Physiology & Behavior 266 (2023) 114184

Contents lists available at ScienceDirect

Physiology & Behavior

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

Full Length Article

Sequential treadmill exercise and cognitive training synergistically increase adult hippocampal neurogenesis in mice

F. Ávila-Gámiz [a,b], A.M. Pérez-Cano [a,b], J.M. Pérez-Berlanga [a,b], R.M. Mullor-Vigo [a,b], E.N. Zambrana-Infantes [a,b], L.J. Santín [a,b,*], D. Ladrón de Guevara-Miranda [a,b,*]

[a] Departamento de Psicobiología y Metodología de las Ciencias del Comportamiento, Universidad de Málaga, Spain
[b] Instituto de Investigación Biomédica de Málaga (IBIMA), Spain

ARTICLE INFO

Keywords:
Combined interventions
Forced running
Spatial learning
Water maze
Working memory

ABSTRACT

Combining physical and cognitive training has been suggested to promote further benefits on brain and cognition, which could include synergistic improvement of hippocampal neuroplasticity. In this paper, we investigated whether treadmill exercise followed by a working memory training in the water maze increase adult hippocampal neurogenesis to a greater extent than either treatment alone. Our results revealed that ten days of scheduled running enhance cell proliferation/survival in the short-term as well as performance in the water maze. Moreover, exercised mice that received working memory training displayed more surviving dentate granule cells compared to those untreated or subjected to only one of the treatments. According to these findings, we suggest that combining physical and cognitive stimulation yield synergic effects on adult hippocampal neurogenesis by extending the pool of newly-born cells and subsequently favouring their survival. Future research could take advantage from this non-invasive, multimodal approach to achieve substantial and longer-lasting enhancement in adult hippocampal neurogenesis, which might be relevant for improving cognition in healthy or neurologically impaired conditions.

1. Introduction

Engagement in physical and cognitive activities has been shown to benefit brain structure and function, revealing potential therapeutical applications [1,2]. Preclinical evidence shows that exercised rodents outperform their sedentary peers in tasks involving processes such as spatial learning [3] or working memory [4], while those receiving cognitive training (e.g. complex learning) perform better than controls upon subsequent cognitive challenges [5,6]. In the same line, human research have reported cognitive gains associated with aerobic exercise, with robust effects on memory and executive function [7]. Training based on exercising cognitive skills has also been linked to selective improvements in human cognition, rather transferred to the targeted cognitive domain [8]. Building on this promising foundation, growing evidence has emerged suggesting that combining physical and cognitive training yields better results than either treatment alone on counteracting cognitive decline, brain injury or neurological disorders in human [9–11] and animal models [12–14], highlighting the clinical potential of this multimodal approach.

At the neural level, both physical and cognitive stimulation promote neuroplastic phenomena that may underlie their positive effects on brain and cognition [15,16]. One of the structures targeted by such effects is the hippocampus [17,18], a limbic region known to play a key role in learning and memory [19]. Behavioural experiences sculpt the hippocampus through several adaptations including the continuous addition of dentate granule cells (DGCs) throughout lifespan, so-called adult hippocampal neurogenesis (AHN) [20]. The role of AHN in hippocampal-related processes such as learning, memory or mood regulation has been well established in animal studies [21], thus stimulating research on methods to modulate, eventually potentiate this phenomenon in order to improve cognitive function [22] or recovery from neurological disorders [23–26]. It is widely known that aerobic exercise upregulates AHN, mainly by extending the pool of proliferative precursor cells in the dentate gyrus (DG) [27,28], which in turn underlies enhanced learning and memory [29–32]. By contrast, stimuli that putatively elicit hippocampal activity, such as environmental enrichment (EE) or learning complex tasks, rather promote survival of adult-born DGCs [33,34]. Considering such divergent cellular pathways,

* Corresponding authors at: Facultad de Psicología y Logopedia, Universidad de Málaga, C/Doctor Ortiz Ramos 12, Ampliación de Teatinos, 29010, Málaga. Spain.
E-mail addresses: luis@uma.es (L.J. Santín), david.lgm@uma.es (D. Ladrón de Guevara-Miranda).

https://doi.org/10.1016/j.physbeh.2023.114184
Received 12 January 2023; Received in revised form 26 March 2023; Accepted 4 April 2023
Available online 6 April 2023

the sequential combination of physical exercise and hippocampal-related cognitive stimulation may lead to a synergistic improvement of AHN [35,36]. Evidence in this regard have been reported in exercised rodents thereupon submitted to EE [37] or hippocampal-dependant learning [38], but studies specifically designed to address this issue are, however, still scarce.

In order to be functional, enhancement of AHN should not only involve quantitative but also lasting effects [35,36]. This requires preventing newly-generated DGCs from undergoing programmed cell death, which mainly occurs one-to-two weeks after neural birth [39]. It is known that facing cognitive challenges (e.g. learning complex tasks) favours the rescue of DGCs within this critical window of survival [40–43]. At this stage, cognitive stimulation may take advantage of an exercise-induced expansion of proliferative DGCs to rescue more young, potentially recruitable neurons into the hippocampal network upon future environmental demands [44–47].

Following this rationale, we set out to test whether combining physical and cognitive training leads to synergistic effects on AHN and hippocampal plasticity, particularly on rescuing a cohort of DGCs within a critical window of survival. For this purpose, we submitted mice to ten days of treadmill exercise to increase a pool of proliferative DGCs, which were labelled with bromodeoxyuridine (BrdU) during the first three sessions of running. By the time these marked cells reached ~1 week of age, animals were trained in a working memory task in the Morris water maze (MWM) that has been proposed to be more 'cognitively demanding' and better supports survival of adult-born DGCs compared to the classical (i.e., hidden platform) version of the test [48].

2. Material and methods

2.1. Animals

C57BL/6 J male mice ($N = 36$) aged 10-week-old were obtained from Janvier (Le Genest-St-Isle, France) and housed in groups of three/four per cage on a 12-h light-dark cycle (lights on at 8 am). Animals were maintained in a room with controlled ventilation, temperature (22 ±2°C) and humidity (~40%) in standard laboratory cages provided with nesting material, water, and food ad libitum. All experiments were carried out in accordance with the European (Directive 2010/63/UE) and Spanish (Real Decreto 53/2013 and 1386/2018, and Ley 32/2007) regulations for animal research and were approved by the Experimentation Ethics Committee of the University of Málaga (CEUMA no. 29–2017-A).

2.2. Scheduled exercise

All behavioural procedures were performed between 8 am and 3 pm and mice received habituation to the testing rooms for at least 30 min prior any assessment began. After two weeks of acclimatation, animals (12 weeks-old) were randomly assigned to sedentary (SED) or exercise (EXE) conditions sacrificed 24 h (SED+24 h, $n = 6$; EXE+24 h, $n = 6$) or 9 days (SED+CAGE, $n = 6$; EXE+CAGE, $n = 6$) after the last running session. The exercise protocol employed has been described previously [49] and was carried out using a rodent treadmill (model 47,300 from Ugo Basile, Varese, Italy) with four individual lanes (45 cm length, 5.5 cm width) settled to 0° inclination. To avoid novelty stress, EXE animals were previously habituated for two days in which they were placed into one of the lanes of the treadmill and allowed to explore the apparatus for 5 min. Then, they were subjected to running for 30 min/day at increasing low speeds (from 3 m/min to 15 m/min). After habituation, EXE animals were trained for ten consecutive days with 45 min running sessions at a constant speed of 15 m/min (Fig. 1a,b), which is considered of moderate intensity in untrained C57BL/6 J mice (~75–80% maximal oxygen uptake [50,51]). When necessary, animals were encouraged to run by a gentle tap in the tail using a plastic pipette. The treadmill was carefully cleaned with a solution containing 30% ethanol between mice batches to remove odour cues. SED animals were maintained at home cage inside the testing room during exercise sessions.

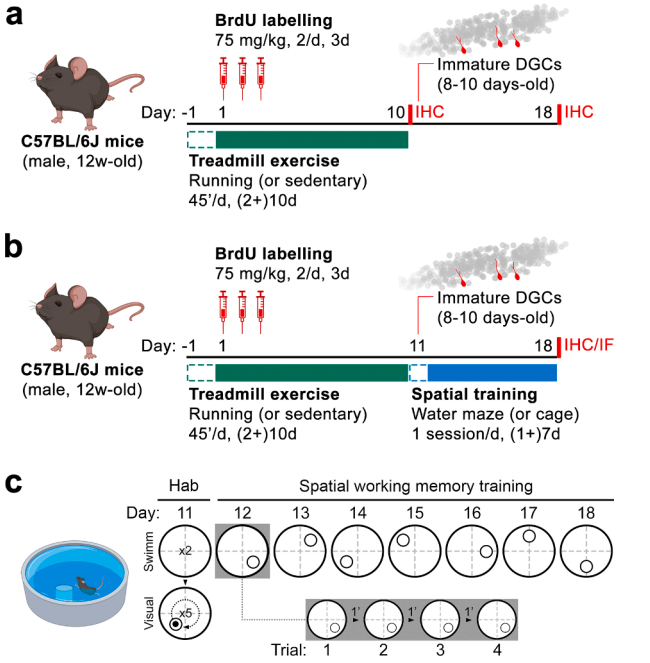

Fig. 1. Experimental design of the study. (a-b) Schematic of the procedures to evaluate the acute/delayed effects of forced running (a) and the effects of combined exercise and spatial training (b) on adult hippocampal neurogenesis. Discontinued lines on treatments represent habituation sessions (days). (c) Diagram of the water maze protocol. *BrdU*: bromodeoxyuridine; *d*: day; *DGCs*: dentate granule cells; *Hab*: habituation; *IF*: immunofluorescence; *IHC*: immunohistochemistry.

2.3. Bromodeoxyuridine administration

Mice were administered with BrdU (dissolved in 0.9% NaCl) to label a cohort of DGCs generated *de novo* during the first three sessions of treadmill exercise (experimental days 1 to 3; Fig. 1a,b). Animals received intraperitoneal (i.p.) injections of BrdU for three consecutive days at a rate of two daily doses (75 mg/kg) separated by six hours.

2.4. Spatial working memory training

Twenty-four hours after the last treadmill session, SED and EXE mice were randomly assigned to conditions either trained in a MWM task (SED+MWM, $n = 6$; EXE+MWM, $n = 6$) or maintained at home-cage inside the testing room (SED+CAGE, $n = 6$; EXE+CAGE, $n = 6$; Fig. 1b). The MWM protocol employed was based on published reports [48] and is depicted in Fig. 1c. The apparatus consisted of a circular pool of 120 cm diameter filled with water (22±1°C) which was made opaque using non-toxic white paint. A white plastic platform of 11 cm diameter was used in cued and training sessions. Eight pseudorandom start locations (North, South, East, West, Northwest, Southwest, Northeast, Southeast) were assigned for each trial to avoid egocentric learning. Distal spatial cues were made visible around the room to facilitate spatial orientation. On the first day, animals received habituation consisting of two exploration trials of the empty pool (i.e., no platform) of 60 s each. Then, mice underwent five consecutive trials of visual-cued training in which the platform protruded from the water surface and was relocated each trial. A black polystyrene object (12 cm height) was placed on top of the platform as a cue to enhance its visibility. In each trial, mice were allowed to explore the pool until reaching the platform or for a maximum of 60 s. Animals that did not find the platform within 60 s were guided to it. Inter-trial intervals (ITI) lasted 1 min during

which mice were returned to home cage and allowed to dry. The working memory training took place over the next seven consecutive days at a rate of four trials per day with 1 min ITI. The platform was submerged 1 cm below the water surface and relocated every day but remained in the same place during the four trials of each session. At the beginning of each trial, animals were released into the pool from one of the pseudorandom starting locations and could navigate until finding the platform or for a maximum of 60 s. Once reached the platform, animals stayed on its surface for 15 s before being removed from the apparatus. The mice which could not find the platform in 60 s were manually driven towards it and remained there for 30 s to favour spatial orientation. All MWM sessions were recorded with a digital video camera fixed to the ceiling of the room and connected to a computer tracking system (RECORD-IT! MEDIA; Panlab, Barcelona, Spain) placed in an adjacent room. Data were analysed using the Ethovision XT 12 software (Noldus, Wageningen, The Netherlands). Measurements of latency to escape (s) and cumulative distance (cm) to the platform, as well as swim speed (cm/s) were registered for each trial. Mice's navigational strategies for finding the platform were registered automatically using the *Rtrack* package [52]. The frequency of use of allocentric (i.e., goal directed search, corrected search, direct path, or perseverance, grouped into 'spatial') and egocentric strategies (i.e., thigmotaxis, circling, random, scanning, or chaining, grouped into 'non-spatial') was calculated for each trial.

2.5. Tissue collection and immunoassays

Twenty-four hours after the last treadmill session (SED+24 h and EXE+24 h groups; $n = 6$/group; Fig. 1a) or the last MWM session (SED+CAGE, SED+MWM, EXE+CAGE and EXE+MWM groups; $n = 6$/group; Fig. 1b) animals were anaesthetised with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS 0.1 M pH 7.4) and 4% paraformaldehyde (PFA). Brains were collected and post-fixed for 48 h at 4°C in 4% PFA and later maintained for 72 h at 4°C in PBS with 30% sucrose. Brain hemispheres were then splitted through the midline and cut into 40 μm coronal sections using a vibratome (Microm H650V; Thermo Fisher Scientific, MA, USA). Free-floating immunohistochemistry (IHC) and immunofluorescence (IF) studies were performed following published protocols [26] (details are provided in Suppl. Methods). The following primary antibodies were employed for IHC procedures: rat monoclonal anti-BrdU (1:500; Abcam, Cambridge, UK) to detect $BrdU^+$ cells; mouse monoclonal anti-proliferating cell nuclear antigen (PCNA, diluted 1:1500; MERCK Sigma-Aldrich, St. Louis, USA) to identify cells undergoing division; rabbit polyclonal anti-doublecortin (DCX, diluted 1:400; Abcam) to detect immature neurons (up to ~3/4 weeks-old); rabbit monoclonal anti brain-derived neurotrophic factor (BDNF, diluted 1:500; Abcam) or mouse monoclonal anti-synapsin Ia/b (1:500; Santa Cruz Biotechnology, TX, USA) to detect the expression of BDNF and synapsin I (SYN1) in the hippocampus, respectively. Staining was visualized using the biotin-avidin method with 5% diaminobenzidine peroxidase as a chromogen. For MWM and CAGE conditions, neuronal differentiation of $BrdU^+$ DGCs was assessed by double IF using rat monoclonal anti-BrdU (1:500; Abcam) and rabbit polyclonal anti-DCX (1:400; Abcam) as primary antibodies and a mixture of Alexa Fluor®-conjugated secondary antibodies (488 goat anti-rat and 594 donkey anti-rabbit, diluted 1:500 in PBS; Abcam).

2.6. Quantification procedures

The number of $BrdU^+$ and $PCNA^+$ DGCs were determined manually employing an Olympus BX41 microscope (Olympus, Solms, Germany) with a 100x oil immersion lens. One of every six sections containing the DG were quantified (dorsal portion covering −1.34 mm to −2.80 mm relative to Bregma; ventral portion covering −2.94 mm to −3.80 mm relative to Bregma) [53]. Preliminary results yielded no detectable inter-hemispheric differences in the number of $BrdU^+$ or $PCNA^+$ DGCs (data not shown), thus the left hippocampus was randomly chosen for IHC/IF analyses. DCX^+ cell count was performed manually in six sections representative of the DG along the dorsoventral axis (covering −1.46 mm to −3.40 mm relative to Bregma) using the ImageJ software (http://imagej.nih.gov/ij/) and high-resolution images taken at 10x magnification in an Olympus BX53 microscope equipped with an Olympus DP73 camera (Olympus). Attending their dendritic morphology, DCX^+ neurons were classified into three different categories: proliferative stage (type-1), intermediate stage (type-2) and postmitotic stage (type-3) [54]. The area of the granular cell layer (GCL) of the DG was measured using the ImageJ software and high-resolution images taken in the Olympus BX53/DP73 set up. Data were expressed as number of cells per area (mm^2). Double labelling of BrdU and DCX was analysed in six sections representative of the DG along the dorsoventral axis (covering −1.46 mm to −3.40 mm relative to Bregma) employing a Stellaris 8 confocal microscope (Leica Microsystems; Wetzlar, Germany) with a 40x oil immersion lens. The percentage of $BrdU^+$ DGCs that co-expressed DCX was estimated on the basis of at least 40 $BrdU^+$ DGCs counted per animal. The hippocampal expression of BDNF and SYN1 was estimated by densitometry in four sections per animal using the ImageJ software (details are provided in Suppl. Information).

2.7. Data analysis

Statistics were conducted in Statistica 8 (StatSoft Power Solutions Inc., OK, USA) and IBM SPSS 20 (IBM, NY, USA). Between and intra-group differences were determined by performing Student's t tests, one/two-way or repeated measures ANOVAs as required. When appropriate, the Duncan's Multiple Range Test (DMRT) was conducted for *post-hoc* comparisons. Relationships between variables were tested by using Pearson's bivariate correlations. A Principal Component Analysis (PCA) with varimax rotation was performed to extract independent dimensions (i.e., factors) from MWM and IHC data. Eigenvalue ≥ 1 was chosen as criterion for component extraction and a factor score (i.e., a standardised value indicating the relative position of each animal in each factor) was computed by the regression method. Only significant analyses ($p \leq 0.05$) are shown.

3. Results

3.1. Effects of treadmill exercise on adult hippocampal neurogenesis

To evaluate the effect of the treadmill protocol on hippocampal cell proliferation and survival, a batch of mice was sacrificed 24 h after the last exercise session (SED+24 h and EXE+24 h groups) and were compared to animals submitted (or not) to running and perfused nine days later (SED+CAGE and EXE+CAGE groups; Fig. 1a). Results from two-way ANOVAs yielded significant *EXE* x *time* interactions for both BrdU [$F_{EXE\ x\ time}(1,20) = 12.009$, $p = .002$, $\eta^2_p = .375$] and PCNA staining [$F_{EXE\ x\ time}(1,20) = 4.458$, $p = .048$, $\eta^2_p = .182$] in the DG. *Post-hoc* analyses revealed that exercised mice sacrificed one day after the last treadmill session showed higher amount of $BrdU^+$ ($p < .01$ vs. all other conditions; Fig. 2a,c) and $PCNA^+$ DGCs ($p < .05$ vs. all other conditions; Fig. 2d,f), with no significant differences found between the other groups (Fig. 2a-f). Further analyses indicated that such increase of $BrdU^+$ and $PCNA^+$ DGCs in EXE+24 h animals were significant in the dorsal DG [BrdU: $F_{EXE\ x\ time}(1,20) = 14.253$, $p = .001$, $\eta^2_p = 0.416$; PCNA: $F_{EXE\ x\ time}(1,20) = 5.423$, $p = .031$, $\eta^2_p = .213$] but not in ventral DG. *Post-hoc* multiple comparisons revealed that the EXE+24 h mice displayed more $BrdU^+$ ($p < .01$ vs. all other conditions; Fig. 2b) and $PCNA^+$ cells ($p < .05$ vs. all other conditions; Fig. 2e) in the dorsal DG, while no significant differences were found between the other groups (Fig. 2d,e). Overall, these results indicate that treadmill exercise enhanced cell proliferation/survival in a transient manner, as both BrdU and PCNA levels returned to baseline after nine days of cage rest.

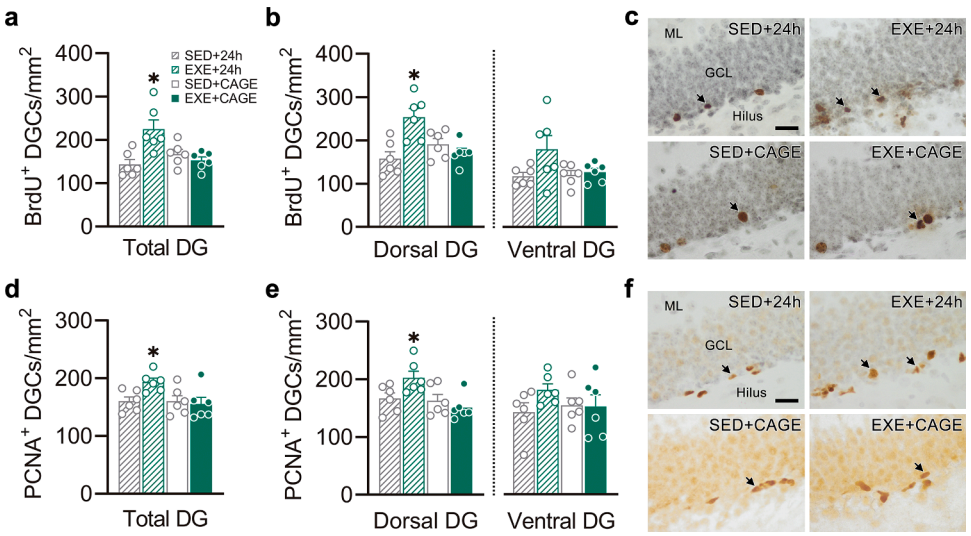

Fig. 2. Acute and delayed effects of treadmill exercise on adult hippocampal neurogenesis. (a) Forced running induced a short-term increase in the number of bromodeoxyuridine $(BrdU)^+$ cells in the dentate gyrus (DG). (b) The exercised-induced acute increase in BrdU expression was significant in the dorsal but not the ventral DG. (c) Representative images of BrdU immunostaining in the DG of each group. (d) Cell proliferation in the DG was enhanced shortly after exercise cessation but not after nine days of home-cage rest. (e) Running acutely increased cell proliferation in the dorsal but not the ventral DG. (f) Representative images of proliferating cell nuclear antigen (PCNA) immunostaining in the DG of each group. Scalebars in (c) and (f) represent 20 μm and examples of positive labelling are indicated by black arrows. Data are represented as mean ± SEM, with dots indicating individual values. *Post-hoc* multiple comparisons (DMRT): difference versus all other groups: $^{*}p < .05$. *DGCs*: dentate granule cells; *GCL*: granular cell layer; *EXE*: exercise; *ML*: molecular layer; *SED*: sedentary.

3.2. Effects of treadmill exercise on water maze performance

To evaluate mice's ability to learn the working memory task in the MWM (Fig. 1c), repeated measures ANOVAs (*day x trial* x *group*) were performed including measures of latency to escape and cumulative distance to the platform. The ANOVAs yielded no significant *day x trial x group* interactions, but rather *trial x group* effects, thus the results of trials 1–4 averaged over the seven-day training period (T1–4) are shown

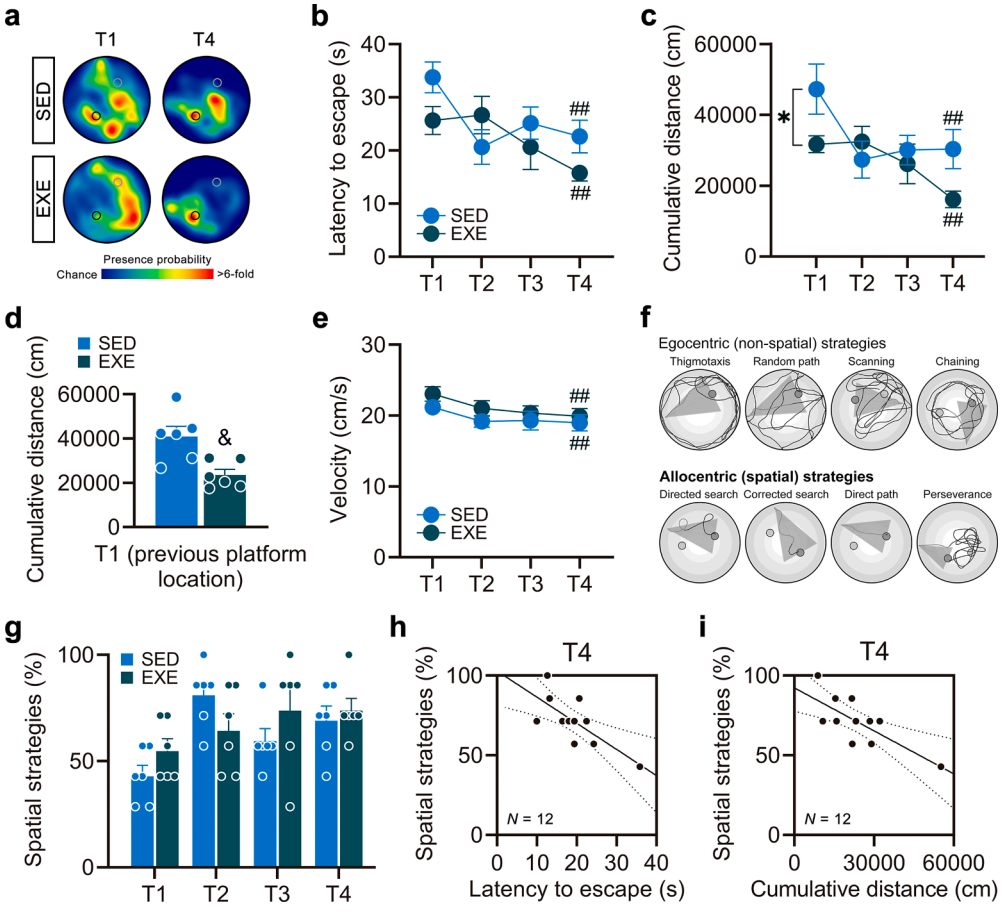

Fig. 3. Mice's performance in the spatial working memory training. (a) Representative heat map plot of sedentary and exercised animals in T1 and T4. Colour scale represent the probability of mice to be present at a given location. (b) Both sedentary and exercised conditions required significantly less time to reach the platform at the end of the training sessions. (c) Sedentary and exercised animals showed a significant reduction in the cumulative distance to the platform at the end of the training sessions, but the values in T1 and T4 were significantly lower in the running group. (d) Exercised animals showed less cumulative distance to reach the previous location of the platform during the first daily trials. (e) No differences were found between groups in swimming velocity during the water maze training. (f) Example images depicting the search strategies assessed in the water maze training ('circling' not shown as animals did not used this strategy in any trial). (g) No differences were found in the use of spatial strategies in T1–4 between sedentary an exercised animals. (h-i) The use of spatial strategies was inversely correlated with the latency to escape from the pool (h) and the cumulative distance to reach the platform (i) in T4. Data in (b-e) and (g) is presented as mean ± SEM. Dots in (d), (g) and (h-i) represent individual values. *Post-hoc* multiple comparisons (DMRT): difference between variables connected by lines: $^{*}p < .05$; difference between T1 and T4 in the same group: $^{\#\#}p < .01$. Student's *t*-test: difference between groups: $^{\&}p < .05$. *EXE*: exercise; *SED*: sedentary; *T1–4*: trial 1–4 (averaged on the 7-day training period).

(daily results are detailed in Suppl. Table 1). Both sedentary and exercised animals required less time to reach the platform in T4 compared to T1 [$F_{\text{trial x group}}(3,30) = 3.412$, $p = .030$, $\eta^2_p = .254$; Fig. 3a,b]. No between-group differences were found within the same trial but there was a non-significant trend of running mice to be faster to find the platform in T1 ($p = .090$) and T4 ($p = .163$; Fig. 3b). Further, both experimental conditions showed a reduction in cumulative distance to the platform in T4 compared to T1 [$F_{\text{trial x group}}(3,30) = 3.528$, $p = .027$, $\eta^2_p = .261$; Fig. 3a,c]. However, *post-hoc* analyses revealed that exercised animals showed a lower cumulative distance in T1 ($p = .039$) and there was also a strong non-significant trend for this group to show a lower cumulative distance in T4 ($p = .073$) compared to the sedentary mice (Fig. 3c). Taken together, our results suggest that both groups of animals were able to learn the task, but running mice outperformed their sedentary counterparts. Moreover, a lower cumulative distance to the previous day's platform location in T1 was observed in exercised animals [$t(10) = 3.331$, $p = .008$, $d = 1.923$; Fig. 3d], indicating that they were better able to retain learned information in the long-term. An additional analysis revealed an overall reduction of swim speed in T2–4 compared to T1 [$F_{\text{trial}}(3,30) = 12.157$, $p < .001$, $\eta^2_p = .549$; Fig. 3e] with no significant group or interaction effects, suggesting that physical fitness of sedentary and exercise conditions was similar during the MWM task and therefore would not account for differences in performance.

Animals' navigational strategies to find the platform were also analysed (Fig. 3f), as they provide valuable information about the degree of hippocampal involvement during MWM performance [55]. No between-group differences were found regarding the percentage of use of 'spatial' strategies in T1–4 (Fig. 3g). Further analyses revealed a significant relationship between the use of allocentric strategies and the performance measures registered in T4 (*latency:* $r = -.735$, $p = .007$; Fig. 3h; *cumulative distance:* $r = -.747$, $p = .005$; Fig. 3i). Overall, these results suggest that better MWM outcomes were associated with the use of spatial (i.e., hippocampal-related) search strategies.

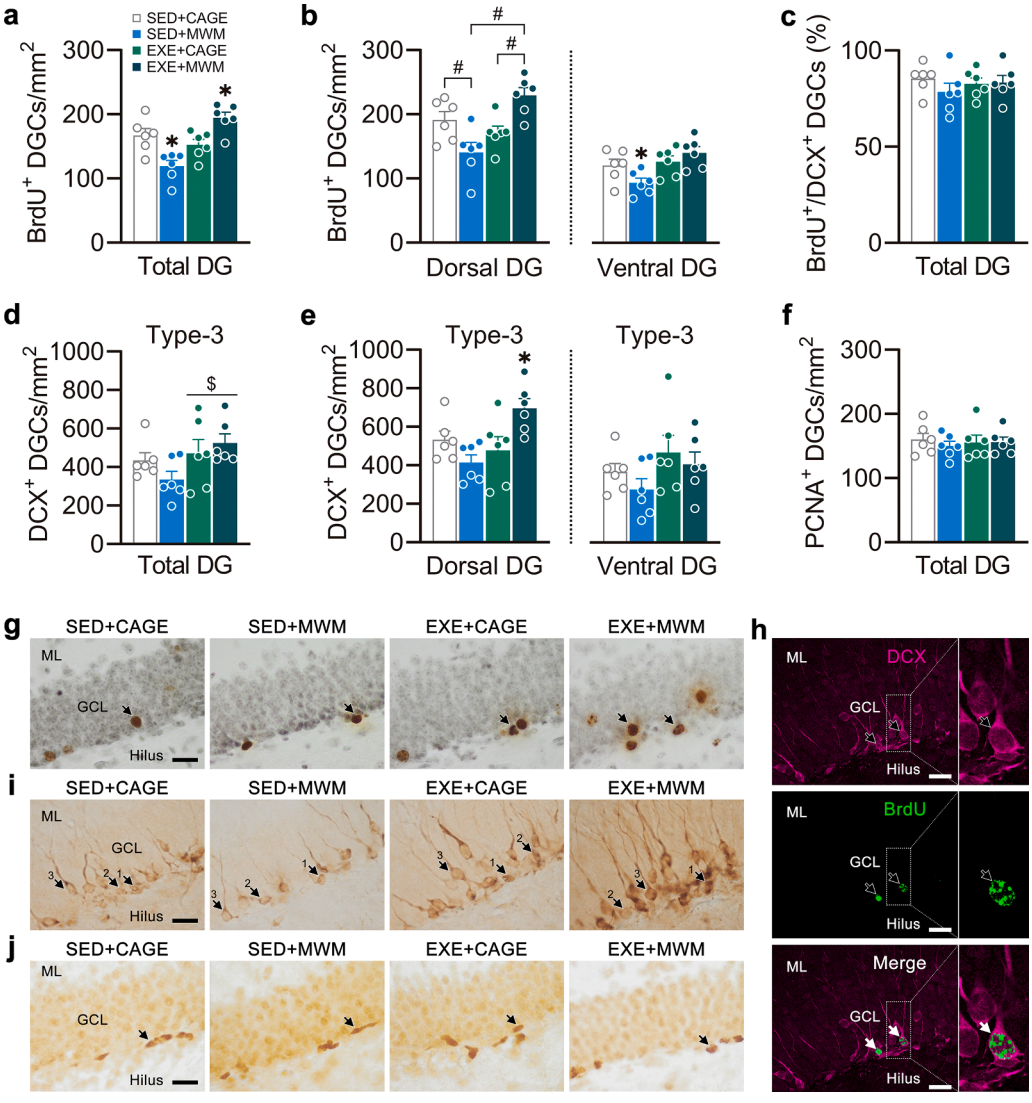

Fig. 4. Effects of combining treadmill exercise and working memory training on adult hippocampal neurogenesis. (a) The combination of running and Morris water maze (MWM) training significantly increased cell survival in the dentate gyrus (DG). In contrast, MWM training downregulated survival of newborn dentate granule cells (DGCs) in sedentary mice, which was evidenced by a reduced population of bromodeoxyuridine $(BrdU)^+$ cells. (b) The combination of exercise and MWM training enhanced cell survival in the dorsal DG. On the contrary, in the sedentary mice that received MWM training the expression of BrdU was reduced in the dorsal and the ventral portions of the DG. (c) No treatment effects were found regarding the neuronal fate of the $BrdU^+$ DGCs. (d) Overall, exercised mice showed more doublecortin $(DCX)^+$ DGCs in postmitotic stage than sedentary groups in the total DG. (e) The animals that received combined treatments expressed significantly more postmitotic DCX^+ DGCs in the dorsal DG, while no differences were found in the ventral DG. (f) No between-group differences were found in cell proliferation at the end of experiment. (g,i,j) Representative immunostaining of BrdU (g), DCX (i) and proliferating cell nuclear antigen (PCNA) (j) of each group. Examples of positive signals are indicated by black arrows (for DCX data, the numbers represent examples of type 1–3 cells); (h) Representative images of DCX and BrdU double immunofluorescence. Examples of positive labelling (DCX and BrdU channels) and co-labelling (merge channel) are indicated by black and white arrows, respectively. Scalebars in g-h represent 20 μm. Data are represented as mean ± SEM, with dots indicating individual values. *Post-hoc* multiple comparisons (DMRT): difference versus all other groups: $^*p < .05$; difference between variables connected by lines: $^{\#}p < .05$; difference versus sedentary groups: $^{\$}p > .05$. *GCL*: granular cell layer; *EXE*: exercise; *ML*: molecular layer; *SED*: sedentary.

3.3. Effects of combined treadmill exercise and water maze training on adult hippocampal neurogenesis and hippocampal plasticity

To determine whether treadmill exercise and MWM training promoted synergic effects on AHN, two-way ANOVAs (*EXE* x *MWM*) were conducted on BrdU, BrdU+DCX, DCX and PCNA immunolabelling levels. A significant interaction effect on the amount of $BrdU^+$ DGCs was found [$F_{EXE\ x\ MWM}(1,20) = 23.739$, $p < .001$, $\eta^2_p = .543$]. *Post-hoc* multiple comparisons revealed that the exercised animals subsequently trained in the MWM showed more BrdU-labelled cells in the DG ($p < .05$ vs. all other conditions), while the sedentary mice submitted to the MWM exhibited a significant reduction in this cell population ($p < .05$ vs. all other conditions). No significant differences were found between the other groups (Fig 4a,g). This suggests that sequential exposure to treadmill exercise and MWM training promoted survival of new-born DGCs to a greater extent than each treatment alone, while isolated MWM training had a negative impact on AHN. Further analyses revealed significant interaction effects on the expression of $BrdU^+$ in the dorsal DG [$F_{EXE\ x\ MWM}(1,20) = 17.413$, $p < .001$, $\eta^2_p = .465$] and the ventral DG [$F_{EXE\ x\ MWM}(1,20) = 5.067$, $p = .036$, $\eta^2_p = .202$]. *Post-hoc* analyses revealed an overall increase in the amount of $BrdU^+$ cells in the dorsal DG in the exercised animals trained in the MWM ($p < .01$ vs. SED+MWM and EXE+CAGE, marginal significance of $p = .052$ vs. SED+CAGE; Fig. 4b). On the other hand, the SED+MWM group showed fewer $BrdU^+$ cells than the SED+CAGE ($p = .016$) and the EXE+MWM mice ($p < .001$) in the dorsal DG and compared to all groups ($p < .05$) in the ventral DG (Fig. 4b). No significant differences were found between the other conditions (Fig. 4b). Together, these results indicate dynamic effects of the MWM training along the dorsoventral axis of the DG depending on the presence/absence of previous exercise. It was also found that $BrdU^+$ DGCs differentiated mainly into neurons as most of them expressed DCX (Fig. 4c,h), with no significant interaction or single-factor effects of the treatments in the percentage of co-labelling of both markers.

Pertaining to DCX immunostaining analysis, we found a significant effect of exercise on the number of type-3 DCX^+ cells in the DG [$F_{EXE}(1,20) = 4.649$, $p = .043$, $\eta^2_p = .189$] in absence of interaction or MWM effects. *Post-hoc* multiple comparisons revealed that, overall, exercised animals showed more type-3 DCX^+ DGCs compared to sedentary mice ($p = .044$; Fig. 4d,i). However, when the analysis was restricted to the dorsal DG, an *EXE* x *MWM* effect was revealed [$F_{EXE\ x\ MWM}(1,20) = 9.903$, $p = .005$, $\eta^2_p = .331$]. The density of type-3 DCX^+ DGCs was increased in the dorsal (but not the ventral) DG of exercised animals trained in the MWM ($p < .05$ vs. all other groups), while no significant differences were found between the other groups (Fig. 4e). Therefore, rather than uncovering an overall effect of exercise, it is likely that the conflicting results observed in the MWM-trained groups masked a global interaction effect in the DG that did reach significance in its dorsal portion. No significant interaction or single-factor effects were found regarding cell counting of type-1/2 DCX^+ cells (Suppl. Fig. 1) indicating that both proliferative and highly immature DGCs populations (respectively) were unaltered at the end of the experiment. There were also no significant differences between groups in the number of DCX^+ neurons in the total DG (data shown in Suppl. Table 2).

To evaluate proliferative activity in the DG shortly after MWM training (preceded or not by exercise), we quantified the level of PCNA immunostaining in the DG of the different groups. A two-way ANOVA yielded no significant interaction or single-factor effects regarding the number of $PCNA^+$ DGCs (Fig. 4f,j), suggesting that the rates of hippocampal cell proliferation remained at basal levels after the behavioural treatments.

To better understand the relationship between treadmill exercise, MWM performance, hippocampal demand, and AHN, a PCA was performed including the following variables: latency to escape in T4, cumulative distance in T4, use of spatial strategies in T4 and number of $BrdU^+$ DGCs per area. Sample adequacy tests revealed that the data was suitable for PCA [Kaiser-Meyer-Olkin test = 0.678; Bartlett's test: $X^2(6) = 32.716$, $p < .001$]. A single factor accounting for 70.76% of variance was extracted from the analysis, with high factor scores indicating low cumulated distances and latencies in T4, high use of spatial strategies in T4 and greater amount of $BrdU^+$ DGCs (Fig. 5a). Further analyses revealed that EXE+MWM mice showed a significantly higher factor score in the dimension extracted in the PCA compared to the SED+MWM condition [$t(10) = 2.374$, $p = .039$; $d = 1.371$; Fig. 5b], suggesting a consistent interrelationship between the combination of the behavioural treatments and the rescue of DGCs in a critical phase of cell survival by the time of facing hippocampal-related cognitive stimulation.

Interestingly, we found no between-group differences regarding the expression of BNDF or SYN1 in CA1, CA3 or the DG of the hippocampus (Suppl. Fig. 2), suggesting that the increase in AHN observed in the EXE+MWM group was not related to changes in plastic processes involving these specific proteins. Probably, the inconsistency of these findings with previous evidence [56,57] may be explained by methodological issues (e.g., delay in tissue collection) [58,59].

4. Discussion

In this work, we provided evidence that the sequential combination of exercise and hippocampal-dependant memory training yields synergetic effects to increase AHN in mice. Our major finding is that animals submitted to ten days of treadmill exercise and then trained in a working memory task in the MWM displayed substantially more surviving DGCs ($BrdU^+$, generated 8–10 days before training) than those subjected to either running or MWM training alone. The combination of treatments also increased the amount of postmitotic DCX^+ neurons in the dorsal DG, possibly reflecting enhanced survival of BrdU-labelled and/or additional populations of DGCs, although accelerated maturation of neurons generated before/during the treatments cannot be ruled out. To the best of our knowledge, this is the first demonstration that combining exercise with subsequent hippocampal-dependant learning synergistically enhances AHN in mice. Studies specifically designed to assess the effects of sequential (not-overlapping) behavioural strategies on adult neurogenesis are scarce. In this regard, additive effects on AHN have been reported using voluntary exercise followed by EE as method for stimulating hippocampal activity and thereby survival of adult-born DGCs [37]. However, whether hippocampus-dependant learning complements the pro-neurogenic effects of exercise in mice remained unknown. In rats, combining wheel running with working memory training in the MWM may extend a population of surviving DGCs (8–10 days old by the time of training) to a greater extent that single treatments or conditions in which the cognitive task was delayed for more than a week or did not

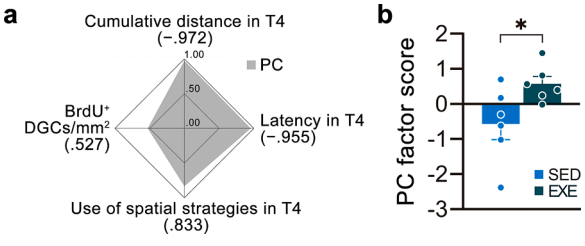

Fig. 5. Multivariate relationship between Morris water maze (MWM) performance and adult hippocampal neurogenesis in sedentary and exercised animals. (a) A principal component (PC) indicative of low cumulative distance and latency in T4, high use of spatial strategies in T4, and increased amount of bromodeoxyuridine $(BrdU)^+$ dentate granule cells (DGCs) was extracted. The scale is represented in absolute values. Numbers represent factor loadings. (b) Exercised mice showed higher scores in the PC extracted by the analysis thus indicating, altogether, better MWM performance, greater use of hippocampal navigational strategies and higher survival of DGCs. Data are expressed as mean ± SEM, with dots indicating individual values. Difference between groups: *$p <$ .05. *T4*: trial 4 (averaged on the 7-day training period).

rely on hippocampal activity [38]. Our findings agree with this evidence, revealing that such multimodal approach for improving AHN can be extended to mice, the most employed species in neurobehavioural research nowadays [60].

Our results support the hypothesis that the positive effects of exercise on AHN could be potentiated if proper hippocampal stimulation is provided during the critical window of cell survival. The cohort of $BrdU^+$ DGCs was generated ~1 week prior to the beginning of the MWM task, thus in the key phase (i.e., 1–3 weeks after neural birth) in which cognitive, hippocampal-related stimuli may prompt cell survival [43,47]. When reaching 1 week of age, immature DGCs begin to establish synaptic contacts within the hippocampal network and express enhanced synaptic plasticity compared to pre-existing mature neurons [45,61]. These properties make adult-born DGCs particularly prone to be rescued from death and subsequently recruited in later hippocampal-dependant learning experiences [43,47]. Exercise would generate a highly plastic microenvironment, including an extended reservoir of new-born cells, that may 'prime' the hippocampus for upcoming relevant stimuli [36]. However, our data revealed that this effect wear off in absence of any pro-survival stimuli. By sequentially introducing a hippocampal-dependant training, we take advantage of the exercise-induced enhanced plasticity to achieve a larger pool of surviving DGCs potentially recruitable upon future cognitive challenges.

Interestingly, we found a significant reduction in survival of $BrdU^+$ DGCs in sedentary mice trained in the MWM, as opposed to previously exercised animals. One study in rats supports that the working memory task employed here promote cell survival to a greater extent than the hidden platform version of the MWM, an effect attributed to increased cognitive demand [48]. It is possible that our findings are contradictory due to different AHN dynamics between rodent species [62]. Nevertheless, studies employing slightly different settings have described no differences in cell survival between rats submitted to working memory training in the MWM (4–7 days) and those untrained or subjected to a reference memory task [38,63], which makes it difficult to draw reliable conclusions. Working memory paradigms in the MWM could also reduce AHN in rats but only when learning is extended over time [63]. By contrast, 8 days of such MWM training may be enough to reduce survival of ~1 week-old DGCs in mice, perhaps as a prerequisite for avoiding interference when learning constantly changing information [64]. However, it would not explain why we and others [38] have found the opposite effect in previously exercised animals. Major methodological inconsistences between studies (e.g., different animal species employed, working memory protocols, etc.) make any comparison difficult. So far, how MWM training interacts with AHN remains unclear. The complex temporal interplay between neural birth and spatial learning [40,42,65] may explain why improvements [33,47] but also reductions [66] and non-significant effects on cell survival [28,67] have been reported, mainly under reference memory paradigms. Likewise, it may also explain why we did not find any significant change in the expression of a wide range of immature (DCX^+) DGCs in the SED+MWM group.

It is nevertheless clear from our results that the MWM training interacted with physical exercise to promote the opposite effect of boosting AHN. This is consistent with evidence indicating that such training favours cell survival in rats under exercise but not sedentary conditions [38]. A possible explanation is that MWM training caused stress-related deleterious effects on AHN [63,68] that were prevented by previous exercise [31,69]. However, our mice received proper habituation to the task, which should have prevented potential stressful effects of MWM training [68]. The increase in AHN showed by the EXE+MWM mice could also be explained by sustained aerobic activity due to swimming in the pool after exercise cessation. Contrary to this idea, such synergistic enhance of AHN may not be replicated employing other (hippocampal-independent) MWM protocols, suggesting that this outcome relies on the presence of a cognitive challenge [38]. Another (non-excluding) explanation relates to the ability of exercise to promote neuroplastic phenomena within the hippocampus that are relevant for learning (e.g. release of neurotrophins or enhancement of synaptic plasticity) [70–72]. Central to the relationship between learning and AHN is not only the degree of cognitive challenge, but the individual experience during the task and the degree of mastery achieved [35]. In our experiment, exercised animals were more efficient at solving the MWM task and better able to retain information than their sedentary peers. It is possible that physical stimulation provided exercised animals with more neurocognitive resources to successfully cope with the MWM training, which may have changed task processing and mastery and thereby AHN regulation. For instance, running facilitates spatial pattern separation [29] as well as learning and memory storage [3], and those processes are in turn related to increased AHN [3,22,33]. Supporting this rationale, the multivariate analysis of our data revealed that improved MWM performance, the use of hippocampal strategies and the survival of new DGCs integrated a single factor that was differentially modulated by physical activity, being significantly greater in exercised animals.

Our results also corroborated that ten days of moderate-intensity treadmill exercise upregulated AHN in the short-term, as previously described [73]. Shortly after exercise cessation (~24 h), we observed a significant increase in the number of recently divided ($PCNA^+$) DGCs in the running mice, in line with studies indicating that it prompts cell proliferation in the hippocampus [28,31,73]. An increase in the number of $BrdU^+$ DGCs (8–10 days-old) was also found in these animals. Whether this reflects additional effects of exercise on cell survival is unclear, as it may rather reflect a net increase in neurogenesis as a result of enhanced cell proliferation [28,74]. Importantly, we observed a significant decay in PCNA and BrdU expression nine days after exercise cessation, suggesting transient and probably activity-dependant effects of running on cell proliferation/survival. While it has been suggested that extended running contributes to maintain high rates of adult neurogenesis [31,74], this is uncertain as some other studies reported non-cumulative [27,75] or even negative [76,77] effects on AHN after long periods of exercise. Interestingly, the DGCs generated *de novo* under treadmill running conditions could be prone to death after exercise cessation [78,79], calling into question their functional relevance upon future cognitive demands. For a sustained improvement of exercise-induced AHN, additional, more specific stimuli might therefore be required [27,36]. In this sense, cells that survive as a result of cognitive challenges seem to play a consistent role in later learning experiences [47,80,81], which suggest functional integration in the hippocampal network. We speculate that our MWM task served as such a source of stimulation promoting long-lasting benefits on AHN greater than those that could be generated by a stand-alone exercise protocol.

Altogether, our results support the notion that combining physical and cognitive interventions promote synergistic effects on AHN. Animal models have revealed that enhancing AHN may be useful to counteract cognitive impairment [3,22,32] and pathological conditions such as neurodegenerative [25], mood [23,24] or substance use [26] disorders. On this basis, the development of more effective and long-lasting strategies to improve AHN is a major goal. In this work, we propose a behavioural, non-invasive method to address this issue, which provides remarkable advantages by avoiding side effects and favouring potential transfer to human research and clinical practice. Strategies based on physical or cognitive training have shown positive results in promoting cognitive function in healthy and cognitively impaired populations [2,15]. Importantly, the combination of both interventions appears to produce even greater benefits [10,82,83] that may correlate with functional changes in brain networks involved in spatial learning, including the hippocampal formation [84]. In humans, whether AHN plays a role in these synergistic pro-cognitive effects remains unknown, but studies in rodents suggest that increased AHN may underly the benefits of combining physical and cognitive training in addressing brain injury or neurocognitive disorders [12–14]. Although the role and features of human AHN are still a matter of intense scientific debate, there is no reason to rule out that the improvement of this phenomenon

may have relevant functional implications for mental health and well-being [85], which might be elucidated in future studies.

5. Conclusions

In this study, we demonstrated synergistic effects of combined physical and cognitive training on increasing AHN in mice. Treadmill exercise transiently expanded the pool of new-born hippocampal cells while improving spatial learning in the MWM. By adding subsequent working memory training, survival of DGCs generated *de novo* during exercise was significantly increased over control and single-intervened conditions. Further research may take advantage of this brief, scheduled behavioural protocol to promote greater and longer-lasting improvements of AHN, which may be useful for enhance cognition or treating neurological disorders.

Data availability statement

Datasets generated during the current study are available from the corresponding authors upon reasonable request.

CRediT authorship contribution statement

F. Ávila-Gámiz: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **A.M. Pérez-Cano:** Investigation. **J.M. Pérez-Berlanga:** Investigation. **R.M. Mullor-Vigo:** Investigation. **E.N. Zambrana-Infantes:** Investigation. **L.J. Santín:** Conceptualization, Funding acquisition, Supervision, Data curation, Methodology, Writing – original draft, Writing – review & editing. **D. Ladrón de Guevara-Miranda:** Conceptualization, Funding acquisition, Supervision, Data curation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge the staff of the Animal Experimentation Centre of the University of Málaga for their valuable assistance during the conduct of the experiments. They also thank David Navas Fernández for his technical support in confocal microscopy and Marina Reina Jerónimo for her assistance in laboratory procedures.

This study received financial support from the Spanish Ministry of Economy and Competitiveness (Agencia Estatal de Investigación, AEI), which was cofounded by the European Regional Development Fund (AEI/FEDER, UE; PSI2017-82604R to L.J.S.); by the Spanish Ministry of Science and Innovation (PID2020-113806RB-I00 to L.J.S); by the Biomedical Research Institute of Málaga (IBIMA; 08-2021-AREA3 to D. L.G.M) and by the University of Málaga (B1-2020_06 to D.L.G-M). Funding for Open Access charge: Universidad de Málaga/CBUA.

Authors F.A-G., A.M.P-C. and J.M.P-B. hold predoctoral grants from the Spanish Ministry of Science and Innovation (PRE2018-085673 to F. A-G), the Spanish Ministry of Universities (FPU20/00908 to A.M.P-C.) and Junta de Andalucía (Consejería de Transformación Económica, Industria, Conocimiento y Universidades, PREDOC_01094 to J.M.P-B.). Author D.L.G-M. holds a postdoctoral grant (Consejería de Transformación Económica, Industria, Conocimiento y Universidades, Junta de Andalucía, POSTDOC_ 21_00222).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2023.114184.

References

[1] C. Gubert, A.J. Hannan, Exercise mimetics: harnessing the therapeutic effects of physical activity, Nat. Rev. Drug Discov. 20 (2021) 862–879, https://doi.org/10.1038/S41573-021-00217-1.
[2] M.S. Keshavan, S. Vinogradov, J. Rumsey, J. Sherrill, A. Wagner, Reviews and overviews mechanisms of psychiatric illness cognitive training in mental disorders: update and future directions, Am. J. Psychiatry. 171 (2014) 510–522.
[3] H. Van Praag, T. Shubert, C. Zhao, F.H. Gage, Exercise enhances learning and hippocampal neurogenesis in aged mice, J. Neurosci. 25 (2005) 8680–8685, https://doi.org/10.1523/JNEUROSCI.1731-05.2005.
[4] K.D. Langdon, D. Corbett, Improved working memory following novel combinations of physical and cognitive activity, Neurorehabil. Neural Repair. 26 (2012) 523–532, https://doi.org/10.1177/1545968311425919.
[5] J.A. Alcalá, J.E. Callejas-Aguilera, J.B. Nelson, J.M. Rosas, Reversal training facilitates acquisition of new learning in a Morris water maze, Learn. Behav. 48 (2020) 208–220, https://doi.org/10.3758/s13420-019-00392-7.
[6] A. Chung, C. Jou, A. Grau-Perales, E.R.J. Levy, D. Dvorak, N. Hussain, A.A. Fenton, Cognitive control persistently enhances hippocampal information processing, Nat. 600 (2021) 484–488, https://doi.org/10.1038/s41586-021-04070-5, 2021 6007889.
[7] C.M. Hoffmann, M.E. Petrov, R.E. Lee, Aerobic physical activity to improve memory and executive function in sedentary adults without cognitive impairment: a systematic review and meta-analysis, Prev. Med. Reports. 23 (2021), https://doi.org/10.1016/j.pmedr.2021.101496.
[8] B. Bonnechère, M. Klass, C. Langley, B.J. Sahakian, Brain training using cognitive apps can improve cognitive performance and processing speed in older adults, Sci. Rep. 11 (2021) 12313, https://doi.org/10.1038/S41598-021-91867-Z.
[9] E.G.A. Karssemeijer, J.A. Aaronson, W.J. Bossers, T. Smits, M.G.M. Olde Rikkert, R. P.C. Kessels, Positive effects of combined cognitive and physical exercise training on cognitive function in older adults with mild cognitive impairment or dementia: a meta-analysis, Ageing Res. Rev. 40 (2017) 75–83, https://doi.org/10.1016/j.arr.2017.09.003.
[10] S. Dhir, W.P. Teo, S.R. Chamberlain, K. Tyler, M. Yücel, R.A. Segrave, The effects of combined physical and cognitive training on inhibitory control: a systematic review and meta-analysis, Neurosci. Biobehav. Rev. 128 (2021) 735–748, https://doi.org/10.1016/j.neubiorev.2021.07.008.
[11] A. Lauenroth, A.E. Ioannidis, B. Teichmann, Influence of combined physical and cognitive training on cognition: a systematic review, BMC Geriatr 16 (2016) 21–23, https://doi.org/10.1186/s12877-016-0315-1.
[12] F. Farokhi-Sisakht, S. Sadigh-Eteghad, G. Mohaddes, A. Ebrahimi-Kalan, P. Karimi, M. Farhoudi, Physical and cognitive training attenuate hippocampal ischemia-induced memory impairments in rat, Brain Res. Bull. 155 (2019) 202–210, https://doi.org/10.1016/j.brainresbull.2019.10.007.
[13] G.F. Hamilton, S.A. Jablonski, F.L. Schiffino, S.A. St. Cyr, M.E. Stanton, A. Y. Klintsova, Exercise and environment as an intervention for neonatal alcohol effects on hippocampal adult neurogenesis and learning, Neuroscience 265 (2014) 274–290, https://doi.org/10.1016/j.neuroscience.2014.01.061.
[14] V. Kapgal, N. Prem, P. Hegde, T.R. Laxmi, B.M. Kutty, Long term exposure to combination paradigm of environmental enrichment, physical exercise and diet reverses the spatial memory deficits and restores hippocampal neurogenesis in ventral subicular lesioned rats, Neurobiol. Learn. Mem. 130 (2016) 61–70, https://doi.org/10.1016/j.nlm.2016.01.013.
[15] K. Hötting, B. Röder, Beneficial effects of physical exercise on neuroplasticity and cognition, Neurosci. Biobehav. Rev. 37 (2013) 2243–2257, https://doi.org/10.1016/j.neubiorev.2013.04.005.
[16] H. Takeuchi, A. Sekiguchi, Y. Taki, S. Yokoyama, Y. Yomogida, N. Komuro, T. Yamanouchi, S. Suzuki, R. Kawashima, Training of working memory impacts structural connectivity, J. Neurosci. 30 (2010) 3297–3303, https://doi.org/10.1523/JNEUROSCI.4611-09.2010.
[17] K.I. Erickson, M.W. Voss, R.S. Prakash, C. Basak, A. Szabo, L. Chaddock, J.S. Kim, S. Heo, H. Alves, S.M. White, T.R. Wojcicki, E. Mailey, V.J. Vieira, S.A. Martin, B. D. Pence, J.A. Woods, E. McAuley, A.F. Kramer, Exercise training increases size of hippocampus and improves memory, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 3017–3022, https://doi.org/10.1073/pnas.1015950108.
[18] A.C.S. Bråthen, Ø. Sørensen, A.M.G. de Lange, A.M. Mowinckel, A.M. Fjell, K. B. Walhovd, Cognitive and hippocampal changes weeks and years after memory training, Sci. Rep. 12 (2022) 1–15, https://doi.org/10.1038/s41598-022-11636-4, 2022 121.
[19] M. Moser, E. Moser, Functional differentiation in the hippocampus, Hippocampus 8 (1998) 608–619, https://doi.org/10.1002/(SICI)1098-1063(1998)8:6<608::AID−HIPO3>3.0.CO;2-7.
[20] J.T. Gonçalves, S.T. Schafer, F.H. Gage, Adult Neurogenesis in the Hippocampus: from Stem Cells to Behavior, Cell 167 (2016) 897–914, https://doi.org/10.1016/j.cell.2016.10.021.
[21] T. Toda, S.L. Parylak, S.B. Linker, F.H. Gage, The role of adult hippocampal neurogenesis in brain health and disease, Mol. Psychiatry. 24 (2019) 67–87, https://doi.org/10.1038/s41380-018-0036-2.
[22] A. Sahay, K.N. Scobie, A.S. Hill, C.M. O'Carroll, M.A. Kheirbek, N.S. Burghardt, A. A. Fenton, A. Dranovsky, R. Hen, Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation, Nature 472 (2011) 466–470, https://doi.org/10.1038/nature09817.Increasing.
[23] A.S. Hill, A. Sahay, R. Hen, Increasing adult hippocampal neurogenesis is sufficient to reduce anxiety and depression-like behaviors, Neuropsychopharmacology 40 (2015) 2368–2378, https://doi.org/10.1038/npp.2015.85.

[24] A. Mateus-Pinheiro, L. Pinto, J.M. Bessa, M. Morais, N.D. Alves, S. Monteiro, P. Patrício, O.F. Almeida, N. Sousa, Sustained remission from depressive-like behavior depends on hippocampal neurogenesis, Transl. Psychiatry. 3 (2013), https://doi.org/10.1038/tp.2012.141.
[25] K. Richetin, C. Leclerc, N. Toni, T. Gallopin, S. Pech, L. Roybon, C. Rampon, Genetic manipulation of adult-born hippocampal neurons rescues memory in a mouse model of Alzheimer's disease, Brain 138 (2015) 440–455, https://doi.org/10.1093/brain/awu354.
[26] D. Ladrón de Guevara-Miranda, R.D. Moreno-Fernández, S. Gil-Rodríguez, C. Rosell-Valle, G. Estivill-Torrús, A. Serrano, F.J. Pavón, F. Rodríguez de Fonseca, L.J. Santín, E. Castilla-Ortega, Lysophosphatidic acid-induced increase in adult hippocampal neurogenesis facilitates the forgetting of cocaine-contextual memory, Addict. Biol. 24 (2019) 458–470, https://doi.org/10.1111/adb.12612.
[27] G. Kronenberg, A. Bick-Sander, E. Bunk, C. Wolf, D. Ehninger, G. Kempermann, Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus, Neurobiol. Aging. 27 (2006) 1505–1513, https://doi.org/10.1016/j.neurobiolaging.2005.09.016.
[28] H. van Praag, G. Kempermann, F.H. Gage, Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus, Nat. Neurosci. 2 (1999) 266–270, https://doi.org/10.1038/6368.
[29] D.J. Creer, C. Romberg, L.M. Saksida, H. Van Praag, T.J. Bussey, Running enhances spatial pattern separation in mice, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2367–2372, https://doi.org/10.1073/pnas.0911725107.
[30] P.J. Clark, W.J. Brzezinska, M.W. Thomas, N.A. Ryzhenko, S.A. Toshkov, J. S. Rhodes, Intact neurogenesis is required for benefits of exercise on spatial memory but not motor performance or contextual fear conditioning in C57BL/6J mice, Neuroscience 155 (2008) 1048–1058, https://doi.org/10.1016/j.neuroscience.2008.06.051.
[31] H. Li, A. Liang, F. Guan, R. Fan, L. Chi, B. Yang, Regular treadmill running improves spatial learning and memory performance in young mice through increased hippocampal neurogenesis and decreased stress, Brain Res. 1531 (2013) 1–8, https://doi.org/10.1016/j.brainres.2013.07.041.
[32] L.N. Codd, D.G. Blackmore, J. Vukovic, P.F. Bartlett, Exercise reverses learning deficits induced by hippocampal injury by promoting neurogenesis, Sci. Rep. 10 (2020) 1–13, https://doi.org/10.1038/s41598-020-76176-1.
[33] E. Gould, A. Beylin, P. Tanapat, A. Reeves, T.J. Shors, Learning enhances adult neurogenesis in the hippocampal formation, Nat. Neurosci. 2 (1999) 260–265, https://doi.org/10.1038/6365.
[34] G. Kempermann, H.G. Kuhn, F.H. Gage, More hippocampal neurons in adult mice living in an enriched environment, Nature 386 (1997) 493–495, https://doi.org/10.1038/386493a0.
[35] D.M. Curlik, T.J. Shors, Training your brain: do mental and physical (MAP) training enhance cognition through the process of neurogenesis in the hippocampus? Neuropharmacology 64 (2013) 506–514, https://doi.org/10.1016/j.neuropharm.2012.07.027.
[36] K. Fabel, G. Kempermann, Physical activity and the regulation of neurogenesis in the adult and aging brain, NeuroMolecular Med. 10 (2008) 59–66, https://doi.org/10.1007/s12017-008-8031-4.
[37] K. Fabel, S.A. Wolf, D. Ehninger, H. Babu, P. Leal-Galicia, G. Kempermann, Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice, Front. Neurosci. 3 (2009) 1–7, https://doi.org/10.3389/neuro.22.002.2009.
[38] L.C. Motta-Teixeira, S.H. Takada, A.V. Machado-Nils, M.I. Nogueira, G.F. Xavier, Spatial learning and neurogenesis: effects of cessation of wheel running and survival of novel neurons by engagement in cognitive tasks, Hippocampus 26 (2016) 794–803, https://doi.org/10.1002/hipo.22560.
[39] A.G. Dayer, A.A. Ford, K.M. Cleaver, M. Yassaee, H.A. Cameron, Short-term and long-term survival of new neurons in the rat dentate gyrus, J. Comp. Neurol. 460 (2003) 563–572, https://doi.org/10.1002/cne.10675.
[40] J.R. Epp, M.D. Spritzer, L.A.M. Galea, Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation, Neuroscience 149 (2007) 273–285, https://doi.org/10.1016/j.neuroscience.2007.07.046.
[41] M.L. Anderson, H.M. Sisti, D.M. Curlik, T.J. Shors, Associative learning increases adult neurogenesis during a critical period, Eur. J. Neurosci. 33 (2011) 175–181, https://doi.org/10.1111/j.1460-9568.2010.07486.x.
[42] M.D. Döbrössy, E. Drapeau, C. Aurousseau, M.Le Moal, P.V. Piazza, D.N. Abrous, Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date, Mol. Psychiatry. 8 (2003) 974–982, https://doi.org/10.1038/sj.mp.4001419.
[43] A. Tashiro, H. Makino, F.H. Gage, Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage, J. Neurosci. 27 (2007) 3252–3259, https://doi.org/10.1523/JNEUROSCI.4941-06.2007.
[44] P. Ambrogini, R. Cuppini, D. Lattanzi, S. Ciuffoli, A. Frontini, M. Fanelli, Synaptogenesis in adult-generated hippocampal granule cells is affected by behavioral experiences, Hippocampus 20 (2010) 799–810, https://doi.org/10.1002/hipo.20679.
[45] S. Ge, C. hao Yang, K. sen Hsu, G. li Ming, H. Song, A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain, Neuron 54 (2007) 559–566, https://doi.org/10.1016/j.neuron.2007.05.002.
[46] V. Ramirez-Amaya, D.F. Marrone, F.H. Gage, P.F. Worley, C.A. Barnes, Integration of new neurons into functional neural networks, J. Neurosci. 26 (2006) 12237–12241, https://doi.org/10.1523/JNEUROSCI.2195-06.2006.
[47] S. Trouche, B. Bontempi, P. Roullet, C. Rampon, Recruitment of adult-generated neurons into functional hippocampal networks contributes to updating and strengthening of spatial memory, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 5919–5924, https://doi.org/10.1073/pnas.0811054106.
[48] I.E.J. Aasebø, A.S. Kasture, M. Passeggeri, A. Tashiro, A behavioral task with more opportunities for memory acquisition promotes the survival of new neurons in the adult dentate gyrus, Sci. Rep. 8 (2018) 1–11, https://doi.org/10.1038/s41598-018-25331-w.
[49] P. Sampedro-Piquero, C. Millón, R.D. Moreno-Fernández, M. García-Fernández, Z. Diaz-Cabiale, L.J. Santin, Treadmill exercise buffers behavioral alterations related to ethanol binge-drinking in adolescent mice, Brain Sci. 10 (2020) 1–13, https://doi.org/10.3390/brainsci10090576.
[50] V. Schefer, M.I. Talan, Oxygen consumption in adult and aged C57BL/6J mice during acute treadmill exercise of different intensity, Exp. Gerontol. 31 (1996) 387–392, https://doi.org/10.1016/0531-5565(95)02032-2.
[51] L.N. Castellani, W.T. Peppler, P.M. Miotto, N. Bush, D.C. Wright, Exercise protects against olanzapine-induced hyperglycemia in male C57BL/6J mice, Sci. Rep. 8 (2018), https://doi.org/10.1038/S41598-018-19260-X.
[52] R.W. Overall, S. Zocher, A. Garthe, G. Kempermann, Rtrack: a software package for reproducible automated water maze analysis, BioRxiv. (2020) 2020.02.27.967372. https://doi.org/10.1101/2020.02.27.967372.
[53] G. Paxinos, K.B.J. Franklin, The Mouse Brain in Stereotaxic Coordinates, 2nd ed., Academic Press, San Diego, 2001 https://doi.org/10.1016/S0306-4530(03)00088-X.
[54] T. Plümpe, D. Ehninger, B. Steiner, F. Klempin, S. Jessberger, M. Brandt, B. Römer, G.R. Rodriguez, G. Kronenberg, G. Kempermann, Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation, BMC Neurosci. 7 (2006) 1–14, https://doi.org/10.1186/1471-2202-7-77.
[55] A. Garthe, G. Kempermann, An old test for new neurons: refining the morris water maze to study the functional relevance of adult hippocampal neurogenesis, Front. Neurosci. 7 (2013) 1–11, https://doi.org/10.3389/fnins.2013.00063.
[56] S. Vaynman, Z. Ying, F. Gómez-Pinilla, Exercise induces BDNF and synapsin I to specific hippocampal subfields, J. Neurosci. Res. 76 (2004) 356–362, https://doi.org/10.1002/JNR.20077.
[57] F. Gómez-Pinilla, V. So, J.P. Kesslak, Spatial learning induces neurotrophin receptor and synapsin I in the hippocampus, Brain Res. 904 (2001) 13–19, https://doi.org/10.1016/S0006-8993(01)02394-0.
[58] A.M. Huang, C.J. Jen, H.F. Chen, L. Yu, Y.M. Kuo, H.I. Chen, Compulsive exercise acutely upregulates rat hippocampal brain-derived neurotrophic factor, J. Neural Transm. 113 (2006) 803–811, https://doi.org/10.1007/s00702-005-0359-4.
[59] M.J.M. Schaaf, R.M. Sibug, R. Duurland, M.F. Fluttert, M.S. Oitzl, E.R. De Kloet, &. E. Vreugdenhil, Corticosterone effects on BDNF mRNA expression in the rat hippocampus during morris water maze training, Stress 3 (1999) 173–183, https://doi.org/10.3109/10253899909001121.
[60] B. Ellenbroek, J. Youn, Rodent models in neuroscience research: is it a rat race? Dis. Model. Mech. 9 (2016) 1079–1087, https://doi.org/10.1242/DMM.026120.
[61] C. Schmidt-Hieber, P. Jones, J. Bischofberger, Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus, Nat. 429 (2004) 184–187, https://doi.org/10.1038/nature02553, 2004 4296988.
[62] J.S. Snyder, J.S. Choe, M.A. Clifford, S.I. Jeurling, P. Hurley, A. Brown, J.F. Kamhi, H.A. Cameron, Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice, J. Neurosci. 29 (2009) 14484–14495, https://doi.org/10.1523/JNEUROSCI.1768-09.2009.
[63] P. Mohapel, K. Mundt-Petersen, P. Brundin, H. Frielingsdorf, Working memory training decreases hippocampal neurogenesis, Neuroscience 142 (2006) 609–613, https://doi.org/10.1016/j.neuroscience.2006.07.033.
[64] Z. Xu, J. Li, F. Zhang, Y. Wu, Y. Gao, J. Liang, C. Zhang, Working memory task decreases the survival of newly born neurons in hippocampus, Neurobiol. Learn. Mem. 95 (2011) 239–247, https://doi.org/10.1016/j.nlm.2010.11.013.
[65] D. Dupret, A. Fabre, M.D. Döbrössy, A. Panatier, J.J. Rodríguez, S. Lamarque, V. Lemaire, S.H.R. Oliet, P.V. Piazza, D.N. Abrous, Spatial learning depends on both the addition and removal of new hippocampal neurons, PLoS Biol. 5 (2007) 1683–1694, https://doi.org/10.1371/journal.pbio.0050214.
[66] P. Ambrogini, L. Orsini, C. Mancini, P. Ferri, S. Ciaroni, R. Cuppini, Learning may reduce neurogenesis in adult rat dentate gyrus, Neurosci. Lett. 359 (2004) 13–16, https://doi.org/10.1016/j.neulet.2003.12.123.
[67] M.F. Trinchero, M. Koehl, M. Bechakra, P. Delage, V. Charrier, N. Grosjean, E. Ladeveze, A.F. Schinder, D.N. Abrous, Effects of spaced learning in the water maze on development of dentate granule cells generated in adult mice, Hippocampus 25 (2015) 1314–1326, https://doi.org/10.1002/hipo.22438.
[68] D. Ehninger, G. Kempermann, Paradoxical effects of learning the Morris water maze on adult hippocampal neurogenesis in mice may be explained by a combination of stress and physical activity, Genes Brain Behav. 5 (2006) 29–39, https://doi.org/10.1111/j.1601-183X.2005.00129.x.
[69] E. Castilla-Ortega, C. Rosell-Valle, C. Pedraza, F. Rodríguez de Fonseca, G. Estivill-Torrús, L.J. Santín, Voluntary exercise followed by chronic stress strikingly increases mature adult-born hippocampal neurons and prevents stress-induced deficits in "what-when-where" memory, Neurobiol. Learn. Mem. 109 (2014) 62–73, https://doi.org/10.1016/j.nlm.2013.12.001.
[70] R.M. O'Callaghan, R. Ohle, Á.M. Kelly, The effects of forced exercise on hippocampal plasticity in the rat: a comparison of LTP, spatial- and non-spatial learning, Behav. Brain Res. 176 (2007) 362–366, https://doi.org/10.1016/j.bbr.2006.10.018.
[71] C. Vivar, B.D. Peterson, H. van Praag, Running rewires the neuronal network of adult-born dentate granule cells, Neuroimage 131 (2016) 29–41, https://doi.org/10.1016/j.neuroimage.2015.11.031.

[72] C. Vivar, M.C. Potter, H. van Praag, All about running: synaptic plasticity, growth factors and adult hippocampal neurogenesis, Curr. Top. Behav. Neurosci. 15 (2012) 189–210, https://doi.org/10.1007/7854_2012_220.
[73] A.F.B. Ferreira, C.C. Real, A.C. Rodrigues, A.S. Alves, L.R.G. Britto, Short-term, moderate exercise is capable of inducing structural, bdnf-independent hippocampal plasticity, Brain Res. 1425 (2011) 111–122, https://doi.org/10.1016/j.brainres.2011.10.004.
[74] J.S. Snyder, L.R. Glover, K.M. Sanzone, J.F. Kamhi, H.A. Cameron, The effects of exercise and stress on the survival and maturation of adult-generated granule cells, Hippocampus 19 (2009) 898–906, https://doi.org/10.1002/hipo.20552.
[75] S.P. Cahill, J.D. Cole, R.Q. Yu, J. Clemans-Gibbon, J.S. Snyder, Differential effects of extended exercise and memantine treatment on adult neurogenesis in male and female rats, Neuroscience 390 (2018) 241–255, https://doi.org/10.1016/j.neuroscience.2018.08.028.
[76] A.S. Naylor, A.I. Persson, P.S. Eriksson, I.H. Jonsdottir, T. Thorlin, Extended voluntary running inhibits exercise-induced adult hippocampal progenitor proliferation in the spontaneously hypertensive rat, J. Neurophysiol. 93 (2005) 2406–2414, https://doi.org/10.1152/jn.01085.2004.
[77] C. Nguemeni, M.W. McDonald, M.S. Jeffers, J. Livingston-Thomas, D. Lagace, D. Corbett, Short- and long-term exposure to low and high dose running produce differential effects on hippocampal neurogenesis, Neuroscience 369 (2018) 202–211, https://doi.org/10.1016/j.neuroscience.2017.11.026.
[78] T. Nishijima, Y. Kamidozono, A. Ishiizumi, S. Amemiya, I. Kita, Negative rebound in hippocampal neurogenesis following exercise cessation, Am. J. Physiol. - Regul. Integr. Comp. Physiol. 312 (2017) R347–R357, https://doi.org/10.1152/ajpregu.00397.2016.
[79] T. Nishijima, M. Llorens-Martín, G.S. Tejeda, K. Inoue, Y. Yamamura, H. Soya, J. L. Trejo, I. Torres-Alemán, Cessation of voluntary wheel running increases anxiety-like behavior and impairs adult hippocampal neurogenesis in mice, Behav. Brain Res. 245 (2013) 34–41, https://doi.org/10.1016/j.bbr.2013.02.009.
[80] M. Lods, E. Pacary, W. Mazier, F. Farrugia, P. Mortessagne, N. Masachs, V. Charrier, F. Massa, D. Cota, G. Ferreira, D.N. Abrous, S. Tronel, Adult-born neurons immature during learning are necessary for remote memory reconsolidation in rats, Nat. Commun. 12 (2021) 1–14, https://doi.org/10.1038/s41467-021-22069-4.
[81] Y. Gu, M. Arruda-Carvalho, J. Wang, S.R. Janoschka, S.A. Josselyn, P. W. Frankland, S. Ge, Optical controlling reveals time-dependent roles for adult-born dentate granule cells, Nat. Neurosci. 15 (2012) 1700–1706, https://doi.org/10.1038/nn.3260.
[82] W.D. Oswald, T. Gunzelmann, R. Rupprecht, B. Hagen, Differential effects of single versus combined cognitive and physical training with older adults: the SimA study in a 5-year perspective, Eur. J. Ageing. 3 (2006) 179–192, https://doi.org/10.1007/S10433-006-0035-Z.
[83] E.G.A. Karssemeijer, J.A. Aaronson, W.J.R. Bossers, R. Donders, M.G.M. Olde Rikkert, R.P.C. Kessels, The quest for synergy between physical exercise and cognitive stimulation via exergaming in people with dementia: a randomized controlled trial, Alzheimers. Res. Ther. 11 (2019) 1–13, https://doi.org/10.1186/s13195-018-0454-z.
[84] K. Holzschneider, T. Wolbers, B. Röder, K. Hötting, Cardiovascular fitness modulates brain activation associated with spatial learning, Neuroimage 59 (2012) 3003–3014, https://doi.org/10.1016/J.NEUROIMAGE.2011.10.021.
[85] G. Kempermann, F.H. Gage, L. Aigner, H. Song, M.A. Curtis, S. Thuret, H.G. Kuhn, S. Jessberger, P.W. Frankland, H.A. Cameron, E. Gould, R. Hen, D.N. Abrous, N. Toni, A.F. Schinder, X. Zhao, P.J. Lucassen, J. Frisén, Human adult neurogenesis: evidence and remaining questions, Cell Stem Cell 23 (2018) 25–30, https://doi.org/10.1016/j.stem.2018.04.004.