Age-Specific Effects of Voluntary Exercise on Memory and the Older Brain

Supplemental Information

Supplemental Methods and Materials

Design

Two experiments were conducted in this study. The first experiment compared the performances of younger (n = 10) and older (n = 10) rats in object and place recognition tasks and in a water maze task. The second experiment used these tasks to assess the effects of exercise (wheel running) in younger and aged rats. In this experiment, younger (n = 20) and older (n = 18) rats were each randomly assigned to a 12-week voluntary running or standard housing condition, tested at baseline and after a first (6 weeks post baseline) and second (8 weeks post baseline) follow-up period.

Subjects

None of the aged animals developed any signs of tumors or any other observable pathology. Aged animals had good body mass (averaging 300 g) and ample subcutaneous and abdominal fat (observed during perfusion). Upon arrival, animals were housed and handled daily for 5 days prior to experimentation. Young animals also had good body mass (averaging 200 g at the initial stage of experimentation and averaging 300 g at the end of experimentation). The ages of young and aged animals at the end of the experiments were 5 months and 24 months respectively. Rats were housed in groups of four in plastic boxes (52 cm length x 35 cm width x 18 cm height) on woodchip bedding, and kept in an air-conditioned colony room (21^{o}C) under a 12-h light:12-h dark cycle (lights on from 07:00 to 19:00). Food and water were continuously

available in the home cage during all phases of the experiments. All experimental procedures were carried out between 7:00 AM and 5:30 PM.

Apparatus

The water maze consisted of a circular pool (150 cm wide and 43 cm tall) constructed from fiberglass and filled with water to a depth of 23 cm. The water in the pool was rendered opaque by the addition of 500 g of non-toxic white Tempera Power Paint (Educational Colors, Australia), and its temperature was maintained at 24 ± 1^{o} C. A circular escape platform (18 cm wide) was submerged 1.5 cm below water level. Four points around the pool circumference were designated north, south, east and west (N, S, E, W), and the pool area was divided into four quadrants (NW, NE, SW, SE). The pool was surrounded by black curtains. Three external cues (a cross, circle, and triangle) were located at the side of the pool. Each cue was made of plastic and painted white. A cross (height and width each 23 cm) was located at the SSW, a wheel (rim 0.5 cm and diameter 23 cm) at WNW, and a equilateral triangle (rim 0.5 cm and side 23 cm) at ENE. A camera (Panasonic, WVBL200) was mounted on the ceiling 164 cm above the middle of the pool. The camera was connected to a DVD recorder in an adjacent room. The pool was illuminated by four light sources (60 watts) on the ceiling with an aluminum shield mounted below to diffuse the light.

Object and place recognition memory tests were conducted in an open field arena (60 cm length x 60 cm width x 50 cm height) constructed from black PVC plastic. Commercially produced objects (e.g., lunch boxes, coke cans, soap dispensers, water bottles), made of aluminum, glass and plastic, and varying in height (11-13 cm), width (6.5-9 cm) and shape (circular, cylindrical, rectangular), were weighted with water or sand to prevent displacement by

rats. All items and locations of objects were counterbalanced between rats. A camera (Panasonic, WVBL200) was positioned centrally 164 cm above the arena and connected to a DVD recorder in an adjacent room.

Running wheels were made out of plastic with a solid back running surface, measuring 11 inches in diameter and 3.5 inches deep (Wodent Wheels, Oregon), and had an attached counter to record revolutions on a weekly basis.

Behavioral Procedures

Experiment 1

Spatial Learning. The protocol used for training and testing in the Morris water maze was based on previous studies (1, 2). *Day 0.* The escape platform was positioned on the center of the pool. Familiarization began by placing the rat on the submerged platform for 30 seconds. Then the rat was removed from the platform and released from the pool edge with its head facing the platform. The rat was guided towards the platform if it had not reached the platform in 60 seconds, or was placed onto the platform after 90 seconds for 10 seconds. This procedure was repeated for two trials with a 5 minute inter-trial period. *Days 1-3*. The escape platform was positioned at the center of the NW quadrant. Each rat received four trials per day over three consecutive days with an inter-trial interval of 5 minutes. Each trial involved the release of the rat from one of four fixed points (N, S, E, W). The starting positions were assigned in random order, to prevent the use of a *praxis* strategy (using a learned sequence of movements), rather than a spatial mapping strategy (2). The time taken to reach the platform was measured, and data from the four daily trials were pooled to determine the daily average. If the rat did not locate the submerged platform in a maximum of 90 seconds, it was placed onto the platform for 10 s. *Day*

5. The platform was removed for testing. Each rat was released from a starting position different to training and allowed to swim freely for 90 seconds. The time spent in the location of the platform, time in quadrant, time to first platform crossing, number of platform and annulus crossings and velocity were scored using EthoVision (Noldus Information Technology, XT 5.1 version, The Netherlands).

Localized Cue Task. Immediately following the Probe trial, rats were given a localized cue test. The submerged platform was moved to the NE quadrant, and signposted by its attachment to a floating buoy (12 cm long, 5 cm wide) with black and white horizontal stripes. The rat was first placed onto the platform for 90 seconds, so that it associated the platform location with the buoy. The rat was then given two familiarization trials (described previously) followed by four test trials. The rat was allowed a maximum of 90 seconds to locate the buoy-marked platform and a 10 second rest period once on the platform. There was a 5 minute interval between trials. Escape latency, velocity and path length was measured.

Object versus Place Recognition Memory. The animals were trained on the Place and Object recognition memory tasks (3, 4). On Days 6-8, rats were familiarized to the arena across daily 10-minute sessions, allowing analysis of spontaneous open field behavior. The arena was cleaned with 70% ethanol after each rat was removed. On Day 9, half the rats received the Object trials and the other half Place trials, and vice versa on Day 10. In the Object trials, each rat was placed in the center of the arena with two identical sample objects for 5 minutes, removed and returned to a cage outside the room for 5 minutes. During this time, the objects were replaced with two new objects, one identical to the sample and the other a novel object. The rat was then returned to the arena for 3 minutes in the retention test. The Place trials followed the same procedure, except that one of the objects was moved to a new location in the retention test

while the other remained in its original location. The proportion of time spent exploring the novel object or place compared with the familiar was measured during the retention test and the exploration ratio, i.e., $time_{novel}/(time_{novel}+time_{familiar})$ was calculated. Exploration was counted if the rat's head was within 4 cm of the object and oriented within 45^{o}, with neck extended and vibrissae moving. Rearing with head oriented upward was also included if at least one forepaw was on the object. Exploration was not counted on the basis of simple proximity, chewing, climbing, sitting or standing on the object. Two observers, one of whom was unaware of the rat's treatment condition, scored the trials of each rat. The agreement between these (i.e., the correlation between the time spent exploring the objects) scores was consistently high, as assessed by Pearson's correlation, $r > 0.9$.

All animals were then sacrificed after behavioral testing on Day 10.

Experiment 2

All animals underwent the same behavioral protocols as described in Experiment 1. However, following the last trial on Day 10, half the rats were provided with running wheels in their home cages (two wheels per cage) while the other half did not have access to a wheel. All animals additionally received two follow up tests. The first follow-up occurred six weeks later, on Day 52, and the second follow-up occurred two weeks after this, on Day 76. The follow-up tests used the same behavioral protocol as previously described. A new set of objects and locations were used for each follow-up test. All items and positions were counterbalanced. At the completion of the behavioral tests on Day 76, rats were sacrificed for neural analyses. Specifically, rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 100 ml of 0.9% saline, containing 1% sodium nitrate and heparin (500 i.u./ml), followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4.

Immunohistochemistry

Synaptophysin

One set of section series was used to assess synaptophysin density. Free floating sections were washed repeatedly in 0.1 M PB, pH 7.4, followed by a 30 min wash in sodium borohydryde at room temperature and 30 min in citrate buffer, pH 6 (Sodium citrate, 10 mM) at 80°C. Sections were incubated in 3% bovine serum albumin (BSA) and 0.25% Triton X-100 in PB, pH 7.4, for 30 mins at 80°C. Sections were incubated in mouse anti-synaptophysin (1:1000; Millipore). This primary antibody was diluted in 0.1 M PB, pH 7.4, containing 3% BSA and 0.25% Triton X-100, and incubations were for 30 mins at 37°C. After washing off unbound primary antibodies, sections were incubated for 30 mins at 37°C in donkey anti-mouse IgG (1:500; Invitrogen) diluted in 0.1 M PB, pH 7.4, containing 3% BSA and 0.25% Triton X-100 (PBT-X).

Doublecortin (DCX) plus Ki67

The next series of sections was used to assess co-expression of Ki67 and DCX. Free floating sections were washed repeatedly in 0.1 M PB, pH 7.4, followed by a 30 min wash in citrate buffer, pH 6 (Sodium citrate, 10 mM) at 80°C. Sections were washed repeatedly in 0.1 M PBS, pH 7.2, followed by a 1 h incubation in PBS, pH 7.2, containing 2% NDS and 0.3% Triton X-100. Sections were then incubated for 24 h at room temperature in rabbit anti-proliferating marker (1:500; Ki67; Abcam) diluted in 0.1 M PBS, pH 7.2, 2% NDS and 0.3% Triton X-100. Sections were repeatedly washed and followed by 2 h incubation at room temperature in donkey anti-rabbit IgG (1:500; Invitrogen) diluted in 0.1 M PB, pH 7.4, containing 2% NDS and 0.3% Triton X-100. Sections were then repeatedly washed and underwent incubation with mouse anti-doublecortin (1:1000; DCX; Millipore) for 24 h at room temperature, followed by a 2 h

incubation at room temperature in donkey anti-mouse IgG (1:500; Invitrogen) diluted in 0.1 M PB, pH 7.4, containing 2% NDS and 0.3% Triton X-100.

Cell Quantification

Presynaptic Density

Separate images of fluorescence synaptophysin staining were captured from nine subregions including the inner, middle and outer portions of the dentate gyrus molecular layer, dentate gyrus polymorph layer, stratum lacunosum moleculare of CA3 and CA2, and stratum radiatum of CA1 of the hippocampus, and the entorhinal cortex at 40x power using an Olympus BX51 microscope and DP72 camera. A 1 in 6 series was used to analyze the inner, middle and outer layers of the molecular layer. All images were acquired in the one session and used identical lighting and scanning parameters. Calculation of optical density was with ImageJ software. Briefly, this involved defining standardized sampling areas of interest within each image, conversion of the image to grayscale, and calculation of the average pixel intensity across these areas for each image. This process was repeated for each hippocampal subregion and intensity measurements from the corpus callous of each animal provided control background levels which were subtracted from regional intensity values. Each animal therefore provided a synaptophysin intensity value in each of the nine regions which were the result of averaging over multiple sampling regions across 18-20 sections. All counts were averaged across both brain hemispheres.

Neurogenesis

Stereological evaluation of non-overlapping cells was conducted throughout the rostro-caudal extent of the hippocampus (between bregma -2.04 mm and bregma -2.92) on sections 200

μm apart by an observer unaware of group allocations. Counting of co-labelled Dapi+Ki67+DCX-positive cells was restricted to the subgranular zone of the dentate gyrus and used a 200 x 200 μm random sampling frame. Positive cells for counting purposes required a complete halo of cytosolic *DCX* fluorescence around a co-labeled Dapi+Ki67 nucleus.

Graphical Network Analysis

Clustering Coefficient

Clustering coefficient measures how interconnected particular nodes are within a network, and is calculated by the ratio of the number of edges between direct neighbors of a node in comparison to the number of all possible edges (1). Formally, clustering coefficient: $C_i = \frac{N_{G_i}}{K_i(K_i-1)/2}$; where G_i is the subgraph which consists of all the direct neighbors of *i*th node, and N_{G_i} is the number of edges in the subgraph G_i. The clustering coefficient for the whole network C_p is then defined as the average clustering coefficient over all nodes in a network: $C_p = \frac{1}{N}\sum_{i \in G} C_i$. Clustering coefficient is hence an index of local structure (2, 5).

Shortest Path Length

The shortest path length (d_{ij}) describes the minimum number of edges that must be traversed to go from one node to another. Formally, $d_{ij} = \sum_{a_{uv} \in P} a_{uv}$; where P is the shortest path length between node i and j.

Global Network Efficiency

Global efficiency represents the ability of a network to transmit information at the global level. For example, a network where each and every node is connected to all other nodes is

representative of maximal global network efficiency, given only one edge needs be traversed to go from any particular node to another. Formally, global efficiency is the inverse of the harmonic mean of the minimum absolute path length between each pair of nodes (6-8), expressed as $E_{global} = \frac{1}{N(N-1)} \sum_{j \in G} \sum_{i \in G, j \neq i} \frac{1}{d_{ij}}$.

Supplemental Results

Experiment 1

Exploration differences in object and place recognition memory in young and aged animals

Aged rats (n = 10) spent significantly less time exploring both object and place objects during object familiarization (i.e., A1 and A2 objects) (ps < 0.05) (Figure 1). Casual observation suggested that aged rats moved slower than younger rats, which may have contributed to the amount of time spent exploring A1 and A2 objects. However, as the exploration time is computed as a ratio (as indexed by the time spent exploring the novel change or novel object over the entire time spent exploring), the amount of time spent exploring A1 and A2 should not affect the amount of time spent exploring the novel object B at test.

Aged rats are impaired at the localized cue task

Aged rats reached the signposted platform slower compared to young rats in the localized cue task. After controlling for individual differences in their performance in an analysis of covariance, there no longer remained any significant age differences on the probe trial on the spatial learning task in time to platform, number of platform crossings, time spent in platform zone or time in target quadrant (ps > 0.05) (Figure S1).

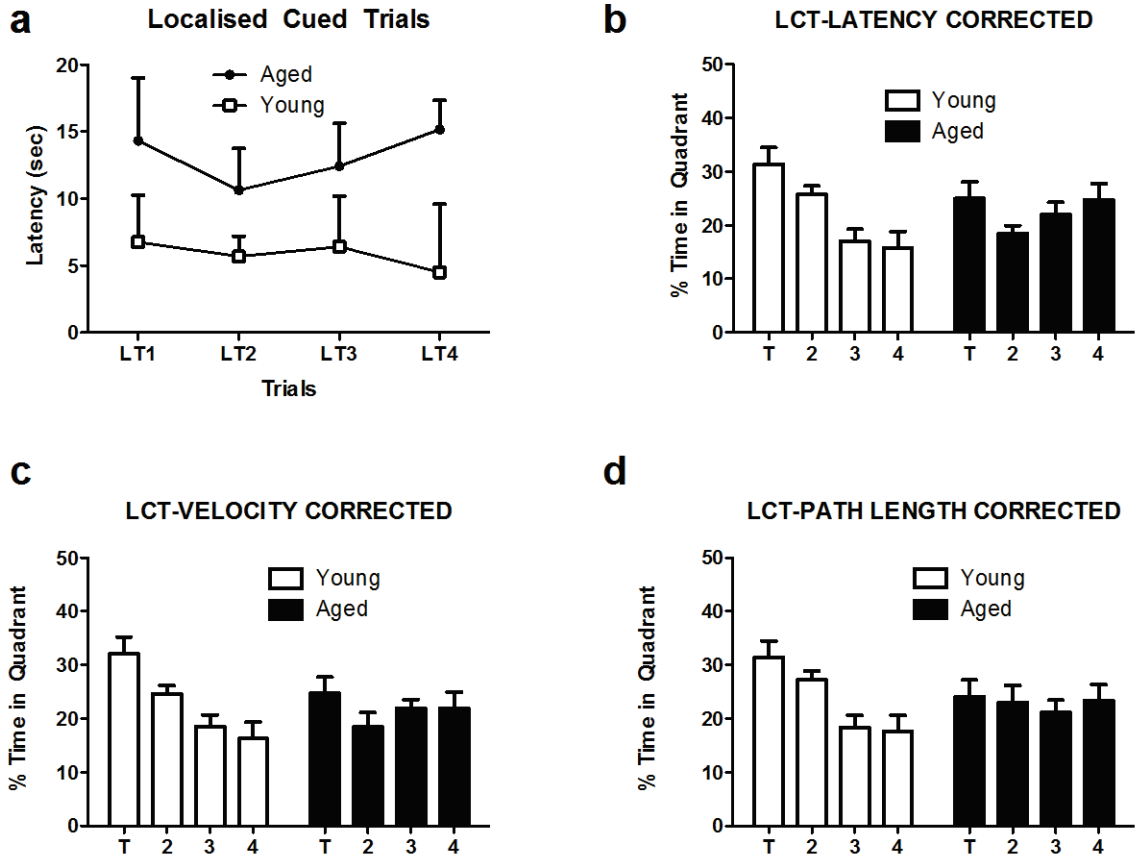

Figure S1. Performance in the hippocampal-independent spatial version of the Morris water maze localized cued task (LCT). (**a**) Individual trials of the localized cue task show no learning curve across time for both young and aged animals. (**b**) Average percentage of time within target (T) and non-target (2-3) quadrants on the delayed probe test in the absence of the platform. After correcting for latency (**b**) or velocity (**c**) or path length (**d**) on the LCT, these differences were eliminated (estimated marginal means).

Experiment 2

Stability of object and place recognition memory

Place and object recognition memory performance was stable over multiple testing sessions in younger and older sedentary rats. In younger rats, within-animal, test-retest reliability over eight weeks produced a coefficient of variation (CV) of 0.15 for place memory and 0.17 for object memory. In older rats, place memory CV was 0.38 and 0.22 for object memory. These CV values were low, indicative of a high level of repeatability for both recognition memory tests over time. However, a general feature of ageing in behavioral studies is an increase in variability, a pattern seen in our data.

Exercise does not affect exploration time in young and aged rats

Exercise did not affect the amount of exploration time at familiarization (A1, A2) or testing (A3) stages in either age groups (*ps* > 0.05) (Figures S2 and S3). However, at first and second follow-up testing, aged exercising rats explored the novel location (B) more than aged sedentary rats (p = 0.02, p = 0.03 respectively) (Figure S2). There were no statistically significant differences between the exploration ratios of younger exercising and sedentary rats (*ps* > 0.05).

All animals exercise consistently

Both groups ran consistently throughout experimentation. Both young and aged rats ran in the wheels with younger rats covering significantly more distance than aged rats when averaged across time ($F_{(1,8)}$ = 79.557, p = 0.0001) (Figure S4A). However, at the end of exercise, there were no significant differences between the two groups (Figure S4B).

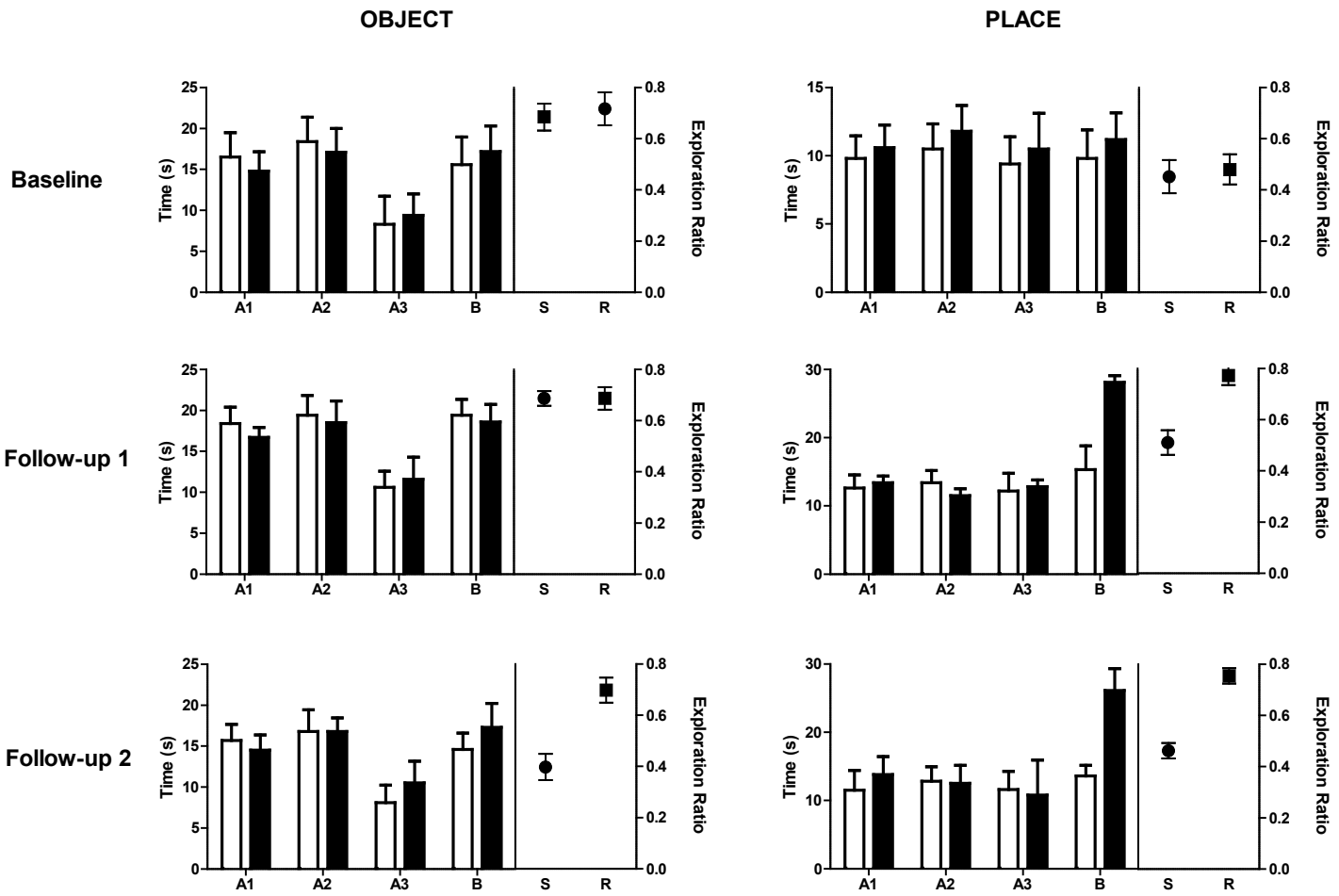

Figure S2. Running rescues place recognition memory performance in aged animals only. Across baseline, follow-up 1 and follow-up 2 testing, there were no differences between exercising and non-exercising groups in the object recognition memory task. However, at first and second follow-up testing, aged exercising rats (R) spent significantly more time exploring the new object location (B) at test than aged sedentary rats (S). This is reflected in a higher exploration ratio. Exercising aged rats did not differ in the amount of time spent exploring the the familiar object or place (A3).

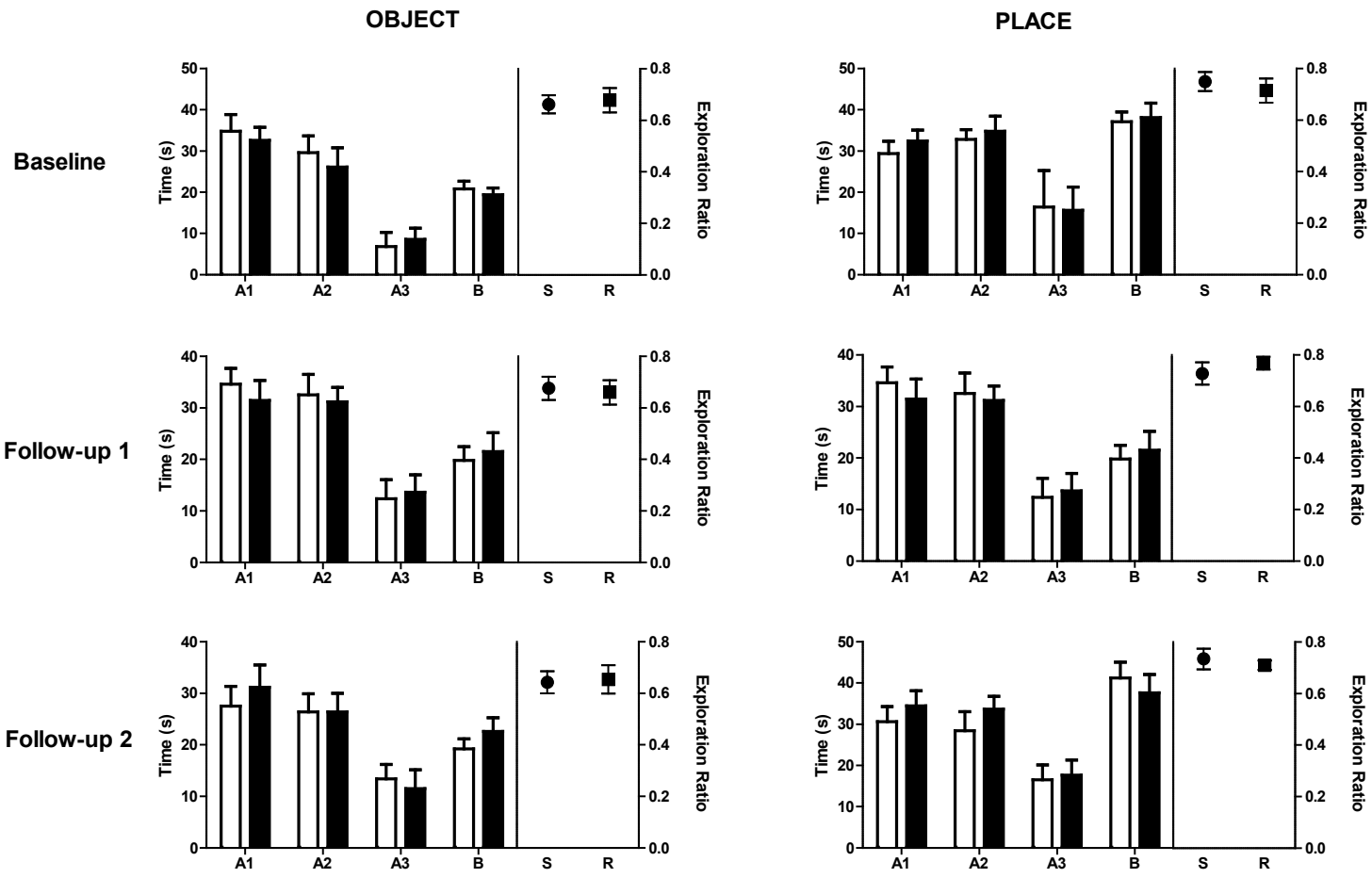

Figure S3. Running has no effect on object or place recognition memory in young animals. Across all stages of testing, there were no significant differences between exercising (R) and non-exercising (S) younger rats in either recognition memory paradigm.

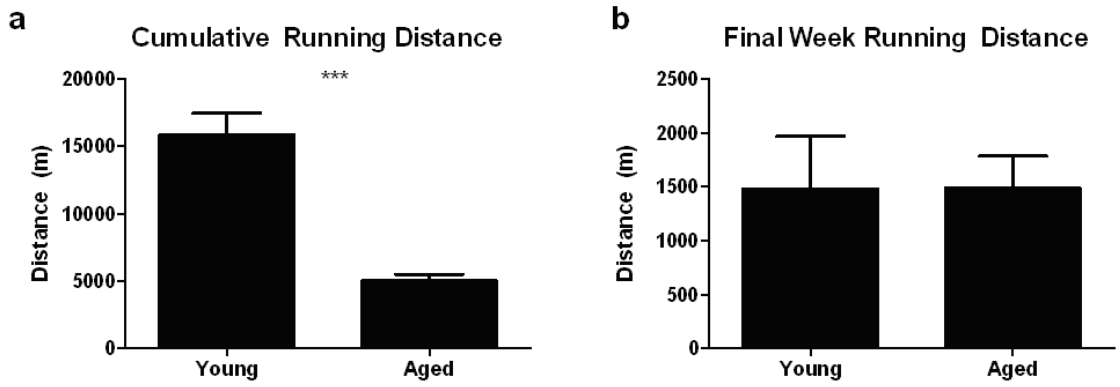

Figure S4. Running distance across experimentation in young and aged rats. Both age groups exercise consistently with young animals running more than aged animals overall. However in the final week of measurement, both young and age groups were exercising equally. *** $p < 0.001$.

Spatial learning and reference memory is not affected by exercise in aged animals

Running improved performance in the Morris maze task but this improvement could not be unequivocally attributed to changes in spatial learning and reference memory (Figure S5). At first follow-up, both young and aged animals learned to locate the hidden platform in the Morris water maze across training, as indicated by a significant linear trend across trials ($F_{1,18} = 14.901$, $p = 0.001$; $F_{1,18} = 17.496$, $p = 0.001$ respectively) (Figure S5A,E). Exercise significantly decreased the latencies to locate the hidden platform in the younger ($F_{1,18} = 5.441$, $p = 0.031$) but not in the older rats ($F_{1,18} = 0.354$, $p = 0.559$) throughout the training trials (Figure S5A,E). At test, there were no statistically significant differences between non-runners and runners in either younger or older rats in: time spent in target quadrant ($F_{1,18} = 1.707$, $p = 0.208$; $F_{1,18} = 1.55$, $p = 0.229$, respectively), number of annulus crossings ($F_{1,18} = 0.085$, $p = 0.774$; $F_{1,18} = 1.692$, $p = 0.21$, respectively), path length ($F_{1,18} = 0.449$, $p = 0.511$; $F_{1,18} = 0.06$, $p = 0.809$, respectively)

and time to first annulus crossing ($F_{1,18}$ = 0.245, p = 0.627; $F_{1,18}$ = 1.671,p = 0.212, respectively) (Figure S5B,F). Additionally, running did not affect performance in the localized cue task by younger or older rats either in the time taken to reach the marked platform ($F_{1,18}$ = 0.456, p = 0.508; $F_{1,18}$ = 2.913, p = 0.105, respectively), speed of travel ($F_{1,18}$ = 0.543, p = 0.470; $F_{1,18}$ = 0.773, p = 0.391, respectively) or mean path length ($F_{1,18}$ = 0.067, p = 0.799; $F_{1,18}$ = 0.296, p = 0.593, respectively) (Figure S5I-K). However, as observed earlier, there were age differences in the ability to find the sign-posted platform, such that averaged across both running and non-running groups, older rats were not only slower to find the marked platform (p = 0.031), but also traversed longer path lengths (p = 0.022), and swam slower (p = 0.04) than younger rats.

At the second follow-up, our data replicated findings from the first follow-up. Exercise improved performance in younger rats across learning trials ($F_{1,18}$ = 7.57, p = 0.013) but this improvement was not detected on any of the measures used to assess memory on the probe trial, suggesting that learning had reached asymptote in both the runners and non-runners (p > 0.05) (Figure S5C-D,G-H). Exercise had no effect on the performance of the older rats (p > 0.05). Localized cue task performance was not affected by exercise on any measures (p > 0.05) but an age difference was present across multiple measures on the localized cue task (p*'s* < 0.05) (Figure S5I-K).

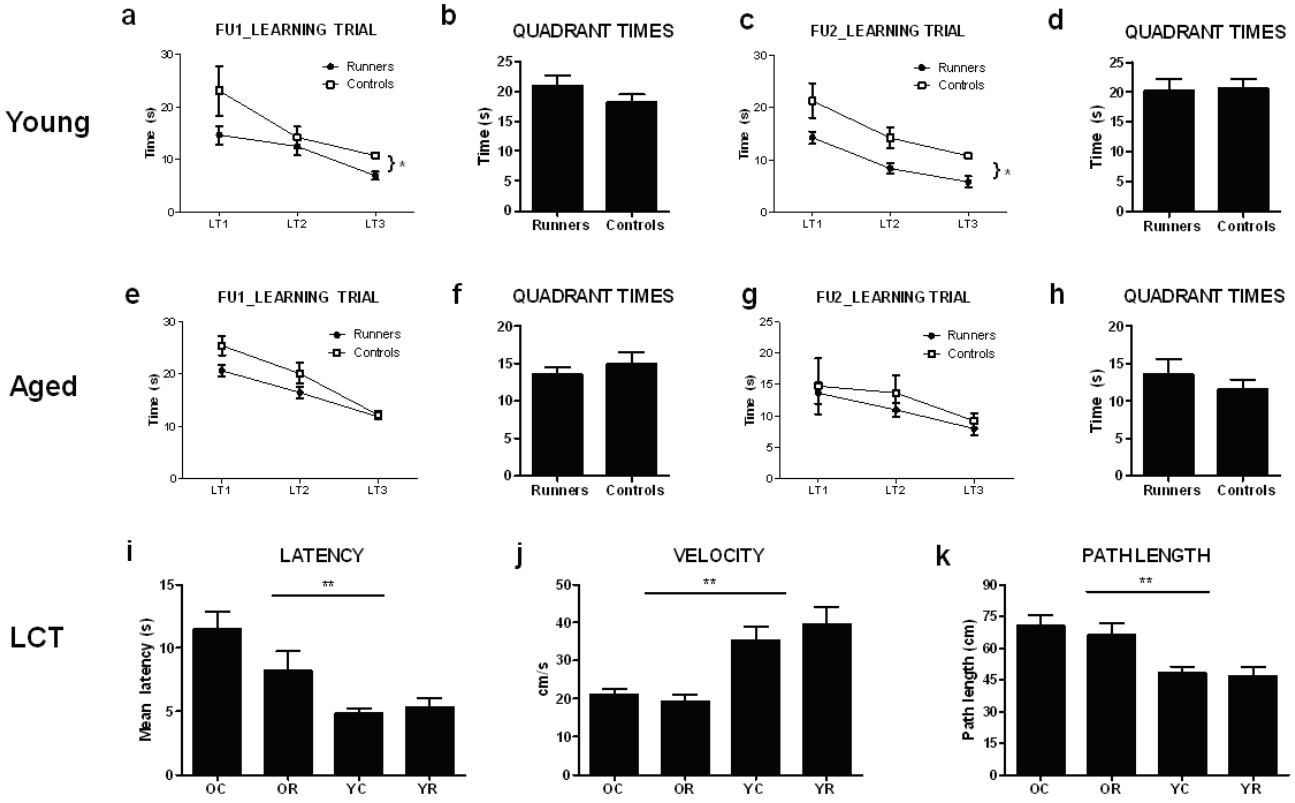

Figure S5. Running rescues spatial memory performance in young animals only. Both young and aged animals exhibited decreased latencies across training trials in first and second follow-up sessions. Running improved performance across training trials at first and second follow-up trials (**a,c** respectively) but not probe trial performance as assessed by time spent in the target quadrant (**b,d** respectively). Running did not improve performance in the older rats at first or second follow-up testing sessions (**e-h**). Running did not affect cued task performance at first follow-up either in time taken to reach the marked platform (**i**), swimming speed (**j**) or path length (**k**). Regardless of exercise condition, older rats remained impaired on this task compared to younger animals. FU, follow-up; LCT, localized cue task; OC, old controls; OR, old runners; YC, young countrols; YR, young runners. * $p < 0.05$; ** $p < 0.01$.

Exercise rescues presynaptic density in aged rats to levels beyond younger animals

Older runners showed significantly higher levels of synaptophysin density than younger runners across the dentate gyrus polymorph layer ($F_{1,14}$ = 6.279, p = 0.025), inner molecular layer ($F_{1,14}$ = 39.68, p = 0.00), middle molecular layer ($F_{1,14}$ = 7.22, p = 0.018) and outer molecular layer ($F_{1,14}$ = 11.411, p = 0.005). There were however no significant differences in hippocampal subfields CA3 ($F_{1,14}$ = 0.909, p = 0.357), CA2 ($F_{1,14}$ = 0.012, p = 0.914) and CA1 ($F_{1,14}$ = 1.801, p = 0.201), but there were higher presynaptic density in the entorhinal cortex of older than younger runners ($F_{1,14}$ = 6.279, p = 0.002) (Figure 4).

Supplemental References

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