# Running to remember: The effects of exercise on perineuronal nets, microglia, and hippocampal angiogenesis in female and male mice

Madeleine G. Maheu[1,2,3], Noah James[2], Zach Clark[1], Alex Yang[2], Ridhi Patel[3], Shawn M. Beaudette[4], Rebecca E.K. MacPherson [2,3], Paula Duarte-Guterman[1,3]

1. Department of Psychology, Brock University, St. Catharines, ON, Canada

2. Department of Health Sciences, Brock University, St. Catharines, ON, Canada

3. Centre for Neuroscience, Brock University, St. Catharines, ON, Canada

4. Department of Kinesiology, Brock University, St. Catharines, ON, Canada

Corresponding authors:

Dr. Rebecca MacPherson, rmacpherson@brocku.ca

Dr. Paula Duarte Guterman, pduarteg@brocku.ca

**Keywords:** aerobic exercise; neuroplasticity; neuroinflammation; angiogenesis; sex differences; dentate gyrus; spatial memory.

**Abstract**

Exercise is accepted as a positive health behaviour; however, a less commonly known benefit of exercise is its role in neuroprotection and cognitive health. The purpose of this investigation was to explore the neurobiological benefits of chronic treadmill exercise in female and male mice through its role in microglial content and morphology, cerebral vascularization, and perineuronal net (PNN). We further examined how these neurobiological changes relate to spatial memory outcomes. C57BL/6J mice were assigned to a sedentary (12F/12M) or exercise group (11F/12M). Mice were treadmill-trained for an hour per day, five days per week, at an increasing speed and incline for eight weeks. During the final week of the exercise intervention, all mice were trained on a spatial memory task (Barnes Maze) and brains were collected for immunohistochemistry. Exercised mice made fewer errors than sedentary mice during the first two days of training as well as the probe. Females, regardless of exercise training, made fewer errors during Barnes maze training, and demonstrated a greater frequency of spatial strategy use compared to males. Exercised mice, regardless of sex, had fewer PNNs in the dentate gyrus of the hippocampus compared to sedentary controls. The number of PNNs in the dorsal dentate gyrus was positively correlated with total errors during training. During the probe, greater errors were correlated with more PNNs among the exercised group only. Microglia count and cerebral vascularization were not affected by exercise, although sedentary mice had significantly greater thick/thin microglia compared to ameboid microglia in the ventral dentate gyrus, which was not observed in the exercised group. It was concluded that exercise decreases PNNs in the dentate gyrus in both sexes and this may be related to better spatial learning and memory.

## Introduction

Exercise is widely accepted as beneficial for health, positively affecting almost every tissue in the body including the brain (Pedersen & Saltin, 2015). However, most literature pertaining to the neurobiological benefits of exercise has been derived from studies utilizing male animal models and sex differences have often been overlooked in studies examining the benefits of exercise on brain health. The mechanisms of how exercise benefits the brain and cognitive health in both sexes are less well understood (Barha et al., 2019). Possible neuroprotective mechanisms of exercise include regulation of inflammation and plasticity through microglia and perineuronal net (PNN) expression, and regulation of angiogenesis. The purpose of this study was to examine the propensity for exercise to influence spatial learning and memory and cellular mechanisms in the brain via neuroplastic, neuroimmune, neurovascular changes in female and male mice.

Perineuronal nets (PNNs) are collections of extracellular matrix molecules, which have a primary role in synaptic plasticity through the stabilization of synapses between neuronal somas and proximal dendrites (Celio et al., 1998). PNNs have a role in neuroprotection and memory formation through the regulation of neuronal excitatory and inhibitory balance, and the removal of PNNs results in decreased synaptic inhibition (Lensjo et al., 2017; Khoo et al., 2019) and improvement in recognition memory (male mice; Gogolla et al., 2009; Romberg et al., 2013). Additionally, PNNs repel neurites and growth cones (Xiao et al., 1996), thereby preventing new synaptic connections (Celio et al., 1998). Synapses with fewer PNNs are thought to be less stable and more plastic than those with greater PNNs (Bozzelli et al., 2018). Although there has been little research on the effects of exercise on PNN distribution, previous studies have found that the number of PNNs is reduced in response to volunteer wheel running. Smith and colleagues (2015) demonstrated lower PNN density in the CA1 region of the hippocampus of adult female rats in response to six weeks of voluntary wheel running. Additionally, voluntary wheel running (for 4 weeks) also reduced PNN cells in the dentate gyrus in

male C57BL/6J mice (Briones et al., 2021). A decrease in PNN expression with exercise may indicate enhanced plasticity, which could influence hippocampal-dependent memory formation, however memory has not been assessed in previous studies. In addition, the relationship between PNNs and memory may be complex, as it has also been postulated that PNN formation is essential for the consolidation of memories (Shi et al., 2019). Moreover, digestion of PNNs (through chondroitinase ABC treatment) increased susceptibility to fear memory erasure (Gogolla et al., 2009). Collectively, changes in PNNs can result in a variety of learning outcomes suggesting a complex relationship between the precise balance of PNNs and their involvement in memory.

Microglia, the immune cells of the central nervous system, have been implicated in learning and memory (Czerniawski et al., 2014; Wadhwa et al., 2017; Nguyen et al., 2020) such as maintenance of synaptic connections and synaptic pruning (Paolicelli et al, 2011), neuronal survival and activity (Sierra et al., 2010), axonal trogocytosis (Lim & Ruthazer, 2021), phagocytosis of cellular waste (Ito et al., 2007), and secretion of immune factors (Zhang et al., 2011; Zhou et al., 2012; Jana et al., 2014, Chen et al., 2019; Koss et al., 2019). In both human and mouse models of neurological disease, PNN components have been found in phagocytosed material within microglia (Crapser, 2020a). Brain regions with high PNN intensities tend to have fewer microglia (Barahona et al., 2022), and decreases in microglia through pharmacological intervention has been associated with increased PNNs in both neurological disease models (Crapser et al., 2020ab) and in healthy adult mouse brains (Liu et al., 2021). Recent research has also directly implicated microglial secretions of inflammatory proteins in reducing PNN integrity (Wegrzyn et al., 2021). Taken together, these studies provide support for the concept that microglial activation is involved in PNN disassembly. However, to date, there have been no studies investigating the relationship between microglia and PNN regulation with respect to exercise intervention. In mouse models of neurological disease (Alzheimer's disease, experimental autoimmune encephalomyelitis, and Parkinson's disease) there is evidence that exercise interventions decrease

microglia number in the substantia nigra pars compacta, striatum, and lumbar spinal cord and produce a hiatus of ameboid activity through a reduction in CD11b expression in males (Sung et al., 2012) and females (Benson et al., 2015). Aerobic exercise-induced neural precursor cell activity is mediated by microglia, and the removal of microglia can eliminate the neurogenic gains from exercise in female mice (Vukovic et al., 2012). Altogether this would suggest that the benefits of exercise on cognitive health are in part produced by the optimization of microglia function; however, it is not clear whether exercise decreases inflammation in healthy wildtype female and male mice. Previous literature also suggests that microglia may regulate PNN expression through inflammatory breakdown of the extracellular matrix (Venturino et al., 2021), and exercise may upregulate the release of inflammatory factors responsible for PNN degradation (Nishijima et al., 2015). Microglia and PNN function can interact, therefore in this study we examined both endpoints following an exercise regimen in females and males.

Exercise is well-known for increasing blood flow through angiogenesis in skeletal muscle (Prior et al., 2004) but less is known about changes that occur in the brain (Pereira et al., 2007; Clark et al., 2009; Mortland et al., 2017). Angiogenesis occurs in response to signaling mediators or increases in tissue size and energy expenditure (activity). Tissues that increase in size or energy expenditure require enhanced exchange with vasculature for oxygen, nutrients, waste processing, and signaling molecules (Prior et al., 2004). In the brain, voluntary wheel running increases blood volume in the mouse dentate gyrus in the hippocampus (sex not reported), and these changes have been associated with increased adult neurogenesis (Pereira et al., 2007). Additional work has demonstrated that exercise enhances blood flow through angiogenesis in the rodent brain by concomitantly acting on multiple stimulating factors (Pereira et al., 2007; Creer et al., 2010; Morland et al., 2017). For example, chronic high intensity interval exercise increases capillary density in the hippocampus in mice, particularly in the dentate gyrus (mixed sexes in combined analysis; Morland et al., 2017). Areas such as the

hippocampus, specifically the dentate gyrus (Pereira et al., 2007; Clark et al., 2009), undergo the greatest vascular changes compared to the cerebral cortex where no changes have been observed (Morland et al., 2017). Furthermore, voluntary wheel running increases vascular density in the dentate gyrus and improves performance on the Morris water maze in male mice (Clark et al., 2009). However, these results are strain dependent, observed in C57BL6J but not B6D2F1/J mice despite neurogenic gains (Clark et al., 2009). Overall, there is evidence that brain angiogenic processes increase in response to exercise (Pereira et al., 2007; Clark et al., 2009; Morland et al., 2017), however this may depend on strain while sex differences have not been examined.

Despite the abundance of research regarding the effect of exercise on various neurophysiological measures, there have been fewer studies focusing on exercise-related sex differences in relation to spatial memory and cellular mechanisms. With this gap in knowledge, the purpose of this study was twofold: (1) to characterize the effects of exercise on spatial memory and dentate gyrus levels of, PNNs, microglia, and angiogenesis; and (2) to determine whether there are any sex differences with respect to these measures.

## Materials and Methods

### *Experimental Design*

This experiment was approved by the Research Ethics Board at Brock University (#21-0-03) and followed the guidelines of the Canadian Council on Animal Care. Young-adult (10-weeks-old) C57BL/6J mice (24 males and 24 females) were obtained from Jackson Laboratory. Mice were housed in groups of four, kept on a 12/12h light/dark cycle, and had ad-libitum access to water and food. Mice were fed a standard mouse chow (2014 Teklad global, 14% protein rodent maintenance diet, Harlan Tekland, Mississauga, ON). Mouse body mass and food intake were tracked on a weekly basis. Weekly

food intake was measured by cage and an average was calculated per mouse. Mice were housed at a temperature of 22-24°C for the duration of the study.

*DXA scan*

A small animal dual-energy X-ray absorptiometry (DXA) scanner (OsteoSys InSIGHT, Scintica) was used to non-invasively measure body composition in anesthetized mice (vaporized isoflurane 5% in $O_2$). Percent lean and fat mass were calculated using total body mass. DXA scans were taken at the beginning (baseline) and the end of the 8-week intervention.

*Exercise by Treadmill Running*

One week after arrival to the animal facility, all mice were exposed to the treadmill for two 5-minute habituation periods in which the mice explored the stationary treadmill, and two 10-minute acclimatization periods in which the mice ran on the treadmill at a speed of 15 m per minute and an incline of 5%. Habituation and acclimatization occurred during the light cycle between 9 and 11 am and took a total of two days, with each day consisting of one habituation period followed immediately by one acclimatization period. Mice were assigned to either the exercise (n=12/sex) or sedentary control (n=12/group) condition.

The training protocol began 72 hours after the last acclimation session. Mice were exercised on two rodent-sized six-lane treadmills for one hour per day, five days per week, for 8 weeks. The training was progressive to consistently achieve moderate exertion of ~65% $VO_2$ max beginning at 20 m/min at 15% incline and ending at 25 m/min at 25% incline for the final two weeks. Exercise sessions occurred between 9:00 am and 11:00 am. One of the female mice was taken out of the exercise group 10 days into the study due to an injury while running was not included in any analyses. During all exercise sessions, the mice in the control group were taken into the treadmill room. The exercise regimen and

assignment to groups were adapted from previous work on inflammation (Castellani et al., 2014) and high fat diets (Snook et al., 2016).

*Barnes Maze Testing*

During the last week of the exercise intervention, both the exercise and sedentary control mice were trained on a spatial memory task using the Barnes maze (height 44 cm, diameter 122 cm, 20 holes). This test involved three phases, a habituation phase, a training phase, and probe phase, all of which were adapted from Barnes (1979). Visual cues were placed around the room and remained in the exact same location and orientation for the duration of testing. During the habituation phase to the Barnes maze, each mouse was individually placed in a clear upside-down beaker in the centre of the maze and guided around the inner perimeter for 30 seconds, ending at the escape hole. They were then allowed to explore the escape hole for 3 minutes within the confines of the beaker. At the end of the three minutes, they were nudged through the escape hole into the escape box (13.5 x 7.75 x 5.75 cm) and allowed to explore the box for one minute. The mouse was then removed from the room and returned into their home cage. Barnes maze training began 24 hours after habituation. Mice were placed in the centre of the maze under a beaker. The beaker was lifted, and mice were allowed to explore for two minutes. If they found the hole within those two minutes, they were left to explore the escape hole for one minute. If they did not find the escape hole, they were guided to it and put inside the escape box to explore for one minute. After exploring the escape box, the mice were removed from the room and returned to their home cage. Training took place every day (one trial per day) over five days. Mice completed this task in a different order every day of training. The probe took place 48 hours after the final training session. Mice were placed in the centre and given three minutes to explore the maze without the escape box present. At the end of the three minutes, the mouse was removed from the room and returned to their home cage. Testing took place at the same time every day (between 1-4 pm).

All sessions (training and probe) were recorded via video. All materials were disinfected between animals using peroxiguard (1:40 dilution).

*Behavioural Analysis*

Overhead videos were taken (Sample Rate: 30Hz; Resolution 1280x720 pixels), and animal tracking was performed using DeepLabCut (DLC v2.2). Specifically, a custom DLC model was trained using user inputs to resolve the locations of all Barnes Maze Targets and the Nose and Tail Base of tracked mice. The DLC model (ResNet 50 architecture) was trained using 20 random frames from 10 input videos for a total of 500,000 epochs, resulting in a mean testing error of 2.35 pixels across all tracked positional landmarks. Tracked XY positional data from DLC were further analysed using custom MATLAB (v2022a) script to scale to real-world units, and to generate discrete performance parameters. Specifically, latency to find the target hole, number of errors (incorrect target visits), number of repeat errors (incorrect repeat visits to target holes), cumulative path length, and average speed, were measured for the training sessions and calculated using MATLAB. Time spent in each quadrant and within the vicinity of the target were added to the probe analysis. Search strategy was categorised manually using an output image of the path taken by each mouse. The following search strategies were included in the analysis: random, serial, random/serial, and spatial. A random strategy was categorised as unsystematic and localized searches of holes by crossing through the centre of the maze (Rosenfield & Ferguson, 2014). A serial strategy was categorised as a systematic search of at least four consecutive holes (Rosenfield & Ferguson, 2014). A random/serial strategy was categorised as having elements of both random and serial strategies (O'Leary et al., 2011). Finally, a spatial strategy was categorised as direct navigation to the correct quadrant, with the exception of crossing the maze centre up to one time, with 3 or fewer errors (Rosenfield & Ferguson, 2014).

*Tissue Collection and Processing*

Immediately after the probe, endpoint DXA scans were performed. The day following the probe and 72 hours after the last exercise bout, mice were deeply and continuously anaesthetized with isoflurane to the extent of reflex loss. Brains were perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in PBS (phosphate buffer saline). Brains were extracted, stored and post-fixed in 4% paraformaldehyde in PBS for 24 hours at 4 °C, after which they were cryoprotected through storage in 30% sucrose in PB solution at 4 °C. Adrenals were collected and weighed as an indicator of stress. Using a freezing sliding microtome (SM2010R, Leica), brains were coronally sectioned in 40 µm increments across the extent of the hippocampus (collected in seven series). Sections were stored in an antifreeze solution containing 0.1 M PBS, 30% ethylene glycol, and 20% glycerol at -20°C.

*Immunohistochemistry*

Iba-1 and Wisteria floribunda agglutinin (WFA) were used to visualize microglia and perineuronal nets, respectively and both stains were used on the same sections. Free-floating sections were rinsed three times for 10 min in 0.1 M phosphate buffer saline (PBS; pH= 7.4) in between each step listed below unless otherwise stated. Sections were blocked with 3% normal goat serum (NGS) and 0.3% Triton-X in PBS for 45 minutes. Sections were incubated with rabbit-anti-Iba-1 primary antibody (1:1000, Wako Chemicals; Cat. No) and WFA (1:400; Sigma; Cat. No) for 23 hours at 4 °C. Sections were rinsed in PBS and then incubated with secondary antibodies goat anti-rabbit Alexa 488 (1:200; ThermoFisher) and streptavidin Alexa 568 (1:400; ThermoFisher) for 20 hours at 4 °C. Sections were rinsed with PBS, counterstained with DAPI, mounted onto frosted microscope slides, and cover slipped with using polyvinyl alcohol with DABCO (PVA-DABCO; Sigma-Aldrich).

On another collection of sections, CD31 was used to visualize the vasculature following the protocol by Rust and colleagues (2020). Sections were washed with 0.1M PB and then incubated with

blocking and permeabilization solution (TNB, 0.1% TBST, 3% normal goat serum) for 30 minutes at room temperature shaking. Sections were rinsed with phosphate buffer three times for 10 minutes each and incubated with primary antibody (rat anti-mouse CD31, BD Biosciences, 550274, 1:100) overnight at 4ºC. The next day sections were rinsed once to remove excess antibody and then three times with phosphate buffer for 10 minutes each. Sections were then immediately incubated with secondary antibody (goat anti-rat Alexa 488, 1:200, ThermoFisher) for 2 hours at room temperature, and then overnight at 4ºC. All antibodies were diluted in the blocking and permeabilization solution (TNB, 0.1% TBST, 3% normal goat serum). Sections were washed, counterstained with DAPI, mounted onto microscope slides, and cover slipped using PVA-DABCO.

*Microscopy Analysis*

A researcher blinded to experimental conditions imaged and analysed Iba-1+ cells, WFA and CD31 expression. Sections were imaged using an epifluorescence microscope (Olympus BX53) at 200X magnification using the same exposure settings for all samples. For each brain, images were taken of four sections of the dorsal and three sections of the ventral hippocampus. Ventral was designated as any section that appeared posterior to Bregma -2.92 (Paxinos & Franklin, 2001). ImageJ Fiji was used for image analysis (Schindelin et al., 2012). A region of interest (0.4597 mm x 0.3448 mm, 0.1585 $mm^2$) was created around the granule cell layer of the dentate gyrus (Figure 1A), from which WFA, Iba-1 and CD31 expression were counted within the region of interest. In addition, Iba-1 morphology was categorized into three groups: ameboid stout/thick, and thick/thin. Categorization was based on the branching of the processes, with an ameboid morphology having no processes, a stout/thick morphology having one to two processes close to the cell body, and a thick/thin morphology having several thin processes extending outwards from the cell body (Figure 1B-D). CD31-labelled

vessels were also analysed by counting number of branches per vessel, and by length of vessel. Vessel length was measured as the longest traceable entity for each vessel within the region of interest.

*Statistical Analyses*

GraphPad Prism (version 8.4.3.686) was used to run all statistical tests. ANOVAs using sex (female, male) and treatment (sedentary, exercised) as between subject factors were performed on the adrenal to body mass ratio, total food intake, total errors, total repeat errors, latency, distance traveled, speed, time spent in the correct quadrant, and time spent near the escape hole during training and the probe session. Two-way independent ANOVAs were also performed on post morphometric parameters and change values in morphometric parameters. Repeated measures ANOVAs with days/weeks as the within subjects' factor and sex and treatment as between subject factors were performed on pre-to-post body mass, latencies, and number of errors during training. For the neurobiological measures, ANOVAs were performed on Iba-1, WFA, and CD31 expression, using sex and treatment as between subject factors. The dorsal and ventral dentate gyri were analysed separately. When a main effect or interaction was found to be significant, a post-hoc Bonferroni comparison was used for all post comparisons. Pearson *r* correlations were performed between CD31, Iba-1 and WFA counts, within the dorsal and ventral regions separately. Pearson *r* correlations were also performed on WFA and Iba-1 counts with behavioural measures of errors and latencies during Barnes maze training and the probe session. We report significance differences ($p \leq 0.05$) and trends ($p \leq 0.08$).

## Results

*Exercise did not alter body composition*

All changes in morphometric parameters with sex and exercise are summarized in Table 1. Exercise did not significantly affect body composition although there were trends - specifically the reduced change

in fat mass among the exercised group compared to the sedentary controls (main effect of exercise: F (1, 43) = 3.625, p = 0.0636)) and the increase in adrenal mass with exercise in females (interaction between exercise and sex: ($F$ (1, 33) = 3.383, $p$ = 0.0749)). Regardless of exercise, we found significant sex differences in body and bone composition across nearly all morphometric parameters. Of note, same-sex groups demonstrated comparable caloric intake and there was no effect of exercise on this parameter.

*Sedentary mice made greater errors than exercised mice during training*

To determine if there were sex or exercise-training dependent differences in spatial learning during the training sessions (Figure 2 and 3) a repeated measures ANOVA was performed for the number of errors incurred during spatial training. The analysis revealed an interaction between training day and exercise ($F$ (4, 171) = 3.630, $p$ = 0.0072). Sedentary mice made significantly more errors than exercised mice on training day one ($p$ = 0.0026) and two ($p$ = 0.0502). From the third day of training onwards, the exercised and sedentary groups had comparable errors before finding the escape hole. Regardless of exercise and training day, males made more errors than females (main effect of sex: $F$ (1, 43) = 10.87, $p$ = 0.002; Figure 2A).

An independent ANOVA was performed on total errors accumulated during training (errors summed across training days). Exercised mice accumulated fewer total errors than their sedentary counterparts (main effect of exercise: $F$ (1, 43) = 16.54, $p$ = 0.0002; Figure 2B). In addition, females accumulated fewer errors than males (main effect of sex: $F$ (1, 43) = 10.34, $p$ = 0.0025).

A repeated measures ANOVA was performed on the number of repeated errors across training day (i.e. the number of times each incorrect target was visited more than once; Figure 2C), which showed an interaction between training day and exercise F (4, 165) = 3.216, p < 0.0001), as well as a main effect of sex (F (1, 43) = 4.261, p = 0.0451), and exercise (F (1, 43) = 8.181, $p$ = 0.0065).

Sedentary mice made greater repeat errors on the first day of training and males made greater repeat errors than females.

We also analysed the total repeat errors accumulated during training (i.e. the total number of times each incorrect target was visited more than once summed over all training days) using an independent ANOVA (Figure 2D) and found that exercised mice made fewer total repeat errors compared to sedentary mice (main effect of exercise: $F$ (1, 43) = 7.990, $p$ =0.0071), and that females made fewer repeated target errors than males (main effect of sex: $F$ (1, 43) = 9.635, $p$ = 0.0034).

*Exercised females spent more time near the escape hole*

A repeated measures ANOVA was performed on the distance of path travelled across training days (Figure 3A). The analysis showed an interaction between training day and exercise (F (4, 165) = 3.551, p = 0.0083), as well as a main effect of training day ($F$ (3.396, 140.1) = 16.96, p < 0.0001), sex ($F$ (1, 43) = 12.42, $p$ = 0.001), and exercise ($F$ (1, 43) = 14.26, $p$ = 0.0005). Sedentary mice travelled a farther distance than exercised mice on training day one and males travelled a farther distance than females before finding the escape hole.

A two-way independent ANOVA was performed on total path distance travelled during training (Figure 3B), which revealed a significant effect of sex ($F$ (1, 43) = 28.93, $p$ < 0.0001), and exercise ($F$ (1, 43) = 15.15, $p$ = 0.0003). Males travelled a longer distance overall compared to females and sedentary mice travelled a farther distance than their exercised counterparts.

A repeated measures ANOVA was performed on the average distance from the escape hole across training days (Figure 3C), which showed an interaction between sex and exercise ($F$ (1, 43) = 5.897, $p$ = 0.0194), as well as a main effect of sex ($F$ (1, 43) = 11.54, $p$ = 0.0015), and exercise ($F$ (1, 43) = 5.845, $p$ = 0.0199). The analysis revealed no effect of training day. To simplify the analysis, a two-way independent ANOVA on the total of the average distance from the escape hole was performed

(Figure 3D), which showed an interaction between sex and exercise ($F$ (1, 43) = 5.063, $p$ = 0.0296) and a main effect of exercise ($F$ (1, 43) = 5.010, $p$ = 0.0304), as well as sex ($F$ (1, 43) = 21.31, $p$ < 0.0001). Multiple comparison tests showed that exercised females had shorter distance travelled to the escape hole, compared to all other groups. None of the other groups significantly differed from each other. To determine whether latencies or errors were affected by systematic differences in the speeds of the groups (Figure 3E), we analysed the average speed for each group on each training day. The analysis demonstrated that sedentary mice moved faster than exercised mice regardless of sex (main effect of exercise: $F$ (1, 43) = 7.360, $p$ = 0.0096; Figure 3E).

*Exercised mice demonstrated greater use of spatial strategy*

We next examined whether there were differences in navigational strategy between the groups (Figure 4) by determining the proportion of each strategy used across all five training days. We found that males used a random strategy significantly more often than females (main effect of sex: F (1, 43) = 11.32, p=0.0016) while females used a spatial strategy more often than males (effect of sex ($F$ (1, 43) = 14.33, $p$ =0.0005; Figure 4B). The proportion of animals using a spatial strategy was affected by exercise ($F$ (1, 43) = 8.463, $p$ =0.0057; Figure 2B) with exercised mice using a spatial strategy more often than sedentary mice. While this effect seemed to be driven by female exercised mice, the interaction between exercise and sex was not significant ($F$ (1, 43) = 1.729, $p$ = 0.1955).

*Exercised mice made fewer errors during the probe trial*

Following training, mice were tested in a probe trial to examine latency to correct hole, correct target visits, errors, and time spent in the target quadrant (Figure 5). There were no significant differences in latency or correct target visits during the probe. However, there was a significant effect of exercise on the number of errors (Figure 5A), with exercised mice visiting fewer incorrect targets

during the probe session compared to sedentary mice (main effect of exercise: F (1, 43) = 5.187, p = 0.0278). There was a significant effect of sex on the time spent in the target quadrant, with females spending more time in this quadrant than males (F (1, 43) = 5.502, p = 0.0237; Figure 5B). Similarly, we also found that females spent significantly more time near the escape hole (F (1, 43) = 9.149, p = 0.0042; Figure 5C).

*Exercised mice had less WFA expression in the dorsal dentate gyrus compared to sedentary controls*

To determine whether there were exercise or sex-dependent effects on the number of perineuronal nets, we examined WFA expression in the dorsal and ventral dentate gyrus (Figure 6A-C). We found that exercised mice had less WFA expression compared to sedentary mice in the dorsal region of the dentate gyrus (main effect of exercise: $F$ (1, 44) = 4.236 $p$ = 0.0455; Figure 6B). There were no significant effects of exercise, sex, nor an interaction in WFA expression in the ventral dentate gyrus (all p's > 0.05; Figure 6C).

*CD-31 and Iba-1 quantity were not affected by exercise*

We determined the effects of exercise on microglia (using Iba-1) and blood vessels (using CD-31) in the dorsal and ventral dentate gyrus (Figure 6D-I). There were no significant effects of sex or exercise, nor any interactions (all p's > 0.05) on Iba-1 counts in the dorsal or ventral dentate gyrus (Figure 6E-F). We next examined changes in blood vessel counts and vessel morphology (i.e., number of vessels, number of branches per vessel, and longest continuous section of each vessel) using CD31. There was a significant effect of sex on branch counts in the ventral dentate gyrus, with females having more branching per vessel than males ($F$ (1, 41) = 4.426, $p$ = 0.0416; Figure 6I), which was not significant in the dorsal region (Figure 6H). There were no significant effects of exercise, sex, nor any interactions regarding vessel lengths or number of vessels in either region (all $p$'s > 0.05).

We also investigated whether exercise training resulted in changes in microglia morphology. There were no differences in morphological proportions in the dorsal dentate gyrus between any of the groups (all p's > 0.05; Figure 7A). In the ventral dentate gyrus, there was a significant interaction between morphology type and exercise ($F$ (2, 90) = 4.982, $p$ = 0.0089). In sedentary mice, there were more thin and stout microglia compared to ameboid microglia in the ventral region. In the exercised animals, the proportions were shifted with similar levels of thin and ameboid microglia (Figure 7B).

*Correlations between neurobiological measures and errors in the Barnes maze*

We performed Pearson r correlations to determine whether there was a relationship between all neurobiological measures in the dentate gyrus. All correlations showed no association between any of the neurobiological measures in either dorsal or ventral region, except for a small negative trend between Iba1 and CD31 counts in the ventral region (r = -0.2863, p = 0.0538). Correlations based on sex and exercise were also performed but no significant correlations were found ($p$'s > 0.05).
We next examined potential associations between neurobiological and behavioural measures. There was a small, positive correlation between total errors during training and WFA expression amongst the whole group ($r$ = 0.2876, $p$ = 0.0500; Figure 8A), but no significant relationship existed within any subgroups (sex or exercise).

We found a significant positive correlation between dorsal WFA expression and errors before finding the escape hole during the probe among the exercised group ($r$ = 0.5969, $p$ = 0.0026, Figure 8B), with the correlation similar among the exercised females ($r$ = 0.6451, $p$ = 0.0321) and exercised males ($r$ = 0.6264, $p$ = 0.0293) but not in the sedentary group ($r$ = -0.2336, $p$ = 0.2718; Figure 8C). There were no significant correlations between dorsal WFA and correct target visits, or time spent in the target quadrant during the probe, amongst the whole group or otherwise.

In the ventral region, there were no significant correlations between WFA and total errors during training. However, there was a moderate negative correlation between ventral WFA expression and average repeat targets among the sedentary group ($r$ = -0.4254, $p$ = 0.0392), which was strongly driven by the sedentary males ($r$ = -0.6858, $p$ = 0.0146) as the sedentary females demonstrated no relationship ($r$ = -0.1196, $p$ = 0.7112) nor did either exercised group (exercised females: $r$ = -0.2357, $p$ = 0.4854; exercised males: $r$ = 0.2652, $p$ = 0.4049).

## Discussion

The purpose of this study was to determine the sex-specific effects of exercise training on spatial memory and neurobiological outcomes, as measured by microglia, PNNs, and vascularisation in the dentate gyrus of the hippocampus. Our findings indicate that exercise improves spatial learning in both sexes, and this was associated with a reduction in PNNs but no significant changes in vascularisation or microglia numbers within the dentate gyrus. We found that female mice performed better on the Barnes maze during training and probe trials and we found sex differences in strategy use. Our results however indicate that treadmill exercise benefits spatial memory in both sexes with changes in the extracellular matrix in the hippocampus.

### *Exercise improved spatial learning and memory in both sexes*

There has been ample previous research relating the benefits of exercise to improved learning and memory (Li et al., 2013; Xiong et al., 2015; Hwang et al., 2016). However, most of the past research has focused on males. We found that treadmill exercising mice, regardless of sex showed improved spatial learning and memory assessed with the Barnes maze. Our findings are consistent with previous work in adult male (van Praag et al., 2005) and female (van Praag et al., 1999) C57BL/6 mice using voluntary wheel running and the Morris water maze. Our results demonstrate that the exercise-

induced benefit was most prevalent during the first two training days, during which time the exercised mice had significantly fewer errors, thereby indicating hastened spatial learning compared to sedentary controls. This may suggest that the exercised mice created a more stable representation of the novel maze environment from the initial habituation session. This is also supported by the finding that the exercise trained mice made fewer repeat errors on the first day of training, which may also indicate enhanced working memory. These findings add to the existing literature as they indicate that exercise might not only improve memory, but also speed of learning. This is supported by our finding that exercised mice incurred fewer errors on the first two days of training, travelled less during the first day of training, had shorter total distances to the target hole across training days (Figure 2 and 3). Previous work has found that voluntary wheel running improves the rate of spatial memory acquisition in young adult male Wistar rats, as measured by distance required to find the escape hole in the Barnes maze (Jacotte-Simancas et al., 2013). Using other spatial memory tasks (radial arm maze, Y maze), other studies have similarly found that wheel running increases speed of learning in female rats (Anderson et al., 2000) and male C57BL/6 mice (van der Borght et al., 2007). In addition to improving speed of spatial learning in the current study, exercise also improved recall ability, as the exercised mice made fewer errors during the probe session compared to their sedentary counterparts. Lastly, the enhanced performance in the Barnes maze among the exercised group does not seem to be the result of enhanced locomotion or fitness, as both groups had comparable speed on all training days, with the exception of day one, during which time the sedentary group demonstrated faster locomotion.

*Females demonstrated enhanced spatial memory, compared to males*

Our results showed that females demonstrated improved spatial learning compared to their male counterparts, as evidenced by fewer total errors observed during training among the female mice. Although contrary to the generally accepted notion of male-favoured spatial memory performance that

is heralded in human literature (Iachini et al. 2005; Lejbak et al., 2011; Yuan et al., 2019), and mirrored in animal research (Gresack & Frick, 2003; Safari et al., 2021), this study demonstrated female-favoured performance in spatial navigation, as measured by fewer errors and increased spatial strategy preference among female mice. Female preference for spatial strategy compared to males is consistent with research conducted in degus using the Barnes maze (Popović et al., 2010), and though not significant in this investigation, the preference for spatial strategy use appeared to be driven by the exercised females. Exercised females also had the shortest average distance to the escape hole during training. Together, these findings indicate that exercise may pose a greater cognitive benefit for female mice compared to male mice. Although the conclusions from these findings are consistent with previous literature, it should be noted that the Barnes maze is only one test of spatial learning and memory, and therefore may not reflect other forms of memory. Previous work has shown that the distal placement of spatial cues in relation to the navigator can affect performance and that this can differ by sex (Saucier et al., 2007). Specifically, females tend to benefit from peri-personal cues (spatial markers that are close to the navigator), while males tend to benefit more so from extra-personal cues (spatial markers that are more distant from the navigator; Saucier et al., 2007). As such, the placement of the spatial cues may have provided greater benefit to females compared to males, and this benefit, combined with that of exercise, may have had a cumulative effect to provide exercised females with the greatest advantage in this spatial memory task.

*Exercise enhanced hippocampal plasticity in both sexes*

Our findings showed that exercise enhances neuroplasticity, as demonstrated by a reduction in PNNs in the dentate gyrus among the exercised mice in both sexes. This is consistent with previous work conducted in female rats using voluntary wheel running (Smith et al., 2015). We were also able to show that this form of neuroplasticity is associated with enhanced spatial memory performance, as total

errors were correlated with the number of PNNs during training. Furthermore, greater PNNs was also associated with poorer spatial memory performance (indicated by errors) during the probe session among the exercised group. To our knowledge, there has been no work regarding the effects of exercise on PNNs with respect to spatial memory outcomes, nor with respect to possible sex differences. Previous work has found that PNN degradation, as well as knockout of genes responsible for PNN composition in the perirhinal cortex results in improved object recognition memory in male mice (Romberg et al., 2013). However, there is additional literature that may demonstrate a complex relationship between PNN expression and memory when factors such as type of memory, brain region, as well as stage of learning and memory (acquisition, recall, retention, and reconsolidation) are considered (Gogolla et al., 2009; Romberg et al., 2013; Shi et al., 2019; Christensen et al., 2021). For example, in macaques, a decrease in parvalbumin-associated PNNs in the retrosplenial cortex is correlated memory impairment on an object recognition test (Gray et al., 2023). Finally, one last factor to be considered is the PNN marker being used. Wisteria floribunda agglutinin (WFA) is the most commonly used marker for PNNs (Härtig et al., 2022). However, recent work has demonstrated that WFA does not bind to all PNNs, nor do other common PNN markers such as those binding aggrecan or chondroitin sulphate proteoglycan link proteins, and this depends on brain region (Hartig et al., 2022). Further work is needed to better understand the different markers and the potential functional implication of different populations of PNNs.

*Exercise did not affect vascularisation and had limited effects on microglia*

Treadmill exercise did not affect the number of microglia in the dentate gyrus. However, we did find that the proportions of microglia type (ameboid, stout/thick, and thick/thin) were regulated by exercise. Exercised mice had a reduction in the proportion of thin microglia. Although we expected that exercise would reduce neuroinflammation, indicated by a decrease in microglia numbers, our results do

not support this hypothesis. Exercise reduces neuroinflammation in the hippocampus in animal models of Alzheimer's disease (Nichol et al., 2008; Ke et al., 2011; Tapia-Rojas et al., 2016; Zhang et al., 2018; Wang et al., 2023) but our work suggests that in healthy adult mice, chronic treadmill exercise does not regulate microglia count in the dentate gyrus. There has been minimal work regarding the impact of exercise on neuroinflammation in conjunction with improved memory outcomes. As such, future work should investigate the propensity for long-term exercise to reduce neuroinflammation through microglia count and phenotype change in various brain regions associated with learning and memory.

Although there is evidence of exercise-induced angiogenesis in the body (Prior et al., 2004), there is limited work regarding this process in the brain. This investigation used a direct approach through the use of CD31 and measurement of blood vessel length, count, and branching, similar to previous work (Staton et al., 2004; Basilio-de-Oliveira & Pannain, 2015). Despite this, we did not find an exercise-induced increase in angiogenesis within the dentate gyrus in female and male mice. It is possible that exercise regulates vascularisation in a brain-specific manner. We focused on the dentate gyrus as it is an area that is strongly related to learning and memory and the site of adult neurogenesis, and both of these measures are regulated by exercise (van Praag et al., 1999; Anderson et al., 2000; van Praag et al., 2005; van der Borght et al., 2007). Previous work has demonstrated increased blood volume in this region in mice and humans (measured by magnetic resonance imaging), as well as an increase in vascular endothelial growth factor in mice (Pereira et al., 2007; Morland et al., 2017). Altogether, other processes may need to be considered to account for the enhancement of blood volume and vascular endothelial growth factor in this area that has been demonstrated by previous work. Finally, the results from this investigation do not preclude the possibility of angiogenesis in other brain regions, and future work should investigate more effective ways of demonstrating the angiogenic process as it relates to the effect of exercise on brain areas underpinning learning and memory.

*Conclusion*

In conclusion, we found an additional mechanism through which exercise favours neuroplasticity in the hippocampus in both sexes. Reduction is PNNs with treadmill exercise were also associated with enhanced spatial memory performance. While we found that females performed better in the Barnes maze, this sex difference in spatial memory was not associated with changes in PNNs in the dentate gyrus. Further work is needed to elucidate underlying mechanisms of sex differences in spatial memory, including other neurobiological measures and brain regions.

**Table 1.** Effects of exercise training and sex on morphometric parameters, adrenal mass, and food intake in male and female mice.

| | Male | | Female | | ANOVA, p | | |
|---|---|---|---|---|---|---|---|
| | Sed. | Ex. | Sed. | Ex. | Ex. | Sex | Intx |
| Body mass (g) | 32.03±2.03 | 30.95±2.22 | 22.32±1.28 | 22.45±0.9 | >0.3475 | **<0.0001** | >0.2295 |
| Fat mass (g) | 3.23±0.89 | 2.83±0.50 | 1.80±0.22 | 1.79±0.22 | >0.2084 | **<0.0001** | >0.2145 |
| % fat mass | 10.1±2.4 | 9.1±1.4 | 8.1±0.9 | 8.0±1.1 | >0.3087 | **0.0017** | >0.3488 |
| Change in fat mass | 0.79±0.57 | 0.54±0.54 | 0.34±0.32 | 0.36±0.14 | >0.1044 | **0.029** | >0.8482 |
| Change in fat % | 0.010±0.015 | 0.002±0.017 | -0.004±0.020 | 0.006±0.014 | **0.0636** | >0.2698 | >0.8082 |
| Lean mass (g) | 28.30±1.82 | 27.62±1.99 | 20.13±1.16 | 20.26±0.96 | >0.5493 | **<0.0001** | >0.3373 |
| % lean mass | 88.4±2.5 | 89.3±1.4 | 90.2±1.2 | 90.2±0.9 | >0.3435 | **0.0057** | >0.3654 |
| Change in lean mass (g) | 4.21±1.40 | 4.62±0.79 | 3.12±0.83 | 2.56±1.14 | >0.7975 | **>0.0001** | 0.1274 |
| Change in lean % | 0.011±0.015 | 0.003±0.018 | 0.003±0.015 | 0.004±0.021 | >0.1458 | 0.1565 | 0.8830 |
| *Adrenal/body mass ratio | 1.175±0.301 | 1.02±0.353 | 1.328±0.393 | 1.66±0.515 | >0.5100 | **0.0054** | **0.0749** |
| Bone mineral density | 0.072±0.002 | 0.074±0.002 | 0.070±0.003 | 0.070±0.002 | >0.5267 | **<0.0001** | >0.2026 |
| Bone mineral content | 0.502±0.037 | 0.494±0.042 | 0.392±0.027 | 0.397±0.018 | >0.9491 | **<0.0001** | >0.4907 |
| Total food intake (g) | 2199±16.04 | 2197±10.29 | 1809±7.77 | 1820±7.45 | >0.4967 | <0.0001 | >0.3338 |

Note: Data are presented as mean ± SD. Two-way ANOVAs were used to assess exercise and sex main effects and interactions on post-measures.
*Data for adrenal/body mass ratio are presented as $x10^{-4}$

## References

Anderson, B. J., Rapp, D. N., Baek, D. H., McCloskey, D. P., Coburn-Litvak, P. S., & Robinson, J. K. (2000). Exercise influences spatial learning in the radial arm maze. Physiology & Behavior, 70(5), 425–429. https://doi.org/10.1016/S0031-9384(00)00282-1

Barahona, R. A., Morabito, S., Swarup, V., & Green, K. N. (2022). Cortical diurnal rhythms remain intact with microglial depletion. *Scientific Reports*, *12*(1), 114–114. https://doi.org/10.1038/s41598-021-04079-w

Basilio-de-Oliveira, R. P., & Pannain, V. L. (2015). Prognostic angiogenic markers (endoglin, VEGF, CD31) and tumor cell proliferation (Ki67) for gastrointestinal stromal tumors. World journal of gastroenterology, 21(22), 6924–6930. https://doi.org/10.3748/wjg.v21.i22.6924

Benson, C., Paylor, J. W., Tenorio, G., Winship, I., Baker, G., & Kerr, B. J. (2015). Voluntary wheel running delays disease onset and reduces pain hypersensitivity in early experimental autoimmune encephalomyelitis (EAE). *Experimental Neurology*, *271*, 279–290. https://doi.org/10.1016/j.expneurol.2015.05.017

Bozzelli, L., Alaiyed, S., Kim, E., Villapol, S., & Conant, K. (2018). Proteolytic remodeling of perineuronal nets: Effects on synaptic plasticity and neuronal population dynamics. Neural Plasticity, 5735789–13. https://doi.org/10.1155/2018/5735789

Briones, B. A., Pisano, T. J., Pitcher, M. N., Haye, A. E., Diethorn, E. J., Engel, E. A., Cameron, H. A., & Gould, E. (2021). Adult-born granule cell mossy fibers preferentially target parvalbumin-positive interneurons surrounded by perineuronal nets. Hippocampus, 31(4), 375–388. https://doi.org/10.1002/hipo.23296

Celio, M. R., Spreafico, R., De Biasi, S., & Vitellaro-Zuccarello, L. (1998). Perineuronal nets: past and present. Trends in Neurosciences (Regular Ed.), 21(12), 510–515. https://doi.org/10.1016/S0166-2236(98)01298-3

Chen, A. Q., Fang, Z., Chen, X. L., Yang, S., Zhou, Y. F., Mao, L., Xia, Y. P., Jin, H. J., Li, Y. N., You, M. F., Wang, X. X., Lei, H., He, Q. W., & Hu, B. (2019). Microglia-derived TNF-α mediates endothelial necroptosis aggravating blood brain-barrier disruption after ischemic stroke. *Cell Death & Disease*, *10*(7), 487. https://doi.org/10.1038/s41419-019-1716-9

Christensen, A. C., Lensjo, K. K., Lepperod, M. E., Dragly, S.-A., Sutterud, H., Blackstad, J. S., Fyhn, M., & Hafting, T. (2021). Perineuronal nets stabilize the grid cell network. Nature Communications, 12(1), 253–253. https://doi.org/10.1038/s41467-020-20241-w

Clark, P. J., Brzezinska, W. J., Puchalski, E. K., Krone, D. A., & Rhodes, J. S. (2009). Functional Analysis of Neurovascular Adaptations to Exercise in the Dentate Gyrus of Young Adult Mice Associated With Cognitive Gain. Hippocampus, 19(10), 937–950. https://doi.org/10.1002/hipo.20543

Crapser, J. D., Spangenberg, E. E., Barahona, R. A., Arreola, M. A., Hohsfield, L. A., & Green, K. N. (2020a). Microglia facilitate loss of perineuronal nets in the Alzheimer's disease brain. *EBioMedicine*, *58*, 102919–102919. https://doi.org/10.1016/j.ebiom.2020.102919

Crapser, J. D., Ochaba, J., Soni, N., Reidling, J. C., Thompson, L. M., & Green, K. N. (2020b). Microglial depletion prevents extracellular matrix changes and striatal volume reduction in a model of Huntington's disease. *Brain*, *143*(1), 266–288. https://doi.org/10.1093/brain/awz363

Creer, D. J., Romberg, C., Saksida, L. M., van Praag, H., & Bussey, T. J. (2010). Running enhances spatial pattern separation in mice. *Proceedings of the National Academy of Sciences - PNAS*, *107*(5), 2367–2372. https://doi.org/10.1073/pnas.0911725107

Czerniawski, J., Miyashita, T., Lewandowski, G., & Guzowski, J. F. (2014). Systemic lipopolysaccharide administration impairs retrieval of context–object discrimination, but not spatial, memory: Evidence for selective disruption of specific hippocampus-dependent memory functions during acute neuroinflammation. Brain, Behavior, and Immunity, 44, 159–166. https://doi.org/10.1016/j.bbi.2014.09.014

Gogolla, N., Caroni, P., Lüthi, A., & Herry, C. (2009). Perineuronal nets protect fear memories from erasure. Science, 325(5945), 1258–1261. https://doi.org/10.1126/science.1174146

Gray, D. T., Khattab, S., Meltzer, J., McDermott, K., Schwyhart, R., Sinakevitch, I., Härtig, W., & Barnes, C. A. (2023). Retrosplenial cortex microglia and perineuronal net densities are associated with memory impairment in aged rhesus macaques. *Cerebral Cortex*, *33*(8), 4626–4644. https://doi.org/10.1093/cercor/bhac366

Gresack, J. E., & Frick, K. M. (2003). Male mice exhibit better spatial working and reference memory than females in a water-escape radial arm maze task. *Brain research*, *982*(1), 98–107. https://doi.org/10.1016/s0006-8993(03)03000-2

Härtig, W., Meinicke, A., Michalski, D., Schob, S., & Jäger, C. (2022). Update on Perineuronal Net Staining With Wisteria floribunda Agglutinin (WFA). *Frontiers in Integrative Neuroscience*, *16*, 851988–851988. https://doi.org/10.3389/fnint.2022.851988

Hawley, W. R., Grissom, E. M., Barratt, H. E., Conrad, T. S., & Dohanich, G. P. (2012). The effects of biological sex and gonadal hormones on learning strategy in adult rats. Physiology & Behavior, 105(4), 1014–1020. https://doi.org/10.1016/j.physbeh.2011.11.021

Hwang, D. S., Kwak, H. B., Ko, I. G., Kim, S. E., Jin, J. J., Ji, E. S., Choi, H. H., & Kwon, O. Y. (2016). Treadmill exercise improves memory function depending on circadian rhythm changes in mice. *International Neurourology Journal*, *20*(Suppl 2), S141–S149. https://doi.org/10.5213/inj.1632738.369

Iachini, T., Sergi, I., Ruggiero, G., & Gnisci, A. (2005). Gender differences in object location memory in a real three-dimensional environment. *Brain and Cognition*, *59*(1), 52–59. https://doi.org/10.1016/j.bandc.2005.04.004

Iaria, G., Petrides, M., Dagher, A., Pike, B., & Bohbot, V. D. (2003). Cognitive strategies dependent on the hippocampus and caudate nucleus in human navigation: Variability and change with practice. The Journal of Neuroscience, 23(13), 5945–5952. https://doi.org/10.1523/JNEUROSCI.23-13-05945.2003

Jacotte-Simancas, A., Costa-Miserachs, D., Torras-Garcia, M., Coll-Andreu, M., & Portell-Cortés, I. (2013). Effect of voluntary physical exercise and post-training epinephrine on acquisition of a spatial task in the Barnes maze. *Behavioural Brain Research*, *247*, 178–181. https://doi.org/10.1016/j.bbr.2013.03.038

Jana, M., Mondal, S., Jana, A., & Pahan, K. (2014). Interleukin-12 (IL-12), but not IL-23, induces the expression of IL-7 in microglia and macrophages: implications for multiple sclerosis. *Immunology*, *141*(4), 549–563. https://doi.org/10.1111/imm.12214

Ke, H.-C., Huang, H.-J., Liang, K.-C., & Hsieh-Li, H. M. (2011). Selective improvement of cognitive function in adult and aged APP/PS1 transgenic mice by continuous non-shock treadmill exercise. Brain Research, 1403, 1–11. https://doi.org/10.1016/j.brainres.2011.05.056

Khoo, G. H., Lin, Y. T., Tsai, T. C., & Hsu, K. S. (2019). Perineuronal nets restrict the induction of long-term depression in the mouse hippocampal CA1 region. Molecular Neurobiology, 56(9), 6436–6450. https://doi.org/10.1007/s12035-019-1526-1

Koss, K., Churchward, M. A., Tsui, C., & Todd, K. G. (2019). In Vitro Priming and Hyper-Activation of Brain Microglia: an Assessment of Phenotypes. *Molecular Neurobiology*, *56*(9), 6409–6425. https://doi.org/10.1007/s12035-019-1529-y

Lejbak, L., Crossley, M., & Vrbancic, M. (2011). A male advantage for spatial and object but not verbal working memory using the n-back task. *Brain and Cognition*, *76*(1), 191–196. https://doi.org/10.1016/j.bandc.2010.12.002

Lensjø, K. K., Lepperod, M. E., Dick, G., Hafting, T., & Fyhn, M. (2017). Removal of perineuronal nets unlocks juvenile plasticity through network mechanisms of decreased inhibition and increased gamma activity. The Journal of Neuroscience, 37(5), 1269–1283. https://doi.org/10.1523/JNEUROSCI.2504-16.2016

Li, H., Liang, A., Guan, F., Fan, R., Chi, L., & Yang, B. (2013). Regular treadmill running improves spatial learning and memory performance in young mice through increased hippocampal neurogenesis and decreased stress. *Brain research*, *1531*, 1–8. https://doi.org/10.1016/j.brainres.2013.07.041

Lim, T. K., & Ruthazer, E. S. (2021). Microglial trogocytosis and the complement system regulate axonal pruning in vivo. *eLife*, *10*, e62167. https://doi.org/10.7554/eLife.62167

Morland, C., Andersson, K. A., Haugen, Ø. P., Hadzic, A., Kleppa, L., Gille, A., Rinholm, J. E., Palibrk, V., Diget, E. H., Kennedy, L. H., Stølen, T., Hennestad, E., Moldestad, O., Cai, Y., Puchades, M., Offermanns, S., Vervaeke, K., Bjørås, M., Wisløff, U., … Bergersen, L. H. (2017). Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. *Nature Communications*, *8*(1), 15557–15559. https://doi.org/10.1038/ncomms15557

Nguyen, P. T., Dorman, L. C., Pan, S., Vainchtein, I. D., Han, R. T., Nakao-Inoue, H., Taloma, S. E., Jerika, J., Molofsky, A. B., Kheirbek, M. A., & Molofsky, A. V. (2020). Microglial remodeling of the extracellular matrix promotes synapse plasticity. Cell, 182(2), 388– 403.e15. https://doi.org/10.1016/j.cell.2020.05.050

Nichol, K. E., Poon, W. W., Parachikova, A. I., Cribbs, D. H., Glabe, C. G., & Cotman, C. W. (2008). Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. *Journal of Neuroinflammation*, *5*(1), 13–13. https://doi.org/10.1186/1742-2094-5-13

Nishijima, T., Kawakami, M., & Kita, I. (2015). A bout of treadmill exercise increases matrix metalloproteinase-9 activity in the rat hippocampus. Neuroscience Letters, 594, 144–149. https://doi.org/10.1016/j.neulet.2015.03.063

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Ragozzino, D., & Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. Science, 333(6048), 1456– 1458. https://doi.org/10.1126/science.1202529

Pedersen, B. K., & Saltin, B. (2015). Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. Scandinavian journal of medicine & science in sports, 25 Suppl 3, 1–72. https://doi.org/10.1111/sms.12581

Pereira, A. C., Huddleston, D. E., Brickman, A. M., Sosunov, A. A., Hen, R., McKhann, G. M., Sloan, R., Gage, F. H., Brown, T. R., & Small, S. A. (2007). in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. *Proceedings of the National Academy of Sciences - PNAS*, *104*(13), 5638–5643. https://doi.org/10.1073/pnas.0611721104

Popović, N., Madrid, J. A., Rol, M. A., Caballero-Bleda, M., & Popović, M. (2010). Barnes maze performance of Octodon degus is gender dependent. *Behavioural brain research*, *212*(2), 159–167. https://doi.org/10.1016/j.bbr.2010.04.005

Prior, B. M., Yang, H. T., & Terjung, R. L. (2004). What makes vessels grow with exercise training? Journal of Applied Physiology, 97(3), 1119–1128. https://doi.org/10.1152/japplphysiol.00035.2004

Romberg, C., Yang, S., Melani, R., Andrews, M. R., Horner, A. E., Spillantini, M. G., Bussey, T. J., Fawcett, J. W., Pizzorusso, T., & Saksida, L. M. (2013). Depletion of perineuronal nets enhances recognition memory and long-term depression in the perirhinal cortex. The Journal of Neuroscience, 33(16), 7057–7065. https://doi.org/10.1523/JNEUROSCI.6267-11.2013

Safari, S., Ahmadi, N., Mohammadkhani, R., Ghahremani, R., Khajvand-Abedeni, M., Shahidi, S., Komaki, A., Salehi, I., & Karimi, S. A. (2021). Sex differences in spatial learning and memory and hippocampal long-term potentiation at perforant pathway-dentate gyrus (PP-DG) synapses in Wistar rats. *Behavioral and brain functions : BBF*, *17*(1), 9. https://doi.org/10.1186/s12993-021-00184-y

Saucier, D., Lisoway, A., Green, S., & Elias, L. (2007). Female advantage for object location memory in peripersonal but not extrapersonal Space. Journal of the International Neuropsychological Society, 13(4), 683–686. https://doi.org/10.1017/S1355617707070865

Shi, W., Wei, X., Wang, X., Du, S., Liu, W., Song, J., & Wang, Y. (2019). Perineuronal nets protect long-term memory by limiting activity-dependent inhibition from parvalbumin interneurons. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(52), 27063–27073. https://doi.org/10.1073/pnas.1902680116

Sierra, A., Encinas, J. M., Deudero, J. J. ., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., Tsirka, S. E., & Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Stem Cell*, *7*(4), 483–495. https://doi.org/10.1016/j.stem.2010.08.014

Smith, C. C., Mauricio, R., Nobre, L., Marsh, B., Wüst, R. C., Rossiter, H. B., & Ichiyama, R. M. (2015). Differential regulation of perineuronal nets in the brain and spinal cord with exercise training. Brain Research Bulletin, 111, 20–26. https://doi.org/10.1016/j.brainresbull.2014.12.005

Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J., & Lewis, C. E. (2004). Current methods for assaying angiogenesis in vitro and in vivo. International journal of experimental pathology, 85(5), 233–248. https://doi.org/10.1111/j.0959-9673.2004.00396.x

Sung, Y.-H., Kim, S.-C., Hong, H.-P., Park, C.-Y., Shin, M.-S., Kim, C.-J., Seo, J.-H., Kim, D.-Y., Kim, D.-J., & Cho, H.-J. (2012). Treadmill exercise ameliorates dsilopaminergic neuronal loss through suppressing microglial activation in Parkinson's disease mice. *Life Sciences*, *91*(25-26), 1309–1316. https://doi.org/10.1016/j.lfs.2012.10.003

Tapia-Rojas, C., Aranguiz, F., Varela-Nallar, L., & Inestrosa, N. C. (2016). Voluntary Running Attenuates Memory Loss, Decreases Neuropathological Changes and Induces Neurogenesis in a Mouse Model of Alzheimer's Disease. Brain Pathology (Zurich, Switzerland), 26(1), 62–74. https://doi.org/10.1111/bpa.12255

van Praag, H., Christie, B. R., Sejnowski, T. J., & Gage, F. H. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. Proceedings of the National Academy of Sciences, 96(23), 13427–13431. https://doi.org/10.1073/pnas.96.23.13427

van Praag, H., Shubert, T., Zhao, C., & Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. The Journal of Neuroscience, 25(38), 8680–8685. https://doi.org/10.1523/JNEUROSCI.1731-05.2005

van der Borght, K., Havekes, R., Bos, T., Eggen, B. J., & van der Zee, E. A. (2007). Exercise improves memory acquisition and retrieval in the Y-maze task: Relationship with hippocampal neurogenesis. Behavioral Neuroscience, 121(2), 324–334. https://doi.org/10.1037/0735-7044.121.2.324

Venturino, A., Schulz, R., De Jesús-Cortés, H., Maes, M. E., Nagy, B., Reilly-Andújar, F., Colombo, G., Cubero, R. J. A., Schoot Uiterkamp, F. E., Bear, M. F., & Siegert, S. (2021). Microglia enable mature perineuronal nets disassembly upon anesthetic ketamine exposure or 60-Hz light entrainment in the healthy brain. Cell Reports, 36(1), 109313–109313. https://doi.org/10.1016/j.celrep.2021.109313

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents. ILAR journal, 55(2), 310–332. https://doi.org/10.1093/ilar/ilu013

Vukovic, J., Colditz, M. J., Blackmore, D. G., Ruitenberg, M. J., & Bartlett, P. F. (2012). Microglia modulate hippocampal neural precursor activity in response to exercise and aging. *The Journal of Neuroscience*, *32*(19), 6435–6443. https://doi.org/10.1523/JNEUROSCI.5925-11.2012

Wadhwa, M., Prabhakar, A., Ray, K., Roy, K., Kumari, P., Jha, P. K., Kishore, K., Kumar, S., & Panjwani, U. (2017). Inhibiting the microglia activation improves the spatial memory and adult neurogenesis in rat hippocampus during 48 h of sleep deprivation. Journal of Neuroinflammation, 14(1), 222. https://doi.org/10.1186/s12974-017-0998-z

Wang, M., Zhang, H., Liang, J., Huang, J., & Chen, N. (2023). Exercise suppresses neuroinflammation for alleviating Alzheimer's disease. Journal of neuroinflammation, 20(1), 76. https://doi.org/10.1186/s12974-023-02753-6

Wegrzyn, D., Freund, N., Faissner, A., & Juckel, G. (2021). Poly I:C activated microglia disrupt perineuronal nets and modulate synaptic balance in primary hippocampal neurons in vitro. *Frontiers in Synaptic Neuroscience*, *13*, 637549–637549. https://doi.org/10.3389/fnsyn.2021.637549

Xiao, Z., Taylor, J., Montag, D., Rougon, G., & Schachner, M. (1996). Distinct effects of recombinant tenascin-R domains in neuronal cell functions and identification of the domain interacting with the neuronal recognition molecule F3/11. The European Journal of Neuroscience, 8(4), 766–782. https://doi.org/10.1111/j.1460-9568.1996.tb01262.x

Xiong, J. Y., Li, S. C., Sun, Y. X., Zhang, X. S., Dong, Z. Z., Zhong, P., & Sun, X. R. (2015). Long-term treadmill exercise improves spatial memory of male APPswe/PS1dE9 mice by regulation of BDNF expression and microglia activation. *Biology of Sport*, *32*(4), 295–300. https://doi.org/10.5604/20831862.1163692

Yuan, L., Kong, F., Luo, Y., Zeng, S., Lan, J., & You, X. (2019). Gender Differences in Large-Scale and Small-Scale Spatial Ability: A Systematic Review Based on Behavioral and Neuroimaging Research. *Frontiers in Behavioral Neuroscience*, *13*, 128–128. https://doi.org/10.3389/fnbeh.2019.00128

Zhang, S., Wang, X.-J., Tian, L.-P., Pan, J., Lu, G.-Q., Zhang, Y.-J., Ding, J. Q., & Chen, S.-D. (2011). CD200-CD200R dysfunction exacerbates microglial activation and dopaminergic neurodegeneration in a rat model of Parkinson's disease. *Journal of Neuroinflammation*, *8*(1), 154–154. https://doi.org/10.1186/1742-2094-8-154

Zhang, J., Guo, Y., Wang, Y., Song, L., Zhang, R., & Du, Y. (2018). Long-term treadmill exercise attenuates Aβ burdens and astrocyte activation in APP/PS1 mouse model of Alzheimer's disease. Neuroscience Letters, 666, 70–77. https://doi.org/10.1016/j.neulet.2017.12.025

Zhou, X., Spittau, B., & Krieglstein, K. (2012). TGF beta signalling plays an important role in IL4-induced alternative activation of microglia. *Journal of Neuroinflammation*, *9*(1), 210–210. https://doi.org/10.1186/1742-2094-9-210

**Figure 1**

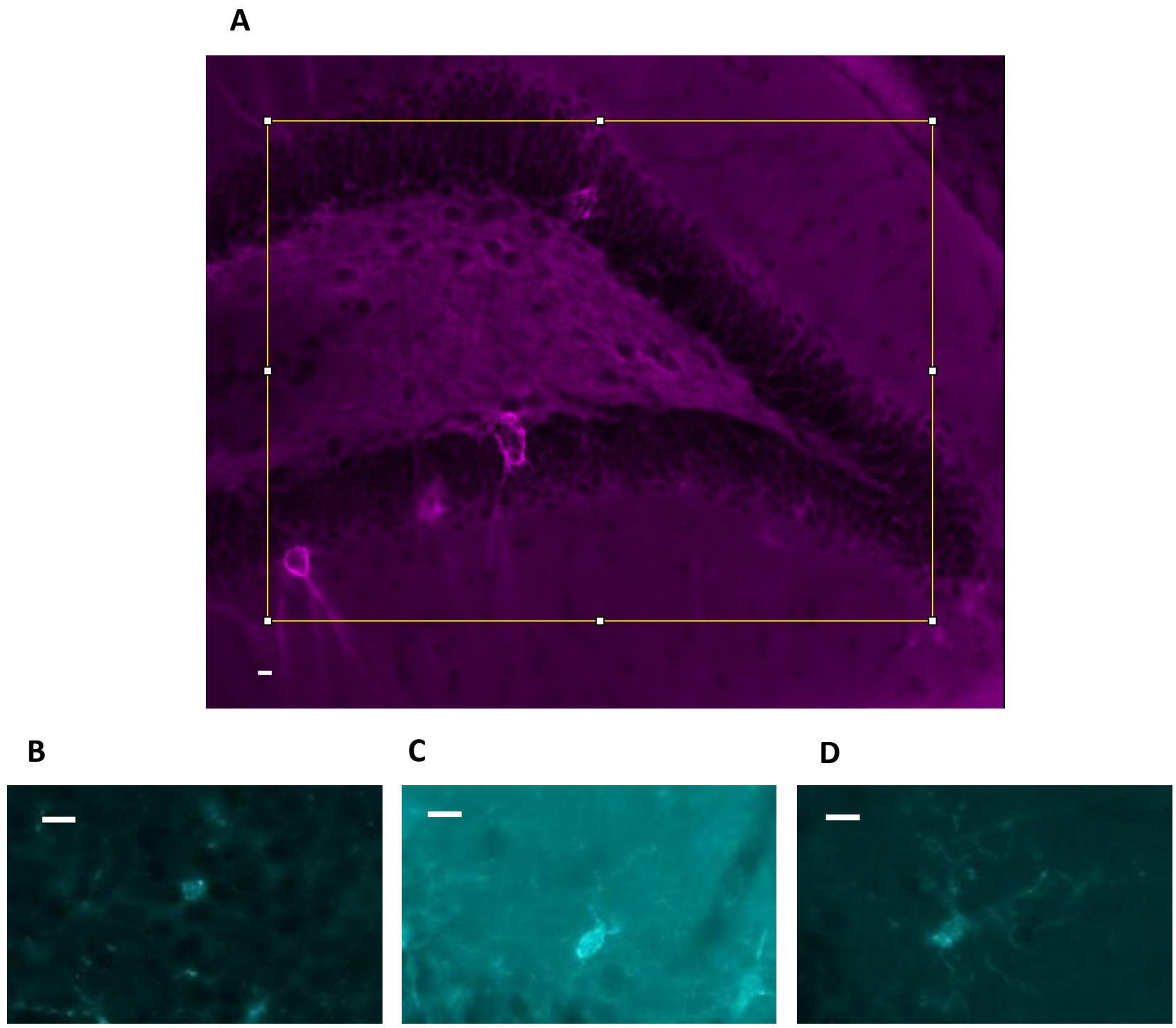


**Fig 1. Region of interest placement in the dentate gyrus (A) and representative images of different microglia morphology (B-D).** (B) Ameboid microglia cell, (C) Stout/thick microglia cell, (D) Ramified/thin microglia cell. Scale bar represents 10 um.

**Figure 2**

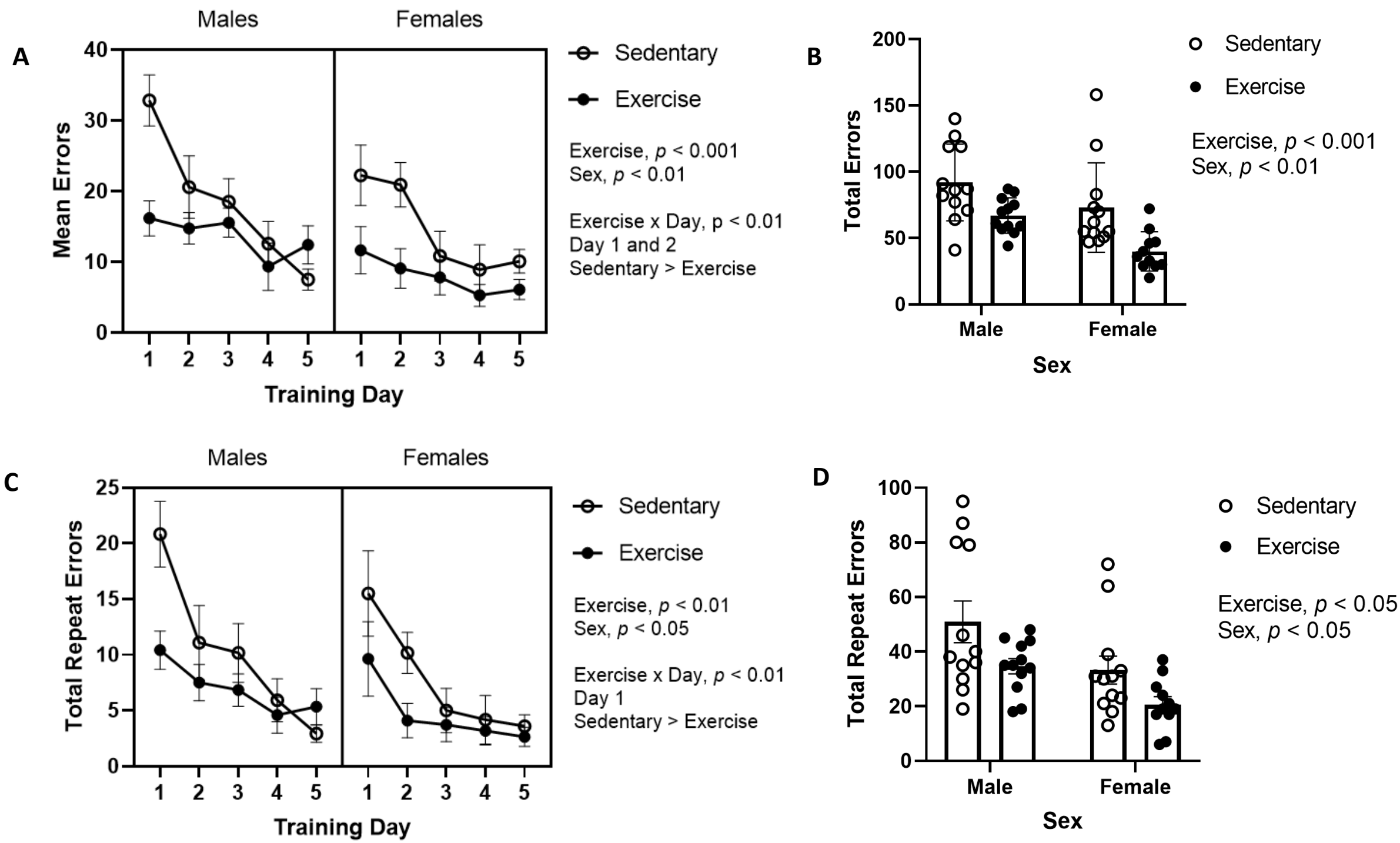


**Fig 2. Effects of exercise on spatial learning in female and male mice in the Barnes maze.** (A) the number of errors by training day, (B) the total number of errors, (C) the total repeated errors by training day, (D) the sum of the total repeat errors. Error bars represent SEM.

**Figure 3**

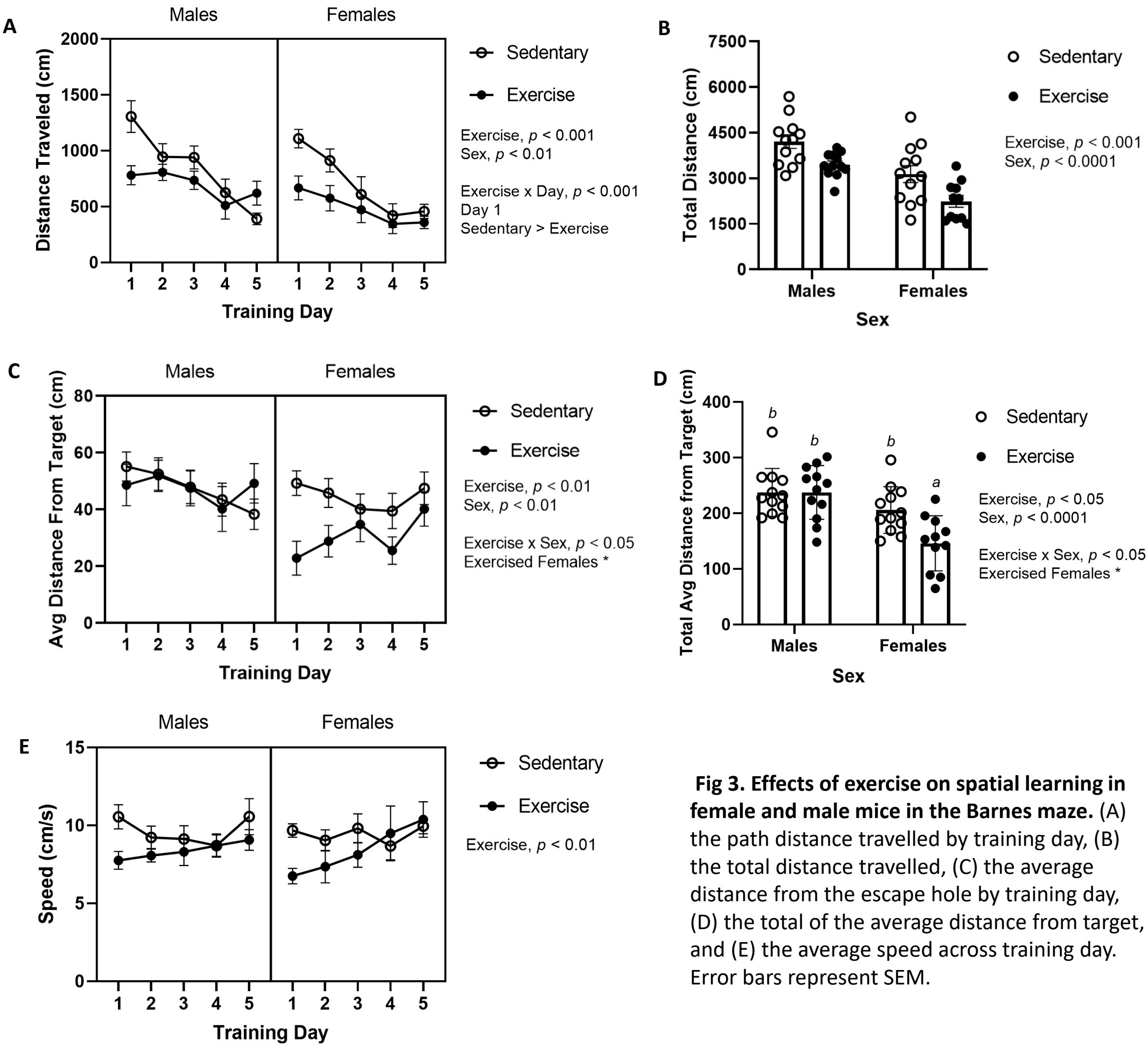


**Fig 3. Effects of exercise on spatial learning in female and male mice in the Barnes maze.** (A) the path distance travelled by training day, (B) the total distance travelled, (C) the average distance from the escape hole by training day, (D) the total of the average distance from target, and (E) the average speed across training day. Error bars represent SEM.

**Figure 4**

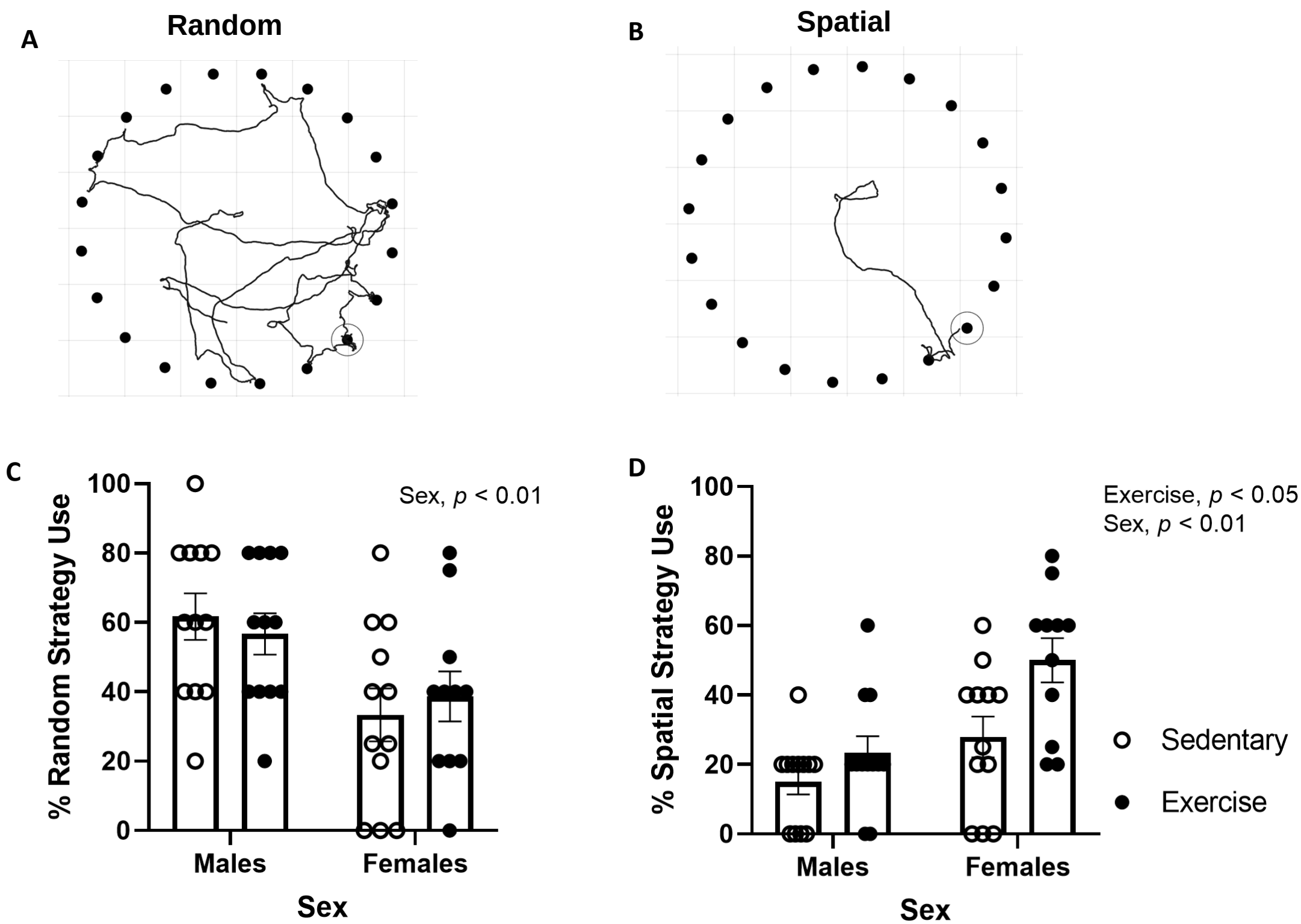


**Figure 4. Effect of exercise training on strategy use in the Barnes maze in female and male mice.** (A) example diagram illustrating a random search strategy, (B) example diagram illustrating a spatial search strategy, (C) percentage of sedentary and exercised mice employing a random strategy, (D) percentage of sedentary and exercised mice employing a spatial strategy. Error bars represent SEM.

**Figure 5**

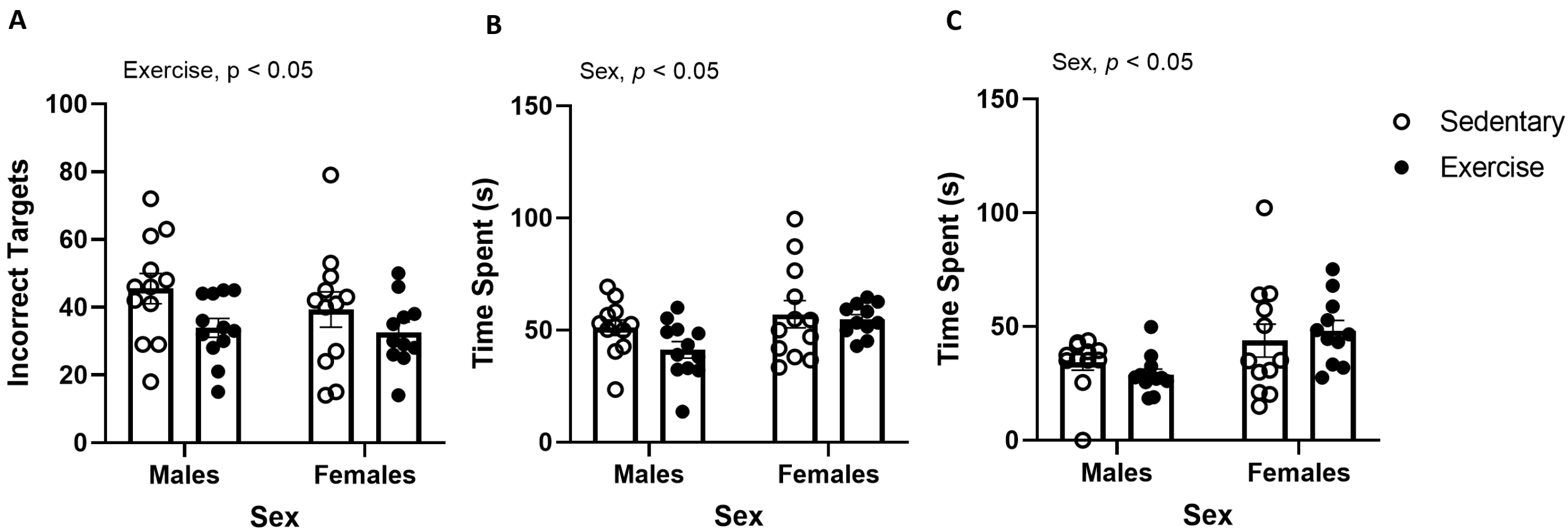


**Fig 5. Effects of exercise on spatial memory performance in female and male mice during the probe session of the Barnes maze.** (A) number of incorrect targets visited, (B) time spent in target quadrant, (C) time spent near the escape hole. Error bars represent SEM.

Figure 6

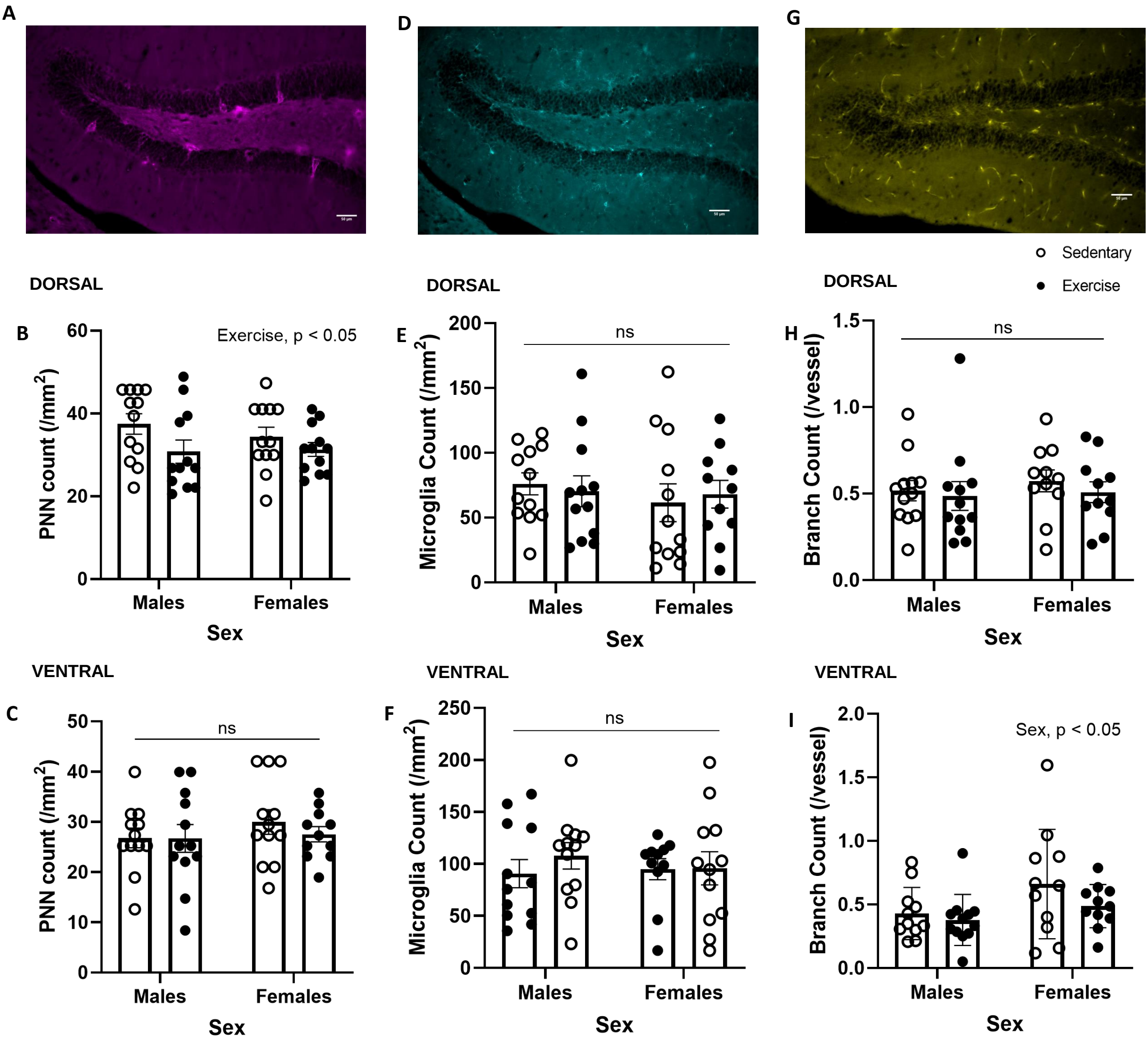


**Figure 6. Effect of exercise training on PNN, microglia, and vascular branch counts in female and male mice.** (A) PNN count in the (B) dorsal and (C) ventral dentate gyrus, (D) microglia count in the (E) dorsal and (F) ventral dentate gyrus, (G) vascular branch count in the (H) dorsal and (I) ventral dentate gyrus. Error bars represent SEM.

**Figure 7**

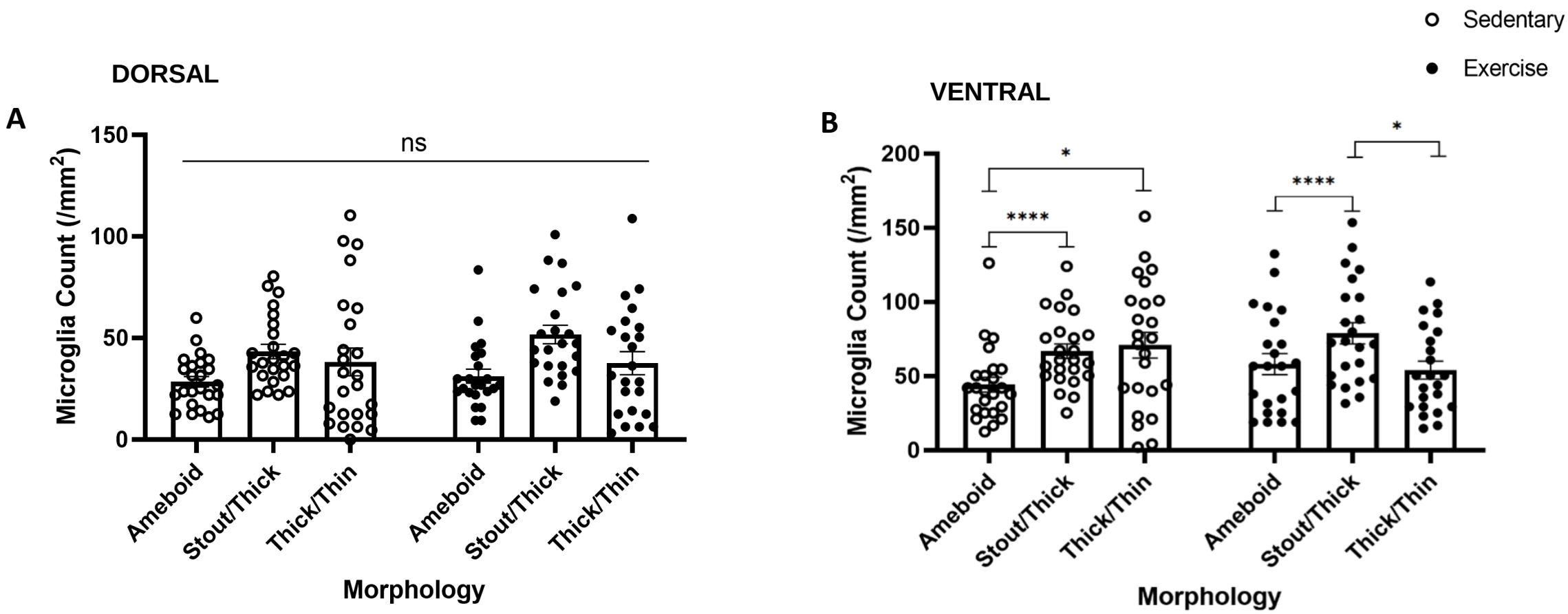


**Figure 7. Effect of exercise training on microglia morphology in the dentate gyrus.** (A) morphology in the dorsal and (B) ventral dentate gyrus. Error bars represent SEM. See Figure 1B-D for morphology definitions.

Figure 8

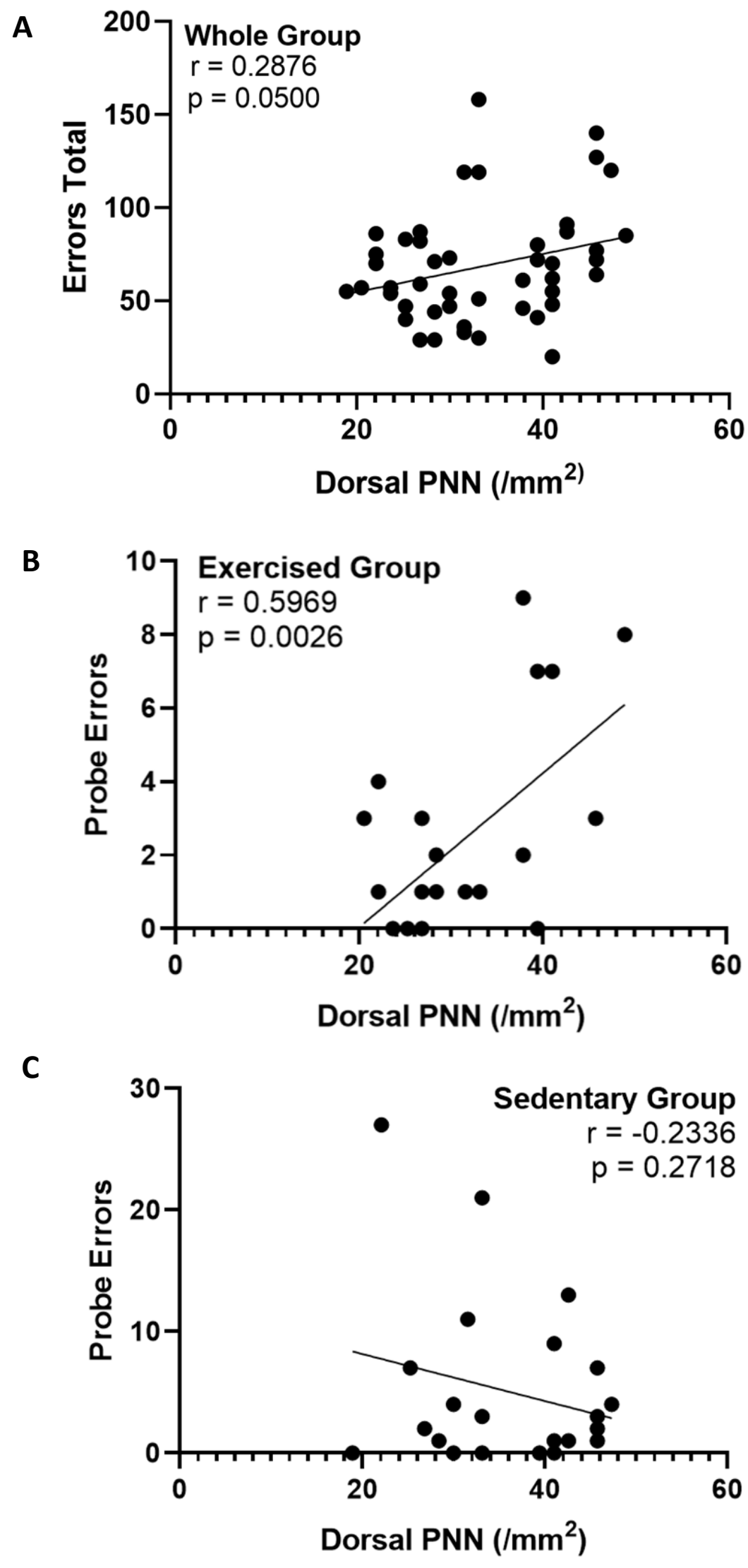


**Figure 8. Correlations between PNN expression and errors during learning and probe**. (A) correlation between dorsal PNNs and total errors, (B) dorsal PNNs and probe errors among the exercised group, (C) PNNs and probe errors among the sedentary group.