

1 **Voluntary running does not increase capillary blood flow but promotes**  
2 **neurogenesis and short-term memory in the APP/PS1 mouse model of**  
3 **Alzheimer's disease**

4

5 Voluntary running does not increase capillary blood flow in APP/PS1 mice

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24 ABSTRACT

25 Exercise exerts a beneficial effect on the major pathological and clinical symptoms  
26 associated with Alzheimer's disease in humans and mouse models of the disease. While  
27 numerous mechanisms for such benefits from exercise have been proposed, a clear  
28 understanding of the causal links remains elusive. Recent studies also suggest that  
29 cerebral blood flow in the brain of both Alzheimer's patients and mouse models of the  
30 disease is decreased and that the cognitive symptoms can be improved when blood flow  
31 is restored. We therefore hypothesized that the mitigating effect of exercise on the  
32 development and progression of Alzheimer's disease may be mediated through an  
33 increase in the otherwise reduced brain blood flow. To test this idea, we examined the  
34 impact of three months of voluntary wheel running in ~1-year-old APP/PS1 mice on short-  
35 term memory function, brain inflammation, amyloid deposition, and cerebral blood flow.  
36 Our findings that exercise led to improved memory function, a trend toward reduced brain  
37 inflammation, markedly increased neurogenesis in the dentate gyrus, and no changes in  
38 amyloid-beta deposits are consistent with other reports on the impact of exercise on the  
39 progression of Alzheimer's related symptoms in mouse models. Notably, we did not  
40 observe any impact of wheel running on overall cortical blood flow nor on the incidence of  
41 non-flowing capillaries, a mechanism we recently identified as one contributing factor to  
42 cerebral blood flow deficits in mouse models of Alzheimer's disease. Overall, our results  
43 replicate previous findings that exercise is able to ameliorate certain aspects of  
44 Alzheimer's disease pathology, but show that this benefit does not appear to act through  
45 increases in cerebral blood flow.

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49 INTRODUCTION

50 BACKGROUND

51 Alzheimer's disease (AD) is the most common neurodegenerative disease, showing increasing  
52 prevalence with age among the elderly population. In addition to a progressive decline in  
53 cognitive ability and memory, the disease is characterized by pathological changes that include  
54 the deposition of the protein fragment amyloid-beta (A $\beta$ ) to form extracellular amyloid plaques,  
55 the aggregation of hyperphosphorylated tau protein in neurofibrillary tangles, increased brain  
56 inflammation, reduced synaptic plasticity, and neuronal cell death [1]. Whereas less than 1%  
57 of AD cases are attributable to genetic mutations in the genes coding for amyloid precursor  
58 protein (APP), presenilin-1 (PS1), or presenilin-2 (PS2) that contribute to the early onset form  
59 of the disease, the vast majority of AD cases are sporadic, and have a later disease onset.  
60 Despite tremendous efforts, there are currently no effective treatments available.

61

62 EFFECTS OF EXERCISE IN ALZHEIMER'S DISEASE

63 Regular physical activity is recognized as providing health-enhancing benefits, including  
64 through reducing the incidence and severity of dementia [2-5]. Aerobic exercise interventions  
65 were shown to maintain and improve neurocognitive function in ageing individuals, particularly  
66 for executive-control function [6-9]. In multiple transgenic mouse models of Alzheimer's  
67 disease, exercise has been shown to affect AD pathology through multiple pathways. In  
68 particular, exercise in the form of wheel running was shown to lead to cognitive improvements,  
69 such as enhanced memory function, a rescue of synaptic plasticity, and an amelioration of  
70 some pathological features, including a decrease in levels of A $\beta$  in some studies [10-17]. The  
71 latter was proposed to be mediated through changes in APP processing [18] and an increase  
72 in clearance of A $\beta$  [19], possibly resulting from an elevation of A $\beta$  clearance proteins [20] and  
73 increased glymphatic clearance [21]. In addition, expression of other genes linked to AD  
74 progression such as  $\beta$ -site APP-cleaving enzyme 1 (BACE1), presenilin 1 (PS1), insulin-  
75 degrading enzyme (IDE), tau, and the receptor for advanced glycation end products (RAGE)  
76 are reduced as a consequence of exercise [22, 23]. This may be due to a decrease in lipid-raft

77 formation [23], activation of SIRT1 (sirtuin (silent mating type information regulation 2 homolog)  
78 1) [12, 24], and an increase in autophagy [19, 25]. In addition, exercise has been proposed to  
79 mitigate mitochondrial dysfunction [11, 26], delay disease-related white matter volume loss  
80 [27-30], reduce neuroinflammation [19, 21, 22, 31-34] and oxidative stress [10, 19, 35], repress  
81 neuronal cell death [36], and favor adult neurogenesis [17, 37-40].

82  
83 BRAIN BLOOD FLOW IN ALZHEIMER'S DISEASE  
84 Cerebrovascular dysfunction has been described as an early feature in the development of  
85 many neurodegenerative diseases, including Alzheimer's disease [41]. For example, cerebral  
86 blood flow (CBF) was shown to be decreased by ~30% in both patients with Alzheimer's  
87 disease and mouse models of APP overexpression [42, 43]. Other structural and functional  
88 cerebrovascular changes that have been described in AD include diminution in blood vessel  
89 diameter [44], loss of microvasculature [45], and impaired blood flow increases in response to  
90 neuronal stimuli or changes in blood pressure [46, 47]. Cardiovascular risk factors, such as  
91 hypertension, type 2 diabetes, and obesity [41], are also among the strongest predictors of AD  
92 risk and severity, again suggesting a tight association between vascular health and dementia.

93 Reduced cerebral blood flow could, in turn, exacerbate aspects of AD progression and  
94 independently contribute to cognitive impairment through, for example, acceleration of A $\beta$   
95 aggregation and plaque growth [48], causing white matter deficits in a tau mouse model [44],  
96 attenuating interstitial fluid flow [48], and a general cellular adaption to dysregulated blood flow,  
97 including pericytes dysfunction [47], metabolic rate contributions [49], and causing cortical  
98 atrophy [44]. Even though a clear understanding of the underlying causes and consequences  
99 of CBF reduction in Alzheimer's disease has not yet emerged, proposed mechanisms for this  
100 hypoperfusion include degeneration of pericytes [47, 50-52], a decrease in nitric oxide [53],  
101 A $\beta$ -mediated constriction of capillaries by pericytes [54], and hypercoagulability due to fibrin  
102 interactions with A $\beta$  [55, 56]. Additionally, a recent study identified an increased number of  
103 cortical capillaries showing obstructed blood flow due to neutrophil adhesion in capillary  
104 segments as a novel cellular mechanism underlying the reduction in cortical blood flow [57].

105 Interestingly, preventing adhesion of neutrophils rapidly increased CBF and led to improved  
106 memory performance within hours [57]. An acute improvement in memory performance due to  
107 CBF increase was seen in APP/PS1 mice as old as 16 months [58], suggesting that increasing  
108 brain blood flow can contribute to improved cognitive performance even in late stages of  
109 disease progression.

110 Exercise has been shown to cause a number of beneficial changes in blood vessels,  
111 including increased baseline velocity [59], enhanced cerebral vasodilator responses [60],  
112 reduced vascular oxidative stress [61], increased vascular density [62], and improved arterial  
113 pressure [63]. Therefore, we hypothesized that the mitigating effect of exercise observed in  
114 Alzheimer's disease may be mediated through an increase in the otherwise reduced brain  
115 blood flow. In particular, we sought to investigate the effects of three months of voluntary wheel  
116 running on CBF, memory function, and brain pathology in ~1-year-old APP/PS1 mice.

117

118 METHODS

119 ANIMALS

120 All animal procedures were approved by the Cornell Institution Animal Care and Use  
121 Committee and were performed under the guidance of the Cornell Center for Animal  
122 Resources and Education (CARE). We used APP/PS1 transgenic mice (B6.Cg-Tg (APPswe,  
123 PSEN1dE9) 85Dbo/J; MMRRC\_034832-JAX, The Jackson Laboratory) as mouse models of  
124 AD. This double transgenic mouse model expresses a chimeric mouse/human amyloid  
125 precursor protein (Mo/HuAPP695swe) and contains a mutant human transgene for presenilin  
126 1 (PSE-dE9). Animals were of both sexes and ranged from 9 to 14 months in age at the  
127 beginning of the study.

128

129 EXERCISE PARADIGM AND MONITORING

130 Mice were randomly assigned to either standard housing (sedentary group) or to a cage  
131 equipped with a low-profile wireless running wheel (ENV-047; Med Associates, Inc.) to which  
132 they had unrestricted access (running group), for a period of three months. A Wireless USB  
133 Hub (DIG-807; Med Associates, Inc.) and Wheel Manager Software  
134 (SOF-860; Med Associates, Inc.) were used to record running data from the wheels. All mice  
135 were single-housed and given free access to water and food.

136

137 BEHAVIOR EXPERIMENTS

138 All experiments were performed under red lighting in an isolated room. Animals were taken  
139 into the procedure room for 1 h on each of two days preceding the experiment and were  
140 allowed to acclimate for 1 h prior to behavior sessions each day. The position of the mouse's  
141 head, body, and tail was automatically tracked by Viewer III software (Biobserve). In addition  
142 to the automatic results obtained by the software, a blinded researcher independently scored  
143 the mouse behavior manually. The testing arena was cleaned with 70% ethanol prior to use  
144 and in between each of the trials to remove any scent clues.

145

146 OPEN FIELD TEST

147 The open field (OF) test was used to evaluate anxiety-like behavior and general locomotor  
148 ability. Mice were placed in the arena individually and allowed to freely explore for a single  
149 30 min period during which time the tracking software recorded movement. Total track length,  
150 average velocity, and time spent in the inner and outer zones (defined as within 5 cm of the  
151 wall of the arena) of the arena were quantified. At the end of the test period, the mice were  
152 returned to their home cage.

153

154 OBJECT REPLACEMENT TEST

155 The object replacement (OR) test was used to measure spatial recognition memory  
156 performance. Mice were presented with two identical objects and allowed to freely explore the  
157 objects for 10 min in a testing arena in which each wall was marked with a different pattern.  
158 Subsequently, mice were returned to their home cage for 60 min. Mice were then returned to  
159 the arena for a 5 min testing period with one of the objects moved to a different location. The  
160 object was moved such that both the intrinsic relationship between the objects and the position  
161 relative to patterned walls were altered. Exploration time for the objects was defined as any  
162 time when there was physical contact with an object or when the animal was oriented toward  
163 the object at a distance of 2 cm or less. Climbing over an object was not considered to be an  
164 explorative behavior unless that action was accompanied by directing the nose toward that  
165 object. The preference score was determined from the testing period data and was calculated  
166 as (exploration time of the replaced object/exploration time of both objects).

167

168 Y-MAZE SPONTANEOUS ALTERNATION TEST

169 The Y-maze task was used to evaluate spatial working memory and willingness to explore new  
170 environments. Testing occurred in a Y-shaped maze consisting of three arms at 120° and  
171 made of light gray plastic. Each arm was 6 cm wide and 36 cm long, and had 12.5 cm high  
172 walls. Mice were placed in the Y-maze individually and allowed to freely explore the three arms  
173 for 6 min, during which time the tracking software recorded the mouse's movement. A mouse

174 was considered to have entered an arm if all four limbs entered it, and to have exited it if all  
175 four limbs exited the arm. The number of arm entries and the number of consecutive entries  
176 into three different arms (alternating triad) was recorded to quantify the percentage of  
177 spontaneous alternation. Because the maximum number of alternating triads is equal to the  
178 total number of arm entries minus 2, the spontaneous alternation score was calculated as  
179 (number of alternating triads/[total number of arm entries -2]).

180

#### 181 NOVEL OBJECT RECOGNITION TEST

182 The novel object recognition test (NOR) was used to evaluate object-identity memory and  
183 explorative behavior. The testing protocol was identical to the object replacement test, except  
184 mice were returned to their home cage for 90 min in between trials and one of the initial objects  
185 was replaced, at the same location, with a novel object for the testing period. The preference  
186 score was analogously determined from the testing period data and calculated was as  
187 (exploration time of the novel object/exploration time of both objects).

188

#### 189 SURGICAL PREPARATION

190 For cranial window implantation, mice were anesthetized under 3% isoflurane and then  
191 maintained at 1.5 – 2% isoflurane in 100% oxygen. Once fully sedated, mice were  
192 subcutaneously administered dexamethasone (0.025 mg per 100 g; 07-808-8194, Phoenix  
193 Pharm, Inc.) to reduce post-surgical inflammation, atropine (0.005 mg per 100 g;  
194 54925-063-10, Med-Pharmex, Inc.) to prevent lung secretions, and ketoprofen (0.5 mg per 100  
195 g; Zoetis, Inc.) to reduce post-surgical inflammation and provide post-surgical analgesia. Mice  
196 were then provided with atropine (0.005 mg per 100 g) and 5% glucose in saline (1 ml per 100  
197 g) every hour while anesthetized. The hair was shaved from the back of the neck up to the  
198 eyes. Mice were placed on a stereotaxic frame over a feedback-controlled heating blanket to  
199 ensure body temperature remained at 37° C. The head was firmly secured and eye ointment  
200 was applied to prevent the animal's eyes from drying out. The operating area was then  
201 sterilized by wiping the skin with iodine and 70% ethanol three times. The mice were given 0.1

202 ml bupivacaine at the site of incision to serve as a local anesthetic. The skin over the top of  
203 the skull was removed and the skull exposed. Using a high-speed drill with different sized bits,  
204 a ~6-mm diameter craniotomy was performed over the cerebral cortex, rostral to the lambda  
205 point and caudal to bregma. The exposed brain was then covered with a sterile 8-mm diameter  
206 glass coverslip which was glued to the skull surface using cyanoacrylate adhesive. Using  
207 dental cement, a small well around the window was created. After completion of the  
208 craniotomy, mice were returned to their cages and were subcutaneously administered  
209 ketoprofen (0.5 mg per 100 g) and dexamethasone (0.025 mg per 100 g) once daily for three  
210 days, and their cages were placed on a heating pad during this time. Animals were given three  
211 weeks to recover from the surgery before imaging experiments.

212

#### 213 IN-VIVO TWO-PHOTON MICROSCOPY

214 For imaging sessions, mice were anesthetized with 3% isoflurane and then maintained at  
215 1.5 – 2% isoflurane in 100% oxygen. Atropine and glucose were provided, as described above.  
216 Eye ointment was applied to prevent the eyes from drying out. Mice were placed on a  
217 stereotactic frame over a feedback-controlled heating pad to keep body temperature at  
218 37° C. To fluorescently label the microvasculature, Texas Red dextran (50 µl, 2.5% w/v,  
219 molecular weight (MW) = 70,000 kDa, Thermo Fisher Scientific) in saline was injected retro-  
220 orbitally. Although we did not use these labels for any analysis, these mice also had  
221 Rhodamine 6G (0.1 ml, 1 mg/ml in 0.9% saline, Acros Organics, Pure) injected into the  
222 bloodstream to label leukocytes and blood platelets and Hoechst 33342 (50 µl, 4.8 mg/ml in  
223 0.9% saline, Thermo Fisher Scientific) to label leukocytes. A custom-built two-photon excitation  
224 fluorescence (2PEF) microscope was used to acquire three-dimensional images of the cortical  
225 vasculature and to measure red blood cell flow in specific capillaries. Imaging was performed  
226 with 830-nm, 75-fs pulses from a Ti-Sapphire laser oscillator (Vision S, Coherent). Lasers were  
227 scanned by galvanometric scanners (1 frame/second) and ScanImage software was used to  
228 control data acquisition [64]. For obtaining broad maps of the cortical surface vasculature, a  
229 4x magnification air objective (numerical aperture of 0.28, Olympus) was used. For high-

230 resolution imaging, a 25x water-immersion objective lens (numerical aperture of 0.95,  
231 Olympus) was used. The emitted fluorescence from Texas Red was detected on a  
232 photomultiplier tube through an emission filter with a 641-nm center wavelength and a 75-nm  
233 bandwidth. Stacks of images were created by repeatedly taking images axially spaced at 1  $\mu\text{m}$   
234 up to a depth of  $\sim$ 300  $\mu\text{m}$ . Additionally, centerline line scans and image stacks across the  
235 diameter of  $\sim$ 15 capillaries within the imaging area were obtained in each mouse.

236

#### 237 ANALYSIS OF CAPILLARY BLOOD FLOW AND VESSEL DIAMETER

238 Blood flow velocities were determined based on the movement of red blood cells (RBCs). Since  
239 the injected dye (Texas Red) labels the blood plasma only, RBCs are seen as dark patches  
240 within the capillaries. We acquired repetitive line scans along the centerline of individual  
241 capillaries, forming space-time images with diagonal streaks due to moving RBCs. As  
242 previously described [65], we used a Radon transform-based algorithm to determine the slope  
243 of these streaks and thus quantify RBC flow speed. Post-hoc sensitivity analysis (G\*Power;  
244  $\alpha=0.05$ ,  $\beta=0.80$ ) with our realized SD and means between running and sedentary groups  
245 suggested we could detect a 30% change in flow speed. Capillary diameters were extracted  
246 from image stacks taken along with each line scan.

247

#### 248 CROWD-SOURCED SCORING OF CAPILLARIES AS FLOWING OR STALLED

249 In the 2PEF microscopy used to take three-dimensional image stacks of the vasculature, the  
250 intravenously injected fluorescent dye labels the blood plasma, but not red blood cells.  
251 Because each capillary segment was visible for multiple frames in the image stack, flowing  
252 segments showed different patterns of fluorescent and dark patches in successive frames due  
253 to moving blood cells. In capillary segments with stalled blood flow, the dark shadows from  
254 non-moving cells remained fixed across all frames where the capillary segment was visible.  
255 We used a purpose-built citizen science data analysis platform, StallCatchers.com, that  
256 enabled volunteers to score  $\sim$ 26,000 individual capillary segments as either flowing or not  
257 using 2PEF image stacks. We recently reported on the methodological details and validation

258 of this StallCatchers based scoring [58]. Briefly, we used a convolutional neural network, called  
259 DeepVess, to segment the 2PEF image stack into voxels that were within vs. outside the  
260 vasculature [66]. Individual capillary segments were identified using standard dilation and  
261 thinning operations to define vessel centerlines [67], with capillary segments defined as the  
262 path between two junctions. To restrict this analysis to capillaries, we excluded all segments  
263 with diameter greater than 10  $\mu\text{m}$ . Image stacks were then created that each had a single  
264 identified capillary segment outlined and these stacks were analyzed by citizen scientists using  
265 StallCatchers to score the identified segment as flowing or stalled. Each segment was scored  
266 by multiple volunteers, each of whom had a sensitivity defined by their performance on capillary  
267 segments we knew to be flowing or stalled, and we computed a weighted “crowd confidence”  
268 score representing the likelihood of each segment being stalled. Laboratory researchers then  
269 looked at these capillary segments as a final validation, starting with segments with the highest  
270 crowd confidence score for being stalled. The crowd confidence score ranged from 0 to 1, and  
271 laboratory researchers examined vessels with a score between 0.5 and 1, a total of 259  
272 capillary segments. For crowd confidence scores between 0.9 and 1, laboratory researchers  
273 concluded 95% were stalled, while for crowd confidence scores between 0.5 and 0.6, only 1  
274 out of 80 vessels were not flowing. The initial scoring by citizen scientists decreased the  
275 number of capillary segments laboratory researchers needed to evaluate by a factor of 100.  
276 We report the density of non-flowing capillaries as stalls per cubic millimeter.

277

## 278 CHARACTERIZATION OF GEOMETRIC PROPERTIES OF CORTICAL CAPILLARIES

279 For each identified capillary segment from the 2PEF image stacks that were segmented using  
280 DeepVess [66], we calculated the diameter (averaged along the length of the segment), the  
281 segment length (distance along the centerline of the vessel between two junctions), and the  
282 tortuosity (segment length divided by the Euclidean distance between the two junctions).

283

## 284 IMMUNOSTAINING OF BRAIN TISSUE

285 For evaluating cell proliferation, mice received intraperitoneal injection of 5-ethynyl-2'-  
286 deoxyuridine (EdU; E10415, ThermoFisher Scientific) at a dose of 25 mg/kg body weight every  
287 day for four days before harvesting the brains. Mice were sacrificed by lethal injection of  
288 pentobarbital (5 mg/100 mg). Brains were extracted and cut in half along the center line. One  
289 hemisphere was snap frozen in liquid nitrogen for future protein extraction and the other half  
290 was kept in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 48 hours at  
291 4° C and subsequently placed in 30% sucrose for histological analysis. Immunohistochemistry  
292 was performed on a set of coronal sections cut on a cryotome with OCT media at a thickness  
293 of 30 µm. From each mouse, every sixth section was mounted, washed with PBS, and blocked  
294 1 hour at room temperature (3% goat serum, 0.1% Triton-X100 in PBS). Sections were then  
295 incubated overnight at 4°C with primary antibodies against Iba1 (1:500, rabbit anti-mouse Iba1;  
296 019-19741, WAKO) and GFAP (1:500, chicken anti-mouse GFAP; ab53554, Abcam) in the  
297 same buffer. Secondary antibodies (goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa  
298 Fluor 594; ThermoFisher Scientific) were used at 1:300 dilution and added to the slides for 3  
299 hours at room temperature. Cell proliferation was detected through use of the Click-iTTM EdU  
300 Cell Proliferation Kit for Imaging (Alexa Fluor 647 dye, ThermoFisher Scientific). Methoxy-X04  
301 was used to counterstain amyloid deposits (1 mg/ml MeO-X04 (5 mg/ml in 10% DMSO, 45%  
302 propylene glycol, and 45% saline) for 15 minutes at room temperature, 4920, Tocris). Hoechst  
303 33342 was used to label cell nuclei (3 µg/ml, ThermoFisher Scientific). Sections were washed  
304 with PBS before mounting with Richard-Allan Scientific Mounting Medium (4112APG,  
305 ThermoFisher Scientific). Images were obtained using confocal microscopy (Zeiss  
306 Examiner.D1 AXIO) operated with Zen 1.1.2 software. Z-stack images of the hippocampal and  
307 cortical regions of each slide were acquired with 1 µm optical section thickness (three adjacent  
308 images between the suprapyramidal blade of the granule cell layer of the dentate gyrus and  
309 CA1, one image in CA3, and two adjacent images in the cerebral cortex taken directly toward  
310 the cortical surface from the hippocampus). Images were then binarized using a manually  
311 determined threshold. Appropriate thresholds varied between mice and were adjusted to  
312 ensure that all morphologically relevant objects were recognized. The fraction of pixels above

313 threshold (%Area) and the integrated density (product of mean gray value and area) was  
314 determined across sections for cortical and hippocampal regions. All sections were stained  
315 and imaged in parallel. Researchers were blinded in this analysis as to whether mice were part  
316 of the running or sedentary group.

317

#### 318 ELISA ASSAY

319 The frozen half-brains were weighed and homogenized in 1 ml PBS containing complete  
320 protease inhibitor (Roche Applied Science) and 1 mM AEBSF (Sigma) using a Dounce  
321 homogenizer. The homogenates were sonicated for 5 min and centrifuged at 14,000 g for 30  
322 min at 4° C. The supernatant (PBS-soluble fraction) was removed and stored at -80° C. The  
323 pellet was re-dissolved in 0.5 ml 70% formic acid, sonicated for 5 min, and centrifuged at  
324 14,000 g for 30 min at 4° C, and the supernatant was removed and neutralized using 1 M Tris  
325 buffer at pH 9 (insoluble fraction). Protein concentration was measured using the Pierce BCA  
326 Protein Assay (ThermoFischer Scientific). The extracts were then diluted to equalize different  
327 protein concentrations. These samples were analyzed by sandwich ELISA for Aβ1-40 and  
328 Aβ1-42 using commercial ELISA kits and following the manufacturer's protocol  
329 (ThermoFischer Scientific). The Aβ concentration was calculated by comparing the sample  
330 absorbance with the absorbance of known concentrations of synthetic Aβ1-40 or Aβ1-42  
331 standards assayed on the same plate. Data was acquired with a Synergy HT plate reader  
332 (Biotek) and analyzed using Gen5 software (BioTek) and Prism8 (GraphPad).

333

#### 334 STATISTICAL ANALYSIS

335 To determine statistical significance of differences between groups, the data was first tested  
336 for normality using the Shapiro-Wilk normality test. In case of normality, the statistical  
337 comparison was performed using an unpaired t-test for comparison between two groups. In  
338 case of non-normal distribution, the Mann-Whitney test (two groups) was used. For the  
339 analysis of blood flow speed and capillary diameter, a nested two-way analysis of variance  
340 (ANOVA) was carried out to account for possible interactions between individual mice.

341 P-values less than 0.05 were considered statistically significant and we used a standardized  
342 set of significance indicators in figures: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .  
343 Boxplots show the median with a black line; the mean is indicated with a red line. The box  
344 spans between the 25th and the 75th percentile of the data, defined as the interquartile range  
345 (IQR). Whiskers of the boxplots extend from the lowest datum within 1.5 times the IQR of the  
346 lower quartile of the data to the highest datum within 1.5 times the IQR of the highest quartile  
347 of the data. Correlation analysis was performed via the Pearson Product-Moment Correlation,  
348 with best fit lines indicated in red. Statistical analysis was performed and graphs were created  
349 using Prism8 (GraphPad).

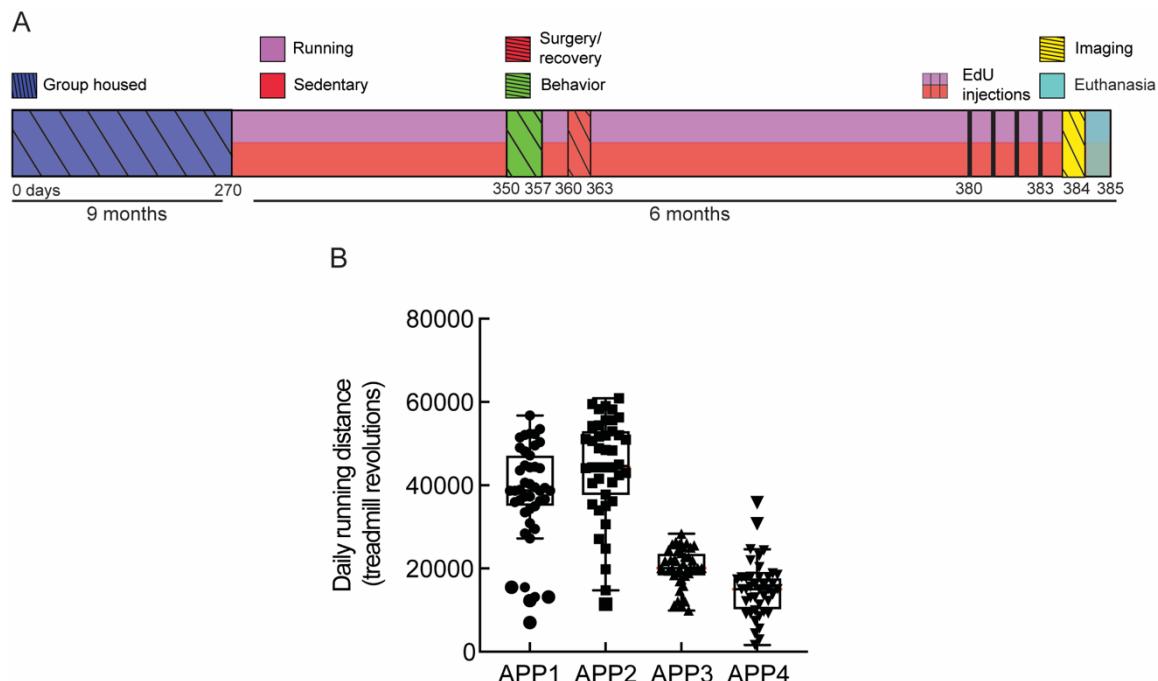
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351

352 RESULTS

353 RUNNING DISTANCE

354 To determine the effects of three months of voluntary exercise in 13 month old APP/PS1 mice,  
355 we provided single-housed mice with unrestricted access to a monitored running wheel inside  
356 their home cages (Fig 1A) [68]. All mice reliably ran on the wheels on a daily basis, with just  
357 under a factor of three variance in the average daily running distance between individual  
358 animals (Fig 1B). Sedentary, control APP/PS1 mice were singly housed for the same duration,  
359 but without access to a running wheel.



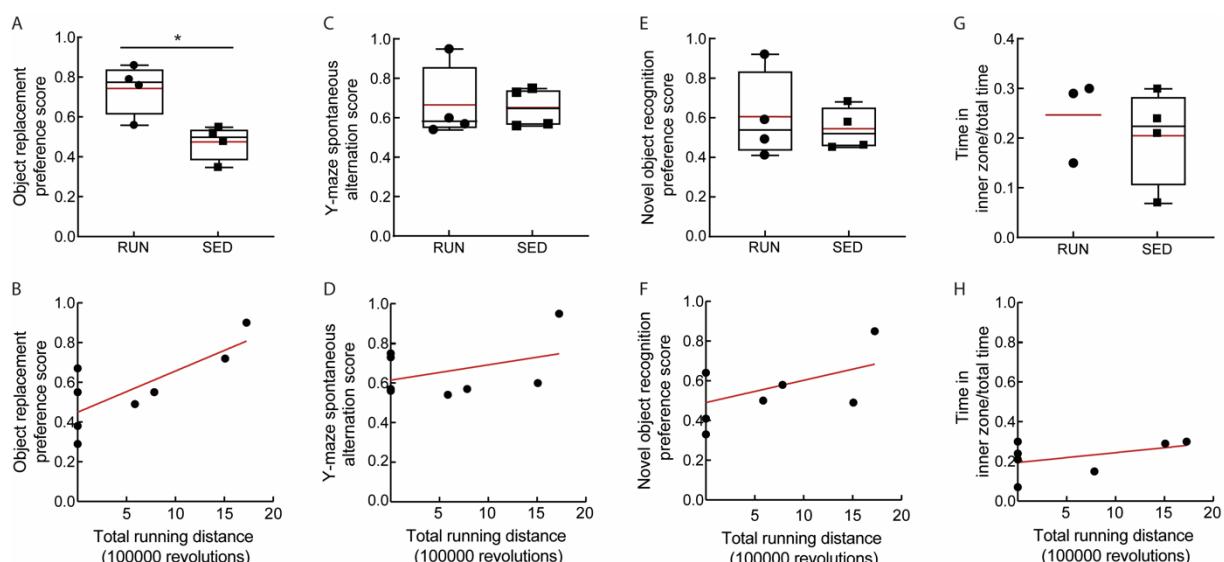
361 **Fig 1. APP/PS1 mice reliably run when given unrestricted access to a running wheel in their home cage. A.**  
362 Timeline of the study. After three months of voluntary exercise on a wheel running (or standard housing for the  
363 sedentary control group), mice underwent behavioral testing. Next, a craniotomy was performed, after which mice  
364 were allowed three weeks to fully recover. The cortical vasculature was then imaged with in-vivo two photon  
365 excitation fluorescence microscopy. Finally, after four consecutive days of EdU-injections, mice were euthanized.  
366 Harvested brain tissue was then used for immunohistochemistry. **B.** Each dot is the number of wheel revolutions  
367 per day over 45 days for the four APP/PS1 mice in the running group. Data show mean + SD.

368

369 RUNNING IMPROVED SOME MEASURES OF SPATIAL MEMORY PERFORMANCE

370 To investigate cognitive function in running versus sedentary APP/PS1 mice, we performed a  
371 variety of behavioral tests aimed at testing different aspects of memory performance. Running  
372 mice achieved a significantly higher preference score in the object replacement test (OR) than  
373 sedentary mice, i.e. they spent significantly more time exploring the replaced object than the

374 object in the familiar location as compared to the sedentary mice (Fig 2A;  $P = 0.014$ ). There  
375 was also a significant correlation between individual running distances and OR performance  
376 (Fig 2B;  $R^2 = 0.58$   $P = 0.028$ ). Conversely, we did not observe an increase in spontaneous  
377 alternation between the three arms of the Y-maze (Fig 2C), nor increased exploration time for  
378 a novel object in the novel object recognition (NOR) test (Fig 2E), nor increased time spent in  
379 the center of the arena in an open field (OF) test (Fig. 2G) in running as compared to sedentary  
380 APP/PS1 mice. Similarly, none of these measures was significantly correlated with individual  
381 running distances (Fig 2D, 2F, and 2H). Additionally, there was no significant difference in time  
382 spent exploring objects in the OR test (Fig S1A) or NOR test (Fig S1B), nor in the number of  
383 arm entries in the Y-maze (Fig S1C), nor in the average track length during the OF test (Fig  
384 S1D) between running and sedentary groups.



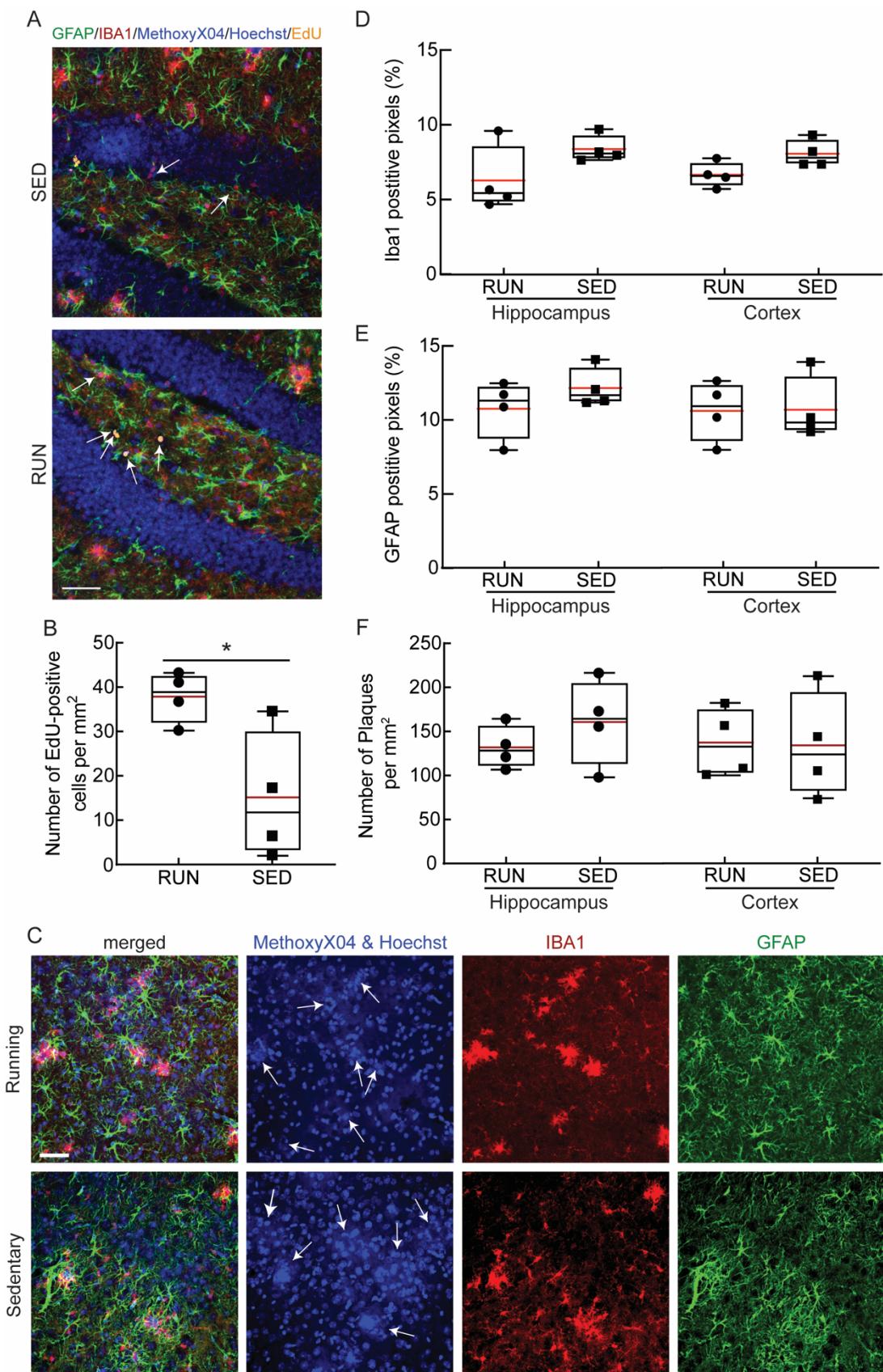
385

386 **Fig 2. Running improved performance in the object replacement test, but did not show strong effects in**  
387 **other short-term memory tests. All behavioral testing results are shown for running (RUN) and sedentary (SED)**  
388 **APP/PS1 mice. A Preference score for OR ( $P = 0.01$ ). B Correlation between OR preference score and total running**  
389 **distance ( $R^2 = 0.6$ ;  $P = 0.03$ ). C Spontaneous alternation score in Y-maze. D Correlation between spontaneous**  
390 **alternation and total running distance. E Preference score in NOR test. F Correlation between NOR preference**  
391 **score and total running distance. G Time spent in the arena center in the open field test. One value was excluded**  
392 **in the running group due to a software failure during the test. H Correlation between time spent in the arena center**  
393 **and total running distance. Animal numbers: RUN:  $n = 4$  (OF: 3); SED:  $n = 4$ .**

394

395 **RUNNING INCREASED HIPPOCAMPAL NEUROGENESIS BUT DID NOT DECREASE**  
396 **BRAIN INFLAMMATION NOR AMYLOID DEPOSITION**  
397 Exercise has been shown to exert its effects through multiple pathways, with increased  
398 hippocampal neurogenesis being one of the most consistently described effects in both

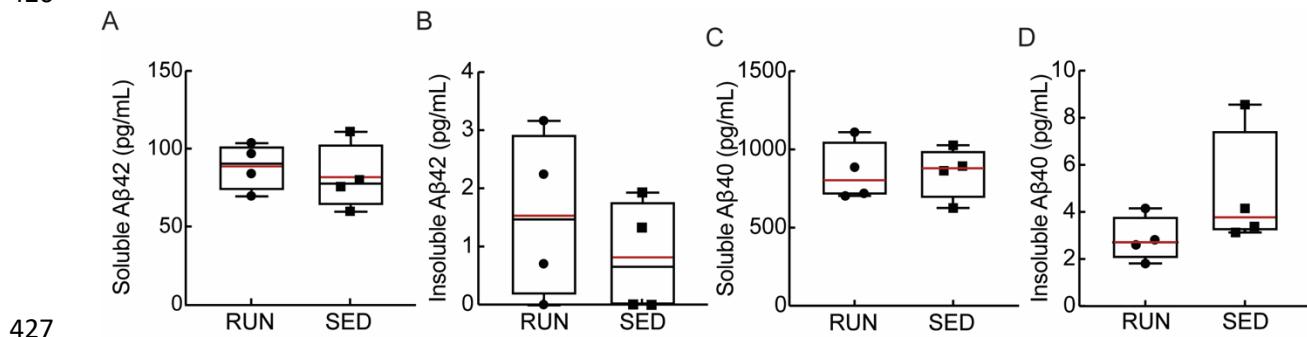
399 patients [69], and in AD mouse models [70, 71]. Consistent with these previous findings, we  
400 found an increased number of proliferating neuronal stem cells in the dentate gyrus of running,  
401 as compared to sedentary, mice, as detected by the number of EdU-positive cells (Fig 3A and  
402 3B;  $P = 0.03$ ). In the same hippocampal tissue sections, as well as in cortical slices (Fig 3C),  
403 we further examined the density of Iba1 and GFAP staining to quantify any differences in the  
404 density of microglia and astrocytes, respectively, providing a measure of brain inflammation.  
405 We also quantified the density of amyloid plaques that were labeled with Methoxy-X04. We  
406 found no notable differences in microglia (Fig 3D) or astrocyte (Fig 3E) density, nor in the  
407 number of amyloid deposits (Fig 3F) between running and sedentary APP/PS1 mice in both  
408 hippocampal and cortical regions. Using ELISA assays, we quantified the concentration of  
409 A $\beta$ 1-40 and A $\beta$ 1-42 in brain lysates and saw no differences between running and sedentary  
410 mice in both the soluble and insoluble fractions (Fig. 4).



411

412 **Fig 3. Running increased neural stem cell proliferation in the dentate gyrus, but did not decrease**  
413 **inflammation nor amyloid plaque burden in APP/PS1. A** Representative confocal images of tissue sections from  
414 the hippocampus of sedentary (top) and running (bottom) APP/PS1 mice, with labeling of astrocytes (anti-GFAP,  
415 green), microglia (anti-IBA1, red), amyloid plaques (Methoxy-X04, blue; no amyloid plaques visible in these fields),  
416 cell nuclei (Hoechst, blue), and proliferating cells (EdU, yellow, indicated with arrows). **B** Boxplot of the density of

417 EdU positive cells in the dentate gyrus from running (RUN) and sedentary (SED) APP/PS1 mice ( $P = 0.03$ , Mann-  
418 Whitney test). **C** Representative confocal images of tissue sections from the cortex from running (top) and sedentary  
419 (bottom) mice. Labeling is the same as in panel A. Images to the right show individual channels. The white arrows  
420 in the second column of images indicate Methoxy-X04 labeled amyloid plaques, which are distinguished from  
421 Hoechst-labeled cell nuclei by morphological differences. **D** Microglial density, represented as the fractional area  
422 that is Iba-1 positive; **E** Astrocyte density, represented as the fractional area that was GFAP positive; and **F** the  
423 number of Methoxy-X04 positive plaques per unit area, from the hippocampus and cortex of RUN and SED  
424 APP/PS1 mice. Animal numbers: RUN:  $n = 4$ ; SED:  $n = 4$ . Scale bars indicates 50  $\mu$ m.  
425  
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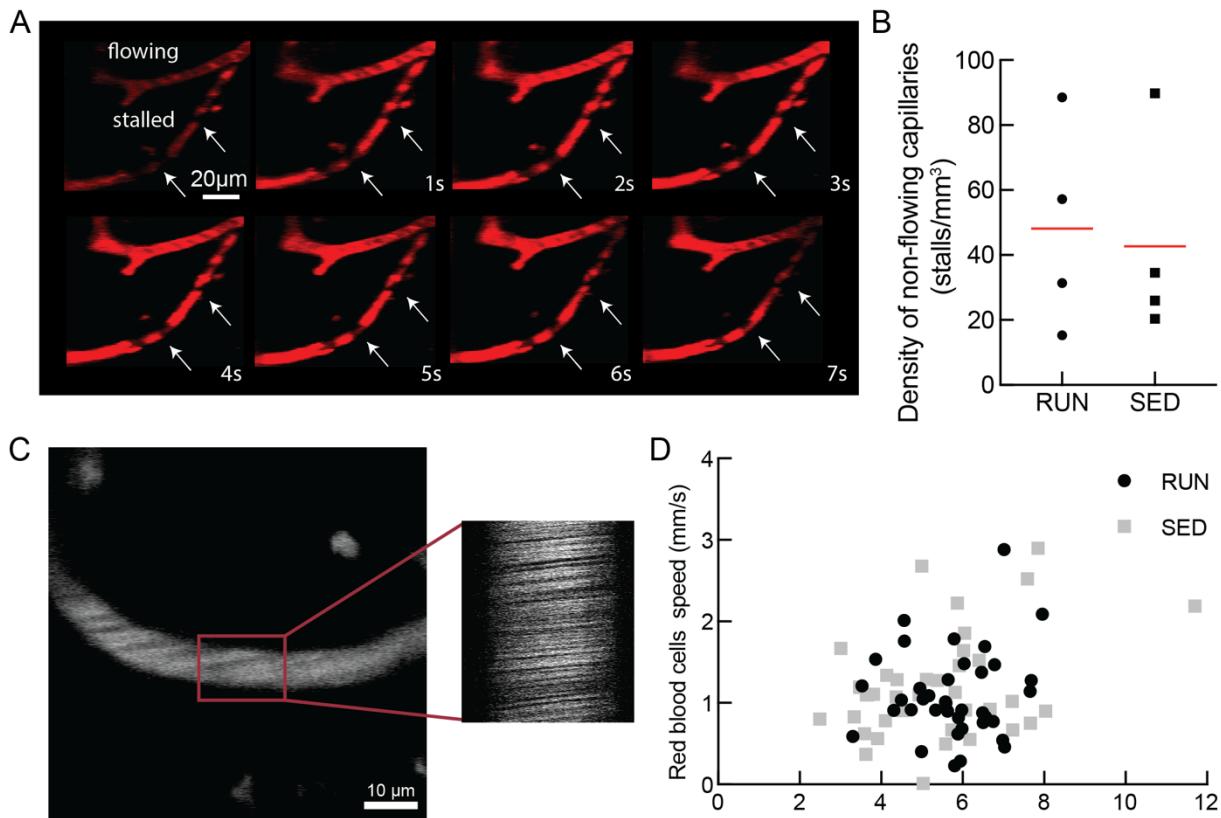


427 **Fig 4. Running did not alter the levels of soluble and insoluble amyloid beta monomers in the brain.** Soluble  
428 (A and C) and insoluble (B and D) concentrations of Aβ1-42 (A and B) and Aβ1-40 (C and D) in brain lysates from  
429 running (RUN) and sedentary (SED) APP/PS1 mice, as measured using ELISA assays.  
430  
431

#### 432 VOLUNTARY RUNNING DID NOT AFFECT CAPILLARY BLOOD FLOW

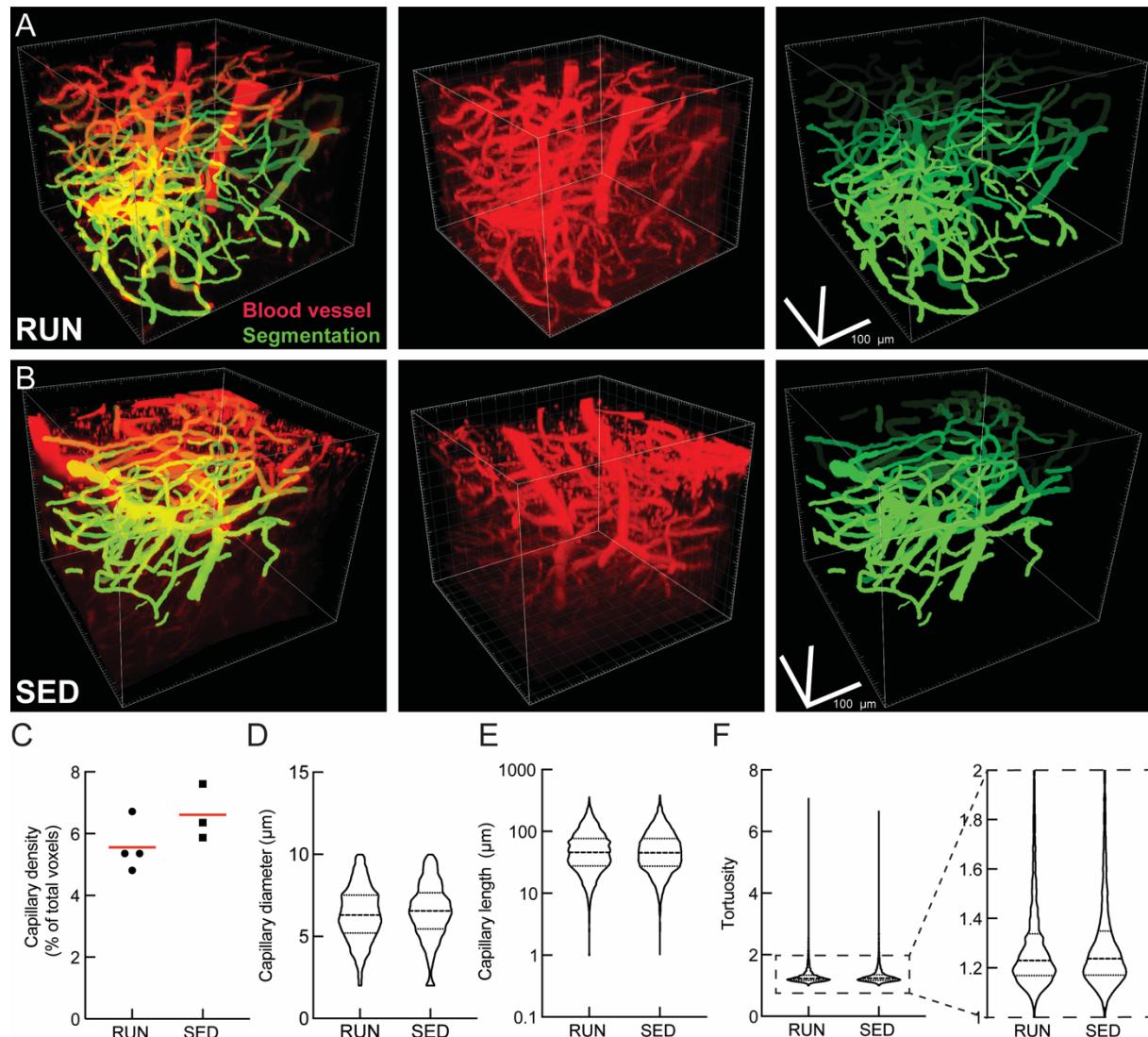
433 The trends toward improved cognitive function (Fig 2), the clear increase in neurogenesis, as  
434 well as the lack of a notable impact on brain inflammation and amyloid deposition (Fig. 3 and  
435 4) that we observed are consistent with previous studies of the impact of exercise on mouse  
436 models of AD [72]. We next sought to determine if these exercise-mediated changes were  
437 correlated with an increase in brain blood flow, perhaps linked to a decrease in the incidence  
438 of non-flowing capillaries. We used a crowd-sourced approach to score individual capillary  
439 segments as flowing or stalled based on the motion of red blood cells (which appear as dark  
440 patches within the fluorescently-labeled blood plasma in 2PEF image stacks) (Fig 5A).  
441 Contrary to our initial hypothesis, we did not observe a decrease in the incidence of stalled  
442 capillaries in running APP/PS1 mice as compared to sedentary controls (Fig 5B). We further  
443 quantified red blood cell flow speed and vessel diameter in cortical capillaries (Fig. 5C), and  
444 saw no differences, on average, between running and sedentary APP/PS1 mice (Fig 5D).  
445 Capillary speed and diameter were also not found to be correlated with the total distance ran  
446 by animals (Fig S2). To further explore potential exercise-induced changes in the brain  
447 vasculature, we used a convolutional neural network-based segmentation algorithm,

448 DeepVess [66], to segment 3D image stacks of the cortical vasculature of running (Fig 6A) and  
449 sedentary (Fig 6B) APP/PS1 mice and we characterized the capillary density (Fig 6C), capillary  
450 diameter (Fig 6D), capillary segment length (Fig 6E), and capillary tortuosity (Fig 6F). None of  
451 these parameters differed between running and sedentary APP/PS1 mice.



452

453 **Fig 5. Running did not decrease the incidence of non-flowing capillaries, not increase capillary blood flow**  
454 **in the brain of APP/PS1 mice. A** Representative 2PEF image sequence showing both flowing and stalled capillary  
455 segments over 7 s. The blood plasma was labeled with Texas Red-dextran and the dark patches in the vessel  
456 lumen were formed by blood cells. The lack of motion of these dark patches in the lower capillary indicates stalled  
457 blood flow. (Stalled capillary is indicated with arrows). **B** Density of capillaries with stalled blood flow in running  
458 (RUN) and sedentary (SED) APP/PS1 mice. **C** Representative 2PEF image of a cortical capillary (left) and a space-  
459 time image from repetitive line scans along the centerline of the capillary segment (right). The diagonal streaks in  
460 the space-time image are formed by moving red blood cells, with a slope that is inversely proportional to the blood  
461 flow speed. **D** Capillary blood flow speed plotted as a function of vessel diameter for cortical capillaries from running  
462 (RUN) and sedentary (SED) APP/PS1 mice.



463

464 **Fig. 6. Running did not alter capillary density or geometry.** A and B Representative 2PEF image stacks of  
465 fluorescently-labeled cortical vasculature (middle) and the segmentation of this image (right) from running (RUN)  
466 and sedentary (SED) APP/PS1 mice, respectively. C Density; D Diameter; E Length and; F Tortuosity of capillary  
467 segments from running (RUN; n = 5514 capillaries) and sedentary (SED; n = 7251 capillaries) APP/PS1 mice.  
468

## 469 DISCUSSION

470 Physical activity is correlated with attenuation of cognitive impairment, amelioration of age-  
471 related changes in the brain, and reduced risk of dementia [73, 74]. While exercise has been  
472 found to have a positive effect on cognition and brain health in aging individuals [75], the  
473 underlying mechanisms have not been fully elucidated. In this study, we investigated the  
474 effects of several months of voluntary wheel running on memory function, AD-related brain  
475 pathology, and cortical blood flow in aged APP/PS1 mice.

476 Impairments in spatial learning and memory in APP/PS1 mice have been reported in  
477 mice as young as seven months of age [76, 77]. We found performance in the object

478 replacement task in 12-month-old APP/PS1 mice was markedly improved by three months of  
479 voluntary wheel running, as compared to sedentary controls, while no improvements were  
480 detected in the novel object recognition task and Y-maze tasks. This exercise-related  
481 improvement in performance on some memory-related tasks but not on others is consistent  
482 with similarly mixed impacts of exercise in previous studies of memory function in mouse  
483 models of AD [72] and in AD patients [78]. While a variety of brain regions are likely involved  
484 in each of these memory tasks, it has been shown that memory of an object's spatial location  
485 (evaluated with object replacement task) is highly dependent on hippocampal regions, while  
486 memory of an object's intrinsic characteristics (evaluated with novel object task) also involves  
487 significant contributions from other brain regions, such as the temporal lobe [79-81]. It is  
488 possible that exercise differentially improves function in different brain regions and this  
489 contributes to the mixed impact of exercise on different memory tasks.

490 Consistent with a broad consensus in the literature [37, 40], we observed increased  
491 neural stem cell proliferation in the dentate gyrus of the hippocampus in exercising APP/PS1  
492 mice, as compared to sedentary controls. Exercise did not, however, lead to changes in brain  
493 inflammation, as assayed by microglia and astrocyte density, or in amyloid pathology, as  
494 assayed by amyloid plaque density and amyloid-beta monomer concentrations. A recent study  
495 in the 5xFAD mouse model of AD similarly found no decreases in measures of brain  
496 inflammation or in the density of amyloid deposits due to exercise [82].

497 We have shown that neutrophils plug a small fraction of brain capillary segments in the  
498 APP/PS1 and 5xFAD mouse models of AD, and that this contributes to the overall brain blood  
499 flow reductions seen in these mice [57]. Contrary to our initial hypothesis that exercise could  
500 decrease the microvascular dysfunction that underlies these capillary stalls, we did not find a  
501 decrease in the number of non-flowing capillaries in running APP/PS1 mice, as compared to  
502 sedentary controls. Further, we did not detect any differences in average capillary blood flow  
503 speeds between running and sedentary APP/PS1 mice. While there is clear consensus that  
504 reduced brain blood flow in both AD patients and mouse models is a key feature of the disease,  
505 the effects of exercise on cortical blood flow in AD remain up for debate. Consistent with our

506 findings in APP/PS1 mice, however, recent studies in patients with dementia (diagnosed as  
507 mild to moderate AD in Refs. [83] and [84]) showed that 3 times per week 60 min of aerobic  
508 exercise led to improved cognition [83, 85, 86], but this effect was not correlated with increased  
509 cerebral blood flow [84, 87]. Finally, we did not observe changes in the density or geometry  
510 of cortical capillaries in APP/PS1 mice that exercised, compared to sedentary controls.

511 While we did not observe an impact of exercise on the capillary stalling phenomena we  
512 recently tied to brain blood flow deficits in AD mouse models, there are other aspects of cortical  
513 microvascular dysfunction that have been shown to occur in mouse models of AD that we did  
514 not examine. For example, mouse models of AD have shown increased blood brain barrier  
515 permeability [88], more contractile pericytes around capillaries [54], attenuation of cerebral  
516 blood flow regulation mechanisms (neurovascular coupling [89] and autoregulation [90]), as  
517 well as hypercoagulability [55, 56]. It is possible that exercise could positively impact some of  
518 these other aspects of microvascular dysfunction in AD, although our observation of no  
519 differences in average capillary flow speeds between exercising and sedentary APP/PS1 mice  
520 suggests that pathologies that impact flow are not likely modulated by exercise.

521

522

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527

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