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The benefits of voluntary physical exercise after traumatic brain injury on rat's object recognition memory: A comparison of different temporal schedules

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Running title: Exercise effects on memory and brain after TBI

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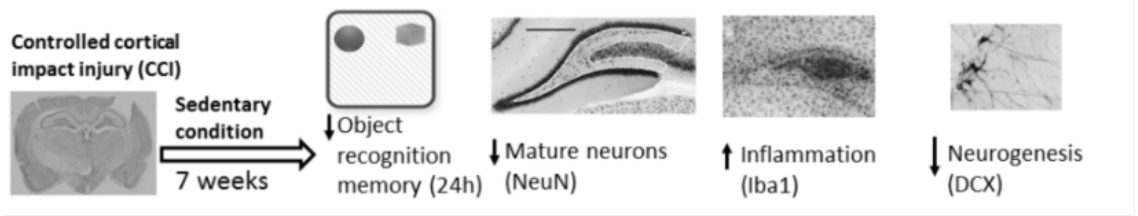
Abstract

Physical exercise can reduce the cognitive decline associated with traumatic brain injury, yet little is known about the optimal administration schedules. Here, different protocols of voluntary wheel running were evaluated for their effects on object recognition memory (ORM), neuroprotection ($NeuN^+$ cells), microglial reactivity (Iba1 staining) and neurogenesis (DCX^+ cells) after controlled cortical impact injury (CCI). CCI-lesioned rats were divided into a sedentary group and three exercise groups: early discontinued exercise (3 weeks of exercise initiated 4 days post-injury, followed by 4 weeks in a sedentary state); delayed exercise (3 weeks of exercise initiated 4 weeks post-injury), and early continuous exercise (7 weeks of exercise starting 4 days post-injury). The deficits induced by CCI in a 24 h ORM test were reversed in the delayed exercise group and reduced in the early discontinued and early continuous groups. The early discontinued protocol also reduced the loss of $NeuN^+$ cells in the hilus, while attenuated microglial reactivity was found in the dorsal hippocampus of both the early exercising groups. Running at the end of the experiment increased the number of DCX^+ cells in the early continuous and delayed groups, and an inverted U-shaped relationship was found between the mean daily exercise time and the amount of neurogenesis. Thus, exercise had benefits on memory both when it was commenced soon and later after injury, although the neural mechanisms implicated differed. Accordingly, the effects of exercise on memory and neurogenesis appear to not only depend on the specific temporal schedule but also, they may be influenced by the amount of daily exercise.

Keywords: Traumatic brain injury; Physical exercise; Object recognition memory; Neuroprotection; Adult neurogenesis; microglial reactivity.

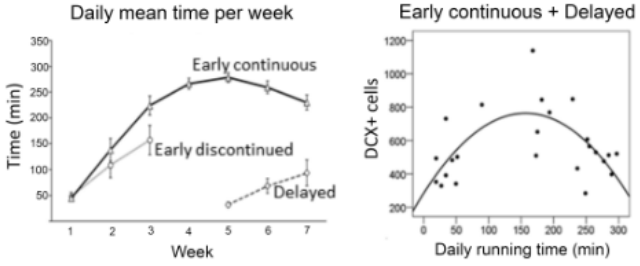
Graphical abstract



Exercise treatment		↓ Memory deficit (ORM)	↑ Protection (NeuN hilus)	↓ Inflammation (Iba1 hippocampus)	↑ Neurogenesis (DCX, Dentate gyrus)
Early discontinued	Exe (3 wks)	+	+	+	
Delayed	Exe (3 wks)	++			+
Early continuous	Exe (7 wks)	+		+	++

Why early continuous exercise treatment did not lead to the best memory outcome?

Related to amount of daily running time?



Introduction

Traumatic brain injury (TBI) is the leading cause of acquired brain damage in young individuals (Thurman, 2016), although it can affect people at any age and its prevalence in older adults has risen in recent years (Faul and Coronado, 2015; Roozenbeek et al., 2013). TBI is associated with a wide variety of short- and long-term effects that may persist for years or decades after the initial insult. The deficits produced can affect the motor, sensory, affective and cognitive domains, with disruptions to executive function and memory amongst the most prevalent cognitive disabilities (Esopenko and Levine, 2017). Trauma or primary injury is also followed by a series of biochemical and cellular events that are referred to as secondary injury (Mckee and Daneshvar, 2015). These events immediately follow the damage that occurs and they are chronic, provoking dynamic histological and functional changes over time (Finnanger et al., 2015; Ramlackhansingh et al., 2011). Indeed, animal studies have highlighted the persistence of the cognitive deficits produced by TBI (Pischiutta et al., 2018), as well as the progressive changes in neurodegeneration that can be perceived in vivo (neuroimaging) and post-mortem (Acosta et al., 2013; Liu et al., 2010; Piao et al., 2013; Pischiutta et al., 2018). Due to the maintenance and/or aggravation of the cognitive deficits over time, additional social, economic and personal burdens develop in association with TBI, making it important to search for suitable therapeutic strategies/interventions that will diminish the long-term changes.

Physical exercise is known to enhance cognition in healthy individuals (Berchtold et al., 2010), and to reduce the memory deficits associated with normal aging and neurodegeneration (Alkadhi, 2018; Voss et al., 2019). There is also evidence that it can reduce the memory deficits associated with acquired brain injury (Crane et al., 2012; Itoh et al., 2011; Jacotte-Simancas et al., 2015). The benefits of physical exercise in acquired brain injury seem to be mediated by several mechanisms, including: the

production of neurotrophic factors; myelin protection; increased cell proliferation; neurogenesis and plasticity; reduced neuron death; and chronic inflammatory responses (Itoh et al., 2011; Jacotte-Simancas et al., 2015; Piao et al., 2013; Chytrova, Ying, & Gomez-Pinilla, 2008; Piao et al., 2013; Ryan & Nolan, 2016). Thus, it would appear that physical exercise favors neuroprotection while enhancing neurogenesis and neuroplasticity.

The neuroprotective effects of physical exercise may also be important in attenuating secondary injury and since this damage arises shortly after the initial insult, it would seem advisable to induce regimes of physical exercise soon after injury. Nevertheless, care must be taken to avoid interfering with the increased metabolic demands associated with injury. As such, initiating exercise too early may be detrimental to the preservation of memory (Griesbach et al., 2009). Conversely, neuroplasticity may have positive effect at any time after injury and thus, both early and late physical exercise protocols may constitute potentially promising treatments for TBI-related defects. However, there is little evidence to support this assumption and what is available is somewhat contradictory. We previously found that voluntary wheel running initiated 4 days after TBI in rats and maintained for 3 weeks until memory testing, reversed TBI-associated memory deficits, attenuating the loss of adult neurons and enhancing neurogenesis (Jacotte-Simancas et al., 2015). By contrast, memory deficits were reduced by delayed wheel running initiated 5 weeks after injury, inducing neuroprotection and enhancing neurogenesis in mice, yet not by an early exercise regime initiated 5 days post-TBI (Piao et al., 2013). However, in these mice exercise ceased 38 days before training and 56 days before sacrifice in the early exercise group, such that the failure to detect any benefits of the early exercise regime could be related to the discontinuation of exercise and not to the time of its initiation after injury. Similarly, forced treadmill exercise was seen to improve memory only after early initiated but not after late initiated exercise

(Chen et al., 2013), while elsewhere, delayed treadmill exercise did reduce the spatial learning deficits of rats that sustained TBI at 4 weeks of age (Ko et al., 2018). Hence, it remains unclear what is the most appropriate temporal schedule of physical exercise to treat cognitive deficits after TBI and how other regimes may influence the benefits obtained. Indeed, many factors remain to be defined, such as the optimal amount of exercise, the minimal duration of exercise, or the best period for exercise discontinuation, etc. These uncertainties, coupled to the small number of studies in patients, which may at times be compromised by their methodology, make it difficult to reach definitive conclusions (Morris et al., 2016). Therefore, further preclinical research is necessary to establish the most appropriate exercise parameters and to define a gold standard exercise regime for TBI patients.

Here, we assessed the effects of different schedules of voluntary physical exercise on object recognition memory (ORM), as well as on the number of mature neurons (neuroprotection), microglial reactivity (neuroinflammation) and neurogenesis (neurorepair) in male rats subjected to TBI in late adolescence. The schedules involved 3 weeks of early discontinued exercise, 3 weeks of delayed exercise and 7 weeks of early continuous exercise. A controlled cortical impact injury (CCI) model was used to induce TBI.

Materials and Methods

Ethics and animal welfare

All procedures were performed in compliance with the European Community legislation for the protection of animals used for experimentation and other scientific aims (2010/63/EU, September 22nd, 2010), and with the Spanish national legislation (Real Decreto 53/2013, February 1st 2013) regulating the care and ethical issues related to animal experimentation. The experimental protocols were approved by the Ethics Committee for Animal and Human experimentation at the Universitat Autònoma de Barcelona and of the Autonomous Government of Catalonia.

In this study, 70 male Sprague-Dawley albino rats were used (Charles River Laboratories; Abresle, France; Supplied by Prolabor; Barcelona, Spain), aged 6-weeks old on arrival. The animals were initially kept in quarantine for one week and they were then housed individually in cages (52 x 28 x18 cm) with *ad libitum* access to water. As such, the animals were 7-weeks-old at the beginning of the experiment, considered to correspond to late adolescence (Schneider, 2013), and their mean initial body weight was 229.75 g (±10.75 g). Throughout the experiments the animals were provided a fixed amount of food (30 g/day, 2014 Teklad global 14% protein rodent maintenance diet: Envigo, Valencia, Spain), which is above the mean daily recommended consumption for adult rats. This procedure does not involve caloric restriction (indeed, the daily voluntary consumption of all the animals, whether sedentary or exercising, was less than that available). In spite of that, in our laboratory we find that this procedure reduces overfeeding and induces better long-term health compared to the standard ad libitum conditions. Body weight is also slightly reduced (for example, around 10% compared to standard ad libitum feeding at 14 weeks of age), and is kept within the age-specific reference values for Sprague-Dawley rats, according to the rat breeder (Charles River Laboratories; https://www.criver.com/products-services/find-model/sas-sprague-

dawley-rat?region=3661#panel1-growth-chart).

The animals were maintained under controlled conditions on a 12 h light-dark cycle (lights on at 8:00 a.m.), temperature 20–22 °C and 40–70 % humidity.

Experimental groups

The animals were randomly assigned to one of the following 5 experimental groups: 1) Sham control group (n=15), the animals in this group were sham operated and remained in a sedentary condition throughout the experiment; 2) Sedentary (Sed) group (n=17), the rats were submitted to CCI and remained in a sedentary state throughout the experiment; 3) Early discontinued exercise (Exe_1-3) group (n=13), these rats were submitted to CCI and they were allowed free access to a running wheel for 3 weeks from day 4 to day 25 post-injury (p.i) (approximately 1 to 3 weeks p.i.), and they remained sedentary from day 26 p.i. until the end of the experiment, for approximately 4 weeks; 4) delayed exercise (Exe_5-7) group (n=13), these rats were subjected to CCI and remained sedentary until day 32 p.i. when they were given free access to a running wheel for 3 weeks until the end of the experiment, exercising during weeks 5 to 7 p.i.; 5) early continuous exercise (Exe_1-7) group (n=12), these rats were subjected to CCI and allowed free access to a running wheel for 7 weeks, from day 4 p.i. until the end of the experiment. The distinct groups of rats were then submitted to the behavioral procedures according to the timeline indicated in Fig. 1.

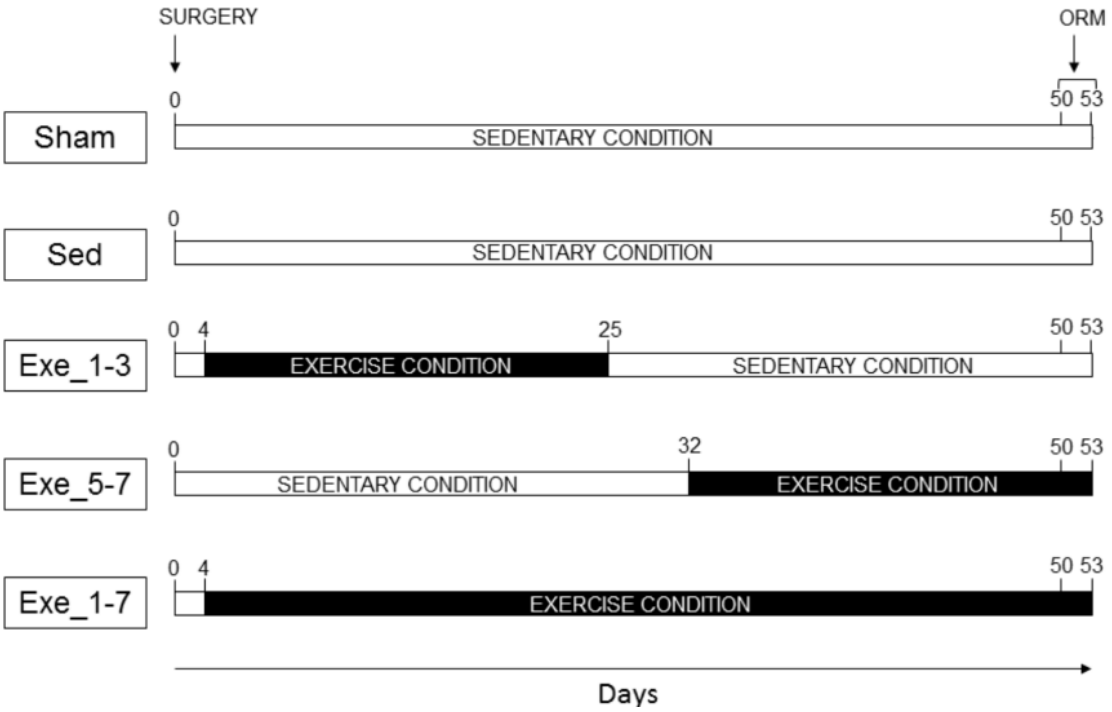


Fig. 1. Timeline of the experimental procedures

Stereotaxic surgery and TBI

TBI was induced by means of CCI, as described previously (Jacotte-Simancas et al, 2015). Impact to the right hemisphere (4.5 mm posterior to Bregma and 3 mm from midline) was made at a velocity of 6.0 m/s, reaching a depth 2.0 mm below the dura matter layer and persisting for 150 msec. Sham animals were operated in a similar way but no impact was applied. To control for post-operative pain, a single subcutaneous injection of buprenorphine (0.02 ml, Buprex: Schering-Plough SA, Barcelona, Spain) was administered.

Physical exercise.

Animals in the Sham and Sed groups were kept in their standard cages throughout the experiment, while the rats in the other groups were only kept in these cages during the sedentary periods. During the periods of wheel running, the animals were maintained in

cages (48 x 26 x 20 cm) containing a 37cm diameter running wheel (Rat Wheel W/Brake, ENV-042: Med Associates Inc, St. Albans, VT, USA). The time and distance run was recorded daily using a bicycle computer (Sigma BC 506: Sigma Elektro GmbH, Neustadt, Germany).

Object recognition memory (ORM) task

All groups started training in the ORM task on day 50 p.i., performed in an open box (65.5 x 65.5 x 35 cm) made of conglomerate coated with brown melamine, situated in a sound-attenuating cage (72 x 72 x 157 cm) made of white melamine, and ventilated by an extractor fan. The illumination on the floor of the box was 30 lux. The objects used in the test varied in color, shape and size, they had no ethological significance and the animals had no previous experience of them. The objects were a Duplo (Lego®) construction, a soft drink can and a wall hanger, and they were available in duplicate copies. The objects were fixed to the floor of the box using double-sided adhesive tape to prevent the animals from moving them. A prior pilot study had shown that rats of the same strain and age had no spontaneous preference for any of these objects. The behavioral sessions were recorded with a video camera mounted above the experimental apparatus and controlled by video tracking software Anymaze (Stoelting Europe, Dublin, Ireland). Object exploration was scored off-line by a trained observer who was unaware of the animal's experimental condition. Object exploration was defined as directing the nose towards the object at a distance ≤ 2cm, while turning around or sitting on the object was not considered exploratory behavior. To avoid the presence of olfactory cues, the apparatus and objects were cleaned thoroughly with a solution of 70% alcohol in distilled water and dried before and after each use.

Training started with 3 sessions of habituation to the experimental box (2 separated by a 2 h interval on the same day, and the third on the following day). Each habituation

session lasted 12 min and in order to test possible anxiety reactions to novel objects, a

neophobia test was conducted 2 h after the last habituation session. In this test, an unfamiliar object was exposed in the centre of the open box and the animal was placed in the box facing away from the object and allowed to explore it for 10 min.

The ORM acquisition session was carried out the day after the neophobia session, and it consisted of a 15 min acquisition session (two identical objects were placed in adjacent corners of the box, 10 cm away from the walls) and 2 retention tests (5 min each). The first retention test was carried out 3 h after acquisition and the second, 21 h later (24 h after acquisition). In the retention tests, one copy of the object used in the acquisition test (familiar) and a novel object were placed in adjacent corners of the cage. The specific objects used as familiar or novel, as well as their positions, were assigned randomly to reduce any potential bias due to a preference for a particular location or object. In the 24 h retention test, the familiar object was always placed in the opposite corner to that used in the 3 h retention test. The time spent exploring each object was recorded, as well as the total object exploration time and total distance moved.

A discrimination index was used to analyze cognitive performance that makes it possible to adjust for any differences in the total exploration time: ([time exploring the novel object – time exploring the familiar object] / total time spent on both objects) x 100 (Akkerman et al., 2012b). Therefore, the values of discrimination indices ranged from -100 (only the familiar object is explored) to +100 (only the novel object is explored). Since the ORM test is based on the natural tendency of rats to explore a novel object over a familiar object, an index significantly higher than zero is considered to reflect good recall of the familiar object (i.e.: animals exploring the novel object more than the familiar one), whereas an index close to zero (i.e.: animals exploring both objects similarly) amounts to chance level and is considered a lack of recall (Akkerman

et al., 2012b). A criterion of ≥10 s of exploration during the acquisition session was established for animals to be included in the statistical analyses of discrimination indices, since low exploration times may distort encoding processes in this task (Akkerman et al., 2012a).

Processing of Brain Tissue

The animals were sacrificed 24 h after the second memory test by administering an overdose of sodium pentobarbital (Dolethal, 200 mg/kg: Vetoquinol SA, Alcobendas, Spain) and they were perfused intracardially with 4% paraformaldehyde (PFA; Sigma-Aldrich; Madrid, Spain). The rat's brain was then dissected out immediately and immersed in the same fixative at 4 ºC for a further 3 h, rinsed with phosphate buffer (PB) and then submerged in a cryoprotective solution (sucrose 30% in PB) for 3-4 days at 4 ºC. Finally, the brains were frozen in isopentane (2-methylbutane: Sigma-Aldrich, Madrid, Spain) and stored at -80 ºC. Parallel coronal cryostat sections (40 μm) were obtained (Shandon Cryotome FSE, Thermo electron corporation, Waltham, MA, USA) between the approximate anteroposterior coordinates -2.28/-2.40 and -5.52/-5.64 from bregma. From each animal, 9 series of free-floating sections and one series of sections mounted on gelatin-coated slides were obtained. This procedure allowed systematic random sampling to be performed.

Cresyl violet staining

The mounted sections were processed for cresyl violet staining and digitized using a slide scanner (Hewlett-Packard Printing and Computing Solutions, HP Scanjet G4050, Sant Cugat del Vallès, Spain). The digital images were calibrated using Fiji image analysis software (Schindelin et al., 2012) and the area of the hippocampus in each slice

was outlined manually to calculate the surface area. The volume of the dorsal hippocampus was estimated by multiplication of the sum of the surface areas measured in all the slices with the section thickness (40 µm). In each section, an interhemisphere ratio was computed for the hippocampal formation [(ipsilateral area / contralateral area) x 100]. The mean ratios for all the sections from each rat were used for more standard comparisons between the TBI groups. These ratios were expected to be close to 100 if there was no loss of volume in the hemisphere ipsilateral to the impact and to differ significantly from 100 if this was not the case.

Immunohistochemical processing

One out of the 9 randomly selected series of free-floating slices was processed to assess the distribution of NeuN, a nuclear protein expressed by mature neurons (Gusel'nikova and Korzhevskiy, 2015). A second series was processed for DCX, expressed by immature neurons (Gonçalves et al., 2016), and a third one for ionized calcium binding adaptor 1 (Iba1), expressed by microglia/macrophage cells (Ito et al., 2001).

For NeuN and DCX immunohistochemistry, free-floating brain sections were washed in tris-buffered saline (TBS) and quenched for 30 min with 0.03% H_2O_2 (Panreac Química SLU, Castellar del Vallès, Spain) in TBS. Non-specific binding was blocked with TBS containing 10% new calf serum (NCS: Sigma-Aldrich, Madrid, Spain), and the sections were then incubated overnight at 4 ºC and for 1 h at room temperature with a primary antibody against NeuN (1:1000 Mouse anti-NeuN, MAB377: Chemicon, Millipore-Merck, Madrid, Spain) or DCX (1:4000 Rabbit polyclonal anti-DCX, 18723: Abcam, Cambridge, UK), diluted in TBS containing 5% NCS. After washing in TBS and TBS with 1% Triton X-100, the sections were incubated for 1 h with biotinylated secondary antibodies at room temperature (1:1000 biotin conjugated Goat anti-mouse IgG

[A16082] or 1:500 biotin conjugated Goat anti-rabbit IgG [A16114]: Thermo Fisher Scientific, Alcobendas, Spain). The sections were then washed with TBS and incubated for 2 h with a streptavidin-biotin horseradish peroxidase complex (1:3600, SA-HRP conjugate, NEL 750001EA: Perkin Elmer, Tres Cantos, Spain), and the reaction product was visualized with diaminobenzidine (DAB) and nickel following the manufacturer's instructions (3,3'-Diaminobenzidine tetrahydrochloride hydrate, SK-4200, DAB kit: Vector laboratories, Palex Medical SA, Sant Cugat del Vallès, Spain). Finally, the sections were mounted on slides, dehydrated through increasing alcohol concentrations, cleared with Histoclear (National Diagnostics™; Nottingham, UK) and cover-slipped with Histomount (National Diagnostics™, Notthingham, UK). One well in each well plate did not contain primary antibody and was used as a negative control. Immunohistochemical processing for Iba1 was carried out on 7 animals per group using a similar procedure to that for NeuN and DCX immunohistochemistry. Endogenous peroxidase was quenched with 0.06% H_2O_2 (Panreac Química SLU, Castellar del Vallès, Spain) in distilled water and 70% methanol for 10 min. The blocking solution was 10% fetal bovine serum (FBS: Sigma-Aldrich, Madrid, Spain) diluted in TBS with 1% Triton X-100. The primary antibody was a rabbit polyclonal antiserum raised against Iba1 (GTX100042 Genetex, AntibodyBCN, Bellaterra, Spain) diluted 1:800 in blocking solution, while the secondary antibody was a biotin conjugated goat anti-rabbit IgG (A1664 Thermo Fisher Scientific, Alcobendas, Spain) diluted 1:500 in the same blocking solution.

Quantification of DCX^+ and $NeuN^+$ cells

Digitized serial images of DCX-stained sections from the whole area of the dorsal dentate gyrus (DG) were obtained at 40x on an Eclipse TE2000-e Nikon microscope with a motorized stage that was driven by Metamorph software (Nikon Instruments

Europe B.V., L'Hospitalet de Llobregat, Spain). Using the same procedures, digitized serial images were obtained at 20x for the hilus and the perirhinal cortex of NeuN-stained sections. Fiji software was used to obtain stitched images of these 3 brain regions for each hemisphere, automatically stitching all the individual frames in the series. Subsequently, 6 stitched images of the granular cell layer of the DG (DCX immunohistochemistry) were quantified per hemisphere and animal (separated by 400 μm), and 3 stitched images (separated by 800 μm) were analyzed for both the hilus and perirhinal cortex (NeuN immunohistochemistry).

Random uniform systematic sampling system was used to quantify the DCX^+ cells in the granule cell layer and $NeuN^+$ cells in the perirhinal cortex. A grid composed of counting frames with an area of 8,303 μm^2 was superimposed on each of the stitched images processed for DCX^+, and the cells were counted manually in every other counting frame. A similar procedure was used to quantify the $NeuN^+$ cells in the perirhinal cortex, although the counting frames of the overlaid grid had an area of 1,245.5 μm^2 and the $NeuN^+$ cells from one in 25 counting frames were quantified. The molecular cell layer of the perirhinal cortex was excluded from the quantification due to the small number of cells in this layer. To avoid any edge effect, all cells touching the right and lower edges of the frame were not counted, while cells touching the left and upper edges were. The measure used for group comparisons was the density of DCX^+ or $NeuN^+$ cells in each stitched image, calculated as (adapted from optical dissector calculations, Yurt et al., 2018):

$$N_V = \sum Q^- / (\sum Sdis \times h)$$

Where Nv, numerical density; $\sum Q^-$, Sum of quantified cells; $\sum Sdis$, sum of quantified dissectors (counting frames) by dissector area; h, height of the slice (given a value of 1 as quantifications were carried out on microphotographs).

A different procedure was used to quantify the cells in the hilus given the relatively few

neurons in this region. Specifically, the area of the hilus was manually outlined and all the $NeuN^+$ cells present within the area outlined were quantified in each of the three slices (not only those in the specific dissectors).

Iba1 analyses

The sections stained for Iba1 were inspected qualitatively, analyzing between 6 and 8 sections per rat in each hemisphere. Each section was visualized on a Nikon Eclipse 80i microscope (Nikon Instruments Europe B.V., L'Hospitalet de Llobregat, Spain) at 4x and 20x magnifications, and scored blind for the presence of reactive $Iba1^+$ cells and for the existence of foci of intense Iba1 staining (dense clusters of activated microglia with or without monocytes). Three sections from each animal (the 3rd, 5th and 7th sections in an anterior to posterior gradient), separated by 800 μm, were also subjected to a densitometric analysis. For each section, approximately 10 microphotographs per hemisphere were captured at 10x magnification with a DXM 1200F Nikon digital camera mounted on a brightfield Nikon Eclipse 80i microscope using the ACT-1 2.20 software. The order of microphotograph acquisition was always the same, starting from the midline and including the entire dorsal hippocampus. Using analySIS® software (Soft Imaging System, GmBH, Münster, Germany), both the percentage of the area occupied by the immunolabelling, as well as the intensity of the immunoreaction (mean grey scale value, from 0 to 255), was recorded for each photograph.

Statistical analyses

The statistical analyses were performed using the statistical programming language R 2.15 (R Development Core Team, 2011). Most of the behavioral data were analyzed through a one-way analyses of variance (ANOVA) using a between-group design. The homogeneity of variances was examined with the Levene test. When between-group

differences were statistically significant, Tukey post-hoc corrections were used to compare between the pairs of groups if homogeneity of variances was fulfilled. Tukey tests were replaced with Games-Howell correction tests to analyze the density of DCX^+ cells due to a lack of homogeneity of variances. One-sample *t*-tests were used to determine whether the mean values of a group differed significantly from a given reference value (0 for the discrimination index and 100 for the interhemispheric ratio). Repeated measures analyses of variance with polynomial contrasts were used to examine the evolution of running behavior over time in each of the exercise groups. A mixed multivariate analysis of variance was applied to examine the group and section differences in the number of $NeuN^+$ cells in the hilus. Finally, correlational analyses and regression function adjustments were also carried out when necessary. Statistical significance was set at the level of $P \leq 0.05$.

Results

Exercise behavior

The mean daily distance (in meters) run in the wheel (A) and the mean daily running time (in minutes, B) was considered for each weekly period of physical exercise undertaken by the 3 relevant groups of rats (Fig. 2). Unfortunately, the data from 1 animal in the Exe_1-3 group were lost due to technical problems and therefore, the statistical analyses were carried out on 12 animals in this group. Polynomial contrasts indicated that the evolution of the mean daily distance covered, and the time spent exercising per week during the relevant period (3 weeks in Exe_1-3 and Exe_5-7 groups and 7 weeks in Exe_1-7 group) fitted a linear ascending function in the Exe_1-3 (distance t=3.98, P=0.02; time t=4.35, P=0.001) and Exe_5-7 (distance t=3.24, P=0.008; time t=2.95, P=0.01) groups. The evolution of the distances and exercise time in the Exe_1-7 group fitted a quadratic function (distance t=-9.38, P<0.001; time -14.76,

P>0.001), indicating a linear increase from the first to the fifth week and a decrease thereafter (see Fig. 2).

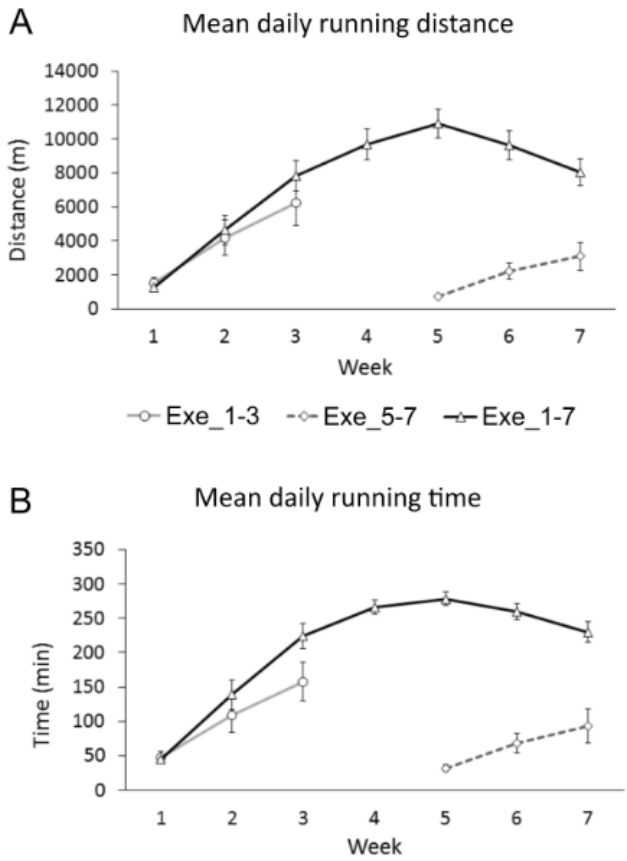


Fig. 2. Mean (+/-SEM) daily distance (m) (A) and time (min) (B) run by each of the 3 exercising groups in each of the respective weeks of exercise treatment.

Two-sample t-tests comparing the mean daily running distance and exercise time between the two groups that commenced their exercise regime shortly after injury (Exe_1-3 and Exe_1-7) indicated no significant differences between these two groups in the first, second and third weeks of running. Two-sample t-tests were also carried out to compare the mean daily running distance and time between the two groups that exercised in the last weeks of the experiment (Exe_5-7 and Exe_1-7 groups), corresponding to the period immediately prior to or concomitant with the training, and close to day of sacrifice (weeks 5, 6 and 7 p.i.). These analyses indicated that the rats in the Exe_5-7 group ran significantly less distance and time than those in the Exe_1-7 group on the 5th [distances $t(23) = -11.75$, $P<0.001$; times $t(23) = -23.12$, $P<0.001$], 6th

[distances t(23) = -7.48, P<0.001; times t(23) = 9.55, P<0.001] and 7th [distances t(23) = -4.00, P<0.001; times t(23) = -4.35, P<0.001] weeks p.i.

Object Recognition Memory (ORM)

Exploration and locomotor activity in the ORM box. The ANOVA indicated between-group differences in locomotion during the neophobia session [F(4,65) = 2.54; P=0.048]. However, post-hoc comparisons failed to detect significant differences between any pair of the experimental groups. One of the animals in the Exe_1-7 group did not explore the object at all in the neophobia test and thus, this animal was excluded from the analyses of latency to initiate object exploration during this test. ANOVA did not reveal statistical differences in latency in the neophobia test among the different experimental groups. As such, no significant between-group differences were found with regards locomotion (total distances moved) in any of the sessions, nor in the total object exploration time, or in the latency to commence object exploration in the acquisition and retention sessions.

Discrimination index in the retention sessions. All the animals fulfilled the minimum exploration criterion to be included in the analyses.

Fig. 3 depicts the mean discrimination index for each of the five experimental groups in the 3 h (Fig. 3A) and 24 h (Fig. 3B) retention sessions. A one-sample *t*-test indicated that the discrimination indices in the 3 h retention test were significantly higher than 0 in the Sham [t(14) = 2.72, P=0.017], Exe_1-3 [t(12) = 3.06, P=0.01], Exe_5-7 [t(12) = 3.22, P=0.007] and Exe_1-7 [t(11) = 2.40, P=0.035] groups, while the discrimination index for the Sed group only approached significance relative to 0 [t(16) = 2.01, P=0.062]. No significant differences among the groups were detected by ANOVA and thus, the Sed group showed only a marginal impairment, while the remaining groups displayed good recall of the familiar object in the 3 h retention test.

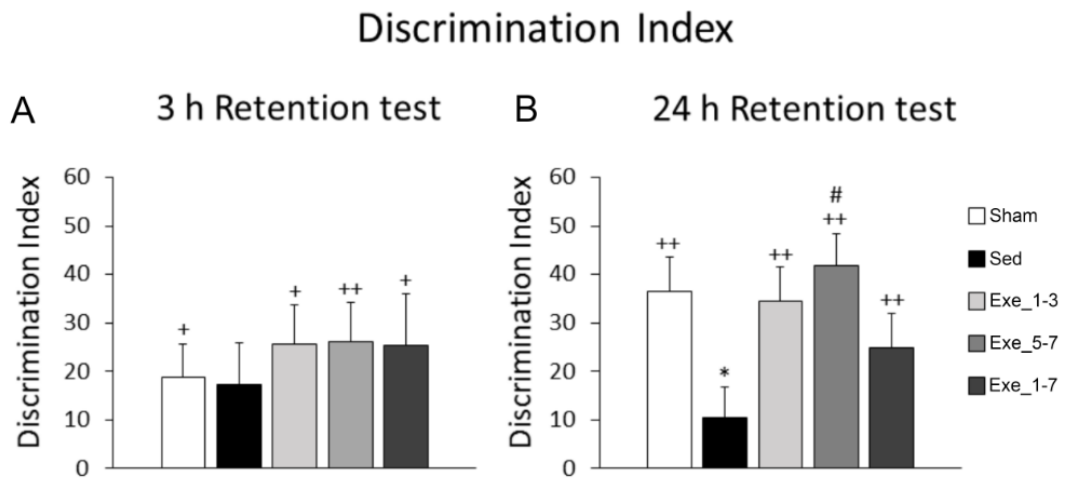


Fig. 3. Mean (+SEM) values of discrimination index for each experimental group in the retention tests of the object recognition memory task carried out 3 h (A) and 24 h (B) after acquisition. + (P<0.05) and ++ (P<0.01): Significant differences with regard to the reference value (0), indicating recall of the familiar object; *(P<0.05): Significant differences compared to Sham group; # (P<0.05): Significant differences compared to L-sed group.

The one-sample t-test indicated that the discrimination indices for the 24 h retention test were significantly higher than 0 in the Sham [$t(14) = 5.16$, $P<0.001$], Exe_1-3 [$t(12) = 4.94$, $P<0.001$], Exe_5-7 [$t(12) = 6.44$, $P<0.001$] and Exe_1-7 [$t(11) = 3.61$, $P=0.004$] groups, indicative of good recall. By contrast, the discrimination index did not differ from 0 in the Sed group, indicative of a lack of recall. Between-group differences were revealed by ANOVA [$F(4,65) = 3.65$; $P=0.01$] and the post-hoc comparisons indicated that there were no significant differences between the Sham, Exe_1-3, Exe_5-7, and Exe_1-7 groups. Conversely, the discrimination index of the Sed group was significantly lower than that of the Sham ($P=0.039$) and Exe_5-7 ($P=0.011$) groups, and there was a trend towards a significant difference between the Sed and Exe_1-3 group ($P=0.089$).

Interhemispheric hippocampal ratio

Cresyl violet staining was analyzed in coronal sections of 56 rats (14 Sham, 11 Sed, 10 Exe_1-3, 11 Exe_5-7, 10 Exe_1-7). A lesion cavity over the parietal lobe was evident in

the ipsilateral hemisphere of all the CCI rats, with a morphological deformation of the dorsal hippocampus and an enlarged lateral ventricle (see Fig. 4A), as seen previously (Amorós-Aguilar et al., 2015; Jacotte-Simancas et al., 2015). The interhemispheric ratios of the dorsal hippocampus (Fig 4B) were significantly lower than the reference value (contralateral) in all the CCI groups [Sed t(10) = -4.07, P=0.002; Exe_1-3 t(9) = -2.69, P=0.025; Exe_5-7 t(10) = -2.46, P=0.034; Exe_1-7 t(9) = -3.42, P=0.008], but not in the Sham group. Between-group differences were detected by ANOVA [F(4,51) = 3.28, P=0.018], with post-hoc comparisons showing that hippocampal ratios were lower in the Sed (P=0.050) and Exe_1-7 (P=0.020) groups relative to the Sham rats, whereas the differences between the latter and the Exe_5-7 or Exe_1-3 groups were not significant.

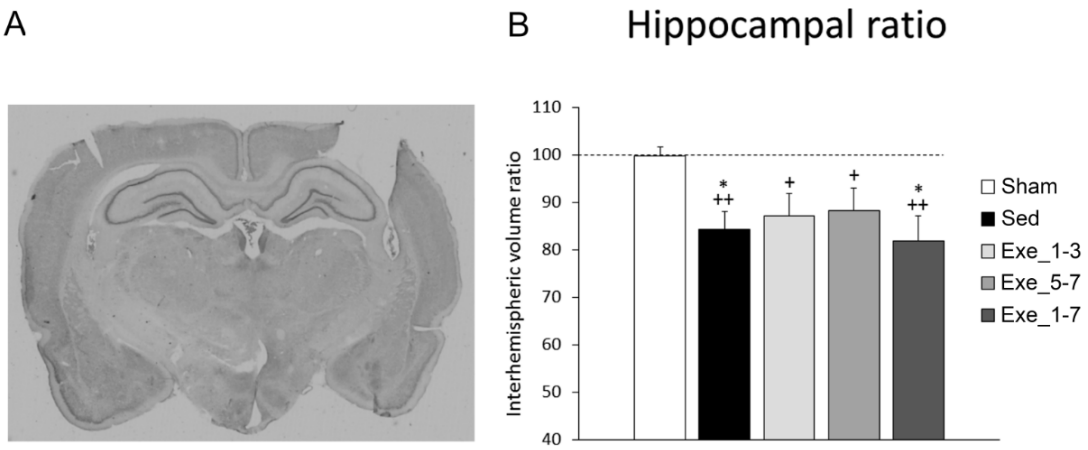


Fig. 4. Representative microphotography of a coronal section stained for cresyl violet in an animal with CCI (A). Mean (+SEM) interhemispheric hippocampal ratios (percent values of the volume of ipsilateral versus contralateral dorsal hippocampus) for each experimental group (B). + (P≤0.05) and ++ (P<0.01): Significant differences with regard to the reference value (100), indicating a lower volume of the dorsal hippocampus of the ipsilateral hemisphere compared to the contralateral hemisphere; *(P≤0.05): Significant differences compared to Sham group.

Density of DCX$^+$ cells in the granular cell layer of the dentate gyrus

Neurogenesis was analyzed in the contralateral hemisphere of 60 animals (12 Sham, 14 Sed, 10 Exe_1-3, 12 Exe_5-7 and 12 Exe_1-7), and in the ipsilateral hemisphere of 59

animals, as was the pooled numerical density of both hemispheres (with 11 animals in the Exe_5-7 group). Stitched images of the granular cell layer of the ipsilateral hemisphere immunostained for DCX were analyzed for each of the 5 experimental groups (for representative images see Fig. 5A; DCX+ cells of a sham rat can also be seen at a higher magnification in Fig. 5B). The mean density of the DCX^+ cells was also calculated for each experimental group in the contralateral (Fig 5C) and ipsilateral hemispheres (Fig. 5D).

Significant between-group differences were detected by ANOVA in the density of DCX^+ cells in the ipsilateral [$F(4,54) = 5.95$, $P<0.001$] and contralateral [$F(4,55) = 6.25$, $P<0.001$] hemispheres, as well as for both hemispheres [$F(4,54) = 7.88$, $P<0.001$]. Post-hoc comparisons showed a significant reduction of the density of DCX^+ cells in the ipsilateral hemisphere of the Sed ($P=0.013$) and Exe_1-3 groups ($P=0.018$) relative to the Sham animals. The DCX^+ cell density in the Exe_1-7 group was significantly higher than in the Sed and Exe_1-3 groups in each hemisphere separately, and in both hemispheres together [Exe_1-7 vs Sed ipsilateral ($P=0.028$), contralateral ($P=0.010$), both hemispheres ($P=0.001$); Exe_1-7 vs Exe_1-3 ipsilateral (0.045), contralateral ($P=0.015$), both hemispheres ($P=0.004$)]. Finally, a significantly higher density of DCX^+ cells was seen in the Exe_5-7 group relative to the Sed and Exe_1-3 groups in the contralateral hemisphere and in both hemispheres together [Exe_5-7 vs Sed contralateral ($P=0.043$), both hemispheres ($P=0.031$); Exe_5-7 vs Exe_1-3 contralateral ($P=0.037$), both hemispheres ($P=0.025$)]. While there appeared to be a higher density of DCX^+ cells in the Exe_5-7 group relative to the Sed ($P=0.069$) and Exe_1-3 ($P=0.077$) rats in the ipsilateral hemisphere, these differences did not reach significance.

In summary, CCI decreased the density of DCX^+ cells in the ipsilateral hemisphere, while exercise in the weeks prior to sacrifice not only restored the degree of neurogenesis in this hemisphere but also, it induced an overall increase in both

hemispheres relative to the sedentary animals after TBI.

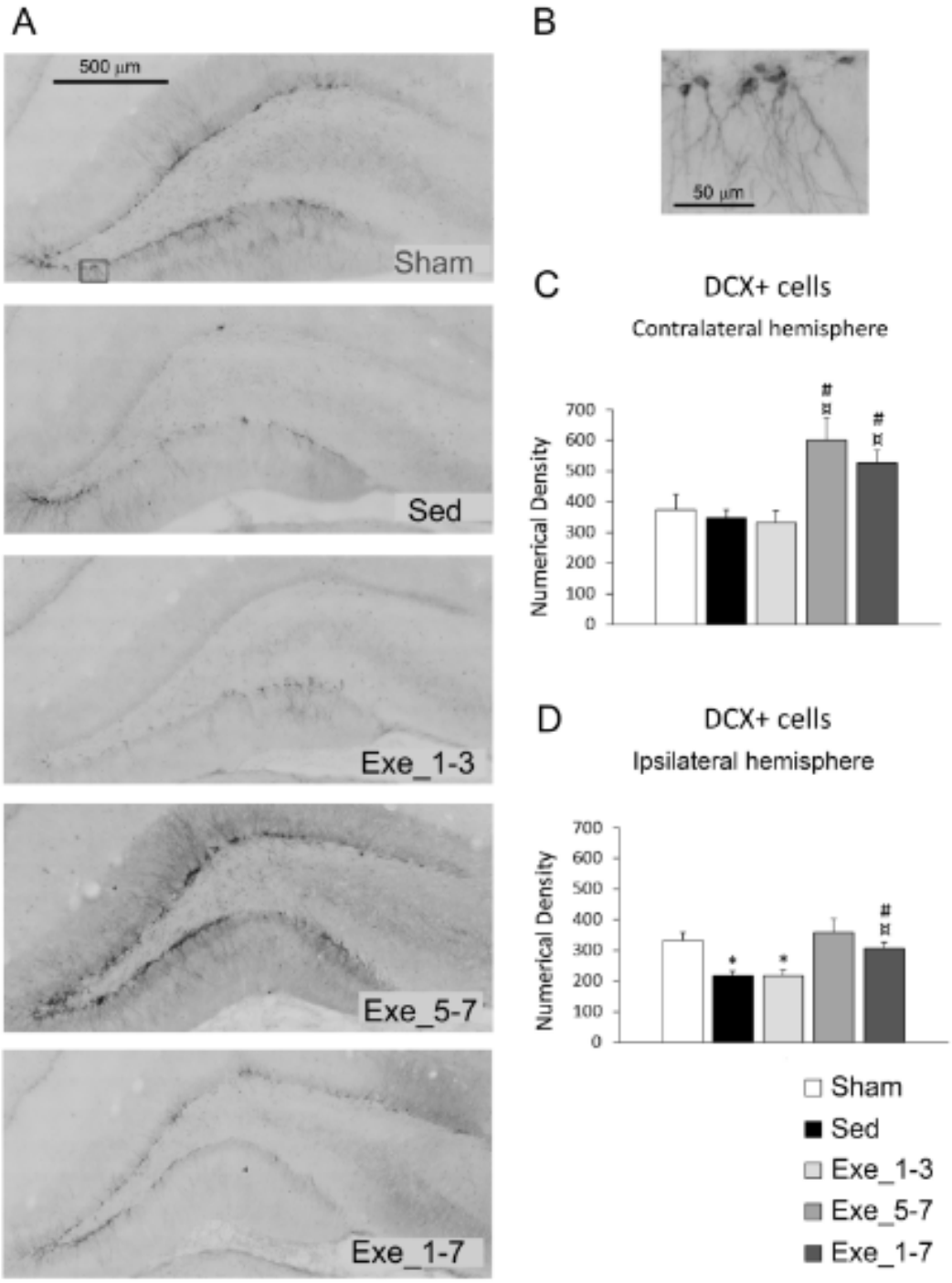


Fig. 5. Representative stitched images of the dorsal dentate gyrus of DCX immunostained slices for each experimental group (A). Larger magnification of DCX+ cells from the region indicated by the rectangle in the image corresponding to a sham rat (B). Mean (+SEM) numerical density of DCX+ cells in the granular cell layer of the dentate gyrus for each experimental group in the contralateral (C) and ipsilateral (D) hemispheres. *($P<0.05$): Significant differences compared to Sham group. # ($P<0.05$): Significant differences compared to L-sed group; ¤ ($P<0.05$): Significant differences compared to L-exe-sed group.

The relationship between exercise, neurogenesis and performance in the ORM task

In the adult rodent DG, DCX is known to label neuroblasts (type 3 cells) and early post-mitotic neurons. Thus, DXC can identify cells that were born a few days earlier and for up to 2-3 weeks after cell birth (Gonçalves et al., 2016; Toni and Schinder, 2015). At around 3-4 weeks of age, these newly generated neurons start to express NeuN (Toni and Schinder, 2015). Given the timing of DCX labelling, we examined whether there were any significant correlations between the number of DCX^+ cells and the mean daily distances and running times of the animals in the last two weeks of the experiment. In the Exe_5-7 group (but not in the Exe-1-7 group) there were significant positive correlations between the density of DCX^+ cells in both the ipsilateral and contralateral hemispheres, and the mean distance run daily [ipsilateral r=0.66, P=0.026; contralateral r=0.78, P=0.002] and the time spent running during this period [ipsilateral r=0.74, P=0.009; contralateral r=0.76, P=0.003]. When the data from the animals in the Exe_5-7 and Exe_1-7 groups were pooled, a significant relationship, which fitted a quadratic U-inverted function (R^2=0.393, P=0.005; see Fig. 6), was found between the mean daily running times in the two weeks prior to sacrifice and the density of DCX^+ cells in the contralateral hemisphere. This suggests that moderate exercise produces more DCX^+ cells than both low and high levels of exercise.

New-born neurons seem to play a significant role in learning and memory (Gonçalves et al., 2016) and hence, we performed a correlational analyses on the discrimination indices from the 3 h and 24 h retention tests, and the DCX^+ cell density. For the whole sample, a positive correlation was found between the density of DCX^+ cells in the ipsilateral hemisphere and the discrimination index in the 24 h but not the 3 h retention test (r=0.38, P=0.003). This correlation was considerably higher for the Exe_5-7 rats (r=0.87, P<0.001), whereas it was not significant for the Exe_1-7 group alone. In addition, the Exe_5-7 group displayed significant correlations between the

discrimination index in the 24 h but not the 3 h retention test and the following exercise measures: total running time in the 3 weeks of wheel availability (r=.556, P=0.049), and mean daily running time in week 6 p.i. (the week prior to ORM training: r=.608, P=0.027). No significant correlations were found between the discrimination index in the 24 h retention test and any variables related to the amount of exercise for the other two exercise groups.

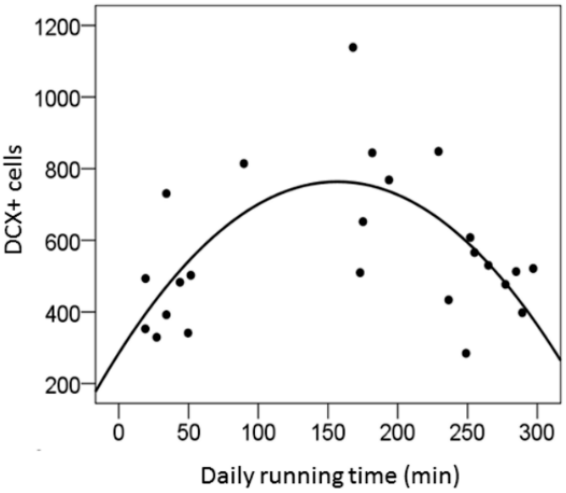


Fig. 6. Relationship between mean daily running time in the last two weeks of the experiment and the numerical density of DCX+ cells in the contralateral hemisphere. This relationship fits a significant inverted-U quadratic function (R^2=0.393; P=0.005), with higher neurogenesis levels being associated to moderate levels of daily running times.

$NeuN^+$ cells in the hilus

*T*he $NeuN^+$ cells in the hilus of the DG were analyzed in 59 animals (14 Sham, 11 L-sed, 12 Exe_1-3, 11 Exe_5-7 and 11 Exe_1-7). In the stitched images depicting the DG immunostained for NeuN (Fig. 7A), the white line (image from a sham rat) corresponds to the area where $NeuN^+$ cells were quantified (hilus). The mean number of $NeuN^+$ cells was quantified in each of the 3 slices of the ipsilateral hilus for each group (Fig. 7B). In the most rostral slice (1) the lesion cavity was small, whereas a large lesion cavity was evident in the most caudal slice (3). We compared the number of $NeuN^+$ cells in each of

the three slices separately for each group using polynomial contrasts. These analyses indicated no significant differences between the three slices in the Sham rats, while the number of $NeuN^+$ cells decreased from the first to the third slice in all the CCI groups, a decrease that fitted a linear function [Sed $t = -5.67$, $P<0.001$; Exe_1-3 $t = -6.86$, $P<0.001$; Exe_5-7 $t = -12.59$, $P<0.001$; Exe_1-7 $t = -8.62$, $P<0.001$].

To examine any possible regional differences in the number of $NeuN^+$ cells between groups, a mixed multivariate analyses of variance was used (group x slice). These analyses indicated significant differences for the two main factors (group and slice) in the ipsilateral hemisphere, as well as their interaction [group $F(54,4) = 30.39$, $P<0.001$; slice $F(108,2) = 125.07$, $P<0.001$; group x slice $F(108,8) = 7.24$, $P<0.001$]. Thus, we analyzed the simple effects for each slice separately, comparing the number of $NeuN^+$ cells in each group to that in the Sed group. These analyses indicated that there were significantly fewer $NeuN^+$ cells in the Sed group than in the Sham group in each of the three slices (slice 1 $t = 2.84$, $P= 0.006$; slice 2 $t = 7.46$, $P<0.001$; slice 3 $t = 11.66$, $P<0.001$). In addition, there were significantly more $NeuN^+$ cells in the first slice of the rats in the Exe_1-3 group than in those of the Sed animals ($t = 2.33$, $P=0.023$), and the difference between these two groups approached significance for the second slice ($P=0.066$) but was not statistically different in the third slice. The Exe_5-7 and Exe_1-7 groups did not show significant differences relative to the Sed rats in any of the slices. Moreover, neither the main factors nor their interaction differed significantly in the contralateral hemisphere.

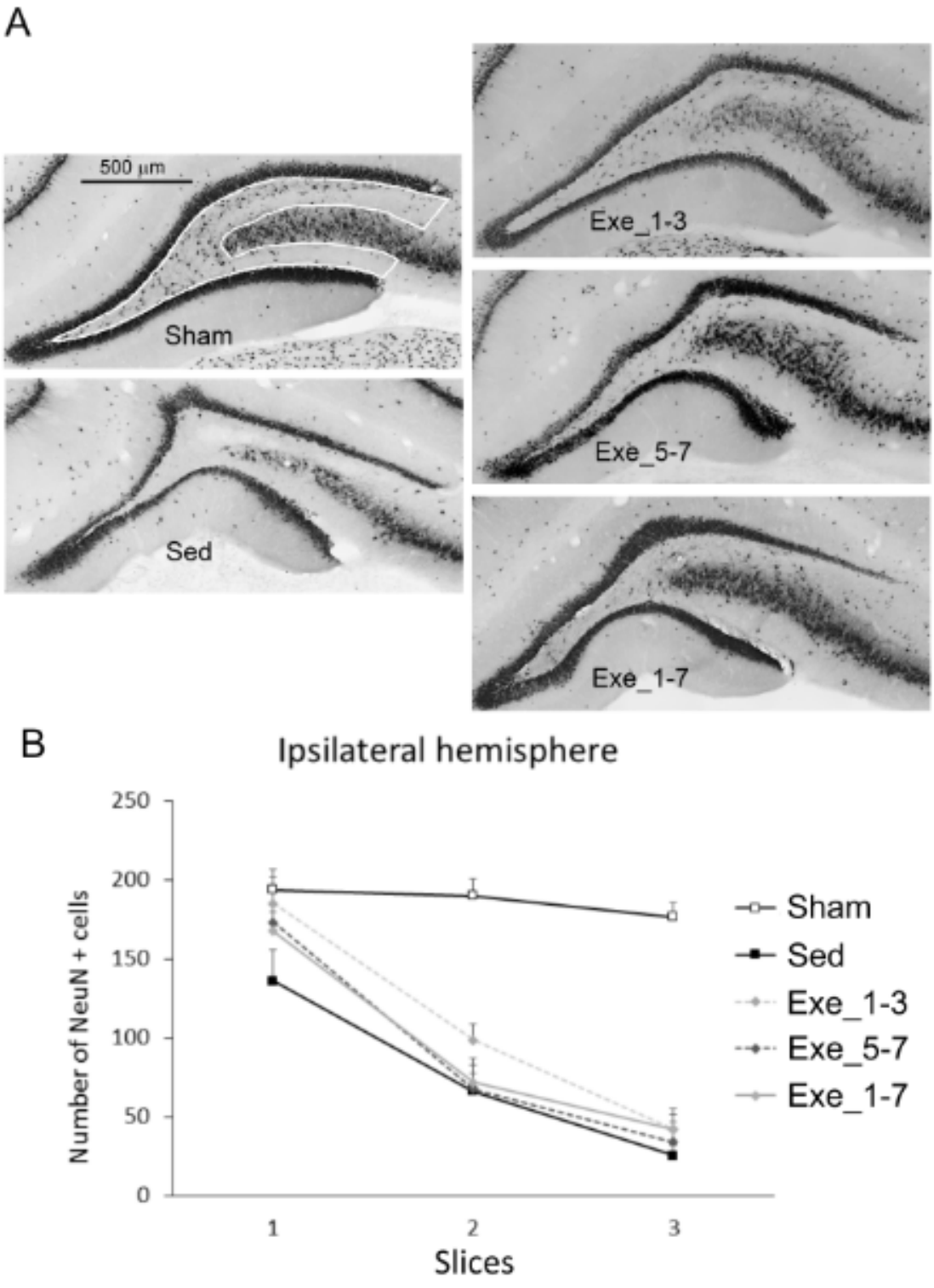


Fig. 7. Representative stitched images of the hilus of the dentate gyrus of NeuN immunostained slices for each experimental group. The white line in the image corresponding to a Sham animal delineates the quantified area (A). Mean (+SEM) number of NeuN+ cells in each of the three slices quantified in the ipsilateral hilus in each experimental group (B). The number of NeuN+ cells in each of the three slices was significantly lower in L-sed group compared to Sham group ($P<0.01$). L-sed group had also a significantly lower number of NeuN+ cells compared to L-exe-sed group in the first slice ($P=0.023$).

In summary, there were substantially fewer mature neurons in the ipsilateral hilus as a consequence of CCI, which was most dramatic in the caudal slices where the lesion cavity was larger, and which was attenuated in the most rostral slice of the Exe_1-3 rats. For the whole sample, the discrimination index in the 24 h retention test was positively correlated to the number of $NeuN^+$ cells in the first slice of the ipsilateral (r=0.41, P=0.001) and contralateral (r=0.33, P=0.010) hilus, and with the total number of $NeuN^+$ cells in the 3 slices of the ipsilateral hilus (r=0.29, P=0.025).

$NeuN^+$ cells in the perirhinal cortex

There did not appear to be any significant differences among the groups in terms of the density of $NeuN^+$ cells in the perirhinal cortex when assessed by ANOVA, neither in the ipsilateral nor the contralateral hemispheres.

$Iba1^+$ cells in the dorsal hippocampus

Iba1 immunohistochemistry was performed on 35 rats (n=7 per group) and analyzed in the ipsilateral hippocampus of rats in each group (for representative images see Fig. 8A), calculating the proportion of animals in each group that presented dense clusters of intensely stained $Iba1^+$ cells (foci: Fig. 8B). Qualitative inspection revealed that the increase in Iba1 immunoreactivity in CCI animals was not homogeneous but rather, it tended to concentrate in certain areas, especially in animals that developed foci of these cells. As expected, none of the Sham animals developed foci in any of the sections analyzed, whereas all the rats in the Sed group and 86% of those in the Exe_5-7 group displayed at least one focus. This percentage fell to 57% in the two groups that initiated physical exercise 4 days after surgery (Exe_1-3 and Exe_1-7 groups).

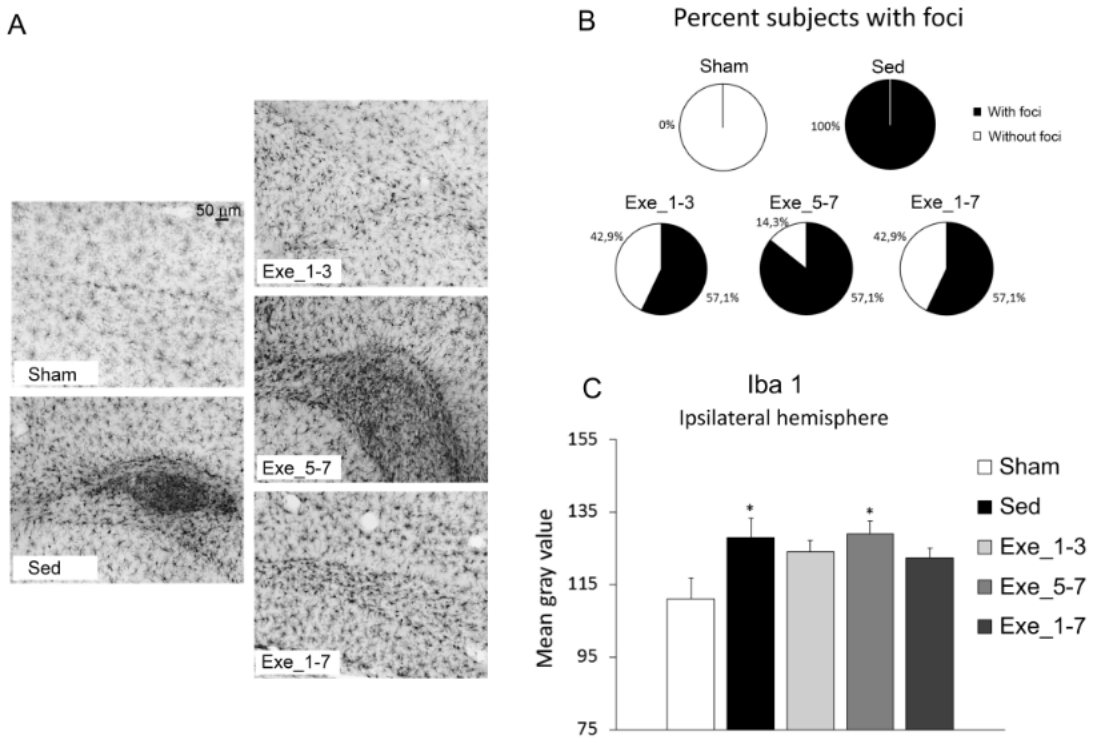


Fig. 8. Representative microphotographs of the hippocampus stained for Iba1 for each experimental group (A). Percent animals in each group with foci of dense clusters of reactive microglia cells (B). Mean (+SEM) of mean gray values of Iba1 immunostaining in each group in the ipsilateral hippocampus (C). * ($P<0.05$): Significant differences compared to Sham group.

The density of these cells was assessed from the mean gray values of Iba1 immunoreactivity in the hemisphere ipsilateral to the impact (Fig. 8C), with higher mean gray values indicating more intense immunolabeling and thus, a higher density of cells. Significant between-group differences were evident in the ipsilateral hemisphere when assessed by ANOVA [($F(4,30) = 2.75$, $P=0.046$], while no differences were found in the contralateral hemisphere. Post-hoc analyses for the ipsilateral hemisphere revealed a significant increase in the mean gray values of the Sed ($P=0.049$) and Exe_5-7 ($P=0.043$) groups relative to the Sham group. By contrast, the mean gray values of the Exe_1-7 and Exe_1-3 groups were not statistically different to those from either the Sham rats or any other CCI group, suggesting an attenuation of Iba1 immunoreactivity. There were no significant differences in the area occupied by the immunolabeling between the different groups.

Discussion

Effects of CCI on ORM

This study shows that CCI induces long-term (53 days p.i.) deficits in ORM in male rats when memory is tested 24 h after acquisition. These deficits are similar to those described previously at 25 and 45 days p.i. (Amorós-Aguilar et al., 2015; Jacotte-Simancas et al., 2015), using the same objects and training conditions, as well as the same CCI parameters. In contrast, only a minor deficit in the 3 h retention test was found, since the discrimination index of L-sed rats was not significantly different from 0, indicative of lack of recall, but their performance did not significantly differ from that of sham animals. Using the same training conditions and CCI severity, previous works have found either impairment (Jacotte-Simancas et al., 2015) or no disturbance (Amorós-Aguilar et al., 2015) in this retention test, suggesting that the performance in this test might vary depending on minor variations in the specific experimental manipulations of the animals, or on the size and location of the lesion cavity. In addition, such differences might also reflect the presence or absence of damage to the perirhinal cortex, a region involved in visual object recognition (Kealy and Commins, 2011). Indeed, when CCI was previously seen to impair performance in the 3 h retention test there was a significant loss of neurons in the perirhinal cortex (Jacotte-Simancas et al., 2015), whereas no neuronal loss was found in this region here, as will be discussed later.

ORM is considered a kind of non-spatial declarative memory task that involves components of episodic-like memory (Cohen and Stackman Jr., 2015). Variations in training and testing procedures may emphasize one or several of these components, and may vary the degree of involvement of different brain structures. Several data suggest that the neural basis of the 3 h and 24 h retention tests differ, with a higher involvement

of the hippocampus in the latter. First, in the 24 h retention the position of the familiar object was changed with respect to its position in the 3 h retention test, increasing the spatial requirements of the task. Second, using a sample trial where two different, rather than two identical, objects were presented, memory of the animals in a test carried out 24 h later was found to depend on plasticity changes in the hippocampus (Clarke et al., 2010). The present training conditions share some of the procedures of the latter work, since the 3 h retention test can be viewed as a second sample phase with two different objects. Third, memory load is higher in the 24 h retention test, which may also require stronger hippocampal participation (Cohen and Stackman Jr., 2015). Finally, we found positive correlations between the discrimination index in the 24 h, but not the 3 h, retention test and both the number of $NeuN^+$ cells in the hilus and the density of DCX^+ cells in the DG.

Histological effects of CCI

CCI is considered mainly a model of focal TBI, because it induces a lesion cavity in the area of the cortex where the impact is applied. However, there is evidence both postmortem as in vivo (magnetic resonance imaging studies), that the pathophysiological changes tend to spread to other brain regions, even to the contralateral hemisphere (particularly with regard to white matter), and may involve widespread axonal degeneration (Bouilleret et al., 2009; Bramlett and Dietrich, 2002; Chen et al., 2003; Hall et al., 2008; Harris et al., 2016; Kochanek et al., 2002; Pischiutta et al., 2018; Turtzo et al., 2013).

In the present work, the lesioned animals had the expected lesion cavity over the parietal lobe seven weeks after injury. In addition, the dorsal hippocampus volume was smaller in animals with TBI that remained sedentary throughout the experiment, in conjunction with largescale neuronal loss in the hilus of the ipsilateral hemisphere. This

neuronal loss was more intense caudally, where the lesion cavity was larger, than in the more rostral section. Stronger microglial reactivity was also evident in the dorsal hippocampus. Thus, all the animals in Sed group had at least one focus of intense microglial reactivity. Increased immunoreactivity was not homogeneous throughout the dorsal hippocampus but rather, it tends to manifest as regions of intense reactivity surrounded by regions with few reactive microglia/macrophages. This pattern might explain the absence of significant differences between the CCI and Sham rats in terms of the proportion of the tissue occupied by the immunolabeling. However, Iba1 immunostaining alone cannot differentiate the specific phenotype of activated microglia/macrophages (M1, M2a, M2b…), a limitation that must be borne in mind. Neurogenesis may be enhanced or impaired by TBI depending on distinct variables, such as the time after injury (Ngwenya and Danzer, 2019). Here, CCI induced a significant decrease in the density of DCX^+ cells in the granule cell layer of the ipsilateral DG 7 weeks after injury.

In consonance with the fact that the damage induced by CCI may spread to distant structures, an earlier study, using the same CCI parameters, found neuron loss in the perirhinal cortex 25 days post-CCI (Jacotte-Simancas et al., 2015). In contrast, in the current work no evidence of reduced number of NeuN+ cells in the perirhinal cortex after CCI has been found. The reasons for these divergences are not known, particularly due to the scarcity of data on damage to this brain area after experimental TBI. Using a different TBI model (lateral fluid percussion), the extent of damage to the perirhinal cortex at 3 (magnetic resonance imaging) and at 13 days (histology) p.i. was found to depend on lesion severity (Ekolle Ndode-Ekane et al., 2017). This suggests that small variations in lesion severity and location might produce distinct effects on the integrity of this region.

The effects of the different regimes of physical exercise on memory and histological variables

Early discontinued exercise (Exe_1-3). It was previously found that 3 weeks of voluntary physical exercise initiated 4 days p.i. reversed the deficits in ORM performance, increased neurogenesis, and reduced TBI-related neuron loss in the hilus and the perirhinal cortex (Jacotte-Simancas et al., 2015). One aim of the present study was to determine whether the benefits of early exercise vanish four weeks after discontinuing exercise. Animals in the early discontinued (Exe_1-3) group retained good memory of the familiar object and their discrimination index in the 24 h retention test did not differ from that of either Sham animals or Sed rats, indicating attenuation of memory deficits. Thus, the benefits on memory persisted for 4 weeks after exercise discontinuation.

The loss of $NeuN^+$ cells in the hilus, the smaller volume of the dorsal hippocampus and the enhanced microglial reactivity induced by CCI were also attenuated in the early discontinued (Exe_1-3) group. Hence, early exercise appears to have neuroprotective effects that persist beyond the moment when exercise is discontinued. In terms of neurogenesis, there was a similar numerical density of DCX^+ cells in both hemispheres of rats subjected to the early discontinued exercise protocol (Exe_1-3 group) and sedentary lesioned rats, fewer than in the ipsilateral hemisphere of sham animals. This indicates that the enhanced neurogenesis that may occur during 3 weeks of physical exercise initiated soon after injury (Jacotte-Simancas et al., 2015) does not persist for 4 weeks after exercise discontinuation. Indeed, there is evidence from healthy mice that interrupting physical exercise for 5 weeks not only checks the enhanced neurogenesis induced by exercise but furthermore, neurogenesis is reduced to levels even lower than those in sedentary animals (Nishijima et al., 2017).

Therefore, the recovery of memory function by early discontinued exercise might be

mediated by regional neuroprotection of mature hilar neurons (an assumption further supported by the positive correlation between discrimination in the 24 h retention test and the number of mature neurons in the hilus), as well as by dampened microglial reactivity. In addition, a role for neurons thought to be born during the exercising period cannot be ruled out. Some such neurons might be incorporated into functional circuits and reach maturity (no longer expressing DCX), contributing to memory performance.

Delayed exercise (Exe_5-7). The animals that initiated physical exercise four weeks after injury performed very well in the 24 h ORM test, similar to Sham group and significantly better than the sedentary TBI animals that performed poorly in this test. Thus, physical exercise might not only reduce but it may even reverse memory deficits, even when it is initiated at more chronic stages, long after the cascade of events leading to secondary injury has been triggered.

Our results are consistent with the reversion of ORM deficits in mice that initiated voluntary physical exercise 5 weeks after CCI (Piao et al., 2013), or when subjected to delayed forced exercise (Ko et al., 2018). However, they apparently contradict the benefits of post-TBI physical exercise when initiated early but not late after injury (Chen et al., 2013). Nevertheless, since that latter data was obtained using a closed head injury model of TBI, the optimal time to initiate exercise may depend on the specific kind of TBI induced, among other variables.

The animals of the Exe_5-7 group, which had been exercising during the last three weeks of the experiment, had a higher density of DCX+ cells than Sed animals as well as than Exe_1-3 rats, which had remained sedentary for 4 weeks before sacrifice. More importantly, in the delayed (Exe_5-7) group the amount of exercise correlated positively with the numerical density of DCX+ cells, and in turn both variables correlated positively with the discrimination index in the 24h retention test. Positive correlations

between neurogenesis and performance in the ORM task had also been reported after 3 weeks of early continuous exercise (Jacotte-Simancas et al., 2015). Newly generated neurons are endowed with electrophysiological properties that render them highly plastic, and they are thought to be involved in learning and memory (Gonçalves et al., 2016). Therefore, the data presented support the notion that enhancement in the number of novel neurons was involved in the benefits of the delayed physical exercise protocol on ORM.

The delayed exercise regime (Exe_5-7 group) also attenuated the loss of dorsal hippocampal volume in the ipsilateral hemisphere relative to the contralateral one, as reflected by the interhemispheric ratio. By contrast, this protocol did not reduce the loss of mature neurons in the hilus, the intensity of Iba1 staining, or the foci of reactive microglia in the dorsal hippocampus. These latter results contrast with previous findings following delayed physical exercise in mice (Piao et al., 2013).

Early continuous exercise (Exe_1-7). We hypothesized that exercise initiated early after injury would reduce the degree to which some biochemical and cellular cascades provoke secondary damage, and that exercise around the time of learning and memory training could improve cognition through increased neurogenesis. Accordingly, we expected that physical exercise initiated early after injury and maintained until completion of the memory tests would exert the strongest effects on memory. However, while such animals had good memory of the familiar object in the 24 h retention test, their performance did not differ significantly from that of sedentary or sham-operated animals. Thus, the ORM deficits of these rats were attenuated to a similar extent as in rats following the early discontinued regime, but to a lower extent than in the delayed exercise group.

With regard to neurohistological data, early continuous exercise (Exe_1-7 group)

increased neurogenesis and reduced the inflammatory response. Thus, Iba1 immunostaining was attenuated in these animals (with no significant differences in mean gray scale values between sham-operated rats and animals in this group), consistent with a substantial reduction in the proportion of rats with foci of intense Iba1 staining (57% as opposed to 100% in TBI sedentary rats).

The early continuous (Exe_1-7) schedule of physical exercise restored neurogenesis, but in this group no significant correlations were found between neurogenesis and the discrimination index in the 24 retention test or between amount of daily exercise and neurogenesis, unlike the positive correlations detected in the delayed exercise protocol (Exe_5-7). It must be noted that daily running distance and time of the Exe_1-7 group were higher compared to the delayed exercise group, which is consistent with data on healthy rats with long-term access to running wheels (Mondon et al., 1985; Nguemeni et al., 2018). Interestingly, when the data of animals of the two groups that exercised at the end of the experiment (Exe_5-7 and Exe_1-7 groups) were pooled, a significant inverted-U shaped function was found between mean daily running times and numerical density of DCX+ cells. This indicates that more neurogenesis was provoked by medium running times than by high running times, which is in concordance with data from healthy rats (Nguemeni et al., 2018).

In healthy rodents there is evidence that the greatest benefits in memory performance are found with moderate levels of voluntary wheel running (Diederich et al., 2017; Garcia-Capdevila et al., 2009). Similar results were found after forced treadmill exercise in a rat model of ischemic stroke (Shih et al., 2013). This led us to wonder whether the high levels of running shown by Exe_1-7 rats might partly explain the suboptimal effects of this regime on memory recovery, although the present results do not allow us to solve this intriguing question. In addition, one must be aware that duration of exercise treatment (considerably longer in the Exe_1-7 group), as well as other

variables, may have also influenced the outcomes found in the early continuous condition. On any instance, the data found underscore the importance of carrying out further studies aimed specifically at testing the relationship between amount of exercise and memory recovery after TBI.

Limitations

The main limitation of this study is that only male rats were used. While the prevalence of TBI is substantially higher in human male subjects than females during adolescence and youth (Faul and Coronado, 2015), this does not imply that animal studies should not address potential gender differences in terms of both TBI outcome or the effect of therapeutic interventions (Späni et al., 2018). While some works have examined gender differences in the effects of either TBI or exercise separately, to our knowledge there are not previous studies examining whether there are sex/gender differences in the cognitive effects of exercise after experimental TBI. Preliminary data obtained in our laboratory indicate that CCI during late adolescence induces similar ORM deficits in male and female rats, although the latter display more intense locomotor activity and object exploration in the training cage. Whether exercise exerts gender-related effects on cognitive performance or histopathological parameters associated to TBI remains unclear and is currently being assessed.

In addition, it is important to take into account that the present results have been obtained with animals that sustained TBI during late adolescence, and for a specific memory task.

Conclusions

The study presented here shows that voluntary physical exercise can reduce the deficits in object recognition memory associated to the CCI model of TBI (focal cortical injury

with progressive spreading of damage to other gray and white matter regions). The benefits are produced both when exercise is initiated soon (4 days) or late (4 weeks) after injury, although the most pronounced effects were found with the delayed protocol. The effects of early exercise were maintained after the exercise regime was discontinued and it appears that the mechanisms mediating the effects of the different exercise protocols differed. As such, the reversion of the memory deficits induced by delayed exercise was mainly associated with enhanced neurogenesis. Early continuous exercise attenuated microglial reactivity in the dorsal hippocampus and enhanced neurogenesis. In turn, discontinued exercise produced some neuroprotection (attenuation of the loss of hilar neurons and of hippocampal volume in the ipsilateral hemisphere). These data have promising implications for patients, at least when TBI is suffered during late adolescence/early youth, since they suggest that exercise can contribute to reduce deficits of non spatial hippocampus-dependent memory at different stages post-TBI. They also underscore the need to clarify the possible relationship between amount of exercise and degree of memory effects after TBI.

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Competing interests

The authors declare that they have no competing interests.

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