Brain, Behavior, and Immunity 98 (2021) 13–27

Contents lists available at ScienceDirect

Brain Behavior and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

# Exercise protects from hippocampal inflammation and neurodegeneration in experimental autoimmune encephalomyelitis

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ARTICLE INFO

*Keywords:*
Multiple Sclerosis (MS)
Synaptic plasticity
Long term potentiation (LTP)
Neurodegeneration
Parvalbumin-positive (PV+) GABAergic interneuron
Microglia
Inflammation
Tumor necrosis factor (TNF)
Interleukin 1 beta (IL-1β)
Voluntary running wheel

ABSTRACT

Exercise is increasingly recommended as a supportive therapy for people with Multiple Sclerosis (pwMS). While clinical research has still not disclosed the real benefits of exercise on MS disease, animal studies suggest a substantial beneficial effect on motor disability and pathological hallmarks such as central and peripheral dysregulated immune response. The hippocampus, a core area for memory formation and learning, is a brain region involved in MS pathophysiology. Human and rodent studies suggest that the hippocampus is highly sensitive to the effects of exercise, the impact of which on MS hippocampal damage is still elusive.

Here we addressed the effects of chronic voluntary exercise on hippocampal function and damage in experimental autoimmune encephalomyelitis (EAE), animal model of MS. Mice were housed in standard or wheel-equipped cages starting from the day of immunization and throughout the disease course. Although running activity was reduced during the symptomatic phase, exercise significantly ameliorated motor disability. Exercise improved cognition that was assessed through the novel object recognition test and the nest building in pre-symptomatic and acute stages of the disease, respectively. In the acute phase exercise was shown to prevent EAE-induced synaptic plasticity abnormalities in the CA1 area, by promoting the survival of parvalbumin-positive (PV+) interneurons and by attenuating inflammation. Indeed, exercise significantly reduced microgliosis in the CA1 area, the expression of tumour necrosis factor (TNF) in microglia and, to a lesser extent, the hippocampal level of interleukin 1 beta (IL-1β), previously shown to contribute to aberrant synaptic plasticity in the EAE hippocampus. Notably, exercise exerted a precocious and long-lasting mitigating effect on microgliosis that preceded its neuroprotective action, likely underlying the improved cognitive function observed in both pre-symptomatic and acute phase EAE mice.

Overall, these data provide evidence that regular exercise improves cognitive function and synaptic and neuronal pathology that typically affect EAE/MS brains.

## 1. Introduction

Proper lifestyle and physical activity have been invariably associated with wellness, well-being and long-lasting remodelling of body functions in both humans and rodents (Hou et al., 2020). Of paramount interest in the context of neuroscience are the effects of exercise on brain plasticity and functioning. Chronic exercise is reported to improve cognition and memory (Creer et al., 2010; Van Praag et al., 1999) and to exert antidepressant and anxiolytic effects (Rebar et al., 2015; Siteneski et al., 2020). Data from human and animal studies suggest that the hippocampus is the brain area most sensitive to exercise (Hamilton and Rhodes, 2015). Regular aerobic activity and voluntary running-wheel

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https://doi.org/10.1016/j.bbi.2021.08.212
Received 15 February 2021; Received in revised form 30 July 2021; Accepted 7 August 2021
Available online 12 August 2021
0889-1591/© 2021 Published by Elsevier Inc.

have been shown to increase the volume of the hippocampus in healthy humans and in rodents, respectively (Biedermann et al., 2012; Erickson et al., 2011). The neurobiological mechanisms underlying these effects include improvement of neurogenesis in the Dentate Gyrus (DG) with new granule cell placement in the granule cell layer, potentiation of synaptic plasticity, and synaptic remodelling and release of neurotrophic factors (reviewed in Cooper et al., 2018).

Overall, the unquestionable beneficial effects of exercise for human health, supported by animal studies, have paved the way for the investigation of exercise as a prevention/supportive therapy for aging and chronic pathologies, including neurodegenerative diseases like Multiple Sclerosis (MS) (Motl, 2020). MS is a neurological disorder of the central nervous system (CNS), characterized by a dysregulated immune response against myelin epitopes, resulting in a diffuse demyelination, axonal pathology and neuronal dysfunction and death (Dendrou et al., 2015). MS lesions are spread throughout the spinal cord and the brain, including the hippocampus (Rocca et al., 2018). Functional dysconnectivity and dysregulated synaptic plasticity of the hippocampus have been proposed to contribute to symptoms of cognitive impairment and mood alterations in people with MS (pwMS) (Colasanti et al., 2016; Costers et al., 2021; Hammond et al., 2020; Novkovic et al., 2015; Rocca et al., 2018). Inflammation is proposed to drive white and grey matter damage in a parallel way, thus leading to changes in synaptic transmission even independently of demyelination (Mandolesi et al., 2015). In particular, in the hippocampus of mice with experimental autoimmune encephalomyelitis (EAE), animal model of MS, inflammation was shown to induce a profound subversion of synaptic plasticity with a significant potentiation of long-term potentiation (LTP) in the Cornu Ammonis area 1 (CA1), as a result of the detrimental synaptic effects of inflammation and the concomitant reduction of the GABAergic transmission (Nisticò et al., 2013). This latter was mainly associated with the loss of inhibitory parvalbumin-positive (PV+) interneurons, which, of note, is a neuropathological hallmark of MS brain (Clements et al., 2008; Dutta et al., 2011).

Different types of exercise have been shown to improve functional outcomes, like motor disability, and to dampen peripheral and central immune response in EAE (Rossi et al., 2009; Einstein et al., 2018; Xie et al., 2019; Souza et al., 2017). In addition, in EAE mice voluntary running wheel was shown to counteract dendritic spine loss in the striatum (Rossi et al., 2009), to attenuate apoptosis and improve neurogenesis in the DG, and to alleviate memory dysfunction (Kim and Sung, 2017). However, the effects of exercise against neurodegeneration and synaptic plasticity changes in the EAE hippocampus have not been explored so far and this is the aim of the present study.

## 2. Materials and methods

### 2.1. Animals

Female C57BL/6 mice (5–6 weeks of age) were purchased from Charles-River (Italy). Mice were kept under standard housing conditions with a light/dark cycle of 12 h, in a temperature-controlled environment (22 °C, 50–60% humidity) and free access to food and water. Animal experiments described in this study were conducted according to the guidelines set by the Internal Institutional Review Committee, the European Directive 2010/63/EU and the European Recommendations 526/2007 and the Italian D.Lgs 26/2014. All efforts were made to minimize the number of animals used and their suffering. The choice of female sex is of relevance for MS, due to the high prevalence of the disease in female versus male subjects (Sellner et al., 2011). All experiments except for nest building were performed blinded. A total of four independent immunizations of a minimum size of 6 animals per group were performed.

### 2.2. EAE induction and exercise protocol

EAE was induced in 9-week-old female C57BL/6 mice after at least two weeks of acclimatization. Exercise was started the day of immunization (day 0). Mice were randomly assigned to standard or running wheel-equipped cages. Running activity was monitored through a revolution counter provided with each cage which counts whole revolutions of the activity wheel. Briefly, mice were injected subcutaneously with an emulsion containing 200 μg of myelin oligodendrocyte glycoprotein 35–55 ($MOG_{35–55}$) in Complete Freund's Adjuvant (CFA), followed by intravenous administration of pertussis toxin (500 ng) twice (at days 0 and 2) as previously described (Gentile et al., 2014). Control mice, hereafter referred to as CFA, received the same treatment as EAE mice without the MOG peptide, including complete CFA and Pertussis toxin. Thus, four experimental groups were included in this study: EAE-sedentary (EAE-SED), EAE-exercise (EAE-EX) and relative controls CFA-sedentary (CFA-SED) and CFA-exercise (CFA-EX) (see also Fig. 1A). Mice were daily scored for clinical symptoms of EAE according to the following 0–5 scale: 0 no clinical signs; 1 flaccid tail; 2 hindlimb weakness; 3 hindlimb paresis; 4 tetraparalysis; and 5 death due to EAE; intermediate clinical signs were scored by adding 0.5. For each animal, the onset day was recorded as the day post immunization (dpi) when it showed the first clinical manifestations (score $> 0$). $N = 6–12$ mice per group. For experimental design and timeline of mice observation and behavioral, electrophysiological and biochemical outcome assessment, please refer to Fig. 1A.

### 2.3. Behavioral assessment

Behavioral study was designed to minimize the wheel-free period for the exercise group. Animals were tested during the light period (9:00–12:00 am) in a dedicated room with a constant temperature (26 ± 1 °C). Each session was preceded by at least 1 h habituation in the behavioral room.

#### 2.3.1. Novel object recognition test (NOR)

The NOR test has been developed to study learning and memory in rodents and is based on their spontaneous tendency to have more interactions with a novel than a familiar object (Lueptow, 2017). To avoid any potential factor influencing mouse interest in running wheel that in turn could impact on total amount of exercise, we tested mouse memory recognition skills through a one-day protocol of novel object recognition (NOR) test, according to a published protocol (Borreca et al., 2020). By this task, we analysed cognition ability in EAE-SED and EAE-EX mice during preclinical phase (7–8 dpi). The behavioural paradigm consisted of three phases. During the familiarization period (open field exploration) each mouse was placed in an empty squared open field (40 cm inside) surrounded by 60 cm-high walls and left free to explore it for 10 min. The mouse was returned to its home cage for a 10-min pause during which the arena was equipped with two identical cylindrical green objects. In the second session (training), the mouse was placed in the centre of the open field and allowed to explore left (sx) and right (dx) objects for 10 min. The mouse was returned again to its home cage for a 1 h-pause during which one (sx or dx) familiar object (FO) was substituted with a novel object (NO), a blue cone. In the third session (testing), the mouse was placed again in the centre of the open field and allowed to explore the FO and the NO for 10 min. The objects were cleaned with 10% ethanol between each session. Mouse behavior was recorded by a camera positioned directly above the box and subsequently analysed using Ethovison software (Noldus, Netherlands), drawing a 15 cm diameter circle zone around the objects. The preference index was calculated according to an already published formula (Antunes and Biala, 2012) as the percentage ratio between the time spent at one object divided by the time spent at both objects in the training and test phase. $N = 11–12$ per group.

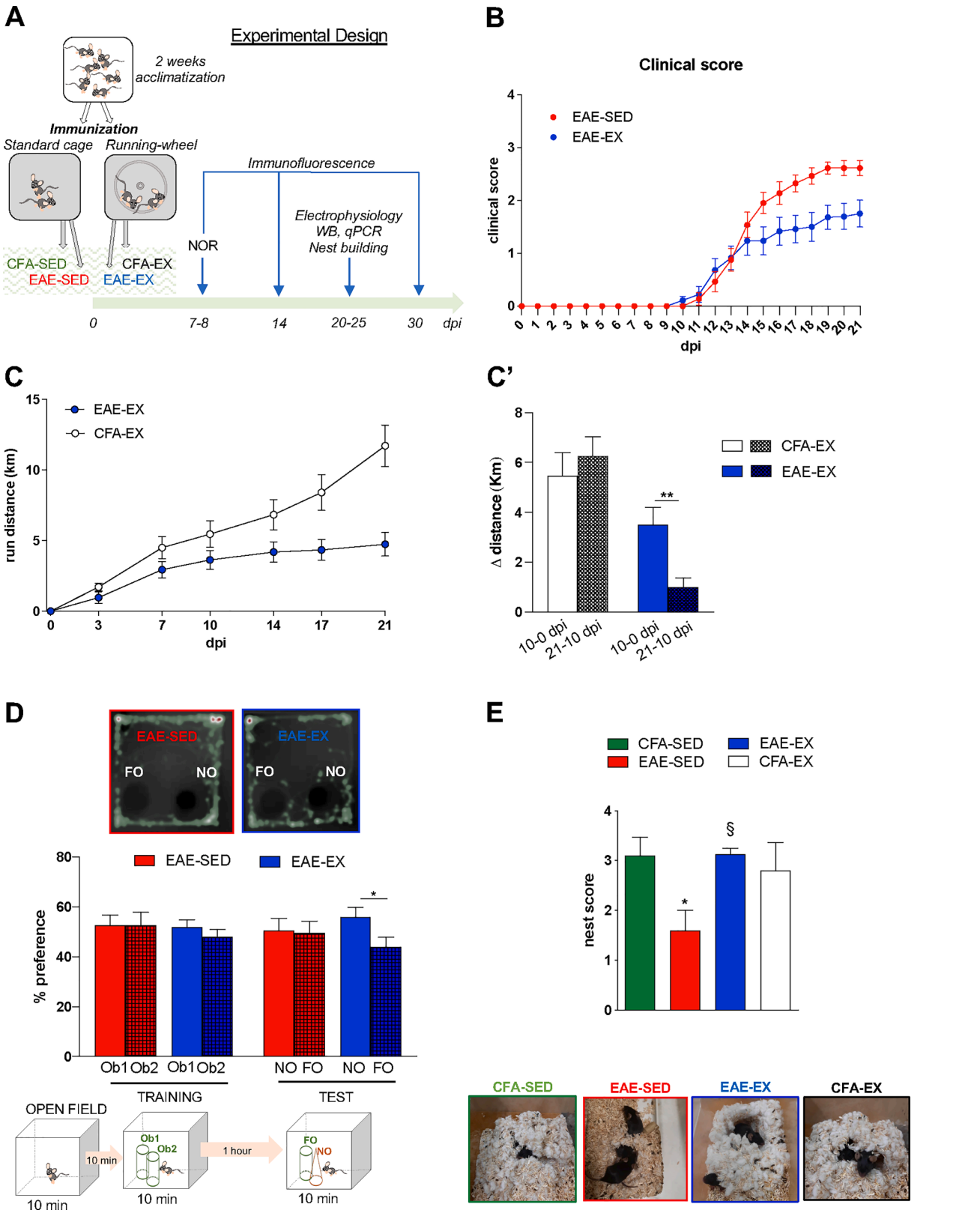


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**Fig. 1. Exercise ameliorates motor and cognitive dysfunctions in EAE mice.** (A) Schematic representation of the experimental design. After two weeks of acclimatization, the day of immunization mice were divided in 4 groups: mice immunized with $MOG_{35-55}$ peptide and housed in standard cage (EAE-SED) or wheel-equipped cages (EAE-EX), control mice free of $MOG_{35-55}$ peptide and housed in standard cage (CFA-SED) or wheel-equipped cages (CFA-EX). After behavioral testing, mice were sacrificed to perform electrophysiology, molecular and biochemical studies at different dpi. (B) The graph shows representative clinical courses of EAE-SED and EAE-EX mice (two immunizations pooled together). Running wheel exposure significantly ameliorated EAE disease progression (time × PA interaction ***$p < 0.001$). N = 18–20 per group. (C) The graph represents the running distance (Km) of CFA-EX and EAE-EX mice over the observation time showing that CFA group keeps running over time while EAE mice reduce their activity from 10 dpi on (time × PA interaction ***$p < 0.001$). N = 18–20 per group. (C') The difference of distance (Δ) covered by EAE mice is reduced soon after the disease onset (dpi 21–10 *vs* 10–0). Mann-Whitney **$p < 0.01$. (D) Exercise rescues short-term memory deficit induced by EAE. The time spent exploring each object was recorded (during both the training and the test phase) calculating the preference index for each object (time exploring one object divided by the time exploring the two objects*100). In both experimental groups, bars on the left show a similar exploration rate for the two objects (ob1 and ob2) during the training phase. The bars on the right show that EAE mice had the same preference index for familiar object (FO) and novel object (NO) while EAE-EX spent more time exploring the NO. On top of the graph, examples of video tracking during the test phase. On the bottom, a cartoon showing the behavioral protocol used. N = 11–12 per group. Unpaired T-test * $p < 0.05$. (E) The histogram shows that exercise prevents the EAE-induced impairment of nest building ability. Photographs on the bottom are examples of nests built by CFA-SED, EAE-SED, EAE-EX and CFA-EX mice. *Mann-Whitney $p < 0.05$ EAE-SED *vs* CFA-SED; §Mann-Whitney EAE-SED *vs* EAE-EX $p < 0.05$. N = 8–10 per group.

#### 2.3.2. Nest building

During the whole experimental procedure, mice were couple housed in cages with a running wheel for exercise groups or no enrichment for standard groups. To evaluate cognitive behavior in a non-invasive manner we evaluated the quality of nest construction in their home cage. To assess the ability of mice in nest building, 1 h before dark phase a pre weighted roll of cotton was posed aside in the cage-top food hopper. The distance between the cotton in the cage-top food hopper and the flat of the cage was lowered by adding the same amount of sawdust in each cage to allow motor impaired-EAE mice to reach the cotton as in Gentile et al., 2014 (Gentile et al, 2014). The morning after the quality of the nest was evaluated using the following scoring system: (1) no nest, (2) platform-type nest consisting of a pallet on the floor of the cage, (3) bowl- or cup-shaped nest with sides, or (4) bowl- or cup-shaped nest with sides and a cover (Bulloch et al., 1982). Animals' behavior was analyzed by trained observers blind to treatment and experimental groups. N = 8–10 per group

### 2.4. Electrophysiology: Extracellular field recordings

EAE-SED, CFA-SED, EAE-EX and CFA-EX mice were sacrificed by cervical dislocation in the acute phase of the disease (21–28 dpi) and their brains were quickly removed and placed in cold artificial cerebrospinal fluid (ACSF, 4 °C) composed of (in mM): NaCl 126, KCl 2.5, $NaH_2PO_4$ 1.25, $CaCl_2$ 2.4, MgCl 1, $NaHCO_3$ 26, D-glucose 11 and saturated with 95% $O_2$, 5% $CO_2$. Sagittal hippocampal slices (300 μm thickness) were prepared using a Leica VT1200S automatic slicer and left to recover for 1 h in oxygenated ACSF at room temperature. After recovering, the slices were transferred to the recording chamber continuously perfused with warm oxygenated ACSF (32 °C, ~3 ml/min) for extracellular recordings. Field excitatory postsynaptic potentials (fEPSPs) were evoked with a tungsten bipolar electrode stimulating the Schaffer Collaterals at 0.033 Hz. Extracellular recording electrode filled with ACSF (2 MΩ) was placed in the stratum radiatum of CA1. At the start of each experiment, the stimulation strength was established to evoke the 50% of the maximum fEPSP amplitude. After a stable baseline recording of 20 min, LTP was induced by a high-frequency stimulation protocol (HFS; single train of 100 Hz, 1 s duration). Signals were amplified (Multiclamp 700B, Molecular Devices) and low pass filtered at 0.3 kHz. In a subgroup of EAE-EX slices, picrotoxin (50 μM, Sigma) was added to the perfusing bath solution to block GABA-mediated transmission. Resulting data were analyzed offline using the Software pClamp 10.6 (Axon Instruments). Statistical significance of fEPSP slopes were evaluated between 50 and 60 min following the delivery of conditioning trains.

### 2.5. Hippocampal protein extraction and western blot (WB)

Animals were sacrificed by cervical dislocation at 21 dpi. Both the left and the right hippocampus were quickly removed and frozen until use. Tissues were homogenized in RIPA buffer plus protease and phosphatase inhibitor cocktails (SIGMA) as in Gentile et al., 2014. After sonication, the homogenates were centrifuged at 13000 × g for 20 min and the supernatant was collected. Protein content was quantified according to the BCA Assay method (Thermofisher). Twenty μg of hippocampal extract were denaturated at 95 °C for 5 min and loaded onto a sodium dodecyl-sulfate polyacrylamide gel. Gel was blotted onto nitrocellulose membrane (Protran; Whatman) and then blocked for 1 h at room temperature (RT) by 5% non-fat dry milk in Tris buffered solution TBS. The following primary antibodies were used: mouse anti-β-actin (1:20000, Sigma) for 1 h room temperature (RT); rabbit anti-synapsin-I (1:20000; Novus Biologicals, 1 h RT). Membranes were incubated with secondary HRP-conjugated IgG anti-mouse (1:10000, 1 h RT) and anti-rabbit (1:10000, 1 h RT). All secondary antibodies were from Amersham GE Healthcare, formerly Amersham Biosciences. After washing, immunodetection was performed by ECL reagent (Amersham GE Healthcare, formerly Amersham Biosciences), and membrane was exposed to film (Amersham GE Healthcare, formerly Amersham Biosciences). Densitometric analysis of protein levels was performed with ImageJ software (http://rsb.info.nih.gov/ij/). Sinapsin-I band density was normalized to β-actin bands. WB results were presented as data normalized to EAE-SED values. N = 5 per group.

### 2.6. RNA extraction and qPCR

Hippocampi of mice sacrificed at 21 dpi were dissected in RNAse-free conditions and total RNA was extracted according to the standard miRNeasy Micro kit protocol (Qiagen). Next, 600 ng of total RNA were reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 6.43–19.8 ng of complementary DNA (cDNA) were amplified in triplicate using the Applied Biosystem 7900HT Fast Real-Time PCR system. SensiMix II Probe Hi-Rox Kit (Bioline; Meridian Life Science) and the following TaqMan gene expression assays were used for the quantification of messenger RNA (mRNAs) coding for TNF, IL-1β, IL-6 and beta-actin (Actb):

Tnf ID: Mm00443258_m1
Il1b ID: Mm00434228_m1
Il6 ID: Mm00446190_m1
Actb ID: Mm00607939_s1

SensiMix SYBR Hi-Rox Kit (Bioline) was utilized for the quantification of messenger RNA (mRNAs) coding for ionized binding protein type-1 (IBA1) and beta-actin (Actb) by using the following primers:

Aif1 mRNA coding for IBA1 (NM_019467): forward GACAGACTGCCAGCCTAAGACAA; reverse CATTCGCTTCAAGGACATAATATCG

Actb (NM_007393.3): forward CCTAGCACCATGAAGATCAAGATCA; reverse AAGCCATGCCAATGTTGTCTCT

For both SYBR and TaqMan qPCR experiments, mRNA relative quantification was performed using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. β-actin was used as endogenous control. All data are expressed relative to CFA-SED. N = 7–14 per group.

### 2.7. Immunofluorescence and confocal microscopy

The immunofluorescence experiments were performed similarly to a method previously described (Gentile et al., 2014). Mice were deeply anesthetized and intracardially perfused with ice-cold 4% paraformaldehyde (PFA) at 7, 14 and 30 dpi (N = 3–6 per group). Collected brains were post-fixed in 4% PFA for 2 h and equilibrated with 30% sucrose for at least one night. Thirty-micrometer-thick coronal sections were serially cut on a frozen microtome to include the whole hippocampus. Brain regions were identified using a mouse brain atlas, and for each animal, at least five serial sections (one section every six in the bregma interval from −1.34 to −2.46) containing the CA1, were processed for immunofluorescence as described above. Slices were permeabilized in PBS with Triton X-100 0.25% (Tx-PBS). All following incubations were performed in Tx-PBS. To block non-specific sites, sections were pre-incubated with 10% normal donkey serum solution for 1 h RT and then incubated with the primary antibody overnight at + 4 °C. Then, after washing, they were incubated with secondary antibodies for 2 h RT and rinsed. Primary antibodies were used as follows: rabbit anti-IBA1 (1:750, Wako); mouse anti-parvalbumin (1:1000, Sigma Aldrich), goat anti-IL-1β (1:300, R&D Systems) and mouse anti-TNF (1:1500, Abcam). These were used in combination with the following secondary antibodies: Cy3-conjugated donkey anti-rabbit or anti-Goat (1:200; Jackson); Alexa-488-conjugated donkey anti-rabbit or anti-mouse (1:200; Invitrogen), and in some experiments Nissl dye. Nuclei were stained with DAPI (1 μl/ml; Sigma Aldrich). Images were acquired using a Nikon Eclipse TI2 confocal laser-scanner microscope with 20x, 40x and 60x objectives. All images had a pixel resolution of 1024×1024. The confocal pinhole was kept at 1.0, the gain and the offset were lowered to prevent saturation in the brightest signals, and sequential scanning for each channel was performed. Z-stack acquisitions (20x objective, zoom 1x with 2 μm interval for a total of 17 steps) were made applying the same intensity and exposure time and large image function that generates a single high-magnification image (capturing 3 or 4 images, according to Bregma level). A 40x objective with Z-stack (zoom 1x with 1 μm interval for a total of 20 steps) was used to detect parvalbumin branching. A 60x objective (zoom 1x with 1 μm interval for a total of 31 steps) was used to detect IL-1β and TNF double staining with IBA1. A z-projection image derived from all captured images was produced. All images were processed using ImageJ software and were adjusted for reducing noise by applying smooth and background subtraction as required by the NIH ImageJ. In the z-projections, the following quantitative analysis were carried out: a PV+ density analysis to evaluate parvalbumin neurodegeneration; morphological analysis of microglia, based on IBA1 staining through three different measurements (microglia total area, microglia density and microglia cell area). A bilateral Roi (region of interest) including CA1 region (stratum oriens and stratum pyramidale) was drawn to measure microglia total area, PV+ and IBA1+ densities. A smaller Roi was designed to analyse IBA1 cell area. To obtain cellular density, the number of PV+ or IBA1+ cells was manually and automatically determined, respectively, and divided by the area covered by the Roi. Data were expressed as the number of cells per $mm^2$. ImageJ software was used to quantify the microglial total area (IBA1+ surface %) in the Roi, generating intensity threshold images (binary images), and the measure of microglial surface was calculated by the software. To quantify microglia cell area, starting from the intensity threshold images, the spatial scale of the image was defined, using the dialog "set scale" of the ImageJ software to obtain measurements in calibrated units (scale: 1,5 pixels/μm). Finally, the pixel square of IBA1+ cells was calculated, and the value converted in $\mu m^2$. Equal intensity thresholds between groups were set for each quantitative analysis. A colocalization mask (mk) was generated on Z-projected images taken at 60x objective to visualize IBA1 and TNF colocalization.

### 2.8. Statistical analysis

Statistical analysis was performed with Prism GraphPad version 9.0. Data distribution was tested for normality by using Kolmogorov–Smirnov test. Non-normally distributed data were analyzed through non-parametric tests. Throughout the text "N" refers to the number of animals. For electrophysiological experiments and quantitative immunofluorescence analysis "n" refers to the number of slices recorded or analysed, respectively. The significance level was established at $p < 0.05$. Differences between two groups were analysed using two-tailed unpaired Student's *t* test. Data were subjected to two-way ANOVA for non-repeated measures to examine the main effect of physical activity (PA) and disease (DIS) and their interaction, time and DIS and their interaction. When the two-way ANOVA reached the statistical significance ($p < 0.05$), Tukey post hoc or unpaired Student's *t* test/Mann-Whitney comparisons were made. Three group comparisons were analysed by one-way ANOVA, followed by Tukey's HSD. The two-way ANOVA for repeated measures was used to assess the interaction between time and PA of clinical score and running activity. All data are expressed as mean ± SEM.

## 3. Results

### 3.1. Exercise ameliorates motor disability and cognitive function in EAE

First, we assessed the effects of exercise on EAE disease onset and progression. EAE induces a progressive paralysis starting around 10 dpi. In line with previous findings (Rossi et al., 2009), voluntary exercise did not change the disease onset (EAE-SED 13.50 ± 0.38 day, EAE-EX 14.22 ± 0.73 day, $p = 0.797$, $U = 171$), but significantly reduced motor disability over the course of disease progression (time × PA interaction $F_{(21,756)} = 5.385$, $p < 0.001$) (Fig. 1B). Furthermore, we measured the distance achieved in each cage to evaluate the amount of running-wheel activity of EAE mice. The run distance of EAE mice over time was significantly reduced compared to CFA-EX mice, as shown in Fig. 1C (time × PA interaction $F_{(6,\ 102)} = 11.44$, $p < 0.001$). Importantly, whereas CFA-EX mice showed a constant running activity, distance travelled by EAE-EX mice was reduced by 3.5-fold starting from 10 dpi, just before disease onset (Fig. 1 C', EAE-EX 10-0 dpi *vs* EAE-EX 21-10 dpi, $p = 0.004$, $U = 9$). This suggests that the beneficial effects of exercise on disability are the consequence of the preventive running activity during the presymptomatic phase rather than a therapeutic effect of exercise in the symptomatic phase of the disease.

Besides motor impairment, EAE induction is followed by emotional and cognitive alterations that are independent of motor disability, as they occur in either presymptomatic or barely motor-impaired mice during the symptomatic phase of the disease (Gentile et al, 2014; Gentile et al., 2015; Planche et al., 2017). To address the effects of exercise on memory function in the presymptomatic phase of the disease, we used the NOR test, which requires unimpaired motor functions. In the training phase both groups showed a similar exploration rate for the two identical objects (Fig. 1D; EAE-SED and EAE-EX discrimination index versus object 1 and object 2, $p > 0.05$). During the test phase, EAE-SED mice showed impaired discriminative skills, since they showed no preference for the novel object, while EAE-EX mice spent more time in exploring the novel object (EAE-SED discrimination index versus familiar object and novel object, $p > 0.05$; EAE-EX discrimination index versus familiar object and novel object $p = 0.036$, $t = 2.24$, $df = 20$). At the peak of the acute phase, when mice reached the maximum clinical score, memory function was tested through the nest building test, which measures a well-conserved natural behavior of rodents that has been linked to proper hippocampal function (Deacon et al., 2002). Irrespective of motor disability, nesting skills have been found significantly impaired in EAE mice (Gentile et al., 2014). The effects of exercise on nesting abilities were assessed in our experimental cohorts. The statistical analysis showed a significant interaction between DIS and PA

($F_{(1,15)}$ = 4.7, p = 0.045) (Fig. 1E). EAE-SED mice confirmed a poor performance in this test compared to CFA-SED (p = 0.039, U = 2.5), while exercise significantly improved nesting skills in EAE mice (p = 0.039, U = 1.5), suggesting beneficial effects of exercise on mice well-being and cognitive function.

Together, behavioral performances at both NOR and nest building suggest that the cognitive deficits that afflict EAE mice since early disease stages were prevented by regular voluntary running-wheel.

### 3.2. Exercise counteracts EAE-induced abnormal synaptic plasticity in the hippocampus

EAE brains are affected by diffuse synaptopathy, which also involves the hippocampus (Mandolesi et al., 2015). To investigate the impact of exercise on hippocampal synaptic plasticity we performed extracellular field recordings of brain slices taken from mice of all experimental groups in the acute phase of the disease (21–28 dpi), which is characterized by an aberrant synaptic plasticity that might underlie cognitive dysfunction in EAE mice (Nisticò et al, 2013). In line with our previous data (Nisticò et al., 2013), LTP magnitude in the CA1 area was significantly enhanced in EAE-SED compared to CFA-SED slices (Fig. 2A). Surprisingly, exercise was able to revert the aberrant synaptic plasticity by restoring the magnitude of LTP. Of note, no significant effect was recorded in the control exercise group (Fig. 2A' one-way ANOVA, Tukey post-hoc p < 0.001, F = 20.62). A significant interaction between PA and DIS factors was calculated by two-way ANOVA ($F_{(1,31)}$ = 31.48, p < 0.0001). Both PA ($F_{(1,31)}$ = 11.96, p < 0.001) and DIS ($F_{(1,31)}$ = 10.05, p < 0.001) showed significant effects.

### 3.3. Exercise restores proper synaptic plasticity in the EAE hippocampus, by preventing GABAergic degeneration

Synaptic activity of CA1 pyramidal neurons is locally regulated by GABAergic interneurons (Basu and Siegelbaum, 2015). We have previously linked defective GABAergic transmission in the CA1 area of the EAE hippocampus to the loss of parvalbumin positive (PV+) interneurons, which affects EAE and MS brains (Mandolesi et al., 2015; Nisticò et al., 2013). Thus, to account for the observed beneficial effects of exercise on synaptic plasticity, we assessed PV+ density in the CA1 area of the hippocampus, where recordings were performed. As shown in the pictures of PV-immunostained hippocampal slices of Fig. 3A-A', exercise fully preserved PV+ interneurons from EAE-induced death (One-way ANOVA p < 0.001, F = 14; EAE-SED *vs* CFA-SED p < 0.001, EAE-EX *vs* EAE-SED p < 0.001; EAE-EX *vs* CFA-SED p > 0.05). The analysis of PV+ cellular density in this region highlighted a gradient of neuronal loss in EAE, with a peak of cell loss at the CA1 midline (white arrows in low magnification pictures). Exercise displayed a homogeneous effect in the whole analysed region. Moreover, while surviving PV+ neurons in the EAE hippocampus showed reduced dendritic branching, PV+ neurons in EAE-EX CA1 area showed a morphology similar to that observed in CFA-SED (Fig. 3-A: white arrows in high magnification images). In addition to this, WB analysis of total hippocampal lysates of EAE-SED and EAE-EX mice showed a significant increase of the presynaptic marker synapsin-I in the EAE-EX hippocampus (Fig. 3B-B', p = 0.024; t = 2.77; df = 8), suggesting an improvement of the synaptic pathology in the whole hippocampus. Indeed, synapsin-I loss has been proven a typical hallmark of synaptic degeneration of the EAE hippocampus (Mori et al., 2014; Ziehn et al., 2012).

Based on the above remarkable neuroprotective effects, we addressed the idea that the rescue effect on synaptic plasticity observed in EAE-EX mice was dependent on the preserved GABAergic activity, given that the outcome of a physiological LTP is modulated by GABAergic inhibition. Indeed, in a subgroup of EAE-EX slices (n = 6) bath application of picrotoxin (50 μM) was able to heavily affect the fEPSP slope parameter (Fig. 3 C-C'; p < 0.001, F = 11.06).

### 3.4. Exercise attenuates microgliosis and inflammation in the hippocampus of EAE mice

Inflammation and microgliosis have been claimed to support synaptic and neuronal damage in the EAE brain (Mandolesi et al., 2015). Thus, we analysed the transcriptional expression of Aif1 coding for the microglia/macrophage marker IBA1 and the main pro-inflammatory cytokines (TNF, IL-1β, IL-6) that typically arise in EAE brains, to address an anti-inflammatory action of exercise in mice sacrificed at 21 dpi. As expected, IBA1 expression was significantly increased in EAE-SED hippocampus compared to both CFA-SED (p = 0.0005) and CFA-EX (p < 0.0001). A significant reduction of IBA1 was observed in EAE-EX hippocampi compared to EAE-SED (p = 0.021) (Fig. 4A). Both PA ($F_{(1,30)}$ = 7.2) and DIS ($F_{(1,30)}$ = 21) factors showed a significant

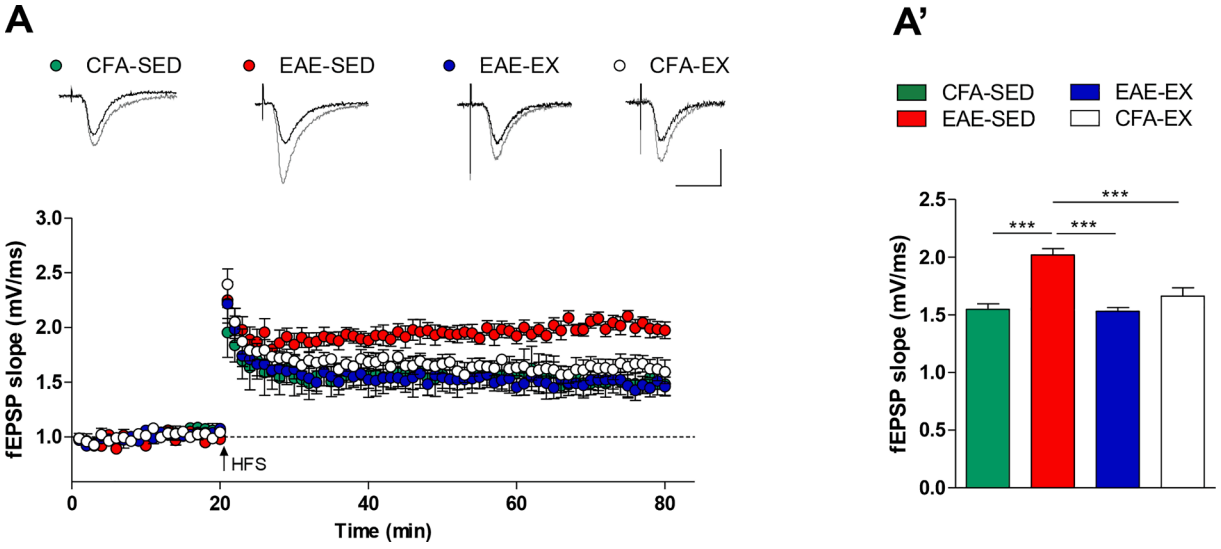



**Fig. 2. The impact of exercise on synaptic plasticity in the hippocampus of EAE mice.** (A) Time course (left panel) and mean (±SEM) fEPSP slope (mv/ms) during baseline and post LTP induction (black arrow indicates high frequency stimulation protocol induction, HFS, 100 Hz, 1 s) in CFA and in EAE slices in both conditions (CFA-SED, green; EAE-SED, red; EAE-EX, blue; CFA-EX, white). Sample traces (upper panel) during baseline (black) and after 1 h post HFS (grey); vertical and horizontal bars represent 0.5 mV and 10 ms, respectively. (A') Bar graph of mean (± SEM) of fEPSP slope between 50 and 60 min post HFS (Post-hoc Tukey comparison ***p < 0.001). n = 6–11 per group, N = 3–5.

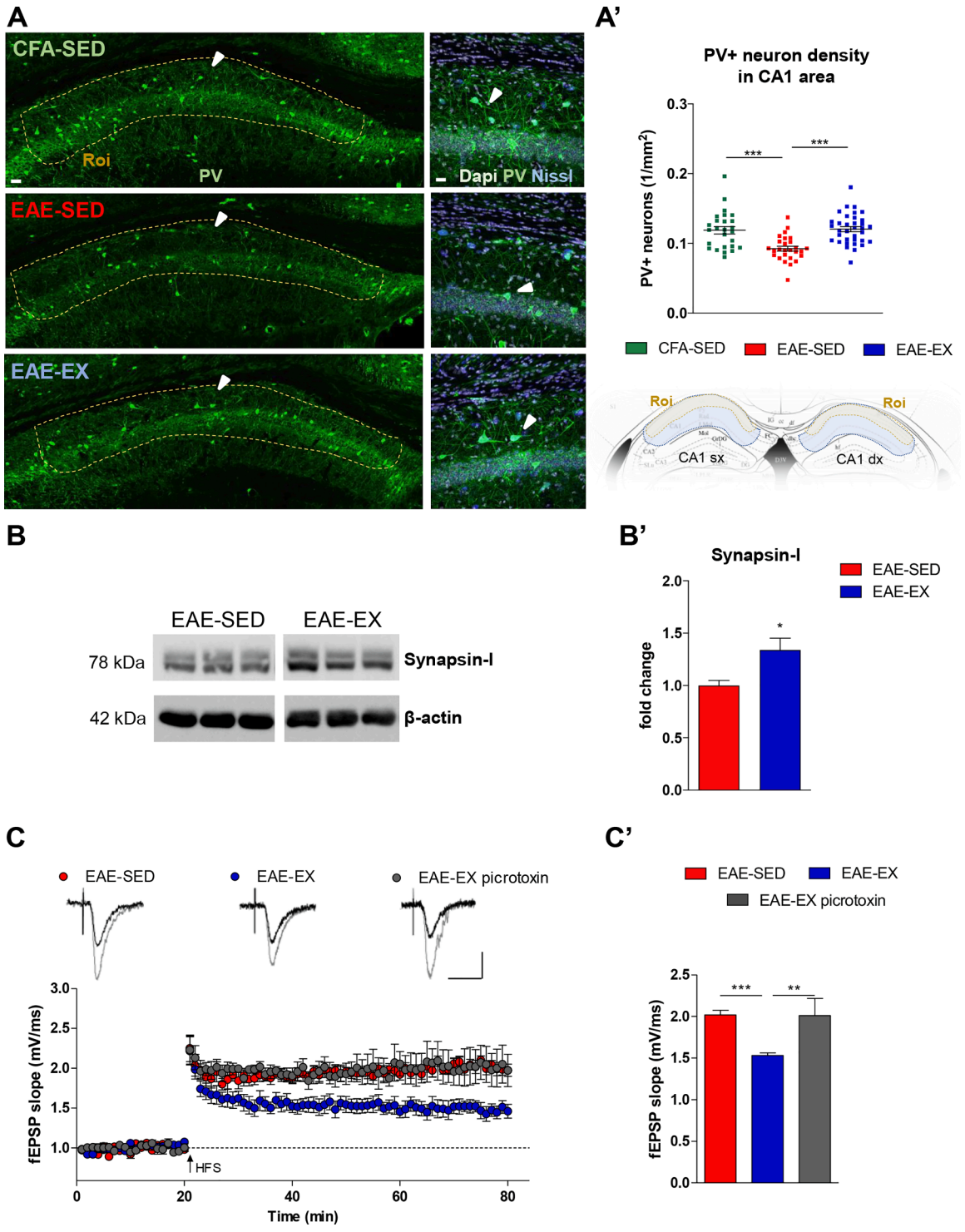



**Fig. 3. Exercise rescues hippocampal plasticity defects induced by EAE counteracting the GABA-ergic neuron degeneration.** (A) Representative confocal microscopy images of hippocampal slices stained with parvalbumin antibody (green fluorescence) showing the GABAergic neuronal death in the CA1 of EAE-SED animals, with a remarkable effect in the CA1 midline (white arrow). Running wheel exposure fully preserved PV-interneuron loss in EAE-EX group. On the right low magnification (40x objective) images highlight the reduced neuronal branching in EAE-SED mice compared to CFA-SED and EAE-EX animals. Slices were stained with parvalbumin (green fluorescence) and Nissl (blue fluorescence) for visualizing selectively interneurons and all neurons, respectively. (A') Mean (± SEM) PV+ density in CA1 Roi (stratum oriens and stratum pyramidale) assessed in both cerebral hemispheres of mice. One-way ANOVA, Post-hoc Tukey comparisons: *** $p < 0.001$. n = 26–35, N = 4–5. Scale bar: 40 μm. Scale bar inset: 20 μm. (B-B') Western blot densitometric analysis shows that the loss of the pre-synaptic marker synapsin-I induced by EAE pathology is prevented in hippocampal lysates of EAE-EX mice. Unpaired T-test $p < 0.05$. N = 6–8 mice per group. (C) Time course (left panel) and mean (± SEM) fEPSP slope (mv/ms) during baseline and post LTP induction (black arrow indicates high frequency stimulation protocol induction, HFS, 100 Hz, 1 s) in EAE-SED, in EAE-EX and EAE-EX in the presence of picrotoxin (50 μM) slices (EAE-SED, red; EAE-EX, blue; EAE-EX picrotoxin, grey). Sample traces (upper panel) during baseline (black) and after 1 h post HFS (grey); vertical and horizontal bars represent 0.5 mV and 10 ms, respectively. (C') Bar graph of mean (±SEM) fEPSP slope between 50 and 60 min post HFS (One-way ANOVA, Post-hoc Tukey comparison **$p < 0.01$ ***$p < 0.001$). n = 6–11 per group, N = 3–5 per group.

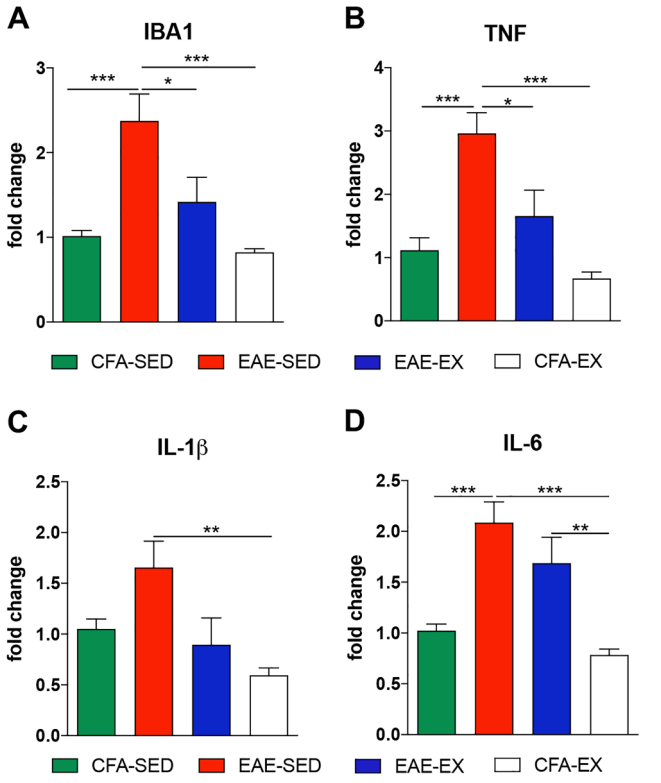



**Fig. 4. Beneficial effects of exercise on hippocampal microgliosis and inflammation.** (A) The mRNA coding for IBA1 was upregulated in EAE-SED hippocampus but rescued in the EAE-EX group (PA $F_{(1,30)} = 7.28$, $p = 0.011$; DIS $F_{(1,30)} = 21.09$, $p < 0.0001$; interaction $F_{(1,30)} = 3.24$, $p = 0.081$). (B) Exercise recovered TNF increased expression in the EAE hippocampus (PA $F_{(1,30)} = 10.80$, $p = 0.0026$; DIS $F_{(1,30)} = 28.27$, $p < 0.0001$; interaction $F_{(1,30)} = 2.61$). (C) IL-β expression was not significantly corrected by exercise (PA $F_{(1,40)} = 8.92$, $p = 0.0048$; DIS $F(1,40) = 4.94$, $p = 0.031$; interaction: $F_{(1,40)} = 0.557$, $p = 0.459$). (D) EAE-SED hippocampus showed a two-fold increase of IL-6 compared to CFA-SED that was not recovered by exercise (PA $F_{(1,40)} = 3.74$, $p = 0.06$; DIS $F_{(1,40)} = 35.23$, $p < 0.0001$; interaction $F_{(1,40)} = 0.245$, $p = 0.625$). For IBA1 and TNF N = 7–10 per group; For IL-1β and IL-6 N = 7–14 per group. Data are expressed as fold change to CFA-SED. Tukey post hoc comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

effect (PA $p = 0.011$, DIS $p < 0.0001$), while the PA × DIS interaction did not reach the statistical significance ($F_{(1,30)} = 3.2$, $p = 0.081$). Similarly, TNF expression was significantly increased in EAE-SED compared to CFA-SED ($p = 0.002$) and CFA-EX ($p < 0.0001$) and reduced by exercise in EAE ($p = 0.017$) (Fig. 4B). PA factor accounted for 14.33% of variation ($F_{(1,30)} = 10.8$), while DIS factor showed higher percent of total variation (37.22%, $F_{(1,30)} = 28.27$), no interaction was observed ($F_{(1,30)} = 2.615$, $p = 0.116$). In another experimental set, in which we confirmed the significant effect of exercise on the IBA1 marker (not shown), we examined the hippocampal expression of IL-1β and IL-6 cytokines. The two-way ANOVA analysis showed significant PA ($F_{(1,40)} = 8.929$. $p = 0.0048$) and DIS ($F_{(1,40)} = 4.94$, $p = 0.031$) effects, without interaction ($F_{(1,40)} = 0.557$, $p = 0.459$) (Fig. 4C). The Tukey post-hoc analysis showed only a significant difference between EAE-SED and CFA-EX ($p = 0.005$). These results were likely due to the mild upregulation of IL-1β in EAE-SED, that, however, was significantly different from CFA-SED (T test $p = 0.043$, $t = 2.129$, $df = 25$) and was reduced, albeit not significantly, by exercise (T test $p = 0.08$, $t = 1.849$, $df = 19$). The analysis of IL-6 expression revealed a highly significant effect of DIS ($F(1,40) = 35.23$, $p < 0.0001$) and an almost significant PA effect ($F_{(1,40)} = 3.747$, $p = 0.06$), without interaction ($F_{(1,40)} = 0.242$, $p = 0.625$) (Fig. 4D). IL-6 showed a high induction in EAE-SED compared to both controls CFA-SED and CFA-EX ($p < 0.0001$). IL-6 expression in the EAE-EX hippocampus was significantly different from CFA-EX ($p = 0.0069$) but not from EAE-SED ($p = 0.368$).

These results indicated that exercise induced a prominent effect of microgliosis and TNF expression reduction and a slight modulation of IL-β in the whole hippocampus.

### 3.5. *Exercise impairs microgliosis and microglial TNF expression in the CA1 area of EAE hippocampus*

We next performed a detailed immunofluorescence analysis of microgliosis extent in the CA1 area, where we previously observed remarkable effects of exercise against EAE-induced aberrant synaptic plasticity and PV+ death. At 30 dpi quantification of microglia density performed by manually selecting the CA1 area (region of interest-Roi), showed a significant reduction of IBA1+ cells in EAE-EX compared to EAE-SED (Fig. 5A, A' green fluorescence IBA1, nuclei in grey stained with Dapi; Tukey post hoc test $p < 0.001$ for all comparisons). PA activity accounted for 4.21% of total variance ($F_{(1,73)} = 20.16$, $p < 0.001$), DIS accounted for 81.93% of total variance ($F_{(1,73)} = 392$, $p < 0.001$), and a faintly significant interaction between PA and DIS ($F_{(1,73)} = 3.75$, $p = 0.057$) was observed.

In line with this, IBA1 immunofluorescence analysis (binary image) showed a significant reduction in the percent area of microglia in the CA1 of EAE-EX hippocampus, suggesting morphological modifications of IBA1+ cells in addition to density changes of the same cellular population (Fig. 5B, C, Tukey post hoc comparisons CFA-SED *vs* EAE-SED $p < 0.001$, CFA-SED *vs* EAE-EX $p < 0.001$, EAE-SED *vs* EAE-EX $p < 0.05$). The central zone of the CA1 (red Roi in Fig. 5B), where we observed the most striking loss of PV+ cells in the EAE-SED group, was selected for morphological analysis. Here, the mean surface area of IBA+ cells was reduced by about 32% in EAE-EX (Fig. 5B''', C') compared to EAE-SED (Fig. 5B'', C'), suggesting attenuated microglia activation (mean cell volume 104.4 $\mu m^2$ in EAE-SED, mean cell volume in EAE-EX 70.69 $\mu m^2$; one-way ANOVA $p < 0.001$; CFA-SED *vs* EAE-SED $p < 0.001$, CFA-SED *vs* EAE-EX $p < 0.001$, EAE-SED *vs* EAE-EX $p < 0.001$; Fig. 5B'-B''', C'). These data indicate that exercise mitigated both microglia activation and density in the CA1 area.

During EAE, microglia fuel local inflammatory reactions by releasing a number of pro-inflammatory cytokines, thereby affecting synaptic transmission and plasticity (Mandolesi et al., 2015). Based on qPCR data we assessed the effects of exercise on microglia expression of TNF and IL-1β in the CA1 area, by performing co-immunolabeling of IBA1 with TNF and IL-1β in hippocampal slices. TNF staining (red color) confirmed the upregulation of TNF in EAE-SED hippocampal slices compared to CFA-SED and its significant reduction in EAE-EX (Fig. 6 A). Remarkably, in both EAE-SED and EAE-EX a strong colocalization of TNF with IBA1 was observed, suggesting that these cells are the main source of this cytokine in the inflamed hippocampus. Of note, exercise noticeably reduced TNF production from microglia, as highlighted by colocalization mask (mk-magenta) in Fig. 6A'. IL-1β pattern of expression looked quite different. Immunofluorescence experiments confirmed qPCR data and further corroborated the exercise-mediated lowering effects in the CA1 area (see red staining Fig. 6B). However, IL-1β fluorescent signal colocalized barely with IBA1 in both EAE-SED and EAE-EX, suggesting that microglia do not provide substantial contribution to IL-1β level in EAE hippocampus.

Together, immunofluorescence studies suggest that exercise reduces hippocampal inflammation with the specific effects on microglia activation and microglia TNF expression.

### 3.6. *Reduced microgliosis anticipates neuroprotection in EAE-exercise hippocampus*

So far, data highlight both anti-inflammatory and neuroprotective effects of exercise in the acute phase of the disease. Whereas this can underlie the recovered synaptic plasticity and cognitive function at this

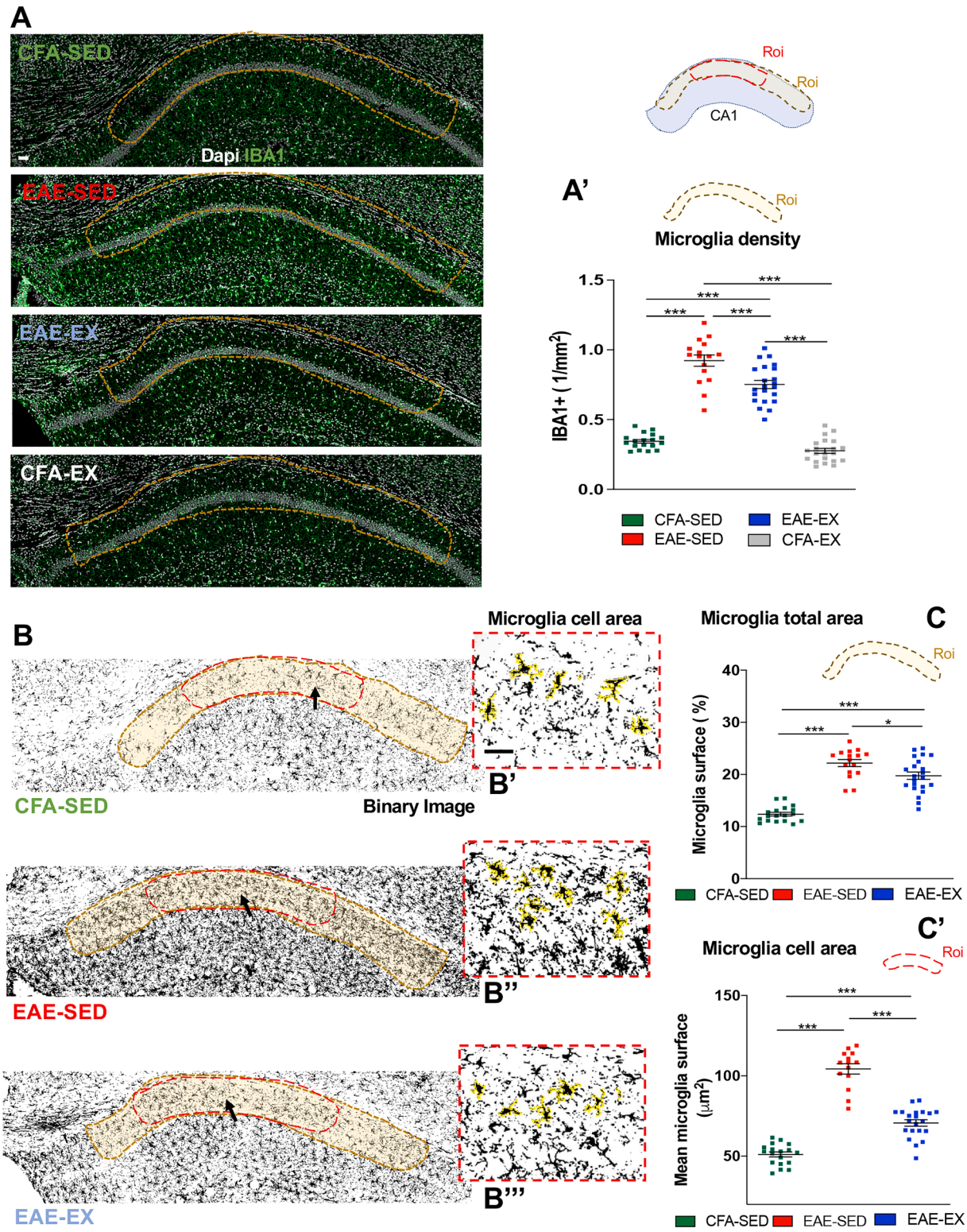



**Fig. 5. Exercise reduces microglia activation in CA1 area of the EAE hippocampus.** (A) Representative confocal microscopy images of hippocampal slices stained with IBA1 antibody (green fluorescence). On the right, schematic representation of the Roi (orange and red) used to perform the analyses. Scale bar: 40 μm. (A') IBA1+ cell density in the orange Roi covering the CA1 region is reduced in EAE-EX mice compared to EAE-SED mice. n = 16–22 per group, N = 4–5 per group. (B) Binary images, which clearly highlight exercise effect on microgliosis, were used to carry out cellular morphological analysis. Scale bar: 40 μm. (B'-B''') Magnification of microglia cells in CA1 midline (red Roi), where the greatest PV + death was previously observed, showing different IBA1 cell morphology in the three experimental groups. Scale bar: 40 μm. (C-C') Percentage of the area occupied by microglia (C) in the Roi covering the CA1 region is reduced in EAE-EX mice compared to EAE-SED mice. n = 16–22 per group. (C') Cell morphology analysis revealed that CFA-SED and EAE-EX animals show a reduced IBA1 cellular area suggesting a decreased microglia activation in exercise condition. n = 14–21 per group, N = 4–5 per group. One-way ANOVA, Post-hoc Tukey comparisons: *$p < 0.05$ *** $p < 0.001$.

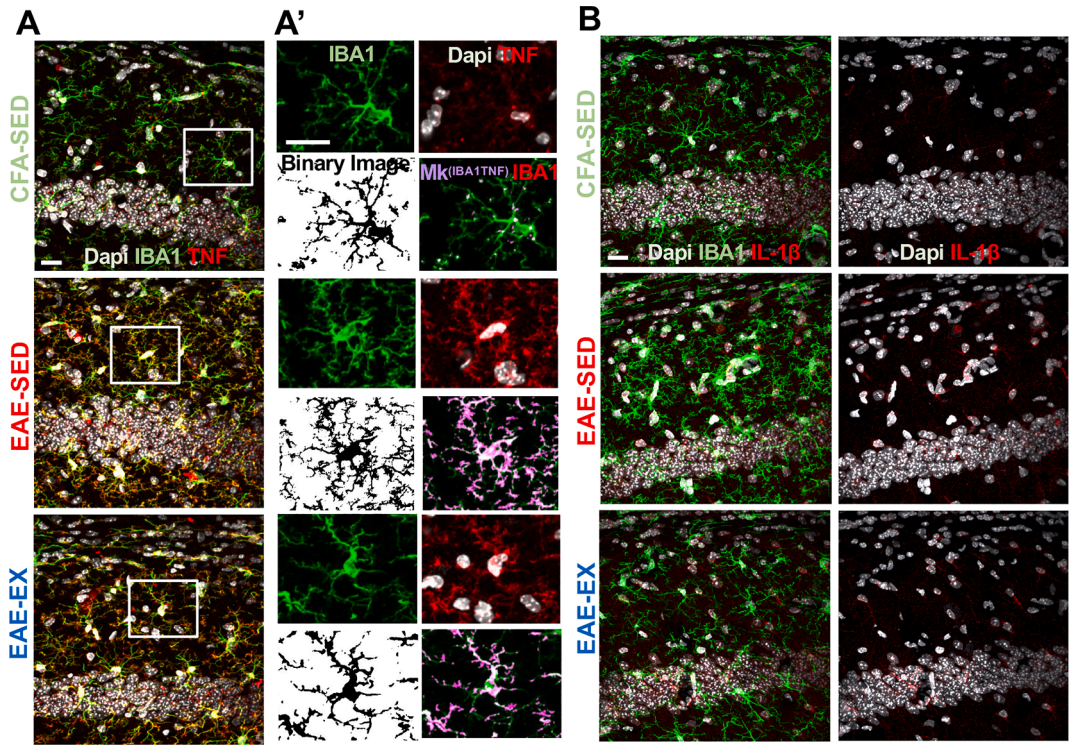



**Fig. 6. Microglia are the main source of TNF in the EAE hippocampus.** Representative confocal microscopy images of hippocampal slices stained with IBA1 (green fluorescence), TNF (red fluorescence) and IL-1β antibodies (red fluorescence). Slices are counterstained with Dapi (grey). (A) Voluntary running wheel decreased TNF expression in CA1 of EAE mice. (A') Of note, the overlapping area (mask-mk, magenta) is clearly reduced in microglia from EAE-EX hippocampus compared to EAE-SED group, further indicating less production of TNF from these cells. (B) IL-1β upregulation induced by EAE is reduced in CA1 of EAE-EX mice but colocalization with IBA1 is not appreciated. Scale bar: 20 μm.

stage, it does not clarify whether the neuroprotective effect of exercise is linked or not to its anti-inflammatory action and whether one or both effects support the EAE-EX preclinical phenotype. To this aim, we quantified the number of IBA1+ cells and PV+ interneurons at two earlier time points, at 7 dpi in EAE-SED and EAE-EX that underwent NOR, and at 14 dpi, the disease onset. Exercise significantly reduced IBA1+ cell density already at 7 and 14 dpi (data and statistics for both time-points are reported in the table of Fig. 7A-A'; two-way ANOVA analysis at 14 dpi: PA effect $F_{(1,59)} = 18.56$, $p < 0.001$; DIS effect $F_{(1,59)} = 57.66$, $p < 0.001$; DIS × PA interaction $F_{(1,59)} = 0.614$, $p = 0.436$).

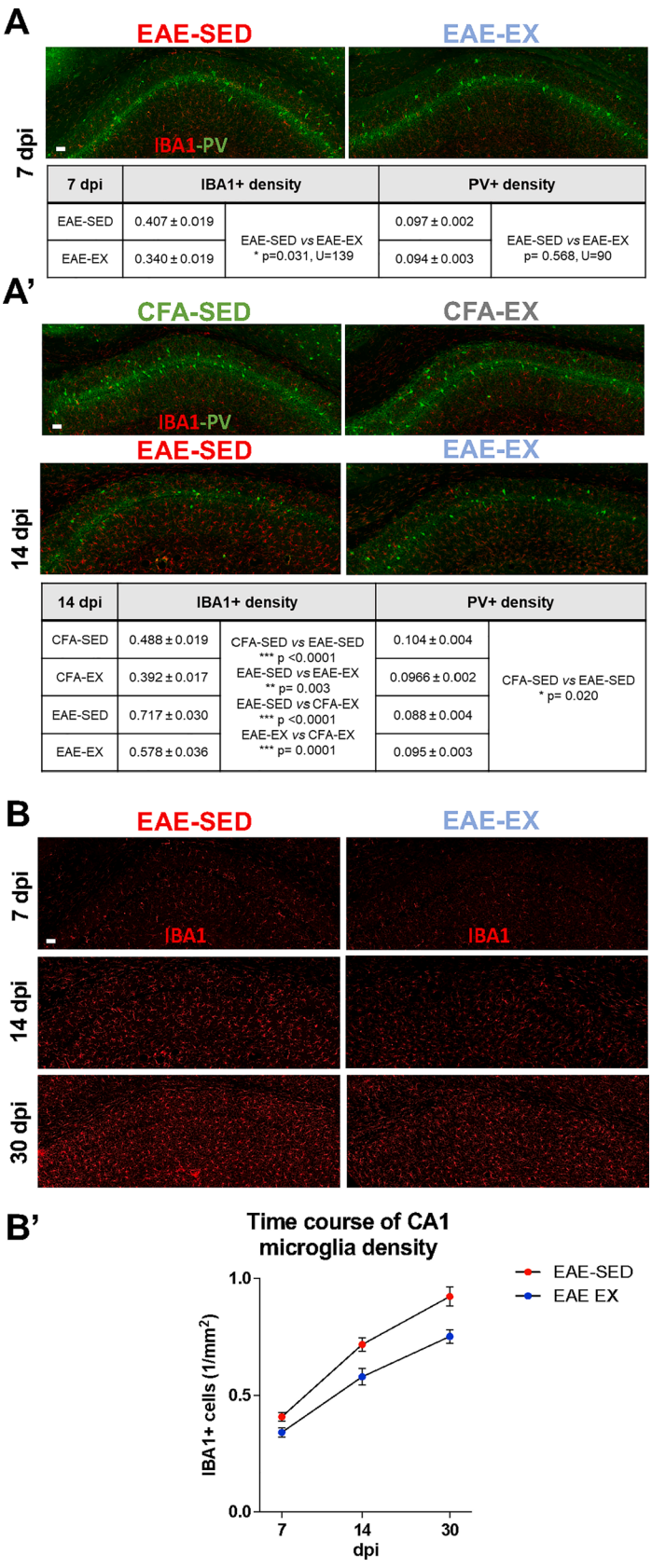
The analysis of PV+ interneuron density showed no differences between the EAE-SED and EAE-EX at 7 and 14 dpi (data and statistics for both time-points are reported in the table of Fig. 7A-A'; two-way ANOVA analysis at 14 dpi: DIS effect $F_{(1,59)} = 4.61$, $p = 0.035$; PA effect $F_{(1,59)} = 0.006$, $p = 0.936$; DIS × PA interaction $F_{(1,59)} = 3.36$, $p = 0.071$). Thus, while the neuroprotective effects of exercise seem to be more evident at later stages, exercise showed a prominent and early impact on microgliosis, as highlighted by the different time course of CA1 microglia density of EAE-SED and EAE-EX (Fig. 7B-B', PA effect $F_{(1,100)} = 25$, $p < 0.001$; Time effect $F_{(2,100)} = 125.6$, $p < 0.001$; PA × Time $F_{(2,100)} = 1.66$, $p = 0.195$).

## 4. Discussion

The most relevant finding of this study is the neuroprotective effect of preventive exercise on a typical hallmark of EAE/MS brains, the loss of PV+ interneurons. We have shown that voluntary running wheel initiated the day of $MOG_{35-55}$ immunization prevents the loss of GABAergic PV+ interneurons in the CA1 area of EAE hippocampus, providing an increased GABAergic input that supports synaptic plasticity in the same hippocampal region. Remarkably, exercise attenuates microgliosis since the presymptomatic phase of the disease, likely accounting for the improved cognition observed at this stage, while the neuroprotective effect of exercise seems to be more pronounced at later stages, suggesting a temporal relationship between the two events. Based on this, mechanistically, we propose that exercise protects neurons through an early and long-lasting anti-inflammatory action as indicated by reduced microglia activation and cytokine expression in the hippocampus. Phenotypically, this translates into improved well-being and cognitive functions in exercise mice.

Exercise is a non-pharmacological intervention increasingly prescribed for pwMS. Clinical studies suggest that exercise induces functional improvements (muscular strength and mobility), reduces fatigue, and improves quality of life (Amatya et al., 2019; Farrell et al., 2020). In reverse, current literature does not uniformly support a beneficial effect of exercise on either cognition or hippocampal volume in pwMS (Gharakhanlou et al., 2020; Hvid et al., 2021). However, addressing the disease-modifying potential of exercise in the human setting can be challenging, due to the lack of standardized protocols and heterogeneity in measured outcomes (Dalgas et al., 2020). Animal studies have the potential to disclose this issue, by deepening molecular and cellular effects of exercise. In this respect, several studies have clearly shown that different types of exercise protocol longitudinally reduce disease symptoms in EAE mice (Rossi et al., 2009; Souza et al., 2017; Einstein et al., 2018), and have also provided evidence for peripheral and central immunomodulatory effects (Souza et al., 2017; Einstein et al., 2018; Xie et al., 2019; Zaychik et al., 2021). In line with our previous work (Rossi et al, 2009), we show that voluntary running wheel reduces motor disability and that this is largely due to the preconditioning effect of running activity in the presymptomatic phase of the disease, since after disease onset the distance run is dramatically reduced.

To date, only one study addressed the impact of exercise on hippocampal pathology. Building on the well-consolidated notion that neurogenesis in the DG is sensitive to exercise (Van Praag et al., 1999). Kim




| 7 dpi | IBA1+ density | | PV+ density | |
|---|---|---|---|---|
| EAE-SED | 0.407 ± 0.019 | EAE-SED *vs* EAE-EX * p=0.031, U=139 | 0.097 ± 0.002 | EAE-SED *vs* EAE-EX p= 0.568, U=90 |
| EAE-EX | 0.340 ± 0.019 | | 0.094 ± 0.003 | |

| 14 dpi | IBA1+ density | | PV+ density | |
|---|---|---|---|---|
| CFA-SED | 0.488 ± 0.019 | CFA-SED *vs* EAE-SED *** p <0.0001<br>EAE-SED *vs* EAE-EX ** p= 0.003<br>EAE-SED *vs* CFA-EX *** p <0.0001<br>EAE-EX *vs* CFA-EX *** p= 0.0001 | 0.104 ± 0.004 | CFA-SED *vs* EAE-SED * p= 0.020 |
| CFA-EX | 0.392 ± 0.017 | | 0.0966 ± 0.002 | |
| EAE-SED | 0.717 ± 0.030 | | 0.088 ± 0.004 | |
| EAE-EX | 0.578 ± 0.036 | | 0.095 ± 0.003 | |

**Fig. 7. Effect of exercise on microgliosis and neurodegenerative events in the CA1 hippocampus during EAE.** (A-A') Representative confocal microscopy images of hippocampal slices stained with IBA1 (red fluorescence) and PV (green fluorescence) antibodies. On the bottom tables reporting the values (mean ± SEM) of IBA1+ and PV+ density in the CA1 region at 7 (A) and 14 (A') dpi and the statistics. Exercise reduced hippocampal microgliosis in EAE mice at 7 dpi (Mann-Whitney test n = 11–21 per group, N = 5–6 per group) and 14 dpi (One-way ANOVA, Post-hoc Tukey comparisons, n = 13–19 per group, N = 3–5 per group). Scale bar: 40 μm. (B) Representative confocal microscopy images of EAE-SED and EAE-EX hippocampal slices stained with IBA1 (red fluorescence) antibody at 7, 14 and 30 dpi. Scale bar: 40 μm. (B') Graph showing the time course of CA1 IBA1 cell density at 7, 14 and 30 dpi in EAE-SED mice and EAE-EX mice.

and colleagues showed that treadmill exercise started in the acute phase of the disease significantly improved neurogenesis, reduced markers of apoptosis in the DG and corrected cognitive dysfunction in the step-avoidance test (Kim and Sung, 2017). Here, we show that voluntary running wheel improved memory and cognitive abilities, as highlighted by mice performance in tests run in preclinical (NOR) and symptomatic (nest building) phase of the disease, suggesting an early effect of exercise. Indeed, memory function assessed through the NOR test, a validated tool to study several aspects of hippocampal-dependent learning and memory in rodents (Lueptow, 2017), is impaired before (Olechowski et al., 2013; present study) and at the onset of EAE (dos Santos et al., 2019). Such dysfunction has been shown to persist in later stages of EAE by means of behavioral tests adapted for motor-impaired mice (Aharoni et al., 2019). In our experiments, to avoid the confounding factor of motor disability, cognition was tested in acute-phase mice by evaluating the nest building behavior, which we showed to be independent of EAE motor impairments (Gentile et al., 2014). Indeed, we found that EAE mice with negligible motor symptoms showed the same nest skills or severely motor impaired EAE mice (Gentile et al., 2014). Thus, the beneficial effect of exercise on nest building here described is likely the consequence of the ameliorated hippocampal pathology that we previously characterized in the acute phase of the disease (Nisticò et al., 2013). Nest building is a natural rodent skill linked to cognitive ability of mice in building a comfortable and safe environment (Deacon, 2006), which depends on proper hippocampal function. Poor nesting behavior has been shown in animal models of cytotoxic hippocampal lesions (Deacon et al., 2002; Jedynak et al., 2012), Alzheimer disease (Filali and Lalonde, 2009; Zhu et al., 2020) and stroke (Yuan et al., 2018). Nesting activity has been proposed to be useful to monitor disease progression, including cognitive deterioration, and drug response in Alzheimer's disease model (Zhu et al., 2020). Notably, cells involved in nest recognition and conceptualization, thereby called nest cells, have been identified in the murine hippocampus (Lin et al., 2007). Thus, nest building, which mirrors daily activities in humans (Lin et al., 2007), seems implicated in hippocampal-dependent executive function.

Concerning the clinical and behavioral study we performed, one criticism to the interpretation of our results could stem from the use of standard non-enriched cages to rear control CFA and EAE groups, as suggested in several studies on the effects of housing conditions on mouse behavior (Joisten et al., 2020; Roemers et al., 2019). However, based on previous literature it can be reasonably argued that the effects of exercise here observed are not the consequence of the lack of minimal physical activity or environmental enrichment in the control group. Indeed, we have previously shown that EAE mice reared in cages with blocked running wheel did not show amelioration of clinical symptoms compared to mice in cages with unblocked running wheel (Rossi et al., 2009). In addition, inactivity and reduced cage sizes have been shown to affect motor coordination and grip strength rather than emotionality and memory function (Roemers et al., 2019).

Different hippocampal regions, including the CA1, and cellular/molecular mechanisms other than neurogenesis, have been shown to contribute to memory formation (Lisman et al., 2017; Schimanski and Nguyen, 2004). Notably, structural and functional connectivity changes in the hippocampus of MS subjects have been proposed to underlie memory/learning dysfunctions in MS (Rocca et al., 2018). In particular, regional hippocampal atrophies, characterized by an early involvement of the DG and a late spread to the CA1, have been shown to underlie specific memory domain impairments over the course of the disease (Planche et al., 2018). Demyelination, synaptic and neuronal loss contribute to hippocampal atrophy in MS through intermingled and parallel pathways (Rocca et al., 2018). Of note, changes in the synaptic compartment have been shown to play a pivotal role in inducing both functional synaptic transmission/plasticity alteration and neurodegeneration (Di Filippo et al., 2018; Mandolesi et al., 2015). In particular, a paradoxical amount of synaptic plasticity has been described in the CA1 area of acute-phase EAE hippocampus (Nisticò et al., 2013). The expression of an aberrant LTP in this region has been linked to the detrimental effects of IL-1β and to the reduced GABAergic tone at excitatory pyramidal neurons as the consequence of PV+ loss. Noteworthy, the death of this neuronal population is a neuropathological hallmark of both MS and EAE brains (Clements et al., 2008; Ziehn et al., 2010).

Here we found that exercise prevented the loss of PV+ neurons in the CA1 area of the EAE hippocampus and restored the expression of normal synaptic plasticity. Voluntary running wheel is reported to exert different regional effects in terms of synaptic plasticity. On one side, it is quite well-established the notion that it increases the magnitude of LTP in the DG of hippocampus in healthy rodents, mainly through neurogenesis induction (Van Praag et al., 1999). On the other side, it has been reported that exercise can exert both null (Van Praag et al., 1999) and enhanced (Miller et al., 2018) effects in modulating LTP in the CA1 area of healthy mice. These disparate findings are likely due to different experimental variables such as distinct patterns of synaptic stimulation protocols. In our experiments, exercise did not change synaptic plasticity in control mice but showed a specific action in the EAE condition. Thus, the evidence that exercise significantly attenuated microgliosis and inflammation, including IL-1β, and preserved the PV+ population, leads us to propose that the synaptic effect of exercise observed in the EAE hippocampus can be ascribed to its anti-inflammatory/neuroprotective action. In particular, we showed that blocking the GABAergic transmission in EAE-EX through picrotoxin application increased the LTP magnitude. These findings suggest that the survival of PV+ interneurons induced by exercise plays a crucial role in modulating the GABAergic influence on EAE synaptic plasticity. Although *ex vivo* picrotoxin application cannot be considered as an accurate pharmacological model of EAE condition, it offers an indirect evaluation of GABAergic alterations and the consequence on excitatory responses. Indeed, EAE-EX mice slices were sensitive to pharmacological GABAergic blockade and readily expressed an aberrant plasticity, mimicking the EAE condition. Of note, these data are consistent with previous experiments where GABA transmission was pharmacologically enhanced (Nisticò et al., 2013).

Local inflammatory reaction sustained by activated microglia has been associated with synaptic and neuronal damage in the EAE hippocampus (Di Filippo et al., 2016; Mancini et al., 2018; Mori et al., 2014; Nisticò et al., 2013). Importantly, microgliosis is an early event that precedes motor disability and that has been correlated with hippocampal alteration and cognitive dysfunction in EAE mice (Habbas et al., 2015; Planche et al., 2017). Our molecular and immunohistochemical data suggest that exercise exerts an early (7 dpi) and long-lasting (14 and 30 dpi) mitigation of microgliosis together with a prominent anti-inflammatory action in the acute phase as highlighted by reduced TNF and IL-1β expression in the hippocampus. The fact that voluntary running wheel attenuates microgliosis in EAE hippocampus is noteworthy. To date, the beneficial effects of voluntary running wheel on microgliosis in animal models of MS has been reported in the spinal cord of EAE mice (Mifflin et al., 2017) and the striatum of cuprizone mice (Mandolesi et al., 2019). Likewise, running wheel has been shown to dampen hippocampal age-related microgliosis (He et al., 2017; Kohman et al., 2012). In contrast, in different genetic models of Alzheimer's disease (AD), running wheel was shown to exert both null and beneficial effects on microgliosis (Belaya et al., 2020; Nichol et al., 2008; Rodríguez et al., 2015; Svensson et al., 2020), suggesting that its effects depend on the pathological context. Here, through a time-course of IBA1 immunostaining at different disease stages we show that exercise attenuates microgliosis since the presymptomatic phase, likely contributing to the improved memory function observed at this stage. In contrast to this, exercise-mediated neuroprotection was prominent in the acute phase. In line with previous findings (Ziehn et al., 2010), we show that the loss of PV+ interneurons in the CA1 area occurs in early symptomatic EAE (14 dpi), but we could not detect a significant effect of exercise on neurodegeneration that, however, was evident later. The

neuroprotective effect of exercise could be underestimated, since the mere count of PV+ cells in this initial stage of neurodegeneration might be insufficient in detecting differences in neuronal suffering status that precedes neuronal death. Also, these data suggest that the attenuated microgliosis might contribute to the later neuroprotective effects of exercise. In line with this idea, studies in MS brains suggest that PV+ death depends on local inflammatory milieu (Magliozzi et al., 2021). In this respect, TNF has been involved in mechanisms of PV+ degeneration in models of traumatic brain injury and in mice receiving intracerebroventricular infusion of TNF (Deng et al., 2020; Wang et al., 2018). Though a causal relationship between microglia-derived TNF and PV+ loss in EAE has not been demonstrated so far, we may speculate that in our experimental setting exercise exerted a preventive anti-inflammatory action that preserved PV+ neuronal population, which in turn contributed to rescue synaptic plasticity and cognitive function in EAE.

## 5. Conclusion

Our data indicate that exercise exerted a preconditioning effect on EAE hippocampal pathology and cognitive performance, by attenuating microgliosis and local inflammation. More importantly, exercise proved to act as an authentic disease modifying therapy for MS. Exercise rescued EAE cognitive deficits similarly to the immunomodulatory drugs fingolimod (dos Santos et al., 2019) and glatiramer acetate (Aharoni et al., 2019) and, more importantly, prevented PV+ loss as previously observed with siponimod (Gentile et al., 2016).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

*Acknowledgements*

The authors thank Massimo Tolu and Monica Nencini for helpful technical assistance. The authors thank Dr. Francesca De Santa from Institute of Cell Biology and Neurobiology (IBCN), National Research Council of Italy (CNR) for her support in qPCR experiments.

Graphical abstract was created with BioRender.com.

*Funding*

The study was supported by: the Italian Ministry of Health (GR-2018-12366154 to AG and FB; GR-2016-02361163 to AM; GR-2016-02362380 to DF and FB; RF-2018-12366144 to DC and GM; Ricerca corrente to IRCCS San Raffaele Pisana; Ricerca corrente to IRCCS Neuromed); the Italian Ministry of University and Research (MIUR)-PRIN 2017- cod. 2017K55HLC to DC; FISM-Fondazione Italiana Sclerosi Multipla- cod. 2019/S/1 and financed or co-financed with the '5 per mille' public funding to DC and FRR; private donation in memory of Chiara Sardi to D.C. FDV was supported by a research fellowship FISM-Fondazione Italiana Sclerosi Multipla- cod. 2018/B/2 and financed or co-financed with the '5 per mille' public funding.

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