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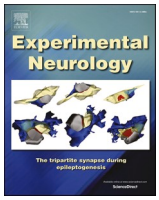

Research paper

Physical exercise as a cognitive rehabilitation treatment after traumatic brain injury: Intensity- and sex-dependent effects

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ABSTRACT

We investigated the effects of forced physical exercise (PE) intensity on cognitive dysfunction and histological changes associated with traumatic brain injury (TBI), in both male and female rats. Controlled cortical impact (CCI) produced similar short- and long-term memory deficits in both sexes, and these deficits were associated with reduced volume and neuronal loss in the hippocampus, but not with changes in neurogenesis. We found sex differences in the effects of intensity of forced PE on cognitive recovery: all PE intensities tested improved short-term memory in both sexes, but to a greater extent in females, while long-term memory benefits were intensity- and sex-dependent. Males benefited most from low-intensity PE, while females showed optimal results at moderate intensity. These optimal PE intensities increased the neurogenesis in both sexes. A neuroprotective effect of low-intensity PE was evident in males, but no effect was observed in females. These findings suggest an intensity- and sex-specific effect of PE post-TBI, emphasizing the need for tailored PE protocols based on sex to enhance therapeutic outcomes.

1. Introduction

Traumatic brain injury (TBI) is a growing public health concern and it is the leading cause of death and disability worldwide among trauma-related injuries (Rubiano et al., 2015). In a significant proportion of patients, TBI results in chronic functional deficits across multiple domains, including sensory and motor deficits, as well as cognitive problems such as attention, episodic memory, executive functions, working memory, information processing speed, language functions, and visuo-spatial processing. Among these cognitive deficits, persistent memory impairment is particularly common (Dikmen et al., 2009; Horneman and Emanuelson, 2009). Consequently, there is a critical need for therapeutic strategies to mitigate these cognitive deficits. The pathophysiology of TBI is complex, involving both primary and secondary injuries. The primary injury is caused directly by external impact to the brain and occurs within seconds. This primary injury triggers secondary injury, which evolves over weeks to months and consists of a molecular, chemical, and inflammatory cascade that cause further brain damage (Galgano et al., 2017). Research on TBI has focused on stabilizing the primary injury site, reducing or preventing secondary damage, and improving the neural network reorganization and functional recovery. Despite significant advancements in understanding the mechanisms of primary and secondary brain injuries, no specific therapeutic protocols for TBI have been shown to be effective (Alves et al., 2019; Galgano et al., 2017).

The less-than-ideal clinical outcomes of treatment have been attributed to several factors, with gender bias in TBI research emerging as a significant contributor. Review studies suggest that being female is a vulnerability factor for developing prolonged post-concussion symptoms (King, 2014). These findings strongly support the need for sex-specific treatments tailored to address such differences. However, there is a sex bias in TBI research that is evident in both clinical trials, where fewer women are recruited, and experimental injury models, which predominantly use male rodents (Gupte et al., 2019). This bias not only impedes the understanding of TBI pathophysiology but also hinders the development of effective treatments, underscoring the urgent need for inclusive and balanced research methodologies that consider sex-specific factors to improve treatment efficacy and promote better clinical outcomes for all TBI patients.

Physical exercise (PE) has emerged as a promising rehabilitative

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treatment for reducing TBI-induced cognitive deficits (O'Carroll et al., 2020; Zhang et al., 2022). The beneficial effects of post-injury PE appear to be linked to a decrease in the neurodegenerative processes, primarily through reducing inflammation (Amorós-Aguilar et al., 2020; Piao et al., 2013) and neuronal death (Amorós-Aguilar et al., 2020; Itoh et al., 2011; Jacotte-Simancas et al., 2015; Kim et al., 2010; Ko et al., 2018; Piao et al., 2013; Yoon and Kim, 2018), as well as increasing regenerative processes, particularly neuroplasticity (Griesbach et al., 2007; Griesbach et al., 2004; Piao et al., 2013) and neurogenesis (Amorós-Aguilar et al., 2020; Jacotte-Simancas et al., 2015; Piao et al., 2013). However, uncertainties remain regarding the optimal PE parameters, including exercise intensity. The few studies assessing the effect of exercise intensity on TBI-associated cognitive deficits in animal models have yielded mixed results, showing beneficial effects at either low (Shen et al., 2013) or moderate (Karelina et al., 2021) intensities. These discrepancies may be due to differences in exercise protocols or strains used. Notably, the only experiment examining the effect of exercise intensity on TBI outcomes in both male and female mice (White et al., 2023) revealed sex-dependent effects. In males, low-to-moderate aerobic exercise improved functional outcomes, whereas high-intensity exercise impaired cognitive recovery. Conversely, in female mice, all the three exercise intensities had an intermediate effect on cognitive recovery. These findings suggest that rehabilitative treatments should be sex-specific and highlight the need to include female subjects in TBI research and potential rehabilitative treatments.

In the present study, we aimed to evaluate how different exercise intensities affect the reversal or attenuation of TBI-induced memory deficits in both male and female subjects.

2. Materials and methods

2.1. Ethics and animal welfare

All procedures were performed in compliance with the local and European legislation regulating the care and ethical issues related to animal experimentation (2010/63/EU; Real Decreto 1386/2018) and were approved by the corresponding ethics committee for animal experimentation (Autonomous Government of Catalonia, 9734/1).

Sixty-eight male and sixty-two female Sprague-Dawley albino rats (Charles River Laboratories; Abresle, France; Supplied by Prolabor; Barcelona, Spain) 7 weeks old on arrival were used. The animals were initially kept in quarantine for one week and then housed in pairs in cages (48 × 26 × 20 cm) with ad libitum access to water. Thus, the animals were 8 weeks old at the beginning of the experiment and their mean initial body weight (mean ± SD) was 185.43 ± 16.93 g for the females and 242.08 ± 25.30 g for the males. Throughout the experiment the animals were provided a fixed amount of food (60 g/cage/day for males and 50 g/cage/day for females, 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, reduces overfeeding and induces better long-term health compared to the standard ad libitum conditions. 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.

2.2. Stereotaxic surgery and TBI

TBI was induced using a controlled cortical impact (CCI) device (Pittsburgh Precision Instruments, Inc., Pittsburgh, PA, USA), as previously described (Jacotte-Simancas et al., 2015). Briefly, an 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 mater layer and persisting for 150 ms. The impactor rod was angled 15 degrees to the vertical axis to maintain a perpendicular position relative to the tangential plane of the brain curvature at the impact surface. Sham animals underwent similar procedures, but without impact. To control for post-operative pain, a non-invasive and stress-free oral treatment with buprenorphine (0.4 mg/Kg body weight; Buprex: Schering-Plough SA, Barcelona, Spain) mixed in Nutella® (2 g/Kg body weight) was administered 1 h pre- and 23 h post-injury. The mixture was offered stuck on a piece of adhesive tape which was attached to the inner home cage wall, a couple of centimetres above the bedding. To ensure that the rats consumed the entire mixture, they were offered the Nutella® two days prior to surgery to habituate them to the new food and avoid food neophobia (Abelson et al., 2012). The estrous cycle of female rats was not evaluated at the time of injury.

2.3. Physical exercise

The animals were randomly assigned to one of the following 5 experimental groups (Fig. 1): Sham control group (Sham): the animals were sham operated and remained in a sedentary condition; Tbi sedentary group (Tbi-sed): the rats underwent a controlled cortical impact (CCI) and remained in a sedentary condition; exercising groups (Tbi-8, Tbi-12, Tbi-16): the rats underwent a CCI and, 4 days later, began PE training (26 min/day in a single session, 5 days a week, until the end of the experiment 4 weeks later). For the exercise sessions, rats were placed in a 37 cm diameter wheel (Rat Wheel, ENV-042, Med Associates, Inc., St.Albans, VT, USA) coupled to a motor controlled by a microprocessor (Arduino® Uno, Arduino SA, Chiasso, Switzerland) that allowed control of the rotation parameters (speed, time, acceleration). During the initial days, the duration, and speed of the exercise were increased daily until reaching the target speed for 20 min per session (8 m/min for the Tbi-8 group, 12 m/min for the Tbi-12, 16 m/min for the Tbi-16 group). After these initial sessions, each 26-min session started with a warm-up (2 min) at low speed, followed by 4 cycles of 5 min at the target speed and 1 min at low speed. Sessions ended with a 1-min cool down at low speed. The different exercise intensities were chosen in a pilot study to determine the running wheel exercise tolerance. This pilot study showed that a substantial proportion of rats did not tolerate intensities higher than 16 m/min. To ensure that all animals in the highest intensity were able to follow the established pace, 16 m/min, 12 m/min (75 % of highest intensity) and 8 m/min (50 % of highest intensity) were chosen as the treatment intensities.

2.4. Object recognition memory (ORM) task

ORM task started 29 dpi as previously described (Amorós-Aguilar et al., 2020). Briefly, sessions were conducted in an open box situated in a sound-attenuating cage ventilated by an extractor fan and illuminated at 30-lx at floor level. The objects used were a Duplo (Lego®) construction, a soft drink can, and a wall hanger, all fixed to the floor of the box using double-sided adhesive tape to prevent movement. The behavioural 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 offline 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 ≤2 cm, while turning around or sitting on the object was not considered exploratory behaviour. To avoid the presence of olfactory cues, the apparatus, and objects were thoroughly cleaned with a 70 % alcohol solution 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 90-min interval on the same day, and the third on the following day). Each habituation session lasted 12 min and the amount of locomotor activity was recorded. To test possible anxiety reactions to novel objects, a neophobia test was conducted 90 min after the last habituation session. In this test, an unfamiliar object was placed 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

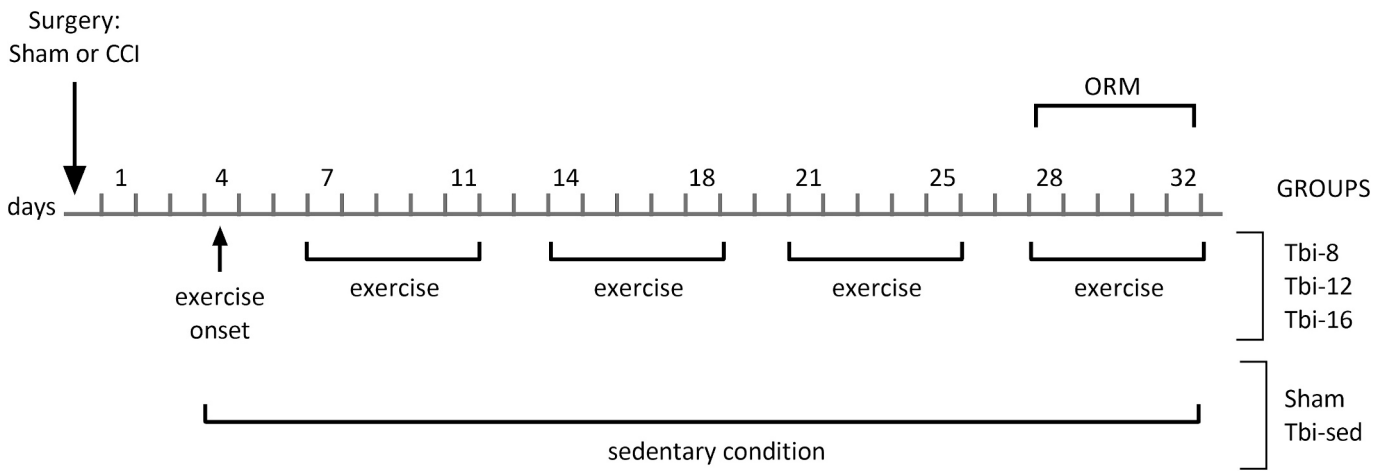

Fig. 1. Timeline of the experimental procedures.

latency to the first exploration was recorded.

The ORM acquisition session was carried out the day after the neophobia session, and consisted of a 15-min acquisition session (two identical objects were placed in adjacent corners of the box, 10 cm away from the walls) followed by 2 retention tests (5 min each). The first retention test was carried out 3 h after acquisition and the second was conducted 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 from that used in the 3-h retention test. The time spent exploring each object was recorded by a researcher blinded to the experimental group.

A discrimination index was used to analyse cognitive performance, allowing adjustment for any differences in total exploration time: ([time exploring the novel object – time exploring the familiar object] / total time spent on both objects) x 100 (Akkerman et al., 2012). Since the ORM test is based on the natural tendency of rats to explore a novel object over a familiar one, an index significantly higher than zero is considered to reflect good recall of the familiar object. A criterion of ≥10 s of exploration during the acquisition session was established for animals to be included in the statistical analyses, as low exploration times may distort encoding processes in this task (Akkerman et al., 2012).

2.5. Processing of brain tissue

The animals were sacrificed 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 brains were immediately dissected out and immersed in the same fixative at 4 °C for an additional 3 h, then rinsed (3 times 20 min each) with phosphate buffer (PB) and submerged in a cryoprotective solution (sucrose 30 % in PB) for 3–4 days at 4 °C. Finally, the brains were frozen in isopentane (2-methylbutane: Sigma-Aldrich, Madrid, Spain) and stored at −80 °C. Parallel coronal cryostat sections (40 μm) were obtained (Shandon Cryotome FSE, Thermo electron corporation, Waltham, MA, USA) between the approximate anteroposterior coordinates −2.76 and − 4.48 from bregma (Paxinos and Watson, 2007). From each animal, 9 series of free-floating sections and one series of sections mounted on gelatine-coated slides were obtained. This procedure allowed for systematic random sampling.

2.6. Cresyl violet staining

The mounted sections were processed for cresyl violet staining and digitised 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 by a researcher blinded to the experimental group to calculate the surface area. The volume of the dorsal hippocampus was estimated by multiplying the sum of the surface areas measured in all the slices by the section thickness (40 μm). An inter-hemisphere ratio was computed for the hippocampal formation [(ipsilateral volume / contralateral volume) x 100]. This ratio was expected to be close to 100 if there was no loss of volume in the hemisphere ipsilateral to the impact.

2.7. Immunohistochemical processing

Free-floating slices from 6 animals per group were processed to assess the distribution of neuronal nuclei (NeuN), a well-recognised marker for mature neurons (Duan et al., 2016). A second series was processed for doublecortin (DCX), which is expressed by immature neurons (Gonçalves et al., 2016).

For NeuN and DCX immunohistochemistry, free-floating brain sections were washed in Tris-buffered Saline (TBS) and quenched for 10 min with 0.3 % H_2O_2 (Panreac Química SLU, Castellar del Vallès, Spain) in TBS. After washing in TBS-T (TBS + 1 % Triton X-100), non-specific binding was blocked by incubating for 1 h with TBS containing 10 % fetal bovine serum (FBS) and 0.3 % bovine serum albumin (BSA) (Sigma-Aldrich, Madrid, Spain). The sections were then incubated overnight at 4 °C and for 1 h at room temperature with a primary antibody against NeuN (Mouse anti-NeuN, MAB377, Sigma, 1:500, Madrid, Spain) or DCX (1:4000 Rabbit polyclonal anti-DCX, 18723: Abcam, Cambridge, UK) diluted in the blocking solution. After washing in TBS and TBS-T, the sections were incubated for 1 h with biotinylated secondary antibodies at room temperature (Goat anti-Mouse IgG (H + L), Biotin Conjugate, SAB 4600004, Sigma, 1:1000 for NeuN or 1:500 biotin conjugated Goat anti-rabbit IgG [A16114]: Thermo Fisher Scientific, Alcobendas, Spain for DCX). 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) diluted in TBS-T, and the reaction product was visualized with diaminobenzidine (DAB) 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™, Nottingham, UK). One well in each well plate did not contain primary antibody and was used as a negative control.

2.8. Quantification of NeuN$^+$ cells

Serial digitised images of NeuN-stained sections were obtained at 10× for the hilus of dentate gyrus (Hil) of hippocampus using a DSLR camera (EOS_6D_Mark II, Canon Europe Amsterdam, The Netherlands) coupled with an Axio Imager A1 microscope (Carl Zeiss Iberia, Madrid, Spain). Fiji (Schindelin et al., 2012) software was used to stitch the partial images of these brain regions to obtain a single image encompassing the whole Hil for each hemisphere.

Due to the relatively low quantity and density of neurons in the Hil, NeuN$^+$ cells in this region were quantified by a researcher blinded to the experimental group by manually outlining the area of the Hil, and all the NeuN$^+$ cells within the outlined area were quantified in each of two or three slices between −2.76 and − 4.20 from bregma (Paxinos and Watson, 2007). For each hemisphere of each slice, neuron density was calculated by dividing the number of NeuN$^+$ cells by the area of the Hil. To compare the density between ipsilateral and contralateral hemispheres to the lesion, the ratio between hemispheres was calculated [(ipsilateral density / contralateral density)*100] for each slice. Finally, the mean of this ratio for the examined slices was calculated. A mean value of 100 indicates that ipsilateral and contralateral hemispheres have the same density of neurons.

2.9. Quantification of DCX$^+$ cells

DCX+ cells in granule cell layer of the hippocampus contralateral to the injury were quantified using the same microscope and camera used in NeuN quantification. In this case, DCX+ cells were manually counted by a researcher blinded to the experimental group under the microscope at 40× on three to four slices between −3.00 and − 3.96 from bregma (Paxinos and Watson, 2007). The mean number of DCX+ cells was calculated for each animal.

2.10. Statistical analyses

The analyses were performed using the statistical programming language R 3.6.3 (R Core Team, 2020) and the graphical interface Jamovi (The Jamovi Project, 2022). Statistical outliers were identified using box-plot analyses, confirmed with a one-sample *t*-test against the group mean and excluded from the corresponding analyses. Differences between habituation sessions were analysed through a repeated measures analysis of variance (ANOVA). Differences between groups were examined using a one-way ANOVA with a between-group design. Multiple post-hoc comparisons between pairs of groups (Sham against all other groups and Tbi-sed against all exercising groups) were conducted using the Finner correction (Finner, 1993). 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). Correlational analyses (r of Pearson) between the ORM discrimination index and the histological variables were performed. Statistical significance was set at a $p < 0.05$ and Cohen's d was used as an indicator of the effect size.

3. Results

No animals died as a result of the surgical intervention.

3.1. Object recognition memory (ORM)

3.1.1. Locomotor activity and neophobia test

Due to technical problems, data from 1 rat (female Tbi-16) in the first session of habituation was lost.

Repeated measures ANOVA for male subjects showed a significant effect of session [$F(2, 126) = 108.66$, $p < 0.001$] and a session × treatment interaction [$F(8, 126) = 2{,}79$, $p = 0.007$]. However, corrected post-hoc contrasts indicated that locomotion decreased from the first to the second session (Sham $p = 0.010$, Tbi-sed $p = 0.010$, Tbi-8 $p = 0.010$, Tbi-12 $p = 0.010$, Tbi-16 $p = 0.010$) and remained stable from the second to the third session in all groups. For female rats, only the session factor was significant ([$F(2,112) = 27.77$, $p < 0.001$) and, similarly to male rats, corrected post-hoc contrast showed that locomotion was reduced in all the groups from the first to the second sessions ($p = 0.002$) and remained stable from the second to the third ones.

Regarding the neophobia test, some data points were identified as outlier values and were excluded from the analyses (male: one from Tbi-sed, two from Tbi-8, one from Tbi-12, one from Tbi-16; female: one from Tbi-sed, one from Tbi-16). No differences between groups in the latency to explore the new object were found in either male or female groups.

3.1.2. Acquisition session

All the animals met the minimum criterion of exploration time required for inclusion in the analyses. ANOVA showed no differences between groups in either male or female.

3.1.3. Retention sessions

Regarding the 3-h retention session (Fig. 2A) two data points were identified as outlier values and were excluded from the analyses (one from male Tbi-8 and one from female Tbi-16).

One-sample *t*-test for male groups indicated that discrimination indices were significantly higher than 0 in all groups except in Tbi-sed (Sham [$t(12) = 6.43$, $p < 0.001$], Tbi-8 [$t(9) = 4.55$, $p = 0.001$], Tbi-12 [$t(14) = 5.51$, $p < 0.001$], Tbi-16 [$t(9) = 4.44$, $p = 0.002$]). Between-groups analysis indicated a significant effect of the treatment factor [$F(4, 59) = 2.60$, $p = 0.045$], and corrected post hoc contrasts showed that the discrimination index was lower for the Tbi-sed than for the Sham group ($p = 0.021$, $d = 1.14$).

Similarly, *t*-test analyses for female rats showed that all groups except Tbi-sed had discrimination indices significantly higher than 0 (Sham [$t(12) = 3.70$, $p = 0.003$], Tbi-8 [$t(11) = 7.39$, $p < 0.001$], Tbi-12 [$t(10) = 4.02$, $p = 0.002$], Tbi-16 [$t(9) = 6.06$, $p < 0.001$]). Between-groups analyses revealed a significant effect of the treatment factor [$F(4, 53) = 5.05$, $p = 0.002$], and corrected post hoc contrasts showed that the discrimination index for all exercised groups was similar to that of the Sham group and higher than that of the Tbi-sed group (Tbi-8 $p = 0.007$, $d = 1.60$; Tbi-12 $p = 0.007$, $d = 1.42$; Tbi-16 $p = 0.007$, $d = 1.37$).

Regarding the 24-h retention session (Fig. 2B) some data points were identified as outlier values and were excluded from the analyses (male: two from Tbi-8, one from Tbi-12; female: one from Tbi-sed, two from Tbi-16).

Male groups, Sham [$t(12) = 4.19$, $p = 0.001$], Tbi-8 [$t(8) = 8.23$, $p < 0.001$] and Tbi-12 [$t(13) = 3.64$, $p = 0.003$] showed discrimination indices higher than 0. ANOVA indicated significant differences between groups [$F(4, 57) = 4.03$, $p = 0.006$] and corrected post-hoc contrast showed that Tbi-sed had a lower discrimination index than Sham ($p = 0.048$, $d = 0.95$) and Tbi-8 ($p = 0.007$, $d = 1.41$) groups.

t-test analyses for females showed that all groups except Tbi-sed had discrimination indices higher than 0 (Sham [$t(12) = 4.72$, $p < 0.001$], Tbi-8 [$t(11) = 2.76$, $p = 0.018$], Tbi-12 [$t(10) = 11.09$, $p < 0.001$], Tbi-16 [$t(8) = 6.26$, $p < 0.001$]). ANOVA showed a significant effect of the treatment factor [$F(4, 51) = 4.03$, $p = 0.006$] and corrected post-hoc contrasts indicated that Tbi-sed had a lower discrimination index than Sham ($p = 0.010$, $d = 1.30$) and Tbi-12 ($p = 0.007$, $d = 1.58$) groups.

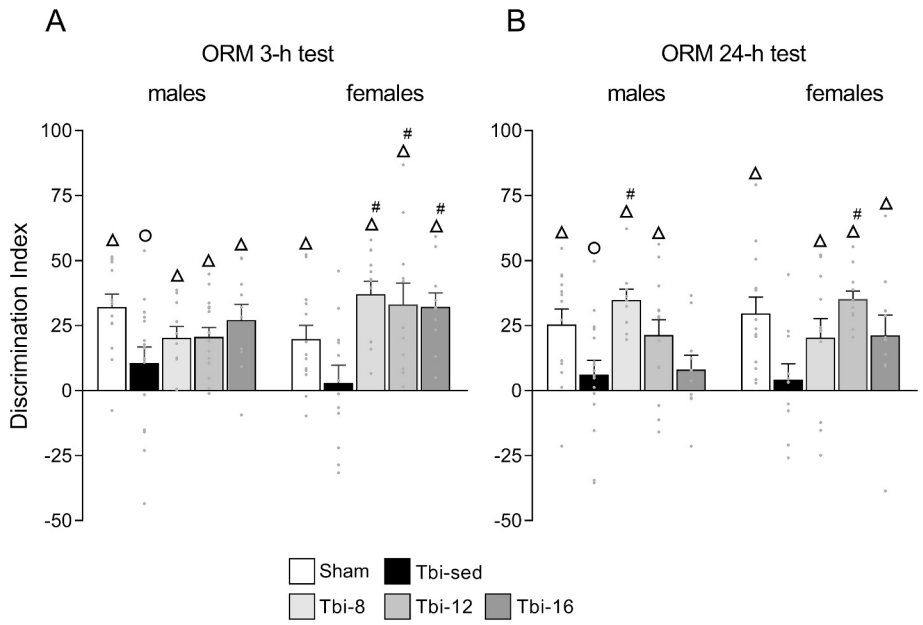

Fig. 2. Mean (+SEM) values of the discrimination index for each experimental group in the retention tests of the object recognition memory task carried out at 3 h (A) and 24 h (B) after acquisition. Δ: Significant differences compared to the reference value (0); o: Significant differences compared to the corresponding Sham group; #: Significant differences compared to the corresponding Tbi-sed.

3.2. Hippocampal volume

Tissue from two subjects from male Tbi-sed group was damaged and therefore excluded from the staining.

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 as seen previously (Amorós-Aguilar et al., 2020; Jacotte-Simancas et al., 2015).

Results were similar for male and female rats. All groups, except Sham, showed a interhemispheric hippocampal volume ratio significantly below 100 [male: Tbi-Sed $t(13) = -4.45$, $p < 0.001$; Tbi-8 $t(11) = -7.92$, $p < 0.001$; Tbi-12 $t(14) = -9.21$, $p < 0.001$; Tbi-16 $t(11) = -8.29$, $p < 0.001$; female: Tbi-sed $t(11) = -14.48$, $p < 0.001$; Tbi-8 $t(11) = -8.85$, $p < 0.001$; Tbi-12 $t(11) = -9.11$, $p < 0.001$; Tbi-16 $t(10) = -8.62$, $p < 0.001$]. ANOVA showed a significant effect of the treatment factor for both sexes [male $F(4, 60) = 10.64$, $p < 0.001$; female $F(4, 56) = 24.59$, $p < 0.001$] and corrected contrast indicated that, in both sexes, all Tbi groups had a lower interhemispheric hippocampal volume ratio than the Sham group [male: Tbi-sed ($p = 0.007$, $d = 1.62$), Tbi-8 ($p = 0.007$, $d = 2.20$), Tbi-12 ($p = 0.007$, $d = 1.68$), Tbi-16 ($p = 0.007$, $d = 2.39$); female: Tbi-sed ($p = 0.007$, $d = 2.78$), Tbi-8 ($p = 0.007$, $d = 2.88$), Tbi-12 ($p = 0.007$, $d = 3.16$), Tbi-16 ($p = 0.007$, $d = 3.18$)], with no differences observed between Tbi-sed and any of the exercised Tbi groups.

No correlation was found between 3-h or 24-h ORM discrimination index and the interhemispheric ratio of hippocampal volume in either males or females.

3.3. Density of NeuN+ cells in the hilus of the dentate gyrus

Fig. 3 depicts the mean interhemispheric ratio of NeuN+ cells density in the Hil. *t*-test analyses showed that Sham group, both male and female, and the male Tbi-8 group had similar amounts of NeuN+ cells in the dentate gyrus of both hemispheres. In contrast, all the other Tbi groups had interhemispheric NeuN+ cells values lower than 100 [male: Tbi-sed $t(5) = -6.7$ $p = 0.001$; Tbi-12 $t(5) = -5.62$, $p = 0.002$; Tbi-16 $t(5) = -5.2$, $p = 0.003$; females: Tbi-sed $t(5) = -9.67$, $p < 0.001$; Tbi-8 $t(5) = -6.22$, $p = 0.002$; Tbi-12 $t(5) = -3.48$, $p = 0.018$; Tbi-16 $t(5) = -2.79$, $p = 0.038$].

ANOVA for the male groups showed a significant effect of treatment [$F(4, 25) = 8.63$, $p < 0.001$] and corrected post hoc contrasts showed that Tbi-sed ($p = 0.007$, $d = 1.87$), Tbi-12 ($p = 0.007$, $d = 2.26$) and Tbi-16 ($p = 0.004$, $d = 3.13$), but not Tbi-8 had lower interhemispheric ratios than the Sham group. No differences were observed between Tbi-sed and any of the exercised Tbi groups.

Regarding female groups, the treatment factor was also significant [$F(4, 25) = 3.74$, $p = 0.016$], and corrected post hoc contrasts showed that all tbi groups had lower scores than Sham group [Tbi-sed ($p = 0.019$, $d = 1.90$), Tbi-8 ($p = 0.047$, $d = 1.36$), Tbi-12 (0.011, $d = 1.88$), Tbi-16 ($p = 0.019$, $d = 1.64$)], with no differences between Tbi-sed and any of the exercised Tbi groups.

No correlation was found between the interhemispheric ratio of NeuN+ cells and the 3-h or 24-h ORM discrimination index in either male or females.

3.4. Number of DCX+ cells in the dentate gyrus of the contralateral hemisphere

Fig. 4 depicts the mean number of DCX+ cells in the dentate gyrus of the contralateral hippocampus.

ANOVA indicated a significant effect of the treatment factor in the male groups [$F(4, 25) = 3.16$, $p = 0.031$]. Corrected post-hoc contrast revealed that only Tbi-8 had a higher number of DCX+ cells than Tbi-sed ($p = 0.021$, $d = 1.93$).

In the female groups, the treatment factor was also significant [$F(4, 25) = 5.63$, $p = 0.002$], but, in this case, only Tbi-12 had a higher number of DCX+ cells than Tbi-sed ($p = 0.014$, $d = 2.01$).

No correlation was found between 3-h or 24-h ORM discrimination index and the number of DCX+ cells in the contralateral dentate gyrus in either males or females.

3.5. Summary of the main results

Table 1 summarizes the main results of the experiment.

4. Discussion

Our findings underscore the importance of considering sex when

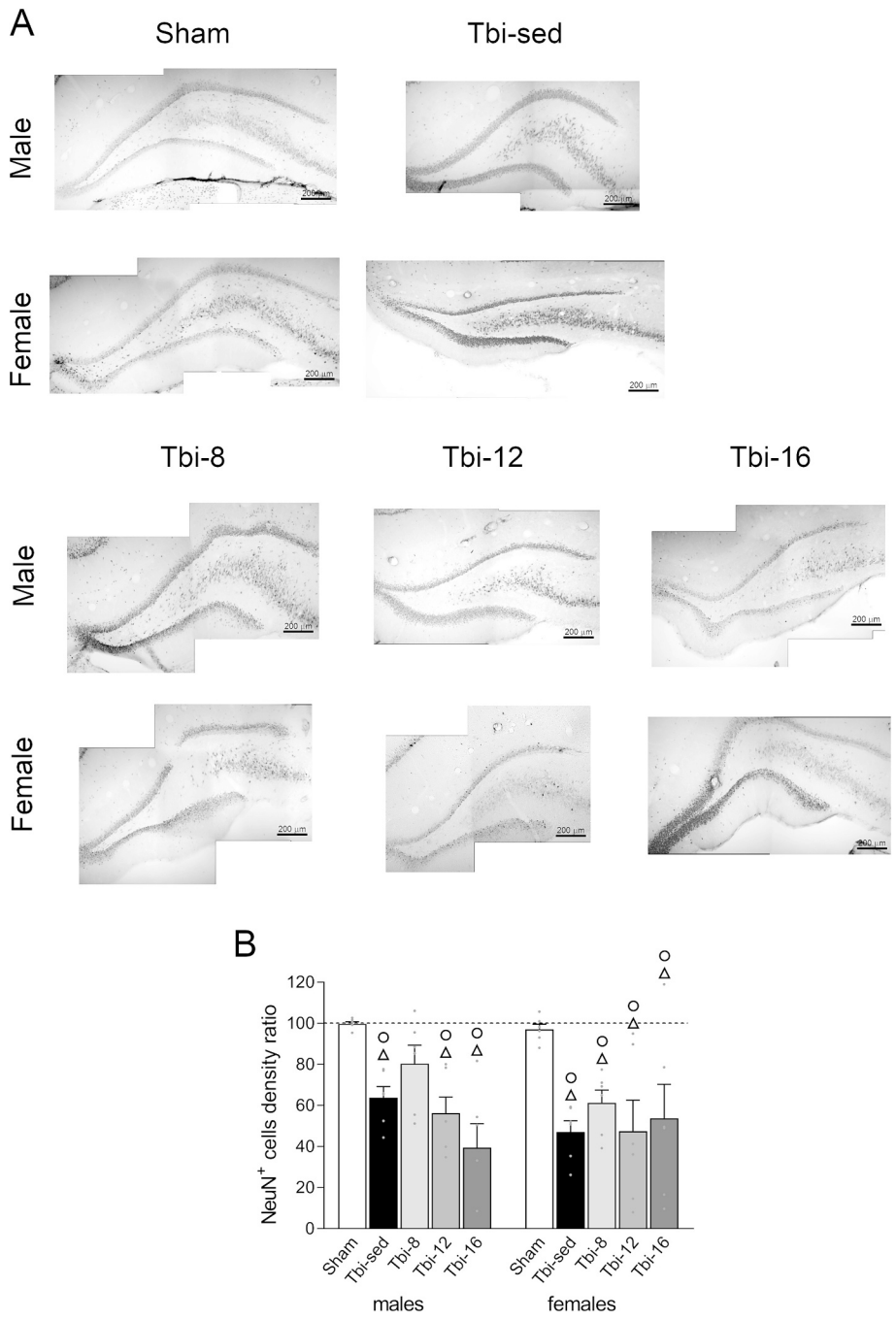

Fig. 3. Representative images of the ipsilateral Hil (approximately −3.24 mm from Bregma), immunostained for NeuN, for each experimental group (A). Mean (+SEM) interhemispheric ratio of NeuN+ cells density in the Hil in each experimental group (B). Δ: Significant differences relative to the reference value (100); o: Significant differences compared to the corresponding Sham group.

using PE to treat cognitive dysfunctions associated with TBI. They also suggest that there are sex differences in the neural mechanisms underlying the beneficial effects of exercise.

4.1. *Effects of controlled cortical impact (CCI)*

CCI led to similar short (3 h) and long-term (24 h) memory deficits in the ORM test in both males and females, assessed at 28 and 29 dpi, respectively. These deficits were not associated with changes in locomotion during habituation to the training cage, latency to explore the new object in the neophobia test, or the amount of object exploration during the acquisition sessions.

CCI also resulted in reduced volume of the dorsal hippocampus and neuronal loss in the Hil, without affecting the number of immature neurons in the dentate gyrus of the hippocampus. Damage to the hippocampus may be related to the observed memory deficits, since this structure is crucial for memory retrieval during retention (Hammond et al., 2004) or when the memory test has a significant spatial component (Clark and Martin, 2005), such as in our 24 h test where the location of the familiar object changes between the first and second tests. Similar memory deficits have been consistently reported using the same CCI and ORM protocol, (Amorós-Aguilar et al., 2020; Jacotte-

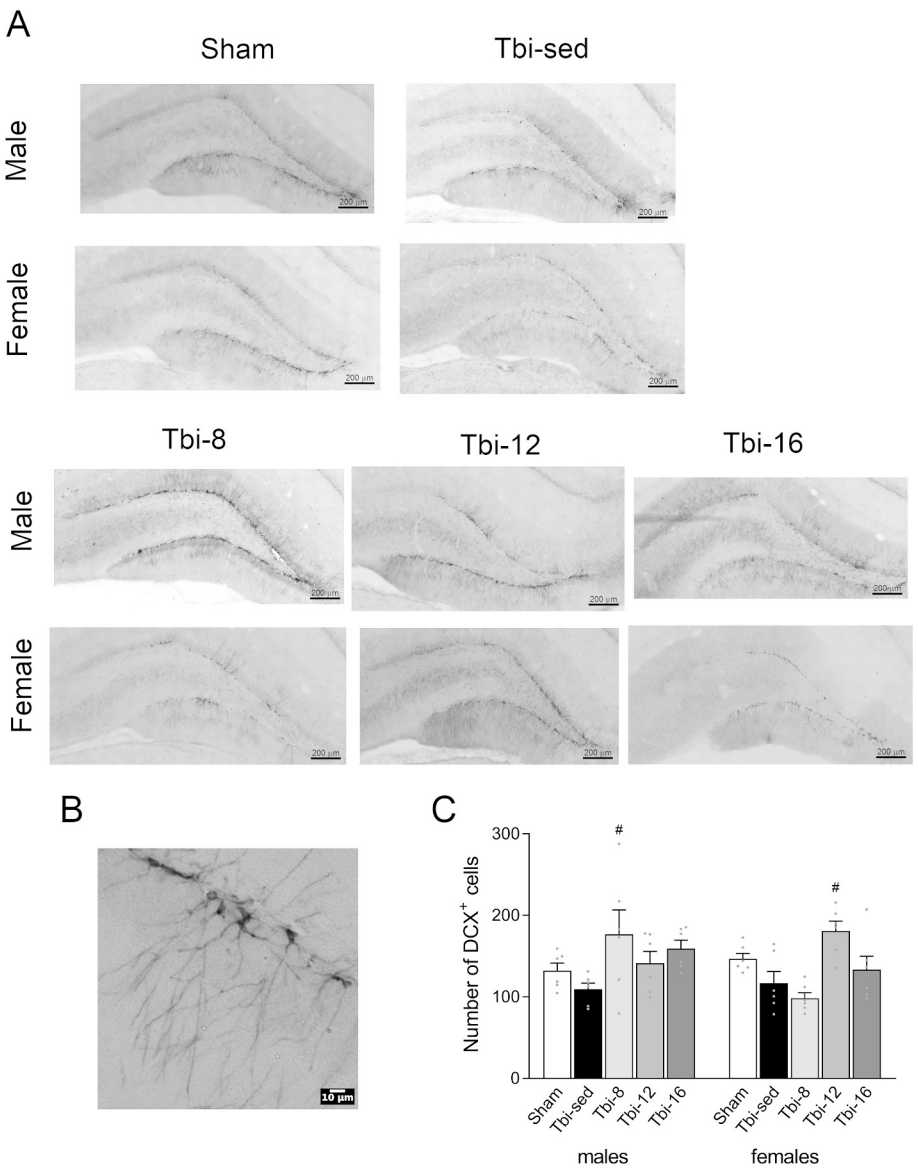

Fig. 4. Representative stitched images (A) and a larger magnification (B) of the contralateral dorsal dentate gyrus of DCX immunostained slices (approximately −3.24 mm from Bregma) for each experimental group. Mean (+SEM) of number of DCX+ cells in the Hil in each experimental group (C). #: Significant differences compared to the corresponding Tbi-sed group.

Simancas et al., 2015). CCI-induced deficits have also been observed in spatial memory tasks, such as the Morris water maze (Free et al., 2017; Tucker et al., 2016; Wagner et al., 2007; Wagner et al., 2004; Xiong et al., 2007). Consistent effects of CCI on neurodegeneration (Amorós-Aguilar et al., 2020; Bruce-Keller et al., 2007; Igarashi et al., 2001; Jacotte-Simancas et al., 2015; Xiong et al., 2007) in the hippocampus have also been found. However, the effects of CCI on neurogenesis are less conclusive, with studies reporting an increase (Xiong et al., 2007), a decrease (Amorós-Aguilar et al., 2020) or no effect (Amorós-Aguilar et al., 2020; Jacotte-Simancas et al., 2015; Piao et al., 2013).

The sex-specific effects of TBI on outcomes are not clear. Generally, human studies report better outcomes in males than in females, while animal studies report the opposite, although these results appear to depend on the severity of the injury (Gupte et al., 2019). Our results support the notion that CCI has similar effects on memory, neurodegeneration and neurogenesis in the hippocampus of both male and female animals.

4.2. Do males and females require the same intensity of physical exercise to obtain cognitive benefits after TBI?

When using PE as a treatment to alleviate cognitive deficits after TBI, several parameters of PE protocol (such as delay after the impact, duration of the treatment, daily amount, intensity, etc) can affect the outcome. Our results underscore the importance of the intensity. Moreover, they also indicate that optimal intensity may vary depending on sex and on the retention interval. In the short-term retention (3 h) session, all tested intensities had a positive effect on ORM in both males and females (as all the exercise groups showed memory of the familiar object), although this effect seems to be greater in females, since their discrimination indices were significantly higher than those of the corresponding Tbi-sed group. Previous studies using the same ORM protocol and CCI severity in male rats have shown that voluntary exercise either improved (Amorós-Aguilar et al., 2020) or had no effect (Jacotte-Simancas et al., 2015) on short-term retention. This suggests that performance in this test may vary due to minor variations in experimental manipulations of the animals, or the size and location of the lesion

Table 1
Summary of the main results.

	3-h ORM test	24-h ORM test	Interhemispheric ratio of hippocampal volume	Interhemispheric ratio of NeuN+ cells density in the hilus	DCX+ cells in contralateral dentate gyrus
Male					
Sham	+	+	=	=	
Tbi-sed	— < Sham	— < Sham	↓ <Sham	↓ <Sham	
Tbi-8	+	+ > Tbi-sed	↓ <Sham	=	>Tbi-sed
Tbi-12	+	+	↓ <Sham	↓ <Sham	
Tbi-16	+	–	↓ <Sham	↓ <Sham	
Female Sham	+	+	=	=	
Tbi-sed	–	— < Sham	↓ <Sham	↓ <Sham	
Tbi-8	+ > Tbi-sed	+	↓ <Sham	↓ <Sham	
Tbi-12	+ > Tbi-sed	+ > Tbi-sed	↓ <Sham	↓ <Sham	>Tbi-sed
Tbi-16	+ > Tbi-sed	+	↓ <Sham	↓ <Sham	

+, remember; —, do not remember; =, no interhemispheric difference; ↓, decrease in ipsilateral compared to contralateral.

cavity. Moreover, comparing forced and voluntary PE is challenging, since parameters such as daily duration and distances can vary considerably, and voluntary exercise intensity can differ greatly among individual animals. In light of our findings, the effects of PE on short-term retention appear to be surprisingly independent of intensity, with a generally beneficial effect observed across all intensities and in both sexes.

In contrast, regarding long-term retention (24 h), our study suggests that the effect of exercise is intensity-dependent, and that the optimal intensity differs between males and females. In male rats, the most pronounced effects were found at low intensity (8 m/min), diminishing at moderate intensity (12 m/min) and absent at high intensity (16 m/min). Conversely, results from female rats indicate that both low (8 m/min) and high intensities (16 m/min) yield intermediate beneficial effect, with the optimal effect associated with moderate intensity (12 m/min). The effects of PE on memory performance cannot be attributed to changes in habituation to the training cage, differences in latency to first explorations in neophobia, or the amount of exploration in the acquisition session in either sex, since there were no differences between groups in these variables.

Based solely on our results, the relationship between PE intensity and memory benefits on the 24-h retention test suggest a linear decreasing trend in males and an inverted U-relationship in females. However, it is possible that a sufficiently low intensity might have no effect in males, supporting an inverted U-shaped relationship in both sexes, but with a sex-specific peak shifted to the right in females compared to males. To our knowledge, only one study (White et al., 2023) has included male and female mice to investigate the effect of exercise intensity on learning and memory deficits related to TBI. In this study, exercise at low, moderate or high intensities had a moderately beneficial effect on the acquisition phase of a Barnes task in females, while in males only low and moderate intensities had a significant positive effect. On the probe session, no effect of any intensity was found in female subjects and, interestingly, low and moderate intensities had no effect on male mice, while high intensity led to profound impairment. Despite significant differences between ORM and Barnes tasks, there are some similarities in the results, especially in male animals, with low to moderate intensities being beneficial and higher intensities having no effect or being detrimental. Previous studies using only male animals have also demonstrated intensity-dependent effects of PE (Karelina et al., 2021; Shen et al., 2013), although comparing specific intensities is challenging due to variations in animal species (rat vs mouse), injury severity and several PE aspects such as the onset delay after injury, apparatus (running wheel vs treadmill), and specific training protocol (progressive, interval, fixed), among others.

4.3. Do males and females share the mechanisms of action of physical exercise on brain after TBI?

CCI reduced the volume of the ipsilateral hippocampus, and this reduction was not attenuated by PE in either males or in females. However, when examining the spared tissue, a sex-specific effect of PE on the density of mature neurons in the Hil was observed. In male rats, the lower intensity, which was most beneficial in the long-term ORM test, was the only one associated with preserved density of neurons in the ipsilateral Hil compared to the contralateral one, displaying intermediate values between those of the Sham and Tbi-sed groups. In contrast, none of the exercise intensities benefited female rats, with all the PE groups showing a similar loss of neurons to the Tbi-sed group. Previous studies have demonstrated the neuroprotective effects of different exercise protocols in male animals (Amorós-Aguilar et al., 2020; Chen et al., 2013; Chio et al., 2017; Gu et al., 2014; Jacotte-Simancas et al., 2015; Ko et al., 2018), but, to our knowledge, there are no previous reports regarding increased neuronal survival due to PE after TBI in female animals. Further research is needed to explore potential sex differences in the neuroprotective effects of exercise.

Consistent with previous studies (Amorós-Aguilar et al., 2020; Jacotte-Simancas et al., 2015; Karelina et al., 2021; Piao et al., 2013), PE increased neurogenesis (number of DCX+ cells) in the dentate gyrus, but the intensity required to achieve this increase differed between males and females. Interestingly, in both sexes, the exercise intensity that led to increased neurogenesis corresponded with the intensity that provided the most benefit in the long-term ORM test, which was the lower intensity for males and the moderate intensity for females.

In summary, our findings from the long-term ORM test, NeuN and DCX suggest that there is a sex-specific optimal PE intensity (lower in males and higher in females) to obtain beneficial effects after TBI. While some neural mechanisms involved in the beneficial effects of PE, such as increased neurogenesis, appear to be common to both sexes, others, like reduced neuronal loss, may differ between sexes. Sex- and intensity-dependent effects of PE after TBI have already been observed in neurometabolic, functional and transcriptional outcome measures (White et al., 2023).

The proposed right shifted dose-response of PE effects for female compared to male rats may partly stem from inherent differences in their running behaviour. Female rats tend to exercise at a higher intensity than males when given full access to a running wheel (Basso and Morrell, 2017). Therefore, when studying the effects of PE intensities on male and female subjects, appropriately adjusting the labelling of intensity levels (low, moderate, high) is crucial. For example, a speed considered moderate in males should be classified as low for females. In our study, we found that low intensities (8 m/min in males and 12 m/min in females) yielded optimal benefits from PE after CCI for both sexes. Moreover, since male rats typically run within a narrower range of intensities compared to females (Basso and Morrell, 2017), it is plausible that the optimal intensity range for males is narrower than for females, as suggested by White et al. (White et al., 2023) and supported by our long-term ORM test results.

5. Conclusions

In conclusion, forced PE after TBI can significantly reduce long-term memory deficits in an intensity-dependent manner. The results suggest that this effect is also sex-dependent, with females showing a right-shifted dose-response curve compared to males. These differences may be due to females' natural inclination to reach higher speeds spontaneously (Basso and Morrell, 2017). In males, this benefit appears to be associated with both neuroprotective and neuroreparative effects, whereas in females, the benefits are primarily neuroreparative. Additionally, more studies are needed to understand the beneficial effect of PE on short-term memory, which appears to be independent of exercise intensity. This study's findings support clinical observations that low to moderate intensity exercise is beneficial after trauma. From a clinical standpoint, it is vital to develop strategies that adjust exercise intensity based on sex. Such tailored interventions will maximize the therapeutic benefits of exercise.

CRediT authorship contribution statement

Ángel Gómez-Porcuna: Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Meritxell Torras-Garcia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Margalida Coll-Andreu:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Soleil García-Brito:** Writing – review & editing, Formal analysis. **David Costa-Miserachs:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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