RESEARCH ARTICLE

A pilot study investigating the effects of voluntary exercise on capillary stalling and cerebral blood flow in the APP/PS1 mouse model of Alzheimer’s disease

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Abstract

Exercise exerts a beneficial effect on the major pathological and clinical symptoms associated with Alzheimer’s disease in humans and mouse models of the disease. While numerous mechanisms for such benefits from exercise have been proposed, a clear understanding of the causal links remains elusive. Recent studies also suggest that cerebral blood flow in the brain of both Alzheimer’s patients and mouse models of the disease is decreased and that the cognitive symptoms can be improved when blood flow is restored. We therefore hypothesized that the mitigating effect of exercise on the development and progression of Alzheimer’s disease may be mediated through an increase in the otherwise reduced brain blood flow. To test this idea, we performed a pilot study to examine the impact of three months of voluntary wheel running in a small cohort of ~1-year-old APP/PS1 mice on short-term memory function, brain inflammation, amyloid deposition, and baseline cerebral blood flow. Our findings that exercise led to a trend toward improved spatial short-term memory, reduced brain inflammation, markedly increased neurogenesis in the dentate gyrus, and a reduction in hippocampal amyloid-beta deposits are consistent with other reports on the impact of exercise on the progression of Alzheimer’s related symptoms in mouse models. Notably, we did not observe any impact of wheel running on overall baseline blood flow nor on the incidence of non-flowing capillaries, a mechanism we recently identified as one contributing factor to cerebral blood flow deficits in mouse models of Alzheimer’s disease. Overall, our findings add to the emerging picture of differential effects of exercise on cognition and blood flow in Alzheimer’s disease pathology by showing that capillary stalling is not decreased following exercise.
Introduction

Background

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

Effects of exercise in Alzheimer’s disease

Regular physical activity is recognized as providing health-enhancing benefits, including through reducing the incidence and severity of dementia [2–5]. Aerobic exercise interventions were shown to maintain and improve neurocognitive function in ageing individuals, particularly for executive-control function [6–9]. In multiple transgenic mouse models of Alzheimer’s disease, exercise has been shown to affect AD pathology through multiple pathways. In particular, exercise in the form of wheel running was shown to lead to cognitive improvements, such as enhanced memory function, a rescue of synaptic plasticity, and an amelioration of some pathological features, including a decrease in levels of Aβ in some studies [10–17]. The latter was proposed to be mediated through changes in APP processing [18] and an increase in clearance of Aβ [19], possibly resulting from an elevation of Aβ clearance proteins [20] and increased lympathic clearance [21]. In addition, expression of other genes linked to AD progression such as β-site APP-cleaving enzyme 1 (BACE1), presenilin 1 (PS1), insulin-degrading enzyme (IDE), tau, and the receptor for advanced glycation end products (RAGE) are reduced as a consequence of exercise [22, 23]. This may be due to a decrease in lipid-raft formation [23], activation of SIRT1 (sirtuin (silent mating type information regulation 2 homolog) 1) [12, 24], and an increase in autophagy [19, 25]. In addition, exercise has been proposed to mitigate mitochondrial dysfunction [11, 26], delay disease-related white matter volume loss [27–30], reduce neuroinflammation [19, 21, 22, 31–34] and oxidative stress [10, 19, 35], repress neuronal cell death [36], and favor adult neurogenesis [17, 37–40].

Brain blood flow in Alzheimer’s disease

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

Reduced cerebral blood flow could, in turn, exacerbate aspects of AD progression and independently contribute to cognitive impairment through, for example, acceleration of Aβ aggregation and plaque growth [48], causing white matter deficits in a tau mouse model [44],
attenuating interstitial fluid flow [48], and a general cellular adaption to dysregulated blood flow, including pericytes dysfunction [47], metabolic rate contributions [49], and causing cortical atrophy [44]. Even though a clear understanding of the underlying causes and consequences of CBF reduction in Alzheimer’s disease has not yet emerged, proposed mechanisms for this hypoperfusion include degeneration of pericytes [47, 50–52], a decrease in nitric oxide [53], Aβ-mediated constriction of capillaries by pericytes [54], and hypercoagulability due to fibrin interactions with Aβ [55, 56]. Additionally, a recent study identified an increased number of cortical capillaries showing obstructed blood flow due to neutrophil adhesion in capillary segments as a novel cellular mechanism underlying the reduction in cortical blood flow [57]. Interestingly, preventing adhesion of neutrophils rapidly increased CBF and led to improved memory performance within hours [57]. An acute improvement in memory performance due to CBF increase was seen in APP/PS1 mice as old as 16 months [58], suggesting that increasing brain blood flow can contribute to improved cognitive performance even in late stages of disease progression.

Exercise has been shown to cause a number of beneficial changes in blood vessels, including increased baseline velocity [59], enhanced cerebral vasodilator responses [60], reduced vascular oxidative stress [61], increased vascular density [62], and improved arterial pressure [63]. Therefore, we hypothesized that the mitigating effect of exercise observed in Alzheimer’s disease may be mediated through an increase in the otherwise reduced brain blood flow. To explore this idea, we conducted a pilot study on the effects of three months of voluntary wheel running on capillary obstruction, CBF, memory function, and brain pathology in ~1-year-old APP/PS1 mice.

Methods

Animals

All animal procedures were approved by the Cornell Institution Animal Care and Use Committee and were performed under the guidance of the Cornell Center for Animal Resources and Education (CARE). We used APP/PS1 transgenic mice (B6.Cg-Tg (APPswe, PSEN1dE9) 85Db/J; MMRRC_034832-JAX, The Jackson Laboratory) as mouse models of AD. This double transgenic mouse model expresses a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and contains a mutant human transgene for presenilin 1 (PSE-dE9). Animals (running: n = 4; sedentary: n = 4) were of both sexes and ranged from 9 to 14 months in age at the beginning of the study (S1 Table).

Exercise paradigm and monitoring

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

Behavior experiments

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

**Open field test**

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

**Object replacement test**

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

**Y-maze spontaneous alternation test**

The Y-maze task was used to evaluate spatial working memory and willingness to explore new environments. Testing occurred in a Y-shaped maze consisting of three arms at 120˚ and made of light gray plastic. Each arm was 6 cm wide and 36 cm long, and had 12.5 cm high walls. Mice were placed in the Y-maze individually and allowed to freely explore the three arms for 6 min, during which time the tracking software recorded the mouse’s movement. A mouse was considered to have entered an arm if all four limbs entered it, and to have exited it if all four limbs exited the arm. The number of arm entries and the number of consecutive entries into three different arms (alternating triad) was recorded to quantify the percentage of spontaneous alternation. Because the maximum number of alternating triads is equal to the total number of arm entries minus 2, the spontaneous alternation score was calculated as (number of alternating triads/[total number of arm entries -2]).

**Novel object recognition test**

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

For cranial window implantation, mice were anesthetized under 3% isoflurane and then maintained at 1.5–2% isoflurane in 100% oxygen. Once fully sedated, mice were subcutaneously administered dexamethasone (0.025 mg per 100 g; 07-808-8194, Phoenix Pharm, Inc.) to reduce post-surgical inflammation, atropine (0.005 mg per 100 g; 54925-063-10, Med-Phar-mex, Inc.) to prevent lung secretions, and ketoprofen (0.5 mg per 100 g; Zoetis, Inc.) to reduce post-surgical inflammation and provide post-surgical analgesia. Mice were then provided with atropine (0.005 mg per 100 g) and 5% glucose in saline (1 ml per 100 g) every hour while anesthetized. The hair was shaved from the back of the neck up to the eyes. Mice were placed on a stereotaxic frame over a feedback-controlled heating blanket to ensure body temperature remained at 37˚ C. The head was firmly secured and eye ointment was applied to prevent the animal’s eyes from drying out. The operating area was then sterilized by wiping the skin with iodine and 70% ethanol three times. The mice were given 0.1 ml bupivacaine at the site of incision to serve as a local anesthetic. The skin over the top of the skull was removed and the skull exposed. Using a high-speed drill with different sized bits, a ~6-mm diameter craniotomy was performed over the cerebral cortex, rostral to the lambda point and caudal to bregma. The exposed brain was then covered with a sterile 8-mm diameter glass coverslip which was glued to the skull surface using cyanoacrylate adhesive. Using dental cement, a small well around the window was created. After completion of the craniotomy, mice were returned to their cages and were subcutaneously administered ketoprofen (0.5 mg per 100 g) and dexamethasone (0.025 mg per 100 g) once daily for three days, and their cages were placed on a heating pad during this time. Animals were given three weeks to recover from the surgery before imaging experiments.

In-vivo two-photon microscopy

For imaging sessions, mice were anesthetized with 3% isoflurane and then maintained at 1.5–2% isoflurane in 100% oxygen. Atropine and glucose were provided, as described above. Eye ointment was applied to prevent the eyes from drying out. Mice were placed on a stereotactic frame over a feedback-controlled heating pad to keep body temperature at 37˚ C. To fluorescently label the microvasculature, Texas Red dextran (50 μl, 2.5% w/v, molecular weight (MW) = 70,000 kDA, Thermo Fisher Scientific) in saline was injected retro-orbitally. Although we did not use these labels for any analysis, these mice also had Rhodamine 6G (0.1 ml, 1 mg/ml in 0.9% saline, Acros Organics, Pure) injected into the bloodstream to label leukocytes and blood platelets and Hoechst 33342 (50 μl, 4.8 mg/ml in 0.9% saline, Thermo Fisher Scientific) to label leukocytes. A custom-built two-photon excitation fluorescence (2PEF) microscope was used to acquire three-dimensional images of the cortical vasculature and to measure red blood cell flow in specific capillaries. Imaging was performed with 830-nm, 75-fs pulses from a Ti-Sapphire laser oscillator (Vision S, Coherent). Lasers were scanned by galvanometric scanners (1 frame/second) and ScanImage software was used to control data acquisition [64]. For obtaining broad maps of the cortical surface vasculature, a 4x magnification air objective (numerical aperture of 0.28, Olympus) was used. For high-resolution imaging, a 25x water-immersion objective lens (numerical aperture of 0.95, Olympus) was used. The emitted fluorescence from Texas Red was detected on a photomultiplier tube through an emission filter with a 641-nm center wavelength and a 75-nm bandwidth. Stacks of images were created by repeatedly taking images axially spaced at 1 μm up to a depth of ~300 μm. Additionally, center-line line scans and image stacks across the diameter of ~15 capillaries within the imaging area were obtained in each mouse.
Analysis of capillary blood flow and vessel diameter

Blood flow velocities were determined based on the movement of red blood cells (RBCs). Since the injected dye (Texas Red) labels the blood plasma only, RBCs are seen as dark patches within the capillaries. We acquired repetitive line scans along the centerline of individual capillaries, forming space-time images with diagonal streaks due to moving RBCs. As previously described [65], we used a Radon transform-based algorithm to determine the slope of these streaks and thus quantify RBC flow speed. Capillary diameters were extracted from image stacks taken along with each line scan.

Crowd-sourced scoring of capillaries as flowing or stalled

In the 2PEF microscopy used to take three-dimensional image stacks of the vasculature, the intravenously injected fluorescent dye labels the blood plasma, but not red blood cells. Because each capillary segment was visible for multiple frames in the image stack, flowing segments showed different patterns of fluorescent and dark patches in successive frames due to moving blood cells. In capillary segments with stalled blood flow, the dark shadows from non-moving cells remained fixed across all frames where the capillary segment was visible. We used a purpose-built citizen science data analysis platform, StallCatchers.com, that enabled volunteers to score ~26,000 individual capillary segments as either flowing or not using 2PEF image stacks. We recently reported on the methodological details and validation of this StallCatchers based scoring [58]. Briefly, we used a convolutional neural network, called DeepVess, to segment the 2PEF image stack into voxels that were within vs. outside the vasculature [66]. Individual capillary segments were identified using standard dilation and thinning operations to define vessel centerlines [67], with capillary segments defined as the path between two junctions. To restrict this analysis to capillaries, we excluded all segments with diameter greater than 10 μm. Image stacks were then created that each had a single identified capillary segment outlined and these stacks were analyzed by citizen scientists using StallCatchers to score the identified segment as flowing or stalled. Each segment was scored by multiple volunteers, each of whom had a sensitivity defined by their performance on capillary segments we knew to be flowing or stalled, and we computed a weighted “crowd confidence” score representing the likelihood of each segment being stalled. Laboratory researchers then looked at these capillary segments as a final validation, starting with segments with the highest crowd confidence score for being stalled. The crowd confidence score ranged from 0 to 1, and laboratory researchers examined vessels with a score between 0.5 and 1, a total of 259 capillary segments. For crowd confidence scores between 0.9 and 1, laboratory researchers concluded 95% were stalled, while for crowd confidence scores between 0.5 and 0.6, only 1 out of 80 vessels were not flowing. The initial scoring by citizen scientists decreased the number of capillary segments laboratory researchers needed to evaluate by a factor of 100. We report the density of non-flowing capillaries as stalls per cubic millimeter.

Characterization of geometric properties of cortical capillaries

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

Estimation of capillary tube hematocrit

Tube hematocrit, \( Hct_{\text{tube}} \), was calculated as previously described [65, 68], where \( Hct_{\text{tube}} = \frac{fV_{\text{RBC}}}{\pi R^2} \), and \( f \) is the RBC flux, \( V_{\text{RBC}} \) is the mean corpuscular volume of mouse RBCs, \( v \) is
centerline RBC speed, and \( R \) is the vessel radius. RBC flux was extracted from line-scan data by counting the number of cells present in the capillary linescans. We only included vessels with single-file RBC motion, where each RBC made a distinct streak in the space-time image. We took the mean corpuscular volume of mouse RBCs to be 45 \( \mu m^3 \) [69].

**Immunostaining of brain tissue**

For evaluating cell proliferation, mice received intraperitoneal injection of 5-ethynyl-2'-deoxyuridine (EdU; E10415, ThermoFisher Scientific) at a dose of 25 mg/kg body weight every day for four days before harvesting the brains. Mice were sacrificed by lethal injection of pentobarbital (5 mg/100 mg). Brains were extracted as previously described [70] and cut in half along the center line. One hemisphere was snap frozen in liquid nitrogen for future protein extraction and the other half was kept in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 48 hours at 4˚C and subsequently placed in 30% sucrose for histological analysis. Immunohistochemistry was performed on a set of coronal sections cut on a cryotome with OCT media at a thickness of 30 \( \mu m \). From each mouse, every sixth section was mounted, washed with PBS, and blocked 1 hour at room temperature (3% goat serum, 0.1% Triton-X100 in PBS). Sections were then incubated overnight at 4˚C with primary antibodies against Iba1 (1:500, rabbit anti-mouse Iba1; 019–19741, WAKO) and GFAP (1:500, chicken anti-mouse GFAP; ab53554, Abcam) in the same buffer. Secondary antibodies (goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa Fluor 594; ThermoFisher Scientific) were used at 1:300 dilution and added to the slides for 3 hours at room temperature. Cell proliferation was detected through use of the Click-iTTM EdU Cell Proliferation Kit for Imaging (Alexa Fluor 647 dye, ThermoFisher Scientific). Methoxy-X04 was used to counterstain amyloid deposits (1 mg/ml MeO-X04 (5 mg/ml in 10% DMSO, 45% propylene glycol, and 45% saline) for 15 minutes at room temperature, 4920, Tocris). Hoechst 33342 was used to label cell nuclei (3 \( \mu g/ml \), ThermoFisher Scientific). Sections were washed with PBS before mounting with Richard-Allan Scientific Mounting Medium (4112APG, ThermoFisher Scientific). Images were obtained using confocal microscopy (Zeiss Examiner.D1 AXIO) operated with Zen 1.1.2 software. Z-stack images of the hippocampal and cortical regions of each slide were acquired with 1 \( \mu m \) optical section thickness (three adjacent images between the suprapyramidal blade of the granule cell layer of the dentate gyrus and CA1, one image in CA3, and two adjacent images in the cerebral cortex taken directly toward the cortical surface from the hippocampus). Images were then binarized using a manually determined threshold. Appropriate thresholds varied between mice and were adjusted to ensure that all morphologically relevant objects were recognized. The fraction of pixels above threshold (%Area) and the integrated density (product of mean gray value and area) was determined across sections for cortical and hippocampal regions. Amyloid plaques were manually traced using the freehand selection tool and the area was extracted. All sections were stained and imaged in parallel. Researchers were blinded in this analysis as to whether mice were part of the running or sedentary group.

**Elisa assay**

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

**Statistical analysis**

To determine statistical significance of differences between groups, the data was first tested for normality using the Shapiro-Wilk normality test. In case of normality, the statistical comparison was performed using an unpaired t-test for comparison between two groups. In case of non-normal distribution, the Mann-Whitney test (two groups) was used. For the analysis of blood flow speed and capillary diameter, a nested two-way analysis of variance (ANOVA) was carried out to account for possible interactions between individual mice. We further used post-hoc sensitivity analysis (G*Power) to determine how large the difference between groups would have had to be for the difference to be detected in cases where differences between groups were not observed. P-values less than 0.05 were considered statistically significant and we used a standardized set of significance indicators in figures: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Boxplots show the median with a black line; the mean is indicated with a red line. The box spans between the 25th and the 75th percentile of the data, defined as the interquartile range (IQR). Whiskers of the boxplots extend from the lowest datum within 1.5 times the IQR of the lower quartile of the data to the highest datum within 1.5 times the IQR of the highest quartile of the data. Correlation analysis was performed via the Pearson Product-Moment Correlation, with best fit lines indicated in red. Statistical analysis was performed and graphs were created using Prism8 (GraphPad).

**Results**

**Running distance**

To determine the effects of three months of voluntary exercise in 13 month old APP/PS1 mice, we provided single-housed mice with unrestricted access to a monitored running wheel inside their home cages (Fig 1A) [71]. All mice reliably ran on the wheels on a daily basis, with just under a factor of three variance in the average daily running distance between individual animals (Fig 1B) and no significant change in distance over time within individual mice (Fig 1C). Sedentary, control APP/PS1 mice were singly housed for the same duration, but without access to a running wheel.

**Running improved some measures of spatial memory performance**

To investigate cognitive function in running versus sedentary APP/PS1 mice, we performed a variety of behavioral tests aimed at getting an initial insight into different aspects of memory performance. Running mice achieved a significantly higher preference score in the object replacement test (OR) than sedentary mice, i.e. they spent significantly more time exploring the replaced object than the object in the familiar location as compared to the sedentary mouse (Fig 2A; P = 0.014). There was also a significant correlation between individual running distances and OR performance (Fig 2B; $R^2 = 0.58 P = 0.028$). Conversely, we did not observe an increase in spontaneous alternation between the three arms of the Y-maze (Fig 2C), nor increased exploration time for a novel object in the novel object recognition (NOR) test (Fig
Voluntary running does not increase capillary stalling in APP/PS1 mice

Running increased hippocampal neurogenesis and decreased hippocampal amyloid deposition but did not affect brain inflammation

Exercise has been shown to exert its effects through multiple pathways, with increased hippocampal neurogenesis being one of the most consistently described effects in both patients [72], and in AD mouse models [73, 74]. Consistent with these previous findings, we found an increased number of proliferating neuronal stem cells in the dentate gyrus of running, as compared to sedentary, mice, as detected by the number of EdU-positive cells (Fig 3A and 3B; \( P = 0.03 \)). In the same hippocampal tissue sections, as well as in cortical slices (Fig 3C), we further examined the density of Iba1 and GFAP staining to quantify any differences in the density of microglia and astrocytes, respectively, providing a measure of brain inflammation. We found no notable differences in microglia (Fig 3D) or astrocyte (Fig 3E) density between running and sedentary APP/PS1 mice in both hippocampal and cortical regions. We also
quantified the density of amyloid plaques that were labeled with Methoxy-X04 (Fig 4A). We found a difference in the volume occupied by amyloid plaques in hippocampal tissue sections (Fig 4B, left; \( P = 0.045 \)), but not in cortical slices (Fig 4B, right). We also did not find a significant difference in number of hippocampal or cortical amyloid plaques (S2 Fig). Using ELISA assays, we quantified the concentration of A\( \beta \)1–40 and A\( \beta \)1–42 in brain lysates and saw no differences between running and sedentary mice in both the soluble and insoluble fractions (Fig 5).

Voluntary running did not affect capillary blood flow

The trends toward improved spatial short-term memory function (Fig 2), the clear increase in neurogenesis, as well as the lack of a notable impact on brain inflammation (Fig 3) and the decrease in hippocampal amyloid deposition (Fig 4) that we observed are consistent with previous studies of the impact of exercise on mouse models of AD [75]. We next sought to determine if these exercise-mediated changes were correlated with an increase in brain blood flow, perhaps linked to a decrease in the incidence of non-flowing capillaries. We used a crowdsourced approach to score individual capillary segments as flowing or stalled based on the motion of red blood cells (which appear as dark patches within the fluorescently-labeled blood plasma in 2PEF image stacks) (Fig 6A). Contrary to our initial hypothesis, we did not observe a decrease in the incidence of stalled capillaries in running APP/PS1 mice as compared to sedentary controls (Fig 6B). There was also no statistically significant difference in the number of stalls between individual animals in each group. We calculated the post-hoc sensitivity of this analysis (G* Power; inequality of proportions; \( \alpha = 0.05, \beta = 0.80 \)) with the incidence of stalling we measured and the number of capillaries characterized in each group, which suggested we...
could detect changes in the incidence of stalled capillaries of 0.3% or larger. We further quantified red blood cell flow speed and vessel diameter in cortical capillaries (Fig 6C), and saw no differences, on average, between running and sedentary APP/PS1 mice (Fig 6D). Again, there was no statistically significant difference in average capillary flow speed or diameter between individual animals in each group. Post-hoc sensitivity analysis (G*Power; Student t-test, $\alpha = 0.05, \beta = 0.80$) with the realized variance of the measurement and the mean speeds from the running and sedentary groups suggested we could detect a 30% change in flow speed. Capillary
speed and diameter were also not found to be correlated with the total distance ran by animals (S3 Fig). In addition, we calculated capillary tube hematocrit and found no difference between running and sedentary mice (S4 Fig). To further explore potential exercise-induced changes in the brain vasculature, we used a convolutional neural network-based segmentation algorithm, DeepVess [66], to segment 3D image stacks of the cortical vasculature of running (Fig 7A) and sedentary (Fig 7B) APP/PS1 mice and we characterized the capillary density (Fig 7C), capillary diameter (Fig 7D), capillary segment length (Fig 7E), and capillary tortuosity (Fig 7F). None of these parameters differed between running and sedentary APP/PS1 mice.

**Discussion**

Physical activity is correlated with attenuation of cognitive impairment, amelioration of age-related changes in the brain, and reduced risk of dementia [76, 77]. While exercise has been found to have a positive effect on cognition and brain health in aging individuals [78], the underlying mechanisms have not been fully elucidated. In this study, we investigated the effects of several months of voluntary wheel running on memory function, AD-related brain pathology, and cortical blood flow in aged APP/PS1 mice.

Impairments in spatial learning and memory in APP/PS1 mice have been reported in mice as young as seven months of age [79, 80]. We found performance in the object replacement task in 12-month-old APP/PS1 mice was improved by three months of voluntary wheel running, as compared to sedentary controls, while no improvements were detected in the novel object recognition task and Y-maze tasks. This inconsistency might be attributed to the small number of mice used in this pilot study, but this exercise-related improvement in performance on some memory-related tasks but not on others is consistent with similarly mixed impacts of exercise in previous studies of memory function in mouse models of AD [75] and in AD patients [81]. While a variety of brain regions are likely involved in each of these memory tasks, it has been shown that memory of an object’s spatial location (evaluated with object...
replacement task) is highly dependent on hippocampal regions, while memory of an object’s intrinsic characteristics (evaluated with novel object task) also involves significant contributions from other brain regions, such as the temporal lobe [82–84]. It is possible that exercise differentially improves function in different brain regions and this contributes to the mixed impact of exercise on different memory tasks. However, we note that we had only a limited number of mice per group, and could not fully assess short-term memory function in the cohort size used in this study and additional experiments would be need to further explore these results.

Consistent with a broad consensus in the literature [37, 40], we observed increased neural stem cell proliferation in the dentate gyrus of the hippocampus in exercising APP/PS1 mice, as compared to sedentary controls. While we saw a reduction in hippocampal amyloid deposits with exercise, we did not observe changes in brain inflammation, as assayed by microglia and astrocyte density, or in amyloid-beta monomer concentrations. A number of studies in

Fig 6. Running did not decrease the incidence of non-flowing capillaries, not increase capillary blood flow in the brain of APP/PS1 mice. A Representative 2PEF image sequence showing both flowing and stalled capillary segments over 7 s. The blood plasma was labeled with Texas Red-dextran and the dark patches in the vessel lumen were formed by blood cells. The lack of motion of these dark patches in the lower capillary indicates stalled blood flow. (Stalled capillary is indicated with arrows). B Density of capillaries with stalled blood flow in running (RUN) and sedentary (SED) APP/PS1 mice. C Representative 2PEF image of a cortical capillary (left) and a space-time image from repetitive line scans along the centerline of the capillary segment (right). The diagonal streaks in the space-time image are formed by moving red blood cells, with a slope that is inversely proportional to the blood flow speed. D Capillary blood flow speed plotted as a function of vessel diameter for cortical capillaries from running (RUN) and sedentary (SED) APP/PS1 mice (points represent individual capillary measurements; 15 vessels were measured per mouse; nested two-way ANOVA).

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different mouse models of AD similarly found differential effects of exercise on measures of brain inflammation or density of amyloid deposits due to exercise [16, 39, 85, 86].

We have shown that neutrophils plug a small fraction of brain capillary segments in the APP/PS1 and 5xFAD mouse models of AD, and that this contributes to the overall brain blood flow reductions seen in these mice [57]. Contrary to our initial hypothesis that exercise could decrease the microvascular dysfunction that underlies these capillary stalls, we did not find a

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Fig 7. Running did not alter capillary density or geometry. A and B Representative 2PEF image stacks of fluorescently-labeled cortical vasculature (middle) and the segmentation of this image (right) from running (RUN) and sedentary (SED) APP/PS1 mice, respectively. C Density; D Diameter; E Length and; F Tortuosity of capillary segments from running (RUN; n = 5514 capillaries) and sedentary (SED; n = 7251 capillaries) APP/PS1 mice.

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decrease in the number of non-flowing capillaries in running APP/PS1 mice, as compared to sedentary controls. Further, we did not detect any differences in average capillary blood flow speeds between running and sedentary APP/PS1 mice.

While there is clear consensus that reduced brain blood flow in both AD patients and mouse models is a key feature of the disease, the effects of exercise on cortical blood flow in AD remain up for debate. Consistent with our findings in APP/PS1 mice, however, recent studies in patients with dementia (diagnosed as mild to moderate AD in Refs. [87] and [88]) showed that 3 times per week 60 min of aerobic exercise led to improved cognition [87, 89, 90], but this effect was not correlated with increased cerebral blood flow [88, 91]. Finally, we did not observe changes in the density or geometry of cortical capillaries in APP/PS1 mice that exercised, compared to sedentary controls.

It is important to note that we used an unrestricted exercise regimen. While the activity pattern on the running wheel tends to consist of several short bursts of high intensity effort, followed by short break [16], which could be taken to resemble interval training in humans, the overall duration of exercise exceeds what would typically be prescribed to a human patient. However, while unrestricted and restricted voluntary wheel running potentially differ in some of their effects on physiological parameters, it has also been shown that major effects such as increased hippocampal neurogenesis are comparable between the two [16].

In addition, it is worth noting that the imaging was done under isoflurane-induced anesthesia. Isoflurane is known to be a vasodilatory substance, which could impact studies of brain vascular function. However, we recently showed that isoflurane did not lead to a difference in the fractions of capillaries that were stalled between awake or anesthetized APP/PS1 mice [57], although there was a subtle impact of isoflurane on capillary stalling in wild-type mice [92]. Furthermore, since both groups of animals were imaged under the influence of isoflurane, any effect would be present in both the running and sedentary groups.

Finally, a major limitation of this pilot study is the small sample size. While we were adequately powered to detect a difference in capillary stalls and resting blood flow, we were underpowered in other aspects of this study, including the behavioral and immunostaining assays, which limits our ability to draw generalizable conclusions from these results.

While we did not observe an impact of exercise on the capillary stalling phenomena we recently tied to brain blood flow deficits in AD mouse models, there are other aspects of cortical microvascular dysfunction that have been shown to occur in mouse models of AD that we did not examine. For example, mouse models of AD have shown increased blood brain barrier permeability [93], more contractile pericytes around capillaries [54], attenuation of cerebral blood flow regulation mechanisms (neurovascular coupling [94] and autoregulation [95]), as well as hypercoagulability [55, 56]. It is possible that exercise could positively impact some of these other aspects of microvascular dysfunction in AD, although our observation of no differences in average capillary flow speeds between exercising and sedentary APP/PS1 mice suggests that pathologies that impact flow are not likely modulated by exercise.

Supporting information

S1 Fig. Running did not affect locomotor activity in behavioral assays. Both running (RUN) and sedentary (SED) APP/PS1 mice exhibited robust exploration, as assayed by the time spent exploring the old and replaced or new objects, in the A object replacement test, and B novel object recognition test. Running and sedentary mice also had C the same number of arm entries, on average, in the Y-maze task, and D the same average total track length in the open field test.

(DOCX)
S2 Fig. No difference was found in number of amyloid plaques. Amyloid plaque density, represented as the number of plaques that were Methoxy-X04 positive; from the hippocampus (left) and cortex (right) of RUN and SED APP/PS1 mice. Animal numbers: RUN: n = 4; SED: n = 4.

S3 Fig. No correlation was found between the total running distance and the A average capillary diameter or B average capillary flow speed. Animal numbers: RUN: n = 4; SED: n = 4.

S4 Fig. Capillary tube hematocrit from cortical microvascular network of running (RUN) and sedentary (SED) APP/PS1 mice. A An example image of a line scan, showing the cells counted in a segment. B Average tube hematocrit in RUN and SED APP/PS1 mice. C Tube hematocrit as a function of capillary diameter. Animal numbers: RUN: n = 4; SED: n = 4.

S1 Table. Characteristics of mice used in this study. Running (n = 4) and sedentary (n = 4) mice of both sexes ranging from 10 to 13 months at the start of the study. Indicated is their average daily running distance in km.

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