.81 ± 0.14
KET + MINO 50 mg/kg	1.40 ± 0.12	1.49 ± 0.12	1.32 ± 0.08#
KET + Risp	1.16 ± 0.08	1.05 ± 0.08	1.26 ± 0.12#

Mice (8–10/group) were administered with KET 20mg/kg or vehicle intraperitoneally (i.p.) for 14 days once a day. Between the eighth and 14th days, these animals additionally received one dose of vehicle, MINO (25 or 50mg/kg, i.p.) or RISP (0.5 mg/kg) 30 min after KET or vehicle. Results are expressed as means ± SEM and analyzed by ANOVA and Tukey's test as a post hoc.
*#$p < 0.05$ *vs*. vehicle and KET groups, respectively.
GSH: glutathione; MDA: malondialdehyde; KET: ketamine; MINO: minocycline; Risp: risperidone; TBARS: thiobarbituric acid reactive substances.

risperidone alone did not promote significant alterations in nitrite levels.

Discussion

Herein we demonstrated that the second generation tetracycline, minocycline, was capable of preventing and reversing the behavioral alterations induced by the repeated administration of ketamine, an animal model of schizophrenia that resembles positive, negative and cognitive symptoms of this mental disorder in humans (Hou et al., 2013). Furthermore, minocycline prevented and reversed several pro-oxidant alterations promoted by ketamine. Therefore, our data suggest that minocycline may have novel therapeutic effects for the acute and maintenance treatment phases of schizophrenia.

Behavioral alterations and schizophrenia symptoms

Positive and negative symptoms as well as cognitive impairments induced by ketamine (Hou et al., 2013) have been partially attributed to the blockade of NMDARs. Indeed, the blockade of

NMDARs located on inhibitory GABAergic neurons in the limbic and subcortical brain regions leads to an increase in neuronal activity in the limbic-striatal circuits through an increase in glutamate and dopamine release; these neurochemical events relate to the positive symptoms of schizophrenia (Chatterjee et al., 2012; Javitt and Zukin, 1991; Lorrain et al., 2003). The blockade of NMDARs in the ventral tegmental area (VTA) promotes a decrease in dopamine release in the PFC, which may be partially responsible for the negative and cognitive symptoms (Neill et al., 2010; Seamans and Yang, 2004; Takahata and Moghaddam, 1998). Therefore, schizophrenia is associated with strongly interconnected abnormalities of glutamatergic and dopaminergic transmission (Laruelle et al., 2003) that are at least in part reproduced by the chronic administration of ketamine in mice (Chatterjee et al., 2012).

Besides mimicking the symptoms of schizophrenia, subchronic ketamine treatment is also known to induce oxidative damage along with nitrergic and GABAergic alterations (Behrens Sejnowski, 2009) which parallel those described in postmortem brains of individuals with schizophrenia (De Oliveira et al., 2009; Keilhoff et al., 2004). Based on the similarities to the pathophysiology of schizophrenia and widespread use of this model (Becker and GreckYou, 2004; Chatterjee et al., 2012; Javitt et al., 2012) it

was chosen and adapted for the present study in order to access the prevention and reversal of ketamine-induced alterations by minocycline.

The face validity of positive symptoms in animal models is questionable in so far as it is impossible to fully mimic this phenomenon. Thus, the literature on animal models evaluating these symptoms has focused on two main categories of behavioral alterations: locomotor hyperactivity and disruptions of PPI (Van Den Buuse, 2010). Indeed, moderate doses of ketamine are able to induce locomotor hyperactivity (Van Den Buuse, 2010). On the other hand, PPI impairment is indicative of a disruption in sensorimotor gating mechanisms, a precognitive process that prevents sensory overload and cognitive fragmentation (Geyer et al., 2002). Accordingly, the PPI alterations have been broadly considered an endophenotype for this disorder with a great translational value (Fendt and Koch, 2013), representing the 'interface of psychosis and cognition' (Arguello and Gogos, 2010).

In this context, our results demonstrate that the administration of minocycline (25 and 50 mg/kg) prevented and reversed ketamine-induced hyperlocomotion and PPI impairment (both tests being correlated with positive symptoms of schizophrenia) with results comparable to risperidone, an atypical antipsychotic. In line with this evidence, minocycline is currently being tested for positive symptoms in a phase IV trial as an add-on therapy with atypical antipsychotics (Levkovitz, 2013) as well as for the treatment targeting the cognitive and negative symptoms of schizophrenia, in the latter case with encouraging results (Chaudhry et al., 2012; Dean et al., 2012; Levkovitz et al., 2010).

To date the scientific literature lacks preclinical studies of minocycline in schizophrenia. Previous studies revealed attenuation of hyperlocomotion and prepulse inhibition of the startle response by the acute administration of minocycline (40 mg/kg) using the model of schizophrenia induced by a single administration of the NMDAR antagonist dizocilpine (Zhang et al., 2007), and also reversion of cognitive deficits (Fujita et al., 2008; Levkovitz et al., 2007) in the same model. In addition, minocycline was able to correct the alterations in behavior and dopaminergic neurotransmission induced by methamphetamine (3 mg/kg), that is, reduced hyperlocomotion, the increased extracellular dopamine levels and the dopamine transporter-immunoreactivity in the striatum (Zhang et al., 2006). These results call for attention to an involvement of dopaminergic neurotransmission in minocycline's antipsychotic effects.

Importantly, our study showed that minocycline prevented and reversed ketamine-induced alterations in the YMT, suggesting an effect of this drug against ketamine-induced deficits in working memory, while risperidone failed to prevent this alteration. In fact, a recent study showed that risperidone did not promote an improvement in working memory and verbal fluency in patients (Remberk et al., 2012). Social withdrawal is one of the core negative symptoms of schizophrenia (Lysaker et al., 2012). In our study minocycline was able to prevent and reverse the decreases in the percentages of social contacts induced by ketamine. Indeed, the negative and cognitive symptoms of this mental disorder have been related to microglial activation and the resulting inflammatory response (Monji et al., 2009). In this regard, minocycline's effects on these symptomatic dimensions may partly result from its anti-inflammatory properties as well as effects on glutamate neurotransmission (Chaudhry et al., 2012), based on findings which show that this drug indirectly modulates NMDAR transmission (Chaves et al., 2009), although the actual mechanism of action is still under active investigation.

Alterations in oxidative stress and nitrite levels and their relationship with schizophrenia

In the present study, the beneficial effects of minocycline on ketamine-induced behavioral alterations were accompanied by alterations in oxidative balance. Hence, the administration of minocycline restored GSH levels and also ameliorated ketamine-induced lipid peroxidation. In fact, GSH is involved in the pathophysiology of schizophrenia and possibly, together with superoxide dismutase and glutathione peroxidase, may work as indicators of schizophrenia severity in acute episodes, being also affected by antipsychotic therapy (Tsai et al., 2013). In addition, GSH-deficient mice have been used as an animal model of schizophrenia and bipolar disorders (Kulak et al., 2012). Interestingly, in the present study risperidone displayed a great increase in GSH levels. Of note, a recent study demonstrated that risperidone treatment restores GSH levels and to a great extent reverses antioxidant defense alterations in the brain of rats treated with phencyclidine perinatally (Stojkovic et al., 2012).

The correction of ketamine-induced oxidative imbalance by minocycline administration as reported in the present study may be an important feature related to the benefits of this drug in schizophrenia. This is reinforced by the fact that an imbalance in the redox-state of the brain is part of the underlying pathophysiology in schizophrenia (Behrens and Sejnowski, 2009). In this context, it was previously reported that the repetitive exposure of adult rodents to ketamine was able to increase the levels of the proinflammatory cytokine IL-6 in the brain, which through activation of the superoxide-producing enzyme NADPH oxidase (Nox2) led to the loss of the GABAergic phenotype of parvalbumin-labeled interneurons and to a decreased inhibitory activity in PFC. In other words, IL-6, found to be altered in schizophrenia patients, can tip the redox balance into a pro-oxidant state (Behrens and Sejnowski, 2009). Importantly, in our study, the oxidative alterations induced by ketamine were observed in all brain areas studied. Therefore, the anti-inflammatory effects of minocycline may in part explain its antioxidant properties observed herein.

Nitrite levels, on the other hand, were increased in ketamine-treated mice only in the ST, which was normalized by minocycline administration at the higher dose (i.e. 50 mg/kg) and risperidone. Recently, a clinical study showed that the levels of superoxide dismutase (SOD) and NO were significantly increased in patients with schizophrenia compared with normal controls (Zhang et al., 2012). In fact, altered neurogenesis may contribute to dysfunction of the dopamine and NO systems and psychosis through convergence of genetic and environmental disease associated factors (Inta et al., 2011). In this regard, a study demonstrated that minocycline was able to potentiate nerve growth factor (NGF) induced neurite outgrowth in PC12 cells by an interaction with IP_3 receptors and several cellular signaling pathways, being, thus, a novel target for the neuroprotective effect of this drug (Hashimoto and Ishima, 2010). To date, enhanced neurogenesis is being related to the neuroprotective effect of atypical antipsychotics (Nandra and Agius, 2012; Peng et al., 2013).

Striatal NO tone also regulates the basal activity and responsiveness of dopamine neurons to cortical and striatal inputs (West and Grace, 2000). For example, the infusion of the NO synthase (NOS) inhibitor, 7-nitroindazole sodium (7-NI) decreased the onset latency and extended the duration of the initial inhibitory phase induced by either orbital PFC or striatal stimulation. On the other hand, in the same study, microdialysis experiment demonstrated that endogenous striatal NO production increases striatal extracellular dopamine levels (West and Grace, 2000). This property of NO to regulate dopamine levels in the ST may partially explain the increase in nitrite levels induced by ketamine, especially in this brain area in the present study, because the repeated administration of ketamine increases dopamine levels in this brain area and this mechanism relates mainly to the positive symptoms of schizophrenia (Chatterjee et al., 2012). Another possible explanation for the decrease in nitrite levels induced by minocycline is its direct chemical scavenging activity against peroxynitrite (Schildknecht et al., 2011).

Currently the role of NO in the pathophysiology of schizophrenia is not completely determined (Coyle, 2013). Studies with drug-free schizophrenic patients showed no significant difference regarding total nitrite plasma/serum concentrations between patients and healthy controls, while patients under antipsychotic drug treatment presented higher levels of plasma/serum total nitrite than controls (Maia-De-Oliveira et al., 2012); beyond that, it was recently reported that the intravenous administration of the NO donor sodium nitroprusside to patients with schizophrenia improved the symptoms of this mental disorder (Hallak et al., 2013).

Strengths and limitations

The strength of the present study was that the ketamine-induced animal model of schizophrenia was able to model all three symptomatic dimensions of schizophrenia in mice, along with oxidative imbalances and nitrergic alterations consistent with those observed in schizophrenia. Furthermore, to our knowledge, we are the first to present preclinical evidences that can partly explain the mechanism of the antipsychotic actions of minocycline.

Some limitations of this study are: 1) the animal model used does not address the developmental component of schizophrenia; 2) the lack of cytokine levels determination to evaluate the anti-inflammatory effects of minocycline as well as the determination of the involvement of dopaminergic and glutamatergic systems.

Conclusion

Here we have shown that minocycline prevented and reversed schizophrenia-like behavioral alterations induced by ketamine. These behavioral effects of minocycline were accompanied by the normalization of ketamine-induced oxidative imbalance in all brain areas studied as well as a restoration of nitrite levels in the ST. These data provide a rationale for the design of clinical trials of minocycline as a stand-alone antipsychotic agent targeting a broad range of schizophrenia manifestations.

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