Minocycline treatment improves cognitive and functional plasticity in a preclinical mouse model of major depressive disorder

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Highlights

- Minocycline treatment improves learning and memory abilities in the short-term
- Minocycline enhances hippocampal functional plasticity in the short-term
- Minocycline does not affect microglial proportion and morphology in the long-term

Keywords

antidepressant; minocycline; neural plasticity; cognition; stress response; microglia

Abstract

Major depressive disorder (MDD) is a chronic, recurring, and potentially life-threatening illness, which affects over 300 million people worldwide. MDD affects not only the emotional and social domains but also cognition. However, the currently available treatments targeting cognitive deficits in MDD are limited. Minocycline, an antibiotic with anti-inflammatory properties recently identified as a potential antidepressant, has been shown to attenuate learning and memory deficits in animal models of cognitive impairment. Here, we explored whether minocycline recovers the deficits in cognition in a mouse model of depression. C57BL6/J adult male mice were exposed to two weeks of chronic unpredictable mild stress to induce a depressive-like phenotype. Immediately afterward, mice received either vehicle or minocycline for three weeks in standard housing conditions. We measured anhedonia as a depressive-like response, and place learning to assess cognitive abilities. We also recorded long-term potentiation (LTP) as an index of hippocampal functional plasticity and ran immunohistochemical assays to assess microglial proportion and morphology. After one week of treatment, cognitive performance in the place learning test was significantly improved by minocycline, as treated mice displayed a higher number of correct responses when learning novel spatial configurations. Accordingly, minocycline-treated mice displayed higher LTP compared to controls. However, after three weeks of treatment, no difference between treated and control animals was found for behavior, neural plasticity, and microglial properties, suggesting that minocycline has a fast but short effect on cognition, without lasting effects on microglia. These findings together support the usefulness of minocycline as a potential treatment for cognitive impairment associated with MDD.

1. Introduction

Major depressive disorder (MDD) is one of the leading causes of global disability worldwide [1] affecting over 300 million people [2]. Although MDD primarily involves mood disturbance, up to 70% of the patients suffer from cognitive deficits [3-7], including impairment in learning and memory, executive functioning, processing speed, attention, and concentration. These symptoms are highly heterogeneous in severity, duration, onset, and treatment response [8] and characterize not only the acute phase of the illness, but may persist in remission and worsen over time with repeated episodes [9-16].

Currently, few pharmacological treatments yielding direct pro-cognitive effects in depressed patients are available [8]. Vortioxetine is the first antidepressant approved by the Food and Drug Administration specifically targeting cognition [17] and able to improve multiple cognitive domains [18-22]. The therapeutic effect of vortioxetine depends on its modulation of a wide range of targets, including serotoninergic, dopaminergic, and noradrenergic neurotransmitters [19, 23]. However, alternatives to this drug are still very limited. Different compounds, from serotonin and norepinephrine reuptake inhibitors to ketamine and psychostimulants, have been explored for their capability to improve cognitive function. However, their efficacy and implementation in clinics are still debated [8].

Compelling evidence indicates that cognitive dysfunctions in mood disorders are characterized by high levels of pro-inflammatory markers and activation of the immune system response [24-26]. Therefore, there is a rising interest in anti-inflammatory drugs as an add-on or stand-alone treatment for cognitive deficits associated with MDD [27, 28]. Minocycline is an antibiotic able to pass the brain-blood barrier that exerts both anti-inflammatory and antidepressant actions [29, 30]. The beneficial effects of this treatment have been reported both in preclinical and clinical studies. Recent evidence shows that minocycline is able to attenuate deficits in learning and memory in animal models of neurodegeneration [31] and cognitive impairment [32]. In addition, its administration as an add-on has been associated with improvement in both negative symptoms and executive functioning in patients with schizophrenia [33, 34]. Thus, we hypothesized that this drug represents a promising treatment for cognitive impairment associated with MDD. To test this hypothesis, we exposed C57BL/6J adult male mice to

two weeks of chronic unpredictable mild stress to induce a depressive-like phenotype. Afterward, the experimental subjects received either vehicle or minocycline for three weeks while living in standard housing conditions. Our prediction was that minocycline treatment favors the restoration restoring of cognitive functions. We measured long-term potentiation and changes in microglia as these cells are reportedly involved in minocycline central action [35]. We performed the behavioral phenotyping by exploiting the IntelliCage system, an apparatus that allows for an automated, high-throughput, and continuous phenotyping of animal behavior in home cages [36, 37].

2. Materials and methods

2.1 Ethical standards

All procedures were carried out in accordance with the European and Italian legislation on animal experimentation (respectively European Directive 2010/63/UE and *Decreto Legislativo* 26/2014). Animals were examined for signs of discomfort as indicated by the animal care and use guidelines ("Guide for the care and use of laboratory animals", National Research Council 2003). The Italian Ministry of Health approved the protocol with permit number D9997.111.

2.2 Animals

Fifty-two C57BL/6J male mice 12–15 weeks old were used (animals for experimental conditions). Mice were obtained directly from Charles River Laboratories located in Calco (Lecco, Italy).

2.3 Housing conditions

Mice were kept under a reversed 12 light-dark cycle at 22–25°C. For the entire duration of the experiment, animals were housed in the IntelliCage system (TSE-system, NewBehavior AG, Zürich, Switzerland), which is an apparatus for automatic monitoring and measuring of mouse behavior. Intellicages are large acrylic cages (20.5 cm high, 58 cm × 40 cm at the top and 55 cm × 37.5 cm at the base, Model 2000 Tecniplast, Buguggiate, VA, Italy) with 4 walls separating each corner from the center so that they form 4 identical triangular conditioning chambers (15 × 15 × 21 cm). Animals have access to each chamber by entering a front hole and only a single mouse can enter a chamber at a time since each one is identified by a transponder. Two drinking bottles were placed in each corner and access to each solution was prevented by a door, thus, to drink mice had to perform a nosepoke. The system is able to collect data about the number and duration of visits as well as the number, duration, and side (right or left) of the nosepokes and licks.

The floor of the cage is covered with bedding while on the top a food rack is filled with standard mouse chow (food *ad libitum*). An additional cage (SocialBox) was used to expand the existing IntelliCage to

a multi-area system, allowing to increase in the number of subjects tested simultaneously. One week before being moved to the IntelliCage, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B Datamars SA, Switzerland). Then, mice were gradually habituated to the IntelliCage environment for 14 days (habituation period) and to 0.1% of saccharin solution. The IntelliCage system allows phenotype individual behavioral responses in a group of socially housed mice without any intervention by the experimenter reducing biases due to animal manipulation and exposure to the testing environment.

2.4 Treatment

After the first 14 days of stressful conditions aimed to induce a depression-like phenotype, mice received for 21 days minocycline or vehicle while they were exposed to the standard environment (Fig. 1). On the day immediately before the beginning of treatment, we split the animals in order to create two experimental groups with an overlapping behavioral profile.

To avoid stress due to the manipulation, minocycline (Minocycline Hydrocloride crystalline, SigmaAldrich, St Louis, MO, USA) was dissolved both in water and saccharin solution. During the treatment period, mice from each experimental group could access only the corner of the IntelliCage administering the treatment to which it was assigned. The solutions were prepared according to the mouse's average weight and daily water consumption to provide an average daily intake of 50 mg/kg of minocycline. The dose was selected based on the literature since it has been reported to suppress the activation of microglia in different mouse models of disease [38-40]. All the solutions were prepared fresh every 2 days because of the instability of minocycline in aqueous solution [41, 42].

2.5 Environmental conditions

Mice were first exposed to the stressful condition for 14 days to induce the depressive-like behavior and, immediately after, received the treatment for 21 days while kept under the standard condition (Fig.1).

Standard condition

In the standard condition, in addition to the different settings (e.g., cage *vs* operant chambers), provided by the IntelliCage system, mice were given Plexiglas shelters of different colors and shapes (e.g., four red transparent Tecniplast plastic nest boxes) and tissue papers [43, 44]. A new tissue paper was provided every five days, and the plastic shelters were cleaned every week.

Stressful condition

After the habituation period, mice were exposed to chronic unpredictable mild stress to induce depressive-like behavior. In stressful conditions, the IntelliCage system was used to constantly (24 hrs) expose animals to different stress procedures previously validated through the assessment of behavioral, cellular, and molecular endpoints [45-47]. The stressful conditions exploited in the present study have been shown to increase corticosterone levels, considered a marker of the stress response [48]. The stresses were delivered for 14 days and, to prevent habituation to the stress procedures, mice were exposed each day to a different stressful stimulus, randomly chosen. The procedures were: short open door: door to access solutions remains open for few sec; delay: door opens randomly with a delay of 1, 1.5, 2, 2.5 sec after the first nosepoke; open door 25%: door opens only following 25% of nosepokes; random air puff: randomly 1, 2, 3, or 4 sec after the first nosepoke, the animal receives an air puff (2 bar). The duration of each paradigm was randomly 12, 18, or 24 hrs.

Finally, during the stressful procedures, no shelter or tissue paper was provided. Thus, we can consider that the animals were under a continuous stress-like condition.

2.6 Behavioral tests

All experimental subjects underwent all testing procedures at all time points. These included liking- and wanting-type anhedonia, and place learning. The experimental procedures used to phenotype the selected behaviors were automatically administered by the IntelliCage avoiding any bias due to the experimenter. It is worth noting that the protocols and the tests we ran through the Intellicage system have been largely validated by us and other research groups [49-53].

Liking-type anhedonia - Saccharin preference

To assess liking-type anhedonia we measured saccharin preference. In each corner of the IntelliCage two bottles were present, one containing tap water and the other containing the saccharin solution; both freely available 24/24 hrs. The position of the water and saccharin bottles in each corner was counterbalanced across the four corners. Saccharin preference was determined as follows: [saccharin solution consumed/ (saccharin solution consumed + water consumed)] × 100. Baseline saccharin preference was measured as the mean of the last two days of the habituation period (i.e., Pre-stress), at the end of the stress exposure, (i.e., Post-stress), and following one (Short-term) and three weeks of treatment (Long-term; Fig. 2).

Wanting-type anhedonia - Progressive Ratio reinforcement schedule

To assess wanting-type anhedonia, i.e., the drive for obtaining a reward, we used the Progressive Ratio reinforcement schedule that utilizes a multiplicative increase in the number of responses (i.e., nosepokes) required to dispense a unit of reinforcement (i.e., saccharin solution). In particular, water was always accessible after one nosepoke while saccharin solution was accessible only after a specific number of nosepokes occurred. This number increases progressively after each series of eight visits according to the following sequence: 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, and 24. Each test session lasted 48 hrs or until mice reached the module with 24 nosepokes. The time for performing the nosepokes increased gradually according to the number of nosepokes requested from one to 24 sec. The test has been run immediately before and after the stress exposure, and following 3 weeks of treatment (Long-term). To make the mice aware of the testing condition, the green LEDs on the top of each door were kept turned on throughout the session.

Cognitive domain – Place Learning test

Place learning consists of a simple spatial task in which mice are allowed to drink in one corner, instead of all four. During the first phase (acquisition), the mice learn which corner is rewarded and which is not, leading to a visiting preference for the rewarded corner (% correct visits). In particular, the doors were closed in all corners but one. Following 24 hrs, the corner in which the bottles are accessible was switched to the respective opposite corner for 24 hrs (reversal). The animals were tested both after 3 days (Short-term) and 3 weeks (Long-term) of treatment.

2.7 Electrophysiology

To perform electrophysiological experiments, at the end of the treatment period, hippocampal slices were collected. Animals were anesthetized with halothane and decapitated. Whole brains were rapidly removed from the skull and immersed for 10 min in ice-cold artificial cerebrospinal fluid (ACSF), continuously oxygenated with 95% O_2 and 5% CO_2 to maintain the proper pH (7.4). Transverse 350 µm slices were cut at 4°C with a vibratome and the appropriate slices were placed in a chamber containing oxygenated ACSF. After their preparation slices were allowed to recover for 1 hr at 30°C. For field recordings, individual slices were then transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) maintained at 30-32°C and constantly superfused at a rate of 2.5 ml/min. At the beginning of each recording, a concentric bipolar stimulating electrode (SNE-100X 50 mm long Elektronik–Harvard Apparatus GmbH) was placed in the *stratum radiatum* for stimulation of Shaffer collateral pathway projection to CA1. Stimuli consisted of 100 µs constant current pulses of variable intensities, applied at 0.05 Hz. A glass micropipette (0.5–1MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200–600 µm from the stimulating electrode, in order to measure orthodromically evoked field extracellular postsynaptic potentials (fEPSP). Stimulus intensity was adjusted to evoke fEPSP of amplitude about 50% of the maximal amplitude with minimal contamination by a population spike. Evoked responses were monitored online, and stable baseline responses were recorded for at least 10 min. Only the slices that showed stable fEPSP amplitudes were included in the experiments. LTP was induced by high-frequency stimulation (HFS, 1 train of stimuli at 100Hz of 1 s duration), repeated after 30 min. To analyze the time course of the fEPSP slope, the recorded fEPSP was routinely averaged over 1 min (n = 3). The fEPSP slope changes following the LTP induction protocol at 25 and 55 min post-tetanus were calculated with respect to those of the baseline (1 min before induction). N/n refers to the number of slices on the total number of mice analyzed. The paired-pulse ratio (PPR) was measured from responses to two synaptic stimuli at 50 ms interstimulus interval. PPR was calculated as the ratio between the fEPSP amplitude evoked by the second stimulus (A2) and that by the first (A1; A2/A1). fEPSP were recorded and filtered (low pass at 1 kHz) with an Axopatch 200A amplifier (Axon Instruments, CA) and digitized at 10k Hz with an A/D converter (Digidata

1322A, Axon Instruments). Data acquisition was stored on a computer using pClamp 9 software (Axon Instruments) and analyzed offline with Clampfit 10 program (Axon Instruments). For each recording, we routinely normalize LTP value to the baseline, measuring the increment of the fEPSP slope 25 min post-tetanus compared to the baseline (1 min before induction). This normalization procedure allows comparing experiments with different basal values of fEPSP slope. Once normalized, we use statistical analysis (one-way ANOVA) to compare the amplitude of LTP at 25 min post-tetanus in all the slices of vehicle-treated versus the values of LTP measured in slices obtained by minocycline-treated animals, for both the first and the second stimulation.

2.8 Tissue collection, section preparation, and immunofluorescence

Mice were placed in a box containing ~ 3 % isoflurane (Iso-Vet) and injected with ketamine (160 mg/kg, Biowet Pulawy) and xylazine (Sedazin, Biowet, 20 mg/kg). Under deep anesthesia, they were perfused transcardially using a perfusion pump (BQ80S Microflow Variable-Speed Peristaltic Pump, Golander) which infused phosphate-buffered saline (PBS) through the left ventricle, until the fluid coming out of the heart was clear. For immunohistochemistry, mice were perfused with cold 4% (w/v) paraformaldehyde (PFA) in PBS (20-30 mL) followed by PBS. Mice were decapitated, then brains were rapidly removed and stored in 4° C PFA for 48 h. Subsequently, brains were transferred into 30% sucrose (w/v) in PBS for 48 hrs and frozen in a Tissue Freezing Medium (Leica) at -80°C.

Brains were cut into 12 μm slices taken rostrocaudally using a cryostat (Microm HM525, Thermo Scientific) at -18ºC and 4-5 sections were placed on polysine™ slides (ThermoScientific). From the prefrontal lobe, sections were prepared from a Bregma 2.34 - 1.94 mm, and from dorsal hippocampi and habenula slices were collected within a Bregma 1.22-2.30 (Paxinos & Franklin, 2001). Sections were stored at -80ºC until further processing.

For immunofluorescence, sections were thawed for 2 hrs, washed 3 times with PBS for 5 min with gentle rocking, then incubated with a blocking serum (D9663, Sigma, 10% in Tris-Buffered Saline, TBS) for 2 hrs. Sections were incubated with the primary antibodies recognizing TMEM119 (1:1000, ab209064 Abcam), Iba1 (1:200, E291118 Novus), Ki-67 (1:1000, ab66155 Abcam), and Doublecortin

(DCX; 1:1000, ab18723 Abcam), diluted in 3% block serum in TBS overnight at 4ºC. After washing three times with PBS, secondary antibodies: donkey anti-rabbit AlexaFluor 488 or donkey anti-goat AlexaFluor 555 (1:1000, Thermofisher Scientific) were applied for 2 hrs at room temperature. After washing 3 times with PBS, cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, 1:1000 in PBS, #D-9542, Sigma), washed and cover-slipped (Medlab, Marienfield) in fluorescence mounting medium (#S3023, Dako).

2.9 Cell quantification and confocal analysis

Fluorescent pictures were acquired using a fluorescent microscope (Leica DM4000B, Leica CTR6500, ebq100) and the Leica App Suite 2.8.1 software. We used 40x objective to obtain 2088 x 1560 pixels counting frames and collected 5 images per area per animal, in particular in three subareas of the medial prefrontal cortex (mPFC), cingulate cortex (CG), prelimbic cortex (PL), and infralimbic cortex(IL), and in three subareas of the dorsal hippocampus, CA1, CA3 and dentate gyrus (DG). We selected to focus on these specific brain regions since it has been reported that the dorsal hippocampus is involved in the response to different antidepressant treatments and that following stress exposure morphological changes occur in this area [47, 54].

Each image was collected in a given channel (blue, red, and green) in order to combine them during the image analysis.

Images were analyzed using the Fiji software (Image J 1.52p Wayne Rasband NIH, USA, 64 bits). A composite image was created with separate channels: green, red and blue for double-stained microglia (TMEM119, Iba1, and DAPI), or green and blue for DCX -positive cells (DCX and DAPI) or Ki-67. Percentages of positive cells were calculated based on the number of DAPI-stained nuclei per image, each area was averaged per animal.

Z-stacks (step size = 0.21 μm) of 20 cells (double-positive for TMEM119 and Iba1) per area/mice (around 2400 pictures) were acquired using the 63x immersion objective of a confocal microscope and numerical aperture 1.4 (LSM800 Zeiss Airyscan) with Zeiss ZEN software. The pictures were analyzed using the Fiji software (Image J 1.52p Wayne Rasband NIH, USA, 64-bits) as described (González Ibáñez et al., 2019). Measurements of the body size and branching area per cell were calculated.

To determine whether the treatment affects the percentage and morphology of microglia we selected two established microglial markers: Ionized calcium-binding adaptor molecule 1 (Iba1) and TMEM119, and we analyzed their immunoreactivity (IR). Iba1 is expressed by microglia and macrophages in health and disease, showing increased expression upon environmental challenges such as chronic stress [55]. TMEM119 is considered a microglia-specific transmembrane protein of unknown function [56]. This marker was shown to be relatively stable across various conditions with some exceptions [57]. We analyzed 20 cells/area/animal using confocal microscopy.

2.10 Statistical methods

The statistical analyses have been performed using the software Statview II (Abacus Concepts, CA, USA) and the R program (version 2022.07.1). All data were analyzed with one-way ANOVA to compare vehicle *versus* minocycline treatment. Stress exposure (Pre- and Post-), time (hrs and min), and sub-regions of each brain area were considered repeated measures within subjects. Concerning the Place learning test, the two phases (acquisition and reversal) have been considered independent measures. *Post-hoc* comparisons were performed using Tukey's test. All mean differences were considered statistically significant at $p < 0.05$. The final version of the graphs has been obtained with Adobe Illustrator software (version CC 2017).

3. Results

3.1 Minocycline improved cognitive abilities in the short-term

Depressive-like behavior

To evaluate the depressive-like phenotype, we assessed liking- and wanting-type anhedonia, previously shown to be affected by exposure to chronic unpredictable mild stress [46, 47, 58]. As expected, we found that following two weeks of chronic stress, all the experimental subjects displayed (i) a significant reduction in saccharin preference [$F(1,40)=39.691$, $p<0.0001$] and (ii) a significant decrease in motivation [$F(1,40)=15.338$, $p=0.0003$, Fig. 2A-B].

The switch to the standard condition led to an improvement of the depressive-like symptoms, both vehicle- and minocycline-treated mice increased their saccharin preference [respectively $F(1,20)=11.639$, $p=0.0028$ and $F(1,20)=4.639$, $p=0.0436$], with no difference between the two treatment groups [$F(1,40)=0.643$, $p=0.4273$]. The prolonged exposure to the standard condition led to an increase in liking-type anhedonia, and the long-term preference for the saccharin solution of both vehicle- and minocycline-treated mice was around 75% with no difference between the two experimental groups [$F(1,35)=0.388$, $p=0.5376$; Fig. 2A]. Accordingly, the wanting-type anhedonia was improved by the exposure to the standard condition for three weeks, although we did not observe a significant difference between vehicle- and minocycline-treated mice [$F(1,33)=0.094$, $p=0.7608$; Fig. 2B].

Cognitive abilities

Within the first week of treatment, we found the main effect of time in the acquisition phase, both vehicle- and minocycline-treated mice progressively increased their percentage of correct visits, [$F(1,120)=6.244$, $p=0.0006$], with no difference between the experimental groups [$F(1,40)=0.048$, $p=0.8285$; Fig. 2C]. A main effect of treatment emerged in the reversal phase, minocycline-treated mice displayed a significantly higher number of correct visits compared with vehicle-treated mice [$F(1,40)=9.827$, $p=0.0032$], suggesting that treatment enhanced cognitive performance. In addition, we found a main effect of time as well, with both groups significantly increasing the number of correct visits [$F(1,120)=9.951$, $p<0.0001$; Fig. 2C]. In both the acquisition and reversal phases of the place learning test at the end of the treatment period, the animals showed a significant increase in their number

of correct visits over time, indicating intact learning abilities and the capability of the animals to learn new spatial tasks [respectively $F(1,90)=7.257$, $p=0.0002$ and $F(1,96)=18.719$, $p<0.0001$; Fig. 2D].

3.2 Functional plasticity has been improved by minocycline treatment in the short-term

We explored LTP stimulating Schaffer collaterals with spaced (30 min apart) high-frequency stimulation (HFS) and analyzing LTP amplitudes 25 min after each stimulation. Following one week of treatment in the standard condition, during the second stimulation, we observed that LTP amplitude was significantly affected by treatment [$F(1,11)=4.572$, $p=0.05$]. In particular, minocycline-treated mice showed higher LTP amplitude (1.877 ± 0.0479, n=7 slice/3 mice) compared to vehicle-treated mice (1.654 ± 0.0984, n=8 slice/3 mice; Fig. 3A). By contrast, at the end of the treatment period, post tetanus LTP amplitude was not significantly affected by treatments (Fig. 3B, vehicle: n=18 slice/8 mice; minocycline: n=16 slice/7 mice).

3.3 Minocycline treatment does not affect microglial proportion and morphology in the long-term

When we analyzed microglial proportion and morphology following three weeks of treatment, we found a co-localization of TMEM119 IR with Iba1 IR. Quantification of the percentage of double-positive Iba1+Tmem119+ cells over all DAPI-positive cells revealed no statistically significant difference between vehicle- and minocycline-treated mice [$F(1,20)=0.001$, $p= 0.9991$ and $F(1,20)=2.390$, $p= 0.1173$] in the three selected subareas of the medial prefrontal cortex, nor in the three selected subareas of the dorsal hippocampus (Fig. 4A).

Double-positive Iba1+TMEM119+ cells were then selected for the quantification of the microglial cell body and branching area. We did not find statistically significant differences in the microglia soma size in the mPFC [$F(1,16)=0.667$, $p= 0.5268$] or in the dorsal hippocampus [$F(1,14)=0.415$, $p= 0.6680$; Fig. 5A].

When the branching of microglial processes was analyzed in the mPFC, there was no difference between vehicle- and minocycline-treated mice [$F(1,16) = 1.685$, $p = 0.2167$]. Similarly, there were no significant differences in the branching area in the hippocampus [$F(1,16) = 2.195$, $p = 0.1437$] between the two experimental groups (Fig. 5B).

These results suggest that minocycline treatment does not interfere with microglial physiological and immune functions, notably pertaining to their surveillance of the parenchyma, in the long run.

4. Discussion

Minocycline significantly enhanced cognitive abilities and neural plasticity in the short-term compared to vehicle. By contrast, both experimental groups equally improved their depressive-like profile, likely because during treatment they were no more exposed to a stressful environment.

As previously reported [46, 47, 58], the exposure to chronic stress was effective at inducing a depressive-like phenotype, as indicated by the significant increase in liking- and wanting-type anhedonia. In the short-term from the end of the stressful condition, all the tested animals showed increased saccharin preference. However, such an increase was no more evident in the long-term (Fig. 2A-B). This observation is in line with previous studies showing that the termination of the stressful condition improves the depressive-like phenotype, but a standard environment is not supportive enough to produce a long-lasting reduction of the anhedonic response [46, 59-61]. In this study, we did not find a significant difference in saccharin preference between minocycline- and vehicle-treated mice. Although a recent systematic review reported that this drug reduces the anhedonic-like behavior in rodents, the studies reporting an increased preference for the sweet solution following minocycline administration did not consider naïve animals, but individuals with an experimentally enhanced inflammatory state, such as those exposed to chronic stress, to olfactory bulbectomized, or affected by type-1 diabetes [62]. These preclinical findings have been confirmed in a recent randomized clinical trial demonstrating that add-on treatment with minocycline is effective only in depressed patients with high baseline levels of inflammation [63].

Minocycline treatment improved learning and memory in the place learning test in the short term as the treatment significantly increased the ability to learn a new spatial task. These results are concordant with those by Naderi and colleagues, who reported that minocycline administration for only seven days helps experimental subjects recover the memory deficits induced by cerebral ischemia/reperfusion in mice [32]. A similar effect has been described in a model of intracerebral hemorrhage: the treatment with minocycline for one week led to an improvement of the neurobehavioral performances and reduced cellular apoptosis and glial cell reactivity [64]. In Alzheimer's disease rodent models, minocycline administration attenuated the memory impairments shown during the Morris Water Maze test and

counteracted neuronal cell death [31]. Finally, the treatment has been proven to counteract the age-associated deterioration of memory and to ameliorate cognitive performance (e.g., spatial learning) in both adult and old mice [65, 66]. Although minocycline's mechanism of action remains unclear, it has been hypothesized that the observed beneficial effects were associated with anti-inflammatory and antioxidant properties [31, 32, 64, 65]. Indeed, the drug can downregulate microglial-driven inflammation and reduce neuronal death triggered by exposure to stress. At three weeks after stress, no difference between the minocycline and vehicle mice was found, all subjects displayed an increase in the number of correct responses during both the acquisition and reversal phases (Fig. 2C), suggesting that both groups recover learning and memory abilities in long-term. The rapid but short effect of minocycline on cognition after exposure to chronic stress could be explained by its ability to reduce microglial proliferation and reactivity when these phenomena are at a high level, which happens during and immediately after stress, when pathological conditions such as oxidative stress, cell damage, and death are in place [67-69]. Accordingly, a number of studies reported both neuronal and behavioral improvements following minocycline administration mostly in preclinical models of diseases associated with persistently increased inflammation [70-75]. These results are in line with the clinical evidence as a study showed that minocycline enhances the learning functions during navigation only in participants with a high body mass index, associated with higher inflammatory levels, while inducing cognitive impairments in participants with low or normal body mass index associated with lower immune activation [76].

To investigate neural modifications underlying the minocycline effects on cognition, we assessed hippocampal neural plasticity measured as LTP, which reflects some mechanisms involved in learning and memory [77-79], and in particular, in place learning, considered a hippocampal-dependent task [80]. We found that minocycline enhanced LTP in the short-, but not in the long-term (Fig. 3). It is worth noting that this temporal profile overlaps with the behavioral effects of minocycline, suggesting that the enhanced performance during the learning and memory task is associated with enhanced plasticity and LTP.

The immunohistochemical results showed a lack of minocycline effect on microglial morphology and proportion in the hippocampus and pre-frontal cortex following three weeks of treatment (Fig. 4A).

This is in line with our behavioral and electrophysiological results indicating a recovery within the first week after stress. Accordingly, previous studies found that minocycline action depends on the inflammatory status and that microglial reactivity is mainly affected in the short-term after stress, when an immune system activation occurs [68, 81-83]. However, here, the relation between inflammatory response and behavioral profile is still speculative because further data on microglial activation at different time points are still warranted.

The present study has some limitations. First, the lack of a non-stressed group did not allow to assess the effect of chronic stress by comparing stressed and non-stressed animals. However, we could measure the stress effect in a within-subjects analysis by comparing the behavioral profile of the animals immediately before and after the stress exposure. Second, we did not measure microglial proportion and morphology following the first week of treatment, when we observed the minocycline effects at behavioral and electrophysiological levels, which hinders potential microglial modifications underlying its efficacy in the short-term. In addition, we did not measure learning and memory immediately after the end of chronic stress to assess the behavioral response before treatment. This choice was motivated by the need to avoid excessive training due to the repetition of the same task.

The rising attention on cognitive symptoms associated with MDD is pointing out the need to develop novel antidepressant treatments able to target this domain in addition to the emotional one. In the present study, we showed that minocycline treatment can enhance neural plasticity and improve learning and memory abilities with a fast but short efficacy suggesting, from a translational perspective, that this drug is a promising candidate to treat cognitive impairments in depressed patients.

These results are of even greater impact when considering that treating cognitive residual symptoms has been shown to be pivotal to preventing new depressive episodes and the reportedly associated increased risk of neurodegenerative disorders [84].

5. Competing interests

Declarations of interest: none

6. Funding

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7. Author contributions

Silvia Poggini: participation in the design of the study, performing most of the experimental procedures, analyzing data, writing of the manuscript; **Maria Banqueri Lopez:** performing experimental procedures, analyzing data, writing of the manuscript; **Naomi Ciano Albanese**: performing experimental procedures and analyzing data; **Maria Teresa Golia** and **Fernando González Ibáñez:** performing part of the experimental procedures; **Marie-Eve Tremblay, Maciej Lalowski** and **Martin Furhmann, Cristina Limatola**: reviewing the manuscript; **Laura Maggi**: discussing data, writing and reviewing the manuscript; **Bozena Kaminska** and **Igor Branchi**: conceptualization of the study, results interpretation, writing of the manuscript.

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10. Figure legends

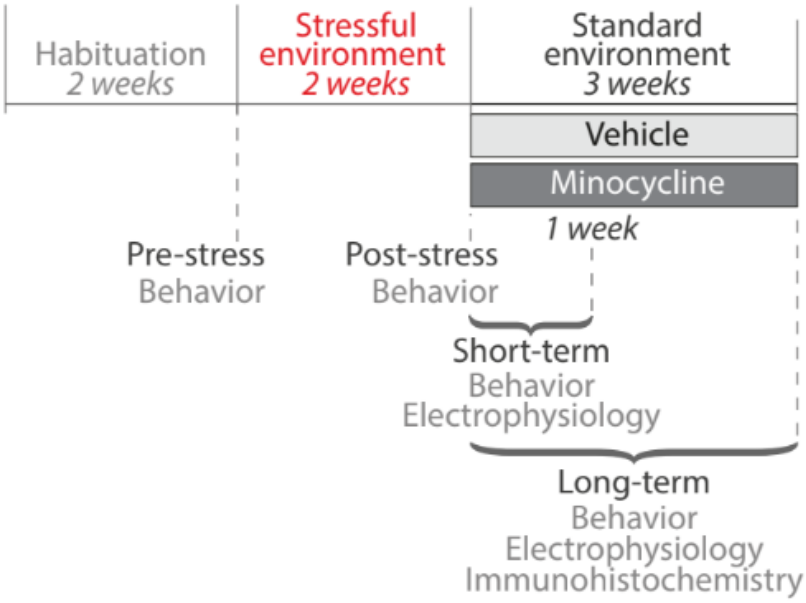

Fig.1 Experimental design. Following two weeks of habituation to the Intellicage, animals were exposed to two weeks of chronic unpredictable mild stress, to induce the depression-like behavior. Then mice were treated with vehicle or minocycline while they were exposed to the standard condition for three weeks.

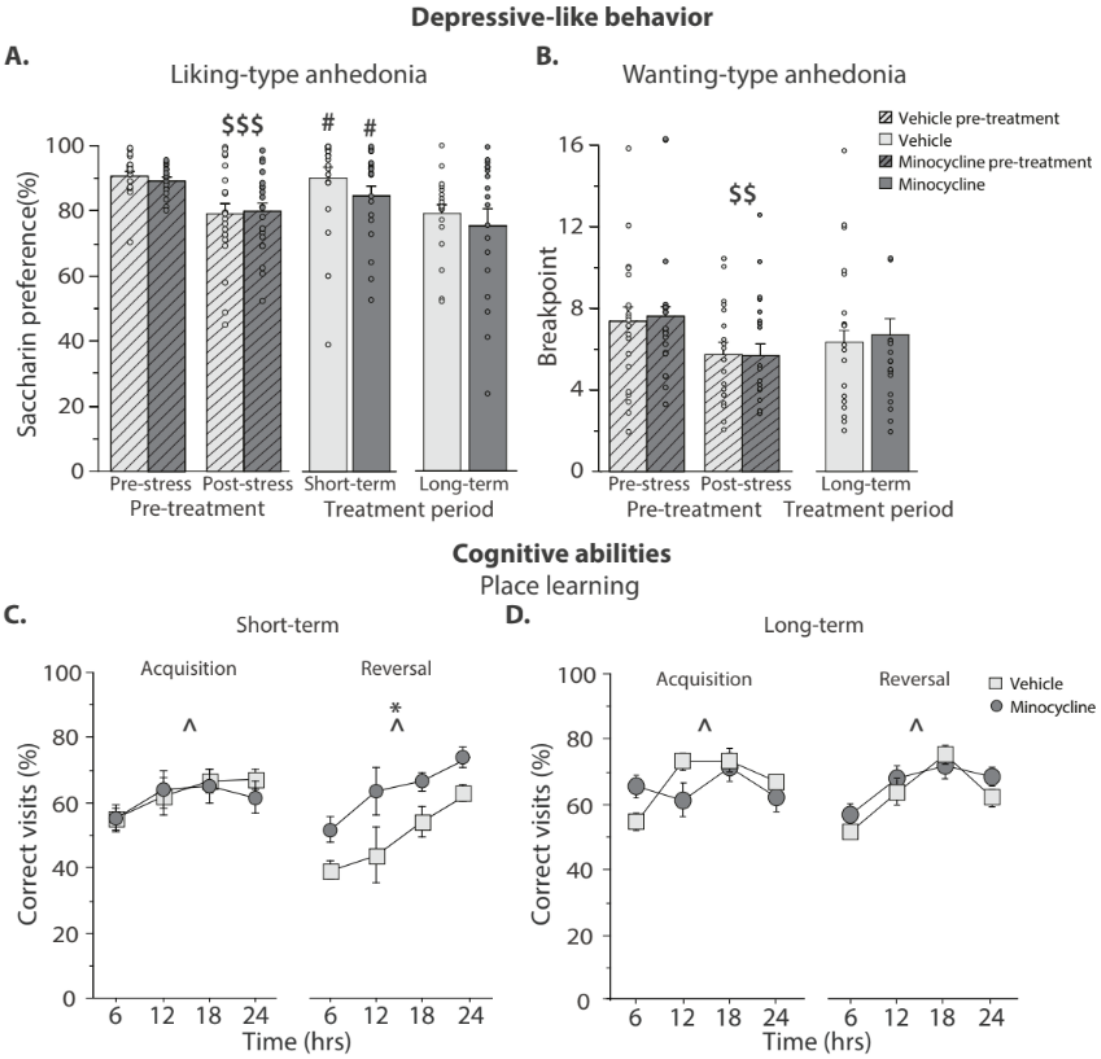

Fig 2. Depressive like-behavior. A. The exposure to two weeks of chronic mild stress induced a depressive-like profile as indicated by the significant decrease of saccharin preference displayed by the animals housed in the Intellicage system. When the animals switched to the standard condition, an increase in their saccharin preference was detected, although such improvement did not last long. n=18-19 per group. **B.** The exposure to chronic mild stress led to a significant increase in wanting-type anhedonia; following three weeks of standard condition, the motivation of the animals increased. n=17-18 per group. **C.** During the acquisition phase of the first place learning test, both vehicle- and minocycline-treated mice progressively increased their visits in the target corner. By contrast, in the reversal phase, minocycline-treated mice displayed a higher number of correct visits. When we run the second place learning test at the end of the treatment period, we observed that all the mice learned and re-learned the correct spatial task with no difference between the two experimental groups. $$ $p<0.05$ and $$$ $p<0.0001$ *vs* pre-stress; # $p<0.05$ *vs* post-stress within the same group; ^ $p<0.05$ main effect of the time, * $p<0.05$ main effect of the treatment, data shown as mean +s.e.m., n=21 per group.

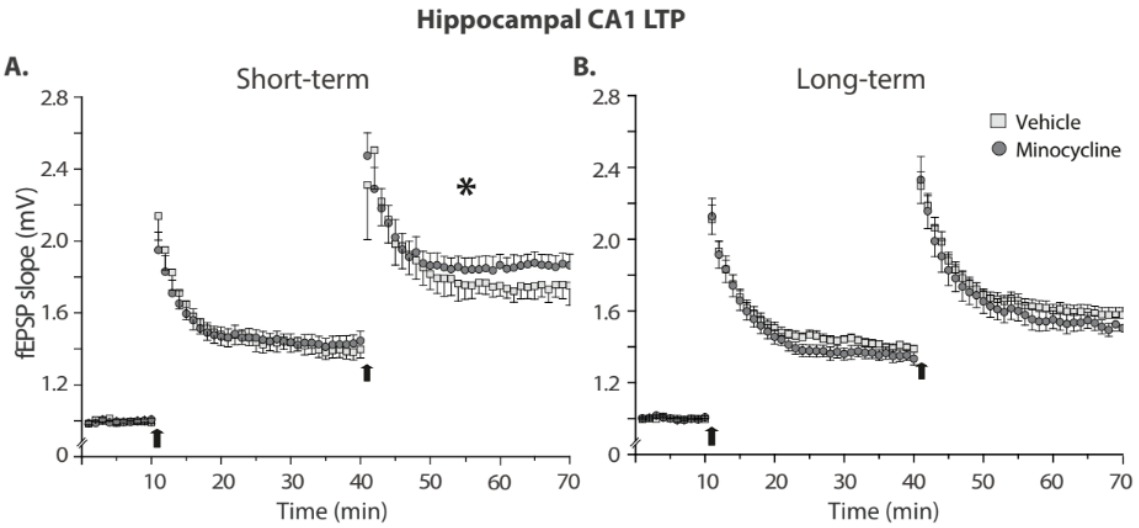

Fig 3. Hippocampal CA1 LTP. LTP of fEPSP slope from extracellular records made from vehicle- (white square; 8 slices /3 mice) and minocycline- (dark circle) treated mice (7 slices/3 mice) following one week (Short-term, **A**) or three weeks (Long-term, **B**) of treatment. Time course of slope values from responses evoked at 0.05 Hz and normalized as detailed in the Methods. Arrows indicate LTP induction (HFS, 1 train of stimuli at 100 Hz, of 1 sec duration). **A.** After one week of treatment, the electrophysiological assessment showed that, following two trains of stimulation, minocycline-treated mice displayed higher LTP compared to vehicle, indicating that minocycline increased neuronal plasticity. **B.** After 3 weeks of treatment, we did not observe any differences between minocycline and vehicle-treated animals, following both first and second stimulation fEPSP, field excitatory post-synaptic potential; *p=0.05 *vs* vehicle, data shown as mean +s.e.m., n=7-8 mice.

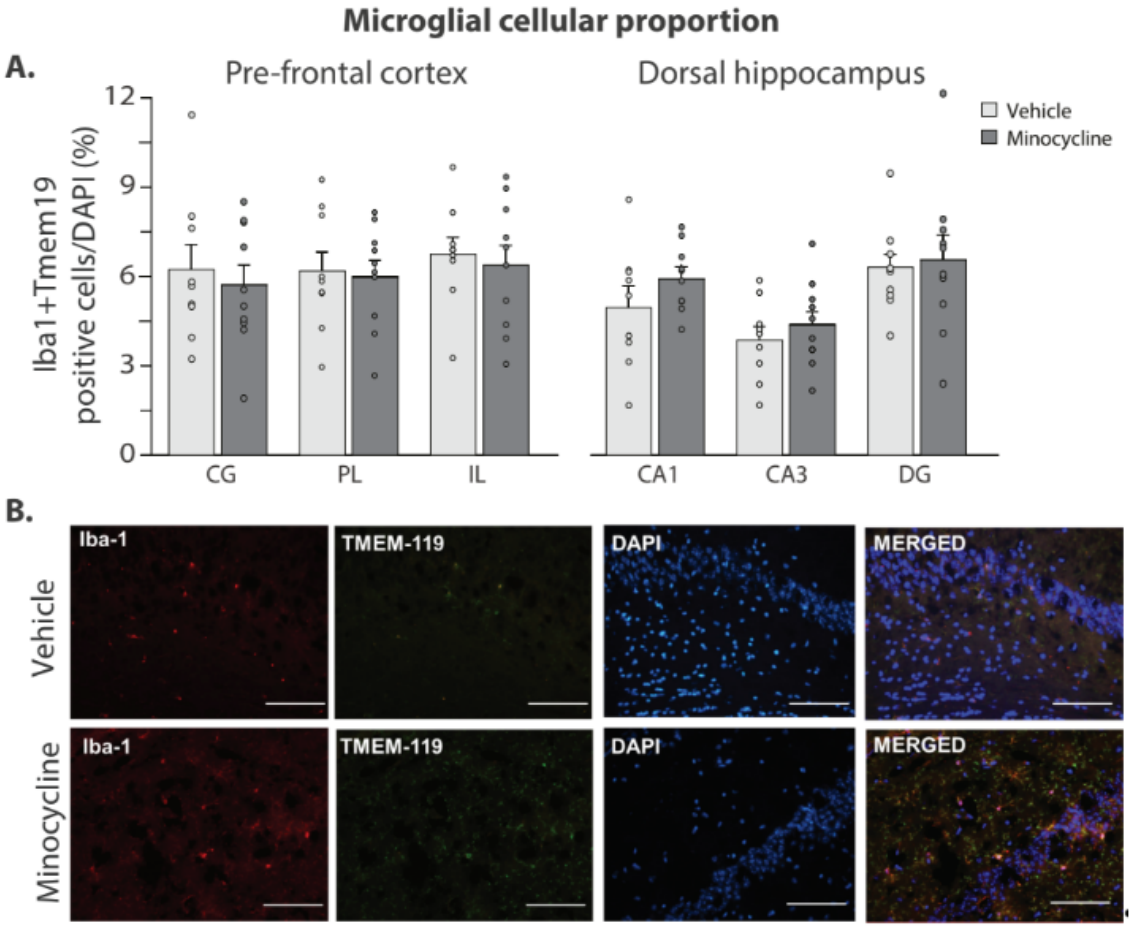

Fig. 4. Microglial cellular proportion. A. Percentages of Iba1 and TMEM119 positive cells (over all DAPI-positive cells) in different areas of the prefrontal cortex and dorsal hippocampus. No significant differences in the number of double-positive cells between vehicle- and minocycline-treated mice were detected at the end of the treatment period in the standard condition. Data shown as mean +s.e.m., n=5 per group. **B.** Representative images for assessment of a number of double-positive microglia. Photomicrographs from the hippocampus of a vehicle- (on the top) and a minocycline- (on the bottom) treated subjects: separate staining for Iba1, TMEM119, DAPI (to visualize nuclei) and merged. scale bar= 100 μm.

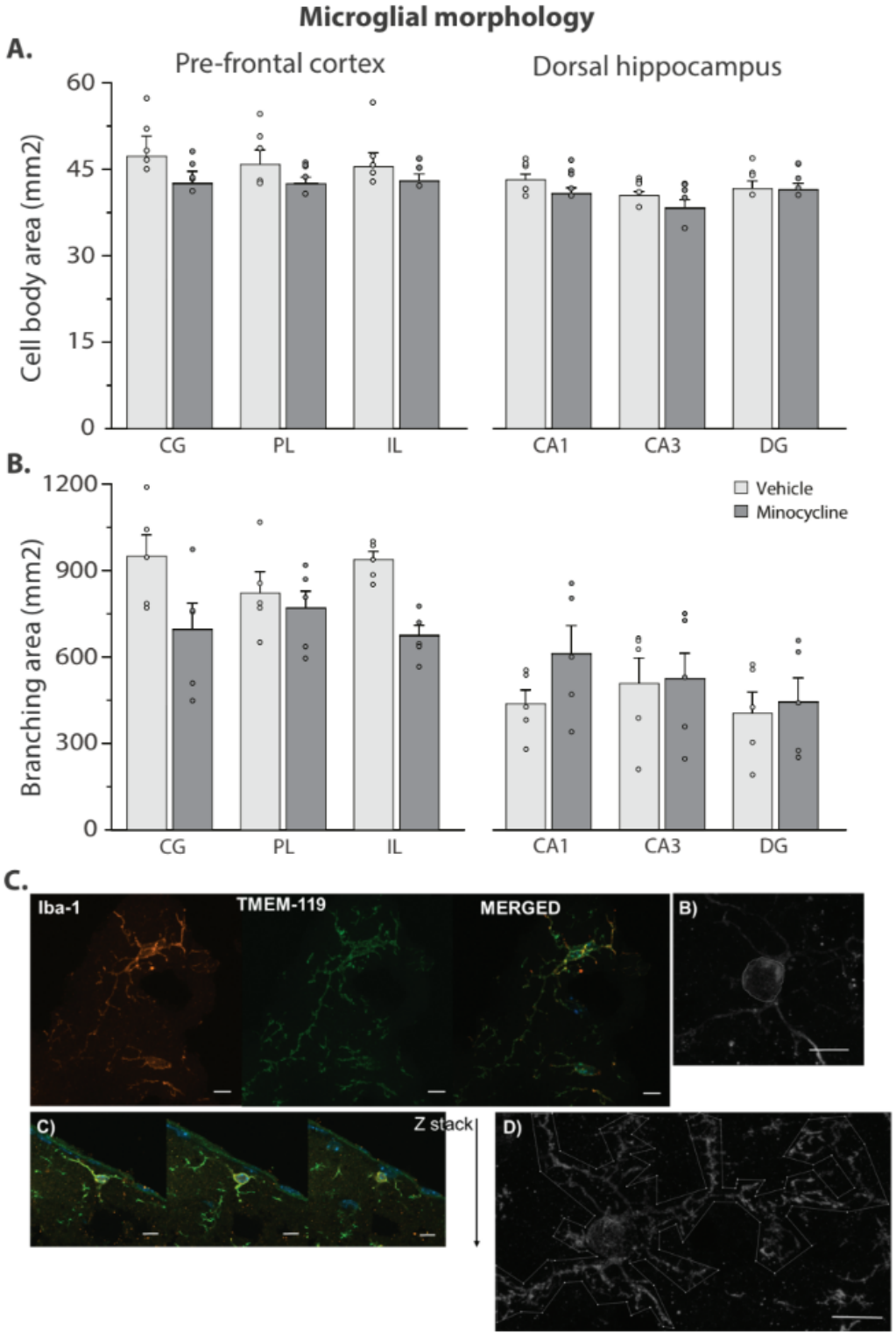

Fig. 5. Microglial morphology. Three weeks after the end of the treatment period, the morphology of microglial cells (TMEM119+Iba1+ cells) was assessed by measuring the size of their cell body area **(A)** and branching area **(B)** in the medial prefrontal cortex (mPFC) and in the hippocampus using confocal microscopy. The analyses did not reveal the statistical difference between the two experimental groups, although we noticed that in the prefrontal cortex minocycline treated mice showed

smaller microglial branching areas compared to vehicle animals. Data shown as mean +s.e.m. n=4-5 per group. **C.** Representative images of microglia for morphology assessment. Photomicrographs of two microglial cells with a large and a small tree size; staining for Iba1, TMEM119, and merged. An example of the morphology assessment shows a drawing encompassing the soma size area to calculate the area covered. View of microglia at the z-axis in the mPFC cortex. An example of a morphology analysis shows drawing the tree size area to calculate the area covered. Scale bar 10 μm. CG, cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; DG, dentate gyrus.

Supplementary

Minocycline did not affect neurogenesis in the dentate gyrus

To assess cell proliferation after three weeks of minocycline treatment, we quantified the Ki-67 staining in the dorsal hippocampus. We did not find a statistically significant difference between the two treatment groups [F (2, 14) =0.190, p= 0.8291; Fig. S1]. In addition, we investigated the expression of Doublecortin (DCX) in the dentate gyrus of minocycline- and vehicle-treated mice. DCX is a microtubule-associated protein expressed in immature neurons and required for neocortical and hippocampal development. This is a widely used marker to investigate adult hippocampal neurogenesis [1] and, in particular, stress-induced changes in the generation of new dentate gyrus granule neurons [2]. No significant difference was found [F (1, 15) =0.595, p= 0.4523; Fig. S1].

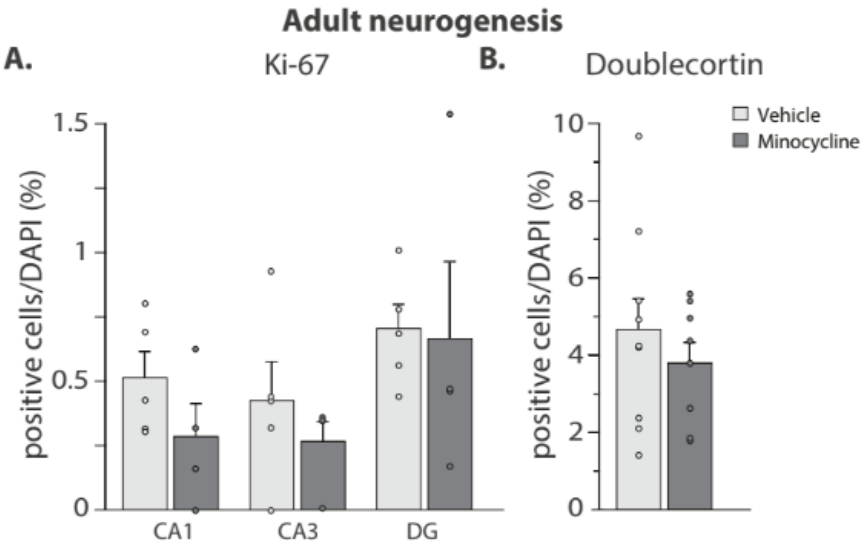

Fig. 1S. Adult neurogenesis.

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