

REGULAR RESEARCH ARTICLE

A Characterization of the Effects of Minocycline Treatment During Adolescence on Structural, Metabolic, and Oxidative Stress Parameters in a Maternal Immune Stimulation Model of Neurodevelopmental Brain Disorders

Diego Romero-Miguel, Marta Casquero-Veiga, Karina S. MacDowell, Sonia Torres-Sanchez, José Antonio Garcia-Partida, Nicolás Lamanna-Rama, Ana Romero-Miranda, Esther Berrocoso, Juan C. Leza, Manuel Desco, María Luisa Soto-Montenegro

Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain (Mr Romero-Miguel, Dr Casquero-Veiga, Mr Lamanna-Rama, Ms Romero-Miranda, Dr Desco, and Dr Soto-Montenegro); CIBER de Salud Mental (CIBERSAM), Madrid, Spain (Drs Casquero-Veiga, MacDowell, Torres-Sanchez, Garcia-Partida, Berrocoso, Leza, Desco, and Soto-Montenegro); Departamento de Bioingeniería e Ingeniería Aeroespacial, Universidad Carlos III de Madrid, Leganés, Spain (Dr Desco); Neuropsychopharmacology and Psychobiology Research Group, Psychobiology Area, Department of Psychology, Universidad de Cádiz, Puerto Real (Cádiz), Spain (Drs Torres-Sanchez, Garcia-Partida, and Berrocoso); Instituto de Investigación e Innovación en Ciencias Biomédicas de Cádiz, INiBICA, Hospital Universitario Puerta del Mar, Cádiz, Spain (Drs Torres-Sanchez, Garcia-Partida, and Berrocoso); Department of Pharmacology and Toxicology, School of Medicine, Universidad Complutense (UCM), IIS Imas12, IUIN, Madrid, Spain (Drs MacDowell and Leza); Centro Nacional de Investigaciones Cardiovasculares, CNIC, Madrid, Spain (Dr Desco); High Performance Research Group in Physiopathology and Pharmacology of the Digestive System (NeuGut), University Rey Juan Carlos (URJC), Alcorcón, Spain (Dr Soto-Montenegro).

Correspondence: Manuel Desco, PhD, Laboratorio de Imagen Médica, Unidad de Medicina y Cirugía Experimental, Hospital General Universitario Gregorio Marañón, Dr. Esquerdo, 46. E-28007 Madrid, Spain (desco@hggm.es).

D.R.-M. and M.C.-V. contributed equally.

Abstract

Background: Minocycline (MIN) is a tetracycline with antioxidant, anti-inflammatory, and neuroprotective properties. Given the likely involvement of inflammation and oxidative stress (IOS) in schizophrenia, MIN has been proposed as a potential adjuvant treatment in this pathology. We tested an early therapeutic window, during adolescence, as prevention of the schizophrenia-related deficits in the maternal immune stimulation (MIS) animal model.

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Methods: On gestational day 15, Poly I:C or vehicle was injected in pregnant Wistar rats. A total 93 male offspring received MIN (30 mg/kg) or saline from postnatal day (PND) 35–49. At PND70, rats were submitted to the prepulse inhibition test. FDG-PET and T2-weighted MRI brain studies were performed at adulthood. IOS markers were evaluated in frozen brain tissue.

Results: MIN treatment did not prevent prepulse inhibition test behavioral deficits in MIS offspring. However, MIN prevented morphometric abnormalities in the third ventricle but not in the hippocampus. Additionally, MIN reduced brain metabolism in cerebellum and increased it in nucleus accumbens. Finally, MIN reduced the expression of iNOS (prefrontal cortex, caudate-putamen) and increased the levels of KEAP1 (prefrontal cortex), HO1 and NQO1 (amygdala, hippocampus), and HO1 (caudate-putamen).

Conclusions: MIN treatment during adolescence partially counteracts volumetric abnormalities and IOS deficits in the MIS model, likely via iNOS and Nrf2–ARE pathways, also increasing the expression of cytoprotective enzymes. However, MIN treatment during this peripubertal stage does not prevent sensorimotor gating deficits. Therefore, even though it does not prevent all the MIS-derived abnormalities evaluated, our results suggest the potential utility of early treatment with MIN in other schizophrenia domains.

Keywords: FDG-PET, inflammatory/oxidonitrosative stress, minocycline, Poly I:C, schizophrenia

Significance Statement

Minocycline (MIN) is a tetracycline with antioxidant, anti-inflammatory, and neuroprotective properties. We aim to evaluate the potential role of MIN administration during peri-adolescence, before the onset of the symptoms, as prevention of the schizophrenia-related deficits in the maternal immune stimulation (MIS) animal model. As far as we know, this is the first study to examine the preventive effect of MIN in brain glucose metabolism and morphology, evaluated by microPET and voxel-based morphometry (VBM) approaches together with behavioral and inflammatory/oxidative stress (IOS) studies. MIN treatment during adolescence partially counteracts morphometric abnormalities and IOS deficits via iNOS and Nrf2–ARE pathways, increasing the expression of cytoprotective enzymes. However, MIN treatment during this peripubertal stage does not prevent sensorimotor gating deficit. Therefore, despite not preventing all the pathological levels assessed, MIN effectivity highlights the usefulness of anti-IOS compounds to slow down the disease course at early stages.

Introduction

Schizophrenia is a chronic multifactorial psychiatric disorder and one of the most disabling mental disorders due to its chronicity, early onset, and high rate of suicide (Correll et al., 2019). Current treatments mainly focus on alleviating the associated symptoms, but their high failure rate (Correll et al., 2019) emphasizes the importance of finding new therapeutic strategies. Recently, there has been growing interest in the prevention of schizophrenia given its well-known association with genetic and environmental risk factors (van Os et al., 2014; Lipner et al., 2019) and its neuroprogressive nature (Zhao et al., 2017). Therefore, the detection of structural and functional brain deficits prior to clinical manifestations suggests the prodrome of this disease as a potential therapeutic window to halt the disease progression or reduce its severity (Millan et al., 2016).

Inflammation and oxidative stress play a key role in the development of schizophrenia. Thus, antioxidant and anti-inflammatory drugs have been proposed as possible therapeutic strategies (Kulak et al., 2013; Muller, 2018). In this respect, minocycline (MIN) (7-dimethylamino-6-dimethyl-6-deoxytetracycline), an antibiotic currently approved for the treatment of some chronic bacterially caused conditions with anti-inflammatory, antioxidant, and neuroprotective properties (Garrido-Mesa et al., 2013b), has received particular attention. Besides its well-recognized antibiotic properties, MIN gained emergent prominence in the late 1990s when a serendipitous neuroprotective effect was discovered in a patient with depression and a non-related bacterial infection (Levine et al., 1996). Since then, its neuroprotective effects have been demonstrated in several animal models of neurological diseases (Romero-Miguel et al., 2021). Thus, it has been hypothesized that MIN might reduce free radicals and pro-inflammatory cytokines released by over-activated microglia

(Zhang and Zhao, 2014). In schizophrenia, the involvement of microglia over-activation led researchers to evaluate the possible neuroprotective effect of MIN. However, until now, the results have been contradictory. Some clinical trials have suggested the beneficial effect of MIN on the negative symptoms in early-phase (Levkovitz et al., 2010; Chaudhry et al., 2012; Liu et al., 2014) and chronic schizophrenia (Khodaie-Ardakani et al., 2014; Zhang et al., 2018). This finding would represent major progress given that current treatments are not effective enough to alleviate such symptoms. Nonetheless, other clinical trials have failed to replicate these improvements (Kelly et al., 2015; Deakin et al., 2018). Therefore, although great progress has been made, the real effect of MIN in schizophrenia remains elusive, especially in the prodromal phase of the disorder.

On the preclinical side, schizophrenia-like animal models may help to decipher the extent to which MIN could be useful as a preventive therapy. In this sense, the well-validated maternal immune stimulation (MIS) animal model is based on epidemiological studies showing the association between a maternal infection during pregnancy and the increased risk of developing schizophrenia in the offspring (Brown and Derkits, 2010). Thus, the stimulation induced by an immunogenic compound (e.g., Poly I:C) during pregnancy results in a sudden release of inflammatory mediators that may trigger long-lasting brain alterations, which become evident at adulthood (Zuckerman and Weiner, 2003). Consequently, adult MIS offspring show reduced hippocampi and enlarged ventricles (Piontkewitz et al., 2011) together with metabolic reductions in cortical regions and hippocampi and metabolic increases in the nucleus accumbens, amygdala, and thalamus (Hadar et al., 2015). In fact, these structural and metabolic disturbances are similar

to those observed in patients with schizophrenia (Tamminga et al., 1992; Seethalakshmi et al., 2006; Haukvik et al., 2013; Berger et al., 2017; Kim et al., 2017). Furthermore, adult MIS animals also show behavioral deficits (Hadar et al., 2015) and biochemical alterations in inflammation/oxidative stress pathways (MacDowell et al., 2017; Casquero-Veiga et al., 2019). This is particularly interesting as inflammatory and oxidative stress (IOS) imbalances play an important role in the pathophysiology of the MIS model (Moller et al., 2015; Talukdar et al., 2020) and schizophrenia (Leza et al., 2015). Moreover, the pathophysiological delay showed by this model makes it a valuable tool for testing preventive approaches. In fact, we recently demonstrated the preventive effect of periadolescent treatments with risperidone (Casquero-Veiga et al., 2019) or omega-3 poly-unsaturated fatty acids (Casquero-Veiga et al., 2021) on schizophrenia-related abnormalities at adulthood in this MIS model.

Here, we aim to evaluate the potential role of MIN administration during peri-adolescence in the MIS rat model of schizophrenia. This work includes studies of brain metabolism, morphometry, behavior, and IOS.

Materials and Methods

Animals

Ninety-three male Wistar rats were maintained at constant temperature ($24^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) under a 12-hour-light/dark cycle with free access to chow/water. Two batches of animals were used: 36 animals underwent behavioral studies, and 57 animals underwent imaging and IOS studies. All animal procedures were conducted in conformity with the European Communities Council Directive 2010/63/EU and approved by the Ethics Committee for Animal Experimentation of our hospital (ES280790000087).

Drug Treatment

Figure 1A presents the drug treatment and study design. Prenatal Poly I:C or vehicle (VH, saline) was i.v. administered. On post-natal day (PND) 21, male offspring were weaned and housed 2-4 per cage. MIN (30 mg/kg, Sigma, M9511) (Levkovitz et al., 2007; Giovanoli et al., 2016) or saline (Sal) was i.p. injected during periadolescence (PND35–49) (Sengupta, 2013). Animals were divided into 4 groups according to the MIS condition (VH, MIS) and treatment (Sal, MIN).

Behavioral Study: Prepulse Inhibition (PPI) Test

PPI of the acoustic startle response was measured at PND70. The session began with 10 minutes of acclimatization to the startle chamber (Cibertec, Spain) with 70-dB background noise followed by 5 trials of startle stimulus (pulse, 120 dB). Next, animals received 10 trials of pseudo-randomly presented stimuli: pulse (120 dB), prepulse (74, 80, or 86 dB)+pulse (120 dB), or no stimulus (background noise). Finally, 5 trials of pulse (120 dB) were conducted. Pulse and prepulse duration was 40 ms, and the prepulse-pulse interval was 100 ms, whereas the intertrial-interval ranged from 10 to 20 seconds.

Imaging Studies

Animals were scanned at adulthood (PND120) (11–17 animals/group) under sevoflurane anesthesia (4.5% induction, 2.5% maintenance in 100% O_2).

Magnetic Resonance Imaging (MRI)—Animals were scanned using a 7-Tesla Biospec 70/20 scanner (Bruker, Germany) (Soto-

Montenegro et al., 2014). A coronal T2-weighted spin-echo sequence was acquired with TE=33 milliseconds, TR=3732 milliseconds, averages 2, and slice thickness 0.4 mm. Matrix size was 256×256 pixels at a FOV of 3.5×3.5 cm^2 .

Positron Emission Tomography (PET)—After 45 minutes of 2-deoxy-2-[^{18}F]fluoro-D-glucose (FDG) uptake (approximately 37 MBq, i.v.), animals were scanned for 45 minutes using a small-animal ARGUS PET/CT scanner (SEDECAL, Spain). Images were reconstructed with a 2D-OSEM algorithm (1.45 mm full width at half maximum [FWHM], voxel size $0.3875 \times 0.3875 \times 0.775$ mm^3 , energy window of 400–700 keV) and corrected for decay and dead time.

Computed Tomography (CT)—Images were acquired using the above-mentioned scanner with the following parameters: 340 mA, 40 KV, 360 projections, 8 shots, and resolution of 200 μm . Images were reconstructed using a Feldkamp algorithm (isotropic voxel size of 0.121 mm) (Abella et al., 2012).

Biochemical Determinations

Frozen tissue samples (-80°C) from the prefrontal cortex, hippocampus, caudate-putamen, and amygdala (5–8 animals/group) were used for the study of biochemical determinations. IOS parameter selection was based on previous works (Leza et al., 2015; MacDowell et al., 2017; Casquero-Veiga et al., 2019, 2021).

Western Blot

Inflammatory mediators (inducible nitric oxide synthase [iNOS] and cyclooxygenase-2 [COX-2]) and the antioxidant pathway (Kelch-like ECH-associated protein 1 [KEAP1], heme oxygenase-1 [HO1], NAD(P)H:quinone oxidoreductase-1 [NQO1]) expression were measured in cytosolic extracts (MacDowell et al., 2013). Protein levels were assessed using the Bradford method.

Proteins were loaded into electrophoresis gel and then blotted onto a membrane with a semi-dry transfer system. Blots were blocked with 5% bovine serum albumin (BSA) (Sigma, Spain) for 1 hour at room temperature and probed overnight at 4°C with rabbit anti-iNOS (sc-650, 1:750 BSA 2%; SCBT), goat anti-COX2 (sc-1747, 1:750 BSA 2.5%; SCBT), mouse anti-KEAP1 (MAB3024, 1:1000; R&D), rabbit anti-HO1 (ab68477, 1:1000; Abcam), goat anti-NQO1 (sc16464, 1:750; BSA 1%, SCBT), and mouse anti- β -actin (A5441, 1:10000; Sigma). Primary antibodies were incubated for 1.5 hours at room temperature with horseradish peroxidase-linked secondary antibodies. Binding was detected by an Odyssey Fc System (LI-COR, Germany). All western blots were performed at least 3 times in separate assays.

Lipid Peroxidation

Lipid peroxidation was determined by the thiobarbituric acid test for malondialdehyde as described (Das and Ratty, 1987).

Nuclear Erythroid-Related Factor Activity

Activation of nuclear erythroid-related factor (NRF2) was measured in nuclear extracts of tissue samples through a commercial ELISA-based kit (600590, Cayman Chemical).

Antioxidant Activity

Tissue samples were sonicated in 400 μL phosphate-buffered saline (pH=7) with a protease inhibitor cocktail (Complete,

Figure 1

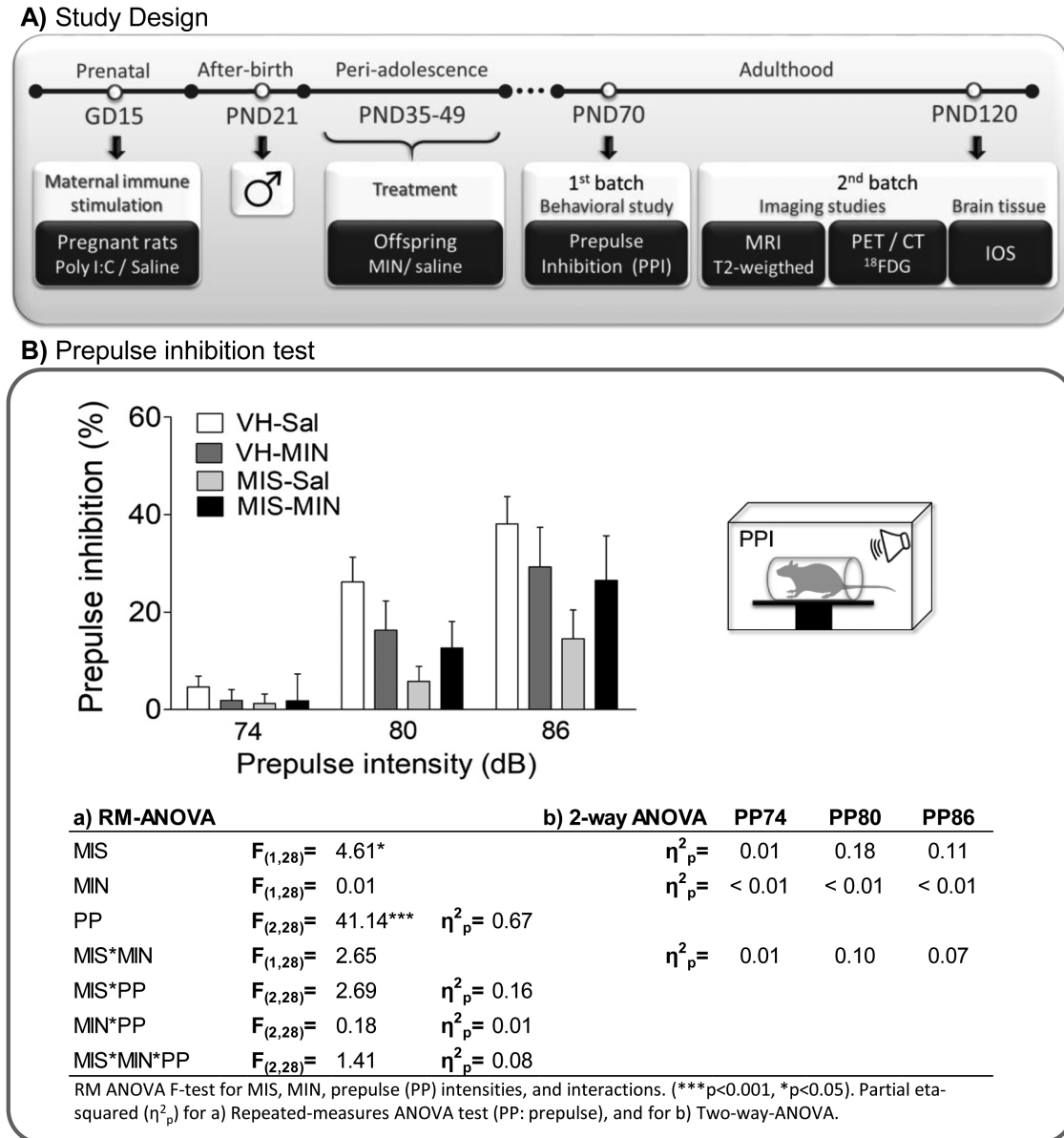


Figure 1. Study design and behavioral changes measured via PPI. (A) Representative diagram of the chronology of the experimental procedures performed during the study according to the age of the animals. Abbreviations: ^{18}F -FDG, [^{18}F]-Fluorodeoxyglucose; CT, computerized tomography; GD, gestational day; IOS, inflammatory/oxidative markers; MIN, minocycline; MRI, magnetic resonance imaging; PET, positron emission tomography; PND, post-natal day; PPI, prepulse inhibition. (B) MIN-related effects on PPI deficits in MIS rats. Each column represents the mean \pm SEM of PPI (%) for each prepulse intensity (74, 80, and 86 dB) of 6–11 animals (VH-Sal 9, VH-MIN 6, MIS-Sal 11, MIS-MIN 6). The table represents the RM-ANOVA results and the effect size (partial eta-squared, η^2_p) of each factor. Furthermore, effect sizes are also reported in a 2-way ANOVA approach to assess the specific effect of MIS and MIN factors on PPI without PP influence.

Roche). Homogenates were centrifuged at 10000 g for 15 minutes at 4°C. Supernatants were used to determine the activity of superoxide dismutase (SOD) (K028-H1, Arbor Assay), catalase (CAT) (K033-H1, Arbor Assay), and glutathione peroxidase (GPx) (703102, Cayman Chemical), and concentration of glutathione (GSH) (K006-H1, Arbor Assay). Results were expressed in U/mg of protein for the enzymes and in $\mu\text{M}/\mu\text{g}$ of protein for GSH.

Data Processing and Analysis

PPI Data—The percent of PPI for each prepulse intensity was calculated as follows: $100 - ((\text{prepulse} + \text{pulse})/\text{pulse}) \times 100$). Note

that prepulse + pulse is the startle response of the 10 PPI trials for each intensity, and pulse is the mean startle response of the 10 pulse-alone trials. Data were analyzed by means of repeated-measurements ANOVA.

MRI and PET Data Processing—A voxel-based morphometry (VBM) approach was performed in MRI data (Casquero-Veiga et al., 2019). Briefly, T2 images were preprocessed, realigned, and resliced to (Valdes-Hernandez et al., 2011) rat brain template space using SPM12 (<http://www.fil.ion.ucl.ac.uk/spm/software/spm12>). A custom brain template was created based on these data (Avants and Gee, 2004), and all resliced images were

registered to the template. Then we obtained gray matter (GM) and cerebrospinal fluid (CSF) segmented and modulated images using, first, the probabilistic maps of the Valdes-Hernandez template as priors, and second, the Jacobian determinants from the spatial normalization process. Modulated images were smoothed by a 10-mm FWHM Gaussian filter and then used for statistical analyses.

In addition, raw T2-images were registered to a common CT reference using the algorithms described in (Gasull-Camos et al., 2017). Then, 5 manual regions of interest (ROIs) were segmented on each MRI image in the hippocampus, ventricles, prefrontal cortex (PFC), cortex, and whole brain according to (Paxinos and Watson, 2008).

PET image post-processing and intensity normalization were performed as described in (Gasull-Camos et al., 2017). Briefly, all PET images were registered to the same stereotactic space as MRI images and normalized in intensity to a cluster of brain regions that do not show statistically significant differences between the groups, following a data-driven approach (Borghammer et al., 2009). Then, images were smoothed by a Gaussian kernel of 2.5 times the voxel size of FWHM and masked to eliminate extracerebral voxels from the voxel-based analysis.

Furthermore, 6 manual ROIs (whole brain, caudate-putamen, hippocampus, cortex, PFC, and amygdala) were segmented on a registered MRI according to (Paxinos and Watson, 2008) and applied to all PET smoothed images.

Biochemical Analysis Processing—Digital images of western blots were analyzed using densitometry (ImageJ, NIH). Values were normalized to the loading control (β -actin) and expressed as a percentage variation from control. Data from the activity assays were normalized to the protein content of each sample.

Voxel-Based Analyses—The statistical analyses were conducted using SPM12. Groups were compared using 2-way ANOVAs, and results were obtained setting a threshold of $P < .01$ uncorrected at voxel-level significance but cluster-based corrected by false discovery rate to avoid type II error. A 1000-voxel clustering threshold was applied to minimize type I error. PET results were obtained setting a threshold of $P < .05$ uncorrected at voxel-level significance but cluster-based corrected. A 50-voxel clustering threshold was applied.

Statistical Analysis of ROI and Biochemical Data—The normality and homoscedasticity of each variable were tested using Shapiro-Wilk's and Levene's test, respectively. Heteroscedastic or abnormally distributed data were transformed through a 2-step approach (Templeton, 2011) to ensure both homoscedasticity and normality. Data were analyzed by means of 2-way ANOVAs followed by Bonferroni post-hoc test ($P < .05$). Data were expressed as mean \pm SEM.

Results

Behavioral Study Results

Figure 1B shows the PPI results. RM-ANOVA reveals a significant MIS and prepulse effect. However, it does not reveal a statistically significant 3-way interaction (prepulse, MIS, and MIN).

Brain Volumetric Results

Manual ROI Analysis—A significant MIS effect was found in ventricular and hippocampal regions, with increased volume in ventricles and reduced volume in the hippocampus in MIS

vs VH animals (Figure 2A). No effects of MIN were observed. An interaction between MIS and MIN was found in the whole brain and hippocampus. Finally, post-hoc tests showed a significant increase of the whole brain and prefrontal cortex in MIS animals after MIN treatment.

VBM Analysis—MIN treatment produced significant GM enlargements in the cerebellum and inferior colliculus in VH animals (Figure 2B–C; supplementary Figure 1A). In addition, MIN enlarged the GM in areas of the cerebellum and brainstem and decreased it in the cortex and the hippocampal-thalamic area. Also, striatal shrinkage became evident in MIN-treated MIS animals compared with the VH-saline group (Figure 2B). Also, MIN diminished the third ventricle in MIS animals (Figure 2C).

Brain Metabolic Results

Manual ROI Analysis—A significant MIS effect was found in the caudate-putamen, with increased metabolism observed. No statistically significant MIN or interaction effects were observed (Figure 3A).

SPM Analysis—In VH animals, MIN did not modify brain metabolism (Figure 3B; supplementary Figure 1B). In MIS animals, MIN significantly diminished metabolism in the brainstem, cortex, and cerebellum, and it increased FDG uptake in the cingulate cortex and nucleus accumbens. Furthermore, similar results were obtained when comparing MIS-MIN animals with the VH-saline group.

Oxidative/Inflammatory Parameters

Table 1 and Figure 4 show the results of IOS analyses.

Prefrontal Cortex—A significant MIS effect was found in iNOS, KEAP1, and malondialdehyde and an almost significant effect was found in NQO1 ($P = .052$). A significant MIN effect appeared in HO1 and an almost significant effect in iNOS ($P = .053$). An interaction was found in iNOS, HO1, and GPx.

Hippocampus—A significant MIS effect was found in SOD. A significant MIN effect appeared in KEAP1, NQO1, GSH_{total} , $GSH_{free}/oxidized$ glutathione (GSSG), and GPx. No interaction was found.

Amygdala—ANOVA showed a significant MIS effect in iNOS, COX2, KEAP1, and HO1. A significant MIN effect appeared in HO1 and COX2. An interaction was found in HO1, SOD, GSH_{free} , and GSH_{total} .

Caudate-Putamen—A significant MIS effect was found in iNOS, KEAP1, and CAT. A significant MIN effect appeared in iNOS and HO1. An interaction was found in iNOS and SOD and almost significant in GPx ($P = 0.054$).

Discussion

In this study, MIN treatment during periadolescence in the MIS model (1) prevents structural abnormalities in the third ventricle, (2) modulates brain metabolism in the cerebellum and nucleus accumbens, (3) reduces the expression of some brain IOS markers, and (4) does not prevent the behavioral deficit in PPI. To our knowledge, this is the first work to tackle this topic using FDG-PET, together with VBM, adapted to rodents.

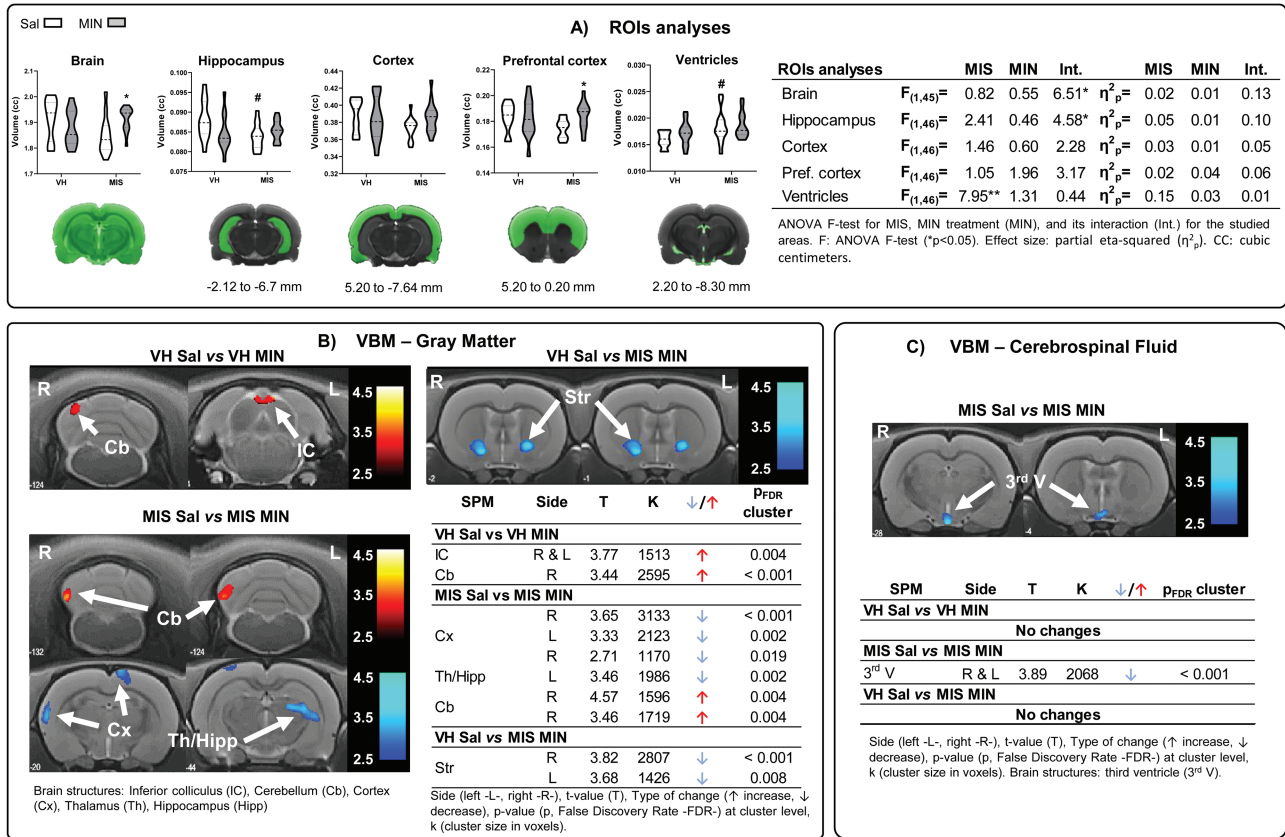


Figure 2. Volumetric changes measured via MRI. (A) ROI analysis: violin plots of global and regional volumetric changes in adulthood after MIN treatment during adolescence in saline and MIS animals (VH-Sal 11, VH-MIN 12, MIS-Sal 13, MIS-MIN 14). Two-way ANOVA followed by Bonferroni post-hoc test (* $P < .05$ vs saline-treated animals; # $P < .05$ vs VH-saline animals). Voxel-based SPM in (B) gray matter (GM) and (C) cerebrospinal fluid (CSF). VBM results in T-maps overlaid on a T2-MR template showing MIN pharmacological effect in VH and MIS animals. The color bars represent the T values corresponding to GM enlargements (warm) and shrinkages (cold). Tables show MIN-related effects on brain volumetric changes in the voxel-based analysis in the VH and MIS animals.

Furthermore, we observed specific MIN-related effects in VH animals (ie, GM enlargements in the cerebellum and inferior colliculus, reduced levels of iNOS and COX-2, and increases in HO1 and NQO1). However, long-term treatment with minocycline is generally safe and well-tolerated in humans (Garrido-Mesa et al., 2013a; Zheng et al., 2019). Furthermore, no pathological implication has been associated with the changes observed in VH animals and, thus, they may be assumable given the potential benefits that minocycline-derived protection may have for the patient.

MIN Does Not Prevent Sensorimotor Gating Deficits

Sensorimotor gating deficits are manifested in the rat MIS model at adulthood as loss of PPI (Hadar et al., 2015; Bikovsky et al., 2016). As expected, our adult MIS animals showed deficits in PPI. However, MIN treatment during adolescence was not effective in halting the progression into a schizophrenia-like behavioral phenotype after infection-mediated neurodevelopmental disturbances. It is noteworthy that the evaluation of other behavioral domains could have provided a broader perspective of the preventive potential of MIN, given the different neurodevelopmental trajectories followed by each specific symptom (Piontkewitz et al., 2012). Therefore, while no successful results were obtained in PPI, MIN could potentially prevent other schizophrenia-like traits shown by the MIS model.

Several preclinical studies have reported that acute MIN treatment decreases hyperlocomotion (Zhang et al., 2007; Giovanoli et al., 2016), improves visual-spatial memory (Levkovitz et al., 2007; Fujita et al., 2008), and improves PPI impairments in MIS models (Mattei et al., 2014; Zhu et al., 2014a, 2014b). In addition, a recent study showed the preventive effect of MIN in a mouse double-hit model of schizophrenia (Giovanoli et al., 2016), showing that MIN administration during the peripubertal stage blocked central inflammatory responses to stress and improved PPI deficits but only in those animals exposed to prenatal immune activation and peripubertal stress. However, as in our study, these improvements did not occur in animals only exposed to MIS (Giovanoli et al., 2016). Several reasons could explain these outcomes: (1) the different animal models used (rats or mice); (2) the regimen of MIN administration, including dose (3–35 mg/kg) and period of time (ie, during adolescence [PND30–50] (Zhu et al., 2014a; Giovanoli et al., 2016) or beginning at late adolescence [PND60] (Mattei et al., 2014); and (3) behavioral studies were conducted after a 2-week drug washout period, whereas examinations in previous studies took place shortly after MIN treatment. This is important, since MIN acts over activated microglia, suggesting the need for activated microglia to obtain a MIN-related benefit on the brain. Thus far, MIN has demonstrated some beneficial effects when it is administered as an adjuvant to risperidone or clozapine. In this sense, MIN improved the negative symptoms in early-phase (Liu et al., 2014) or persistent schizophrenia (Khodaie-Ardakani et al., 2014;

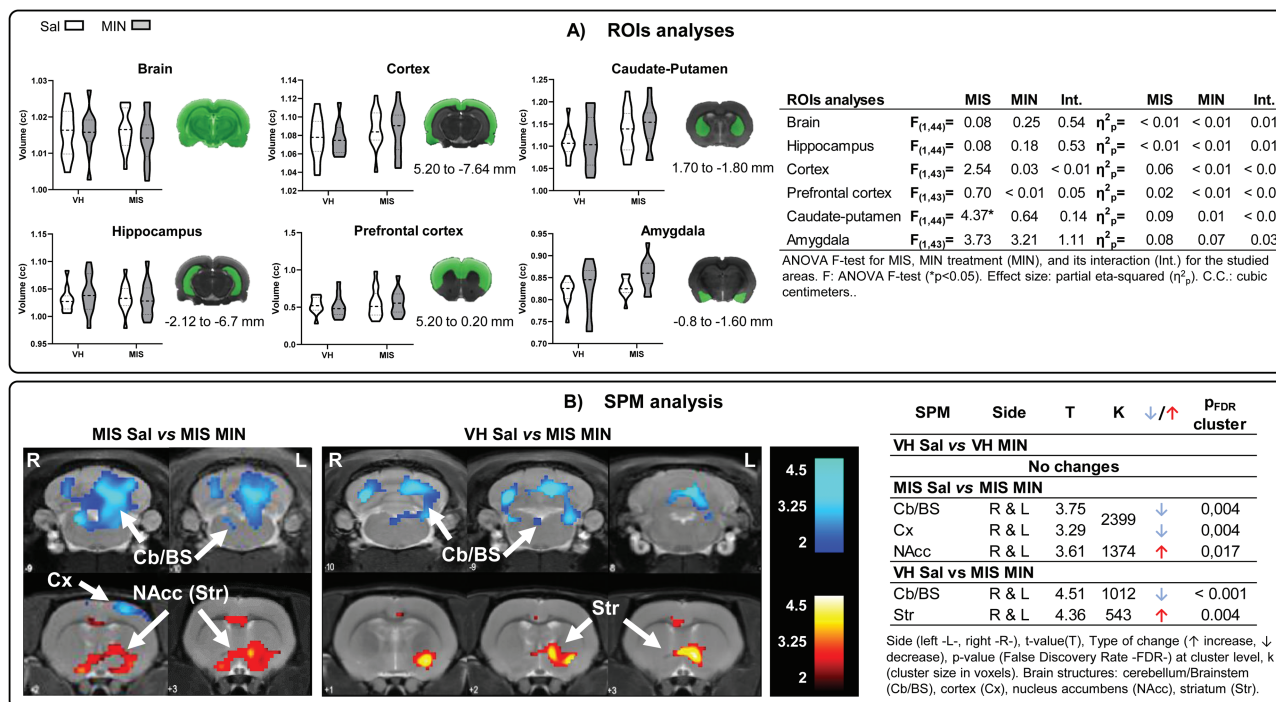


Figure 3. Brain metabolic changes measured via PET. (A) ROI analysis: ROIs were placed by identifying the 3D coordinates of each structure on the rat brain atlas (Paxinos and Watson, 2008) and locating the corresponding position in the MRI. Violin plots show the metabolic changes at adulthood after minocycline treatment during adolescence (VH-Sal 11, VH-Min 11, MIS-Sal 14, MIS-Min 12). Two-way ANOVA followed by Bonferroni post-hoc test was performed (* $P < .05$, MIS effect). Table shows minocycline-related effects on brain metabolism in Sal and MIS animals. (B) SPM analysis: colored PET overlays on the MR reference indicate reduced (blue) and increased (red) FDG uptake after minocycline treatment in MIS animals and compared with control animals (effectivity effect). The color bars represent the T value.

Kelly et al., 2015), and it also improved working memory, anxiety, and depression (Kelly et al., 2015), but its preventive potential has only recently been explored in humans. In this respect, only 1 study addresses a 6-month intervention with MIN added to regular treatment in individuals at risk of developing schizophrenia or psychosis (Qurashi et al., 2017), although no results have been reported yet.

MIN Prevents Volumetric Abnormalities in the Third Ventricle

Two of the volumetric traits induced by MIS are increased ventricular volume and decreased hippocampal volume (Piontkewitz et al., 2011; Hadar et al., 2018; Casquero-Veiga et al., 2019). Here, MIN treatment during peri-adolescence was effective in preventing this enlargement at the third ventricle level, although no hippocampal recovery was observed. Studies addressing structural brain changes in patients with early-phase schizophrenia showed a prevention of GM loss in the mid-posterior cingulate cortex and precentral gyrus after 1 year of MIN treatment as an adjuvant to patients' usual treatment with antipsychotic medication (Chaves et al., 2010). However, the BeneMin trial, performed in a study population comprising people within 5 years of a schizophrenia diagnosis, failed to show GM volume changes after 1 year of treatment (Deakin et al., 2018), which was related to a plateau in GM volume by the time of recruitment. These contradictory results highlight the necessity of future studies considering patients at early stages in which MIN could be effective. However, MIN's side effects (Utari et al., 2010), as well as the fact that not all at-risk people complete the transition to pathology, give the cost/benefit ratio of MIN particular relevance in selecting this kind

of preventive strategy. Therefore, further research in this topic is essential to consider MIN as a plausible strategy in schizophrenia prevention.

At the preclinical side, while several animal studies investigated the MIN effects through MRI techniques, many were performed in animal models of vascular neurological diseases (Tang et al., 2016; Dai et al., 2019), but none of them were conducted in schizophrenia models. Interestingly, one of these studies reported reductions in edema, microglia activation, and lateral ventricular volume, as in our study, in association with MIN use in a model of germinal matrix hemorrhage (Tang et al., 2016). Moreover, the protective effects of MIN were related to the activation of cannabinoid receptor 2 (CB2R) (Tang et al., 2016). In this respect, recent studies highlighted the potential of CB2R alterations in the neurobiology of psychiatric disorders (Ortega-Alvaro et al., 2011; Rodriguez-Munoz et al., 2017). In fact, first episode of psychosis (FEP) and patients with chronic schizophrenia have shown reduced levels of CB2R (Bioque et al., 2013), and the deletion of CB2R in mice has been related to schizophrenia-like behaviors (Ortega-Alvaro et al., 2011). In addition, CB2R are expressed in perivascular microglia, a special microglia with fast turnover and a crucial role in processes such as viral entry to the CNS (Nunez et al., 2004). In this context, our results suggest that MIN treatment may increase CB2R in the microglia, inducing a reduction of edema and lateral ventricular volume in our MIS model. However, further research is necessary to study the effect of MIN on the cannabinoid system in this animal model.

In addition, our VBM analysis revealed that MIN induced significant structural enlargements in the inferior colliculus (IC) in the VH animals, with a similar tendency observed for the MIS animals (supplementary Figure 1), the brainstem in the MIS animals, and the cerebellum in both groups. Reductions

Table 1. Continued

Inflammatory/oxidative markers	MIS	Treatment	Interaction
Hippocampus	$F_{(1,28)} = 1.72$	$F_{(1,28)} = 3.50$	$F_{(1,28)} = 1.01$
Amygdala	$F_{(1,28)} = 0.94$	$F_{(1,28)} = 0.11$	$F_{(1,28)} = 5.64^*$
Caudate-putamen	$F_{(1,28)} = 0.81$	$F_{(1,28)} = 0.26$	$F_{(1,28)} = 3.40$
GSHtotal			
Prefrontal cortex	$F_{(1,28)} = 3.41$	$F_{(1,28)} = 2.59$	$F_{(1,28)} = 0.003$
Hippocampus	$F_{(1,28)} = 1.43$	$F_{(1,28)} = 7.80^{**}$	$F_{(1,28)} = 1.59$
Amygdala	$F_{(1,28)} = 0.48$	$F_{(1,28)} = 1.04$	$F_{(1,28)} = 6.10^*$
Caudate-putamen	$F_{(1,26)} = 2.16$	$F_{(1,26)} = 0.98$	$F_{(1,26)} = 1.85$
GSHfree/GSSG			
Prefrontal cortex	$F_{(1,28)} = 0.002$	$F_{(1,28)} = 0.05$	$F_{(1,28)} = 1.27$
Hippocampus	$F_{(1,28)} = 2.07$	$F_{(1,28)} = 6.47^*$	$F_{(1,28)} = 0.12$
Amygdala	$F_{(1,27)} = 1.91$	$F_{(1,27)} = 0.83$	$F_{(1,27)} = 3.09$
Caudate-putamen	$F_{(1,28)} = 2.80$	$F_{(1,28)} = 0.04$	$F_{(1,28)} = 1.76$
GPx			
Prefrontal cortex	$F_{(1,27)} = 0.03$	$F_{(1,27)} = 0.14$	$F_{(1,27)} = 4.43^*$
Hippocampus	$F_{(1,27)} = 0.74$	$F_{(1,27)} = 7.35^*$	$F_{(1,27)} = 0.43$
Amygdala	$F_{(1,28)} = 0.08$	$F_{(1,28)} < 0.001$	$F_{(1,28)} = 1.38$
Caudate-putamen	$F_{(1,28)} = 1.52$	$F_{(1,28)} = 0.04$	$F_{(1,28)} = 4.01^{0.054}$
MDA			
Prefrontal cortex	$F_{(1,27)} = 6.56^*$	$F_{(1,27)} = 0.53$	$F_{(1,27)} = 0.53$
Hippocampus	$F_{(1,27)} = 2.03$	$F_{(1,27)} < 0.001$	$F_{(1,27)} = 1.62$
Amygdala	$F_{(1,28)} = 0.46$	$F_{(1,28)} = 0.49$	$F_{(1,28)} = 0.03$
Caudate-putamen	$F_{(1,27)} = 1.10$	$F_{(1,27)} = 0.39$	$F_{(1,27)} = 0.14$

Abbreviations: CAT, catalase; COX2, cyclooxygenase-2; F, ANOVA F-test ($P < .05$, $**P < .01$, $***P < .001$); GSH, glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; HO1, heme oxygenase 1; iNOS, inducible nitric oxide synthase; KEAP1, Keich Like ECH associated protein 1; MDA, malondialdehyde; NRE2, nuclear factor-erythroid factor 2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase-1; SOD, superoxide dismutase; η^2_p , partial eta-squared. Each column represents the ANOVA F-test and η^2_p for MIS, minocycline treatment, and its interaction for the studied areas.

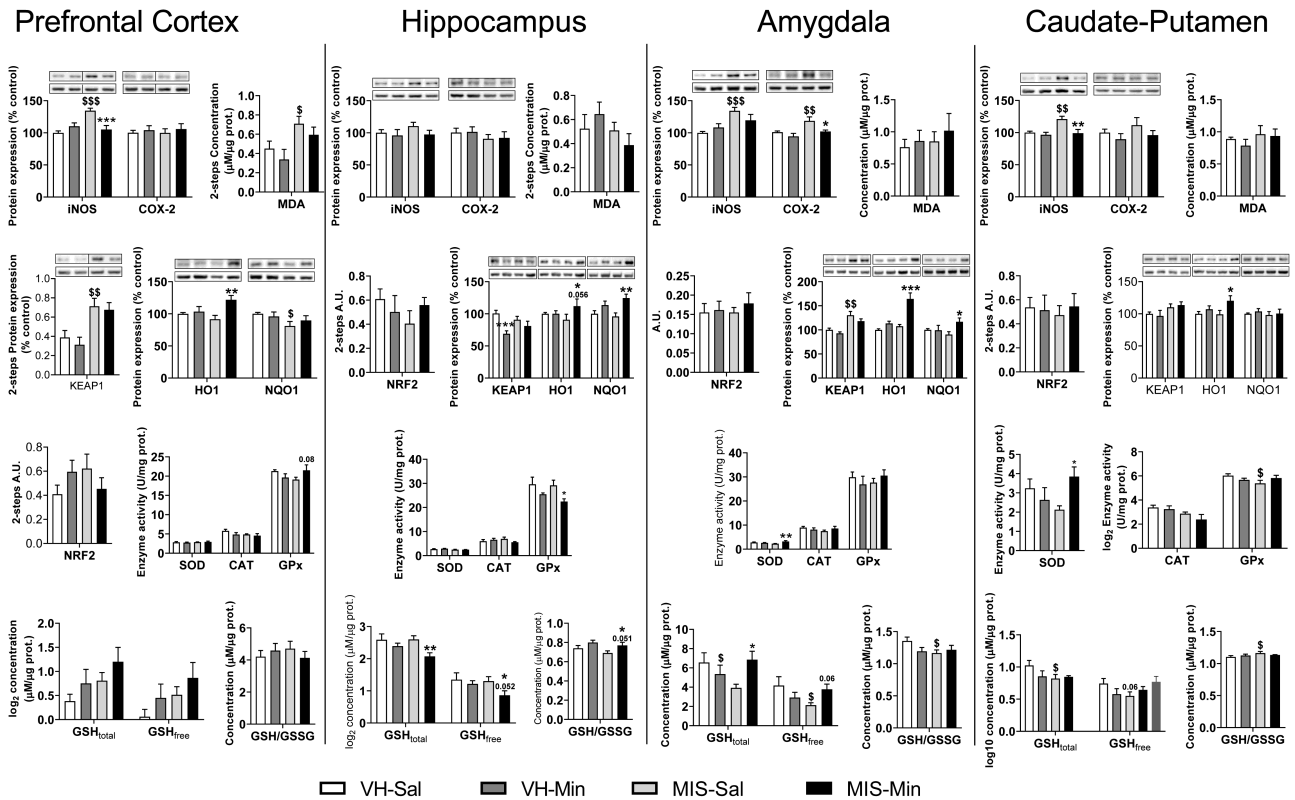


Figure 4. Minocycline-related effects on the expression of IOS markers in VH and MIS animals. Inflammatory markers expression (iNOS, COX2), antioxidant enzyme activity (GPx, CAT, SOD), and oxidative stress markers (MDA, NRF2, KEAP1, HO1, NQO1, GSH_{free}, GSH_{total}, GSSG) in prefrontal cortex, hippocampus, caudate-putamen, and amygdala (n = 7–8 animals). Representative bands of iNOS, COX2, KEAP1, HO1, and NQO1 (upper bands) and the loading control, β-actin (lower bands), are shown above their corresponding graph bars. Each column represents the mean ± SEM of 5–8 animals. Two-way ANOVA followed by a pairwise interaction contrast (*P < .05, **P < .01, ***P < .001, vs Sal-treated animals, &P < .05, &&P < .01, &&&P < .001 vs VH animals).

in the volume of the IC (Kang et al., 2008) and the cerebellum (Yeganeh-Doost et al., 2011) have been reported in patients with schizophrenia, and both structures have been associated with the auditory-cognitive dysfunction seen in this pathology (Horga et al., 2014; Cierpka et al., 2017). Moreover, abnormal glutamatergic neurotransmission was also shown in these structures (Schmitt et al., 2010; Yeganeh-Doost et al., 2011), and MIN acts on the glutamatergic system by modulating the N-methyl-D-aspartate receptor (Garrido-Mesa et al., 2013a), which is altered in schizophrenia (Anderson and Maes, 2013). We have found that MIN partially counteracts the structural shrinkage in these brain areas, probably by enhancing the glutamatergic neurotransmission (Sanchez-Perez et al., 2005; Chaves et al., 2009), reinforcing the idea that MIN could be useful for alleviating the auditory hallucinations in schizophrenia (Shergill et al., 2000).

Surprisingly, MIN-treated MIS animals showed striatal shrinkage compared with VH-saline animals. Striatal alterations are a well-known trait of schizophrenia patients (Ebdrup et al., 2010; Stegmayer et al., 2014; Okada et al., 2016) and also in subjects in the MIS model (Casquero-Veiga et al., 2019). When we explored whether these alterations could be influenced by the MIN treatment, we observed similar results with a less restrictive significance threshold than the one shown before in VH and MIS animals (supplementary Figure 1), suggesting that MIN is not able to counteract this striatal GM reduction compared with antipsychotics such as haloperidol (Konradi and Heckers, 2001; Andersson et al., 2002) or risperidone (Massana et al., 2005; Casquero-Veiga et al., 2019).

MIN Modulates the Cerebellum and Nucleus Accumbens Metabolism

PET studies in schizophrenia patients have shown cortical and subcortical dysfunctions, with reductions in glucose metabolism at global and regional scales compared with controls (Kim et al., 2017). To date, no FDG-PET study has evaluated the effect of MIN on brain metabolism. Nevertheless, huge efforts have been made in understanding the role of microglia and brain plasticity changes in acute psychosis and schizophrenia by using translocator protein-PET tracers (De Picker et al., 2017). However, results are contradictory: while activated microglia has been detected in the frontal and temporal lobes and the hippocampus in patients with ultra-high risk of psychosis (Bloomfield et al., 2016) and schizophrenia (van Berckel et al., 2008; Doorduyn et al., 2009), other authors could not find microglia changes in patients at various clinical stages (Kenk et al., 2015; Coughlin et al., 2016; Collste et al., 2017; Di Biase et al., 2017). The intrinsic limitations of translocator protein-PET could be responsible for these inconclusive data.

In our study, treatment with MIN during adolescence reduced brain metabolism in the cerebellum and increased FDG uptake in the nucleus accumbens in MIS animals, while it did not modify the brain metabolism in VH animals. The cerebellum plays an important role in the pathophysiology of schizophrenia (Moberget et al., 2018). Thus, disruptions in cerebrocerebellar functional connectivity may partially underlie the psychotic symptoms and cognitive deficits seen in schizophrenia (Andreasen et al., 1998) as well as the onset and severity of

neurodevelopmental psychiatric disorders (Sathyanesan et al., 2019; Dong et al., 2020). In vivo PET neuroimaging studies in controls and schizophrenia patients showed that the cerebellum is activated in a variety of mental activities, apart from its classical involvement in motor activity (Andreassen and Pierson, 2008). Preclinical FDG-PET studies showed reduced metabolism in the cerebellum at adulthood after risperidone treatment during adolescence (Casquero-Veiga et al., 2019) or during acute deep-brain stimulation in the medial PFC (Bikovskiy et al., 2016), both in the MIS model. In this sense, our results support the claim that MIN treatment during adolescence could modify cerebellar physiological activity, which could be related to the antidepressant properties attributed to this compound (Pae et al., 2008).

Unexpectedly, MIN treatment in our study increased FDG uptake in the nucleus accumbens in the MIS animals, which may be related to the striatal constriction produced by MIN and previously discussed. Abnormalities of the mesolimbic dopaminergic and reward systems have been reported in psychiatric disorders, such as schizophrenia or depression (Dubol et al., 2018). In the MIS model, abnormal increased glucose metabolism in the nucleus accumbens was also found, and it correlated with high levels of dopamine in this area compared with saline offspring (Hadar et al., 2015). However, we found that MIN treatment during adolescence did not revert the abnormally enhanced metabolic activity in the nucleus accumbens, suggesting a permanence of the exacerbated dopaminergic activity in the mesolimbic system. This fact could be responsible for our not obtaining any improvement in the PPI in the MIS animals, since the nucleus accumbens is involved in sensorimotor gating disruption (Geyer et al., 2001). Therefore, while MIN would not counteract the PPI deficits, schizophrenia could benefit from its use for the treatment of negative symptoms as adjunctive therapy (Khodaie-Ardakani et al., 2014; Liu et al., 2014; Deakin et al., 2018).

MIN May Prevent Brain Inflammatory and Oxidative Alterations

MIN was first shown to be an effective anti-inflammatory and antioxidant drug more than 2 decades ago, when 2 studies demonstrated minocycline's ability to attenuate iNOS expression (Amin et al., 1996; Lee et al., 2004). Here, MIS animals showed higher levels of pro-inflammatory molecules, mainly in the PFC, amygdala, and caudate-putamen, as previously reported (Casquero-Veiga et al., 2019). The 2-week MIN treatment during rat adolescence downregulated iNOS expression in the PFC as well as caudate-putamen and COX-2 expression in the amygdala, with similar trends in the hippocampus and amygdala for iNOS activity. Therefore, our results indicate that MIN exerts its anti-inflammatory action mainly through the iNOS pathway, like antipsychotics with anti-inflammatory activity such as risperidone (Casquero-Veiga et al., 2019). Previous preclinical studies have also demonstrated the iNOS downregulation induced by MIN in different animal models (Wu et al., 2002; Zheng et al., 2014) as well as other pro-inflammatory molecules, such as NF- κ B, in cell culture studies (Sun et al., 2015; Tian et al., 2017). Remarkably, only 1 study has explored MIN as a preventive strategy; it operated with a mouse double-hit model of schizophrenia, also showing beneficial effects of MIN in microglia activation and interleukin-1 β expression in the hippocampus and PFC (Giovanoli et al., 2016). Clinically, there are only 2 studies that have suggested the benefit of MIN in the regulation of negative symptoms and its association with pro-inflammatory cytokine levels (Zhang et al., 2018, 2019). Thus, the addition of MIN to

risperidone during a treatment course of 3 months reduced the serum levels of IL-1 β , IL-6, and TNF- α together with a significant improvement in negative symptoms (Zhang et al., 2018, 2019).

Furthermore, we found that MIN increased the activity/concentration of KEAP1 in the PFC, HO1, and NQO1 in the amygdala, HO1 in the caudate-putamen, and HO1 and NQO1 in the hippocampus only in the MIS animals. One possible explanation for the changes in the MIS animals may be related to the presence of high basal levels of oxidative stress: the bactericidal actions of minocycline prevail over its anti-inflammatory consequences, and hence its antioxidant effect may become more evident in a context of oxidative/nitrosative damage. These molecules are part of the Keap1-Nrf2-ARE (Antioxidant Response Elements) pathway, which is involved in the cellular defense system against oxidative stress. Previous preclinical studies have shown the acute effect of MIN in the modulation of oxidative damage by increasing the Nrf2 pathway (Sakata et al., 2012; Tian et al., 2017). Furthermore, MIN increased antioxidant molecules such as CAT, SOD, and HO1 after nigrostriatal dopaminergic damage in a Parkinson's Disease model (Kumar et al., 2016). However, as far as we know, no study has evaluated the preventive effect of MIN during adolescence on oxidative parameters at adulthood in either the MIS model, schizophrenia patients, or those identified to have a high risk of psychosis. Therefore, our results suggest that MIN improves the oxidative stress imbalance induced by MIS, mainly through increasing the expression of the Nrf2-ARE pathway.

Limitations

This study is subject to some limitations. First, we have evaluated only male animal subjects. The influence of MIS on males is well-validated, which is not the case in females, probably because females develop symptoms later compared with males and due to the need to control the estrous cycle. In this regard, additional studies will be necessary to investigate the effect of sex in the MIS model. Second, the voxel-level statistical analyses in SPM methods were not corrected for multiple comparisons. In this sense, Bonferroni correction results are too conservative for voxel-based image analyses because they consider independence of the voxels, which is not accurate due to the constant presence of spatial correlation between nearby voxels (Lieberman and Cunningham, 2009; Verger et al., 2018). However, we applied cluster-level corrections to prevent type I errors.

Conclusions

Our study demonstrates that MIN treatment during adolescence partially counteracts certain structural abnormalities and inflammatory/oxidative stress deficits via iNOS and Nrf2-ARE pathways in the MIS model, increasing the expression of cytoprotective enzymes. However, MIN treatment during this peripubertal stage does not prevent sensorimotor gating deficits in this model. Therefore, even though it does not prevent all the MIS-derived abnormalities evaluated, our results suggest the potential utility of early treatment with MIN in other schizophrenia domains.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Statement of Interest

None.

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