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A Comparative Study Evaluating the Impact of Physical Exercise on Disease Progression in a Mouse Model of Alzheimer's Disease

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Abstract. Evidence suggests that physical exercise can serve as a preventive strategy against Alzheimer's disease (AD). In contrast, much less is known about the impact of exercise when it is introduced after cognitive deficits are established. Using the TgCRND8 mouse model of amyloidosis, we compared the effects of exercise as an intervention strategy aimed at altering disease progression. Voluntary running for 1 month or 2 months was introduced in 3-month-old TgCRND8 mice, which exhibit amyloid-beta (Aβ) plaque pathology and cognitive deficits at this age. Specifically, we examined Aβ plaque load, spatial memory, and neurogenesis in the dentate gyrus in the hippocampus. After 1 month of running, TgCRND8 mice spent more time in the novel arm of the Y-maze compared to the familiar arms, indicating improved memory. The levels of doublecortin (a marker of immature neurons) were increased in TgCRND8 mice running for 1 month, but with no significant difference in the number of new mature neurons or plaque burden. As the disease progressed, running prevented further deficits in the Y-maze performance and hippocampal neurogenesis and it reduced plaque load pathology in TgCRND8 mice running for 2 months, compared to non-running transgenics. Therefore, the impact of running on memory, neurogenesis, and amyloid pathology was of greater significance when sustained through later stages of the disease.

Keywords: Alzheimer's disease, amyloid pathology, neurogenesis, physical exercise, spatial memory

INTRODUCTION

Alzheimer's disease (AD) is characterized by the progressive accumulation of amyloid-beta peptides (Aβ), neuronal loss, and cognitive impairments [1, 2]. The hippocampus, a neurogenic brain area involved in learning and memory [3], is severely affected in AD [4, 5]. The accumulation of Aβ in the hippocampus can contribute to impaired neurogenesis, neuronal loss, and cognitive deficits [6–8].

Several AD pathologies are lessened by physical exercise when introduced prior to disease onset in mouse models [9–11] and in humans [12, 13]. Recently, Tapia-Rojas et al. [11] evaluated the benefits of exercise introduced prior to the accumulation of amyloid plaques and detection of cognitive deficits in APPswe/PS1ΔE9 mice. Exercise, used a preventative measure, reduced amyloid load and astrogliosis, increased neurogenesis, and improved spatial memory [11].

The benefit of physical activity has also gained recognition as a therapeutic intervention for those who are suffering from mild cognitive impairments (MCI) and are at risk of developing AD [14]. Evidence supports the link between physical exercise

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and improved cognitive ability in pre-clinical dementia and MCI [15–19]. However, for many clinical applications, exercise can be considered as an intervention, i.e., starting after the onset of AD pathology (reviewed in [15, 16]). Nichol et al. [20] showed that three weeks of wheel running improved cognitive performance in aged Tg2576 mice. Other rodent studies where running was introduced in the presence of plaque pathology and cognitive deficits show a lack of significant improvements to amyloidosis, neurogenesis, and spatial memory [21, 22].

To further evaluate the potential of physical exercise as a treatment option, we used the TgCRND8 mouse model of amyloidosis and introduced voluntary running as an intervention strategy in 3-month-old mice, an age where Aβ pathology and spatial memory deficits are present [23, 24]. Memory, neurogenesis and Aβ deposition were evaluated in two independent cohorts of mice, exposed to two different exercise durations. After 1 month of running, TgCRND8 mice showed signs of memory improvement and increased expression of the immature neuronal marker doublecortin (DCX) in the hippocampus relative to non-running transgenic animals. The benefits of exercise after 2 months of running included a significant augmentation in memory performance, increased hippocampal neurogenesis, and decreased amyloid pathology in running TgCRND8 mice compared to non-running transgenics. Overall, our data support the use of physical exercise as relevant disease-modifying intervention, even at stages of established AD pathology.

METHODS

Animals

We used the TgCRND8 mouse model of amyloidosis maintained on the C57BL/6 and C3H hybrid background and expressing the Swedish (KM670/671NL) and Indiana (V717F) mutations of the amyloid precursor protein (APP) [23]. All procedures were conducted in accordance with guidelines established by the Canadian Council on Animal Care and protocols approved by the Sunnybrook Research Institute Animal Care Committee.

Exercise paradigm and monitoring

Three-month-old TgCRND8 mice and non-transgenic littermates (non-Tg) were randomly assigned to either standard housing (non-running group) or to a cage equipped with a spinning disk (running group; BioServ Fast Trac and Mouse Igloo) and bicycle counter (CatEye, Strada Cadence CC-RD200). Figure 1A illustrates the age at which animals enter the study (3-month-old), time at which they were pulsed with 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg) for the first 10 days of the experiment to study neurogenesis, their running paradigm, behavioral testing schedule, and termination ages (4- and 5-month-old). All animals were singly housed and their weight and usage of spinning disks were monitored every other day. Mice were housed in a 12 h (07 : 00–19 : 00) light-dark cycle in a room maintained at 22°C with *ad libidum* access to food and water. A total of 17 animals per cohort were used for behavioral tests, daily activity, and weight monitoring. Brains isolated from 7 animals per cohort were used for immunohistochemistry. Brains from the remaining animals were allocated to other studies. Details on the number and sex of animals used in the current study are provided in Fig. 1B.

Behavioral tests

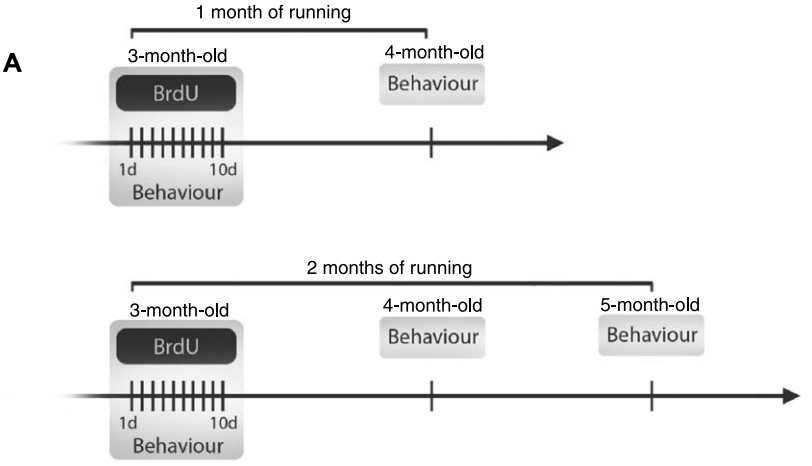
Two separate cohorts of animals were used for the 1 month and 2 months running paradigms in order to conduct all behavior tests on naïve mice.

Exploration of a novel environment

The open field test consists of exploration of a novel environment (cage dimensions: 20×40 cm) for a period of 10 min. Each experiment was video-recorded (Logitech Webcam Pro 9000) and analyzed with the Videotrack Go system (Viewpoint Life Sciences, Montreal, Canada). During video analysis, the area of the open field was divided into a center field (7×26 cm) and a peripheral ring (6.5 cm from the border of the open field); distance travelled and time spent in each area were quantified. Four-month-old animals from all cohorts were evaluated for exploration of the novel environment.

Spatial working memory

Early cognitive deficits in AD patients relate to episodic memory. However, in mice with AD pathology, impairments in spatial working memory are generally the first to be observed and they are also the best studied and modeled [25]. The Y-maze, T-maze [26, 27], and Morris water maze [28], are commonly



B

	N (total: female, male)			
	Running		Non-Running	
	TgCRND8	Non-Tg	TgCRND8	Non-Tg
Daily wheel activity and weight	17: 9, 8	17: 7, 10	17: 8, 9	17: 7, 10
Behaviour	17: 9, 8	17: 7, 10	17: 8, 9	17: 7, 10
Immunohistochemistry	7: 3, 4	7: 3, 4	7: 3, 4	7: 3, 4

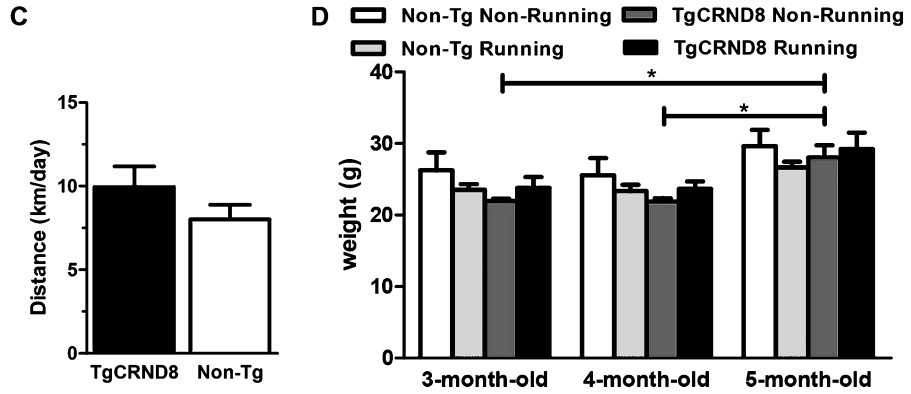


Fig. 1. Experimental paradigm. A) Timeline of the study. At 3 months of age, mice were randomly assigned to either running or non-running groups. Mice were injected with 50 mg/kg BrdU for the first 10 days of the study. Behavioral tests were performed at 3, 4, and 5 months of age in independent cohorts of animals. 4- and 5-month-old mice ran for 1 and 2 months, respectively. B) Table illustrating the number of animals, females and males, in each cohort. C) Average distance ran by TgCRND8 mice (black bar) and non-Tg littermates (white bar). D) Weight of animals at 3, 4, and 5 months of age. Statistics: (C) Student *t*-test, (D) 2-way ANOVA. Significance: $^{*}p < 0.05$. Data represent the mean ± SEM. N = 17 per group.

used for testing reference and spatial working memory in mice. Here, we used the Y-maze task, which has been validated by our group and others in TgCRND8 mice [24, 29, 30]. In comparison to the Morris water maze, the Y-maze is less stressful and requires minimal training, thereby minimizing potential confounds of stress hormones and the learning process on outcome measures [31–34].

Mice are inquisitive in nature [35], and those with intact working memory typically spend more time in the novel arm compared to the two familiar arms of the Y-maze [36]. Here, the Y-maze paradigm con-

sisted of a 10-min acquisition session in which naïve animals explored two arms (familiar arms 1 and 2) of the Y-maze with the third arm blocked, followed by 90 min of rest in a home cage. Animals were then returned to the Y-maze for a 5-min retention session with access to all three arms of the maze, including arm 3, which is referred to as the novel arm. The test was video-recorded (Logitech Webcam Pro 9000) and analyzed with the Videotrack Go system (Viewpoint Life Sciences) to measure the time spent in each arm. Time spent in the center between the three arms was not included in the analysis. Separate cohorts of mice were evaluated with the Y-maze task at 3, 4, and 5 months of age.

Immunohistochemistry for cell survival and differentiation

Mice were deeply anesthetized with a cocktail of 150 mg/kg ketamine and 10 mg/kg xylazine administered with an intraperitoneal injection. Animals were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA for 24 h and equilibrated in 30% sucrose. They were sectioned coronally at 40 μm using a sliding microtome and stored in cryoprotectant at –20°C.

To quantify the extent of neurogenesis, the number of cells immunopositive for BrdU and mature neuronal nuclei (NeuN) antigen were measured. Astrogenesis was quantified as the number of cells immunopositive for BrdU and glial fibrillary acidic protein (GFAP). Immature neurons were characterized as cells immunopositive for DCX.

For BrdU/NeuN/GFAP immunolabeling, sections were incubated in 2N HCl for 30 min at 37°C for antigen retrieval, and then neutralized in borate buffer (pH 8.5) for 10 min. After rinsing with PBS and blocking with 1.5% BSA, 2% donkey serum and 0.15% TritonX-100 for 1 h, sections were incubated with antibodies against GFAP (1 : 500, AbDSerotec, AHP1468), NeuN biotinylated (1 : 200, Chemicon, MAB377B) and BrdU (1 : 400, Serotec, OBT0030) overnight at 4°C. Sections were rinsed and incubated with secondary antibodies appropriate for the primary antibodies used, i.e., donkey-anti-goat IgG Cy5 (1 : 200, Jackson Immunolabs, 705-175-147) for GFAP, streptavidin Alexa Fluor 488 (1 : 200, Jackson Immunolabs, 016-540-084) for NeuN and donkey-anti-rat IgG Cy3 (1 : 200, Jackson Immunolabs, 712-165-153) for BrdU.

For DCX immunolabeling, sections were blocked with 10% donkey serum and 0.25% TritonX-100 for 1 h and incubated with anti-DCX antibody for 48 h at 4°C (1 : 200, Santa Cruz, sc-8066). Sections were washed and incubated with donkey anti-goat Cy3 secondary antibody (1 : 200, Jackson Immunolabs, 705-165-147).

Imaging and quantification

For each animal, three sections (1 in 12 series) containing the dentate gyrus (DG) were used to quantify BrdU cell population and colocalization of BrdU with markers for neurogenesis and astrogenesis. An adjacent set of 3 sections per animal (1 in 12 series) was used for quantitative analysis of DCX immunoreactivity. For each immunostain, all sections were processed at the same time and imaged using the same background and threshold settings.

Immunofluorescence was detected by confocal microscopy at 63X magnification and visualized using the LSM Image Browser (Zeiss Axiovert 100M, LSM510). Fluorochromes DyLight488, Cy3 and Cy5 were excited at 488, 561 and 633 nm wavelengths, respectively. Z-stack images with 1.6 μm optical section thickness were obtained.

BrdU cells in the DG were assessed by counting all BrdU-positive cells in 6 fields of view captured at 63X magnification per section in 3 sections per animal. For the quantification of colocalization, five BrdU-positive cells were imaged for each DG (right and left) on the 3 sections per animal, for a total of 30 cells per animal. BrdU-positive cell was first identified by first detecting Cy3 (Fig. 4, column A) and then NeuN or GFAP (Fig. 4, column B and C) colocalization was determined by combining the sequential visualization of Cy2 and Cy5 channels (Fig. 4).

DCX signal was imaged at 20X magnification with a Zeiss spinning disk microscope (CSU-W1; Yokogawa Electric, Zeiss Axio Observer.Z1 - Carl Zeiss, Don Mills, Ontario, Canada) coupled to Axiocam camera and operated with Zen 1.1.2 software. The Cy3 fluorochrome was excited at a wavelength of 561 nm. Tiled Z-stack images of the entire dentate gyrus were acquired with 0.5 μm optical section thickness, and then projected to obtain a maximum intensity image (Fig. 6). The number of DCX-positive pixels in the dentate gyrus was quantified using ImageJ image analysis software, as previously described [37]. Briefly, the DCX signal was processed using a 10-pixel-wide rolling-ball subtraction, a two-pixel-wide median filter, and an automatic

threshold to determine the number of DCX-positive pixels.

Aβ immunohistochemistry and stereology

Immunostaining against Aβ plaques and quantification using design-based stereology were carried out as described previously [38]. Briefly, sections were incubated in anti-Aβ 6F/3D primary antibody (1 : 400, DakoCytomation, M0872) followed by biotinylated donkey-anti-mouse IgG secondary antibody (1 : 100, Jackson Immunolabs, 715-001-003) and subsequently incubated with streptavidin horseradish peroxidase and 3, 3′-diamidobenzidine (Vectastain Elite ABC Kit and DAB Peroxidase Substrate Kit, respectively, both from Vector Laboratories). Immunolabeled sections were analyzed with StereoInvestigator software (MBF Bioscience, Williston, VT, USA) operating a Zeiss Imager M1 microscope coupled to a digital camera and motorized stage.

For each hemisphere, the hippocampal region was divided into the DG and Cornu Ammonis (CA) (Fig. 7A and B, respectively). Total plaque numbers and mean cross-sectional plaque areas were estimated using the Optical Fractionator and the Nucleator probes, respectively. The mean surface area of Aβ was calculated by multiplying estimates from the Optical Fractionator and the Nucleator probes. Plaque quantification in the CA was based on 6-7 sections per animal sampled at an interval of 1 in 8 with 300 μm × 300 μm sampling grid and 150 μm × 150 μm counting frame. As a result, 25% of CA surface area was sampled with an average of 147 hits per animal and 0.09 coefficient of error (CE Gundersen). Plaque quantification in the DG was based on 12-13 sections per animal at an interval of 1 in 4 with 300 μm × 300 μm sampling grid and 300 μm × 300 μm counting frame. These parameters covered the entire DG for each section, with an average of 207 hits per animal and 0.07 coefficient of error (CE Gundersen).

Statistics

Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis and generation of graphs. All graphs are presented as mean ± SEM. Genotype difference in daily use of spinning disks by the mice was analyzed with the Student *t*-test. Gaussian distribution of data sets was confirmed by Shapiro-Wilk normality test. Differences in Aβ plaque load, DCX, and neurogenesis were assessed with a 2-way ANOVA. Differences in time spent in novel and familiar arms were analyzed with a 1-way ANOVA whereas differences in time spent in novel arm were analyzed with a 2-way ANOVA. Differences in weight were analyzed with a 2-way ANOVA. The Bonferroni correction was used as a *post-hoc* test. Statistical significance (alpha) was set at 0.05, and defined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Running and weight monitoring

On average, the daily distance run (Fig. 1C) by TgCRND8 mice (9.9 ± 1.7 km/day) was not statistically different from their non-Tg littermates (8.0 ± 1.1 km/day; $t_{(32)} = 1.4$, $p = 0.2$). We recorded a 98% compliance to running with 47/48 mice utilizing the spinning disk daily. The weight of TgCRND8 non-running mice increased between 3 and 5, and 4 and 5 months of age (Fig. 1D, $F_{(3,192)} = 11.0$, $^{*}p < 0.05$). No other significant changes were found in weight comparison between groups.

Open field test

No significant differences in the total distance of exploration were found between TgCRND8 and non-Tg littermates, running and non-running, at 4 months of age (Fig. 2A). The distances travelled by 4-month-old mice in the center and peripheral fields were not statistically different between groups (Fig. 2B, C). This data suggests that mice across all groups do not differ in the levels of locomotion, exploration and anxiety-related behavior, supporting the use of the Y-maze as a cognitive task based on exploration.

Y-maze task

Comparing the time spent in novel and familiar arms indicates whether spatial working memory using the Y-maze test is impaired (failure to recognize the novel arm) or preserved (capacity to recognize the novel arm) within each group of mice. At 3 months of age, TgCRND8 mice have no significant preference for the novel arm compared to the familiar arms (Fig. 2D, $t_{(32)} = 1.6$, $p = 0.13$), which is indicative of cognitive deficits. In contrast, non-Tg littermates differentiate the novel arm from the familiar arms (Fig. 2D, $t_{(32)} = 2.3$, $^{*}p = 0.04$).

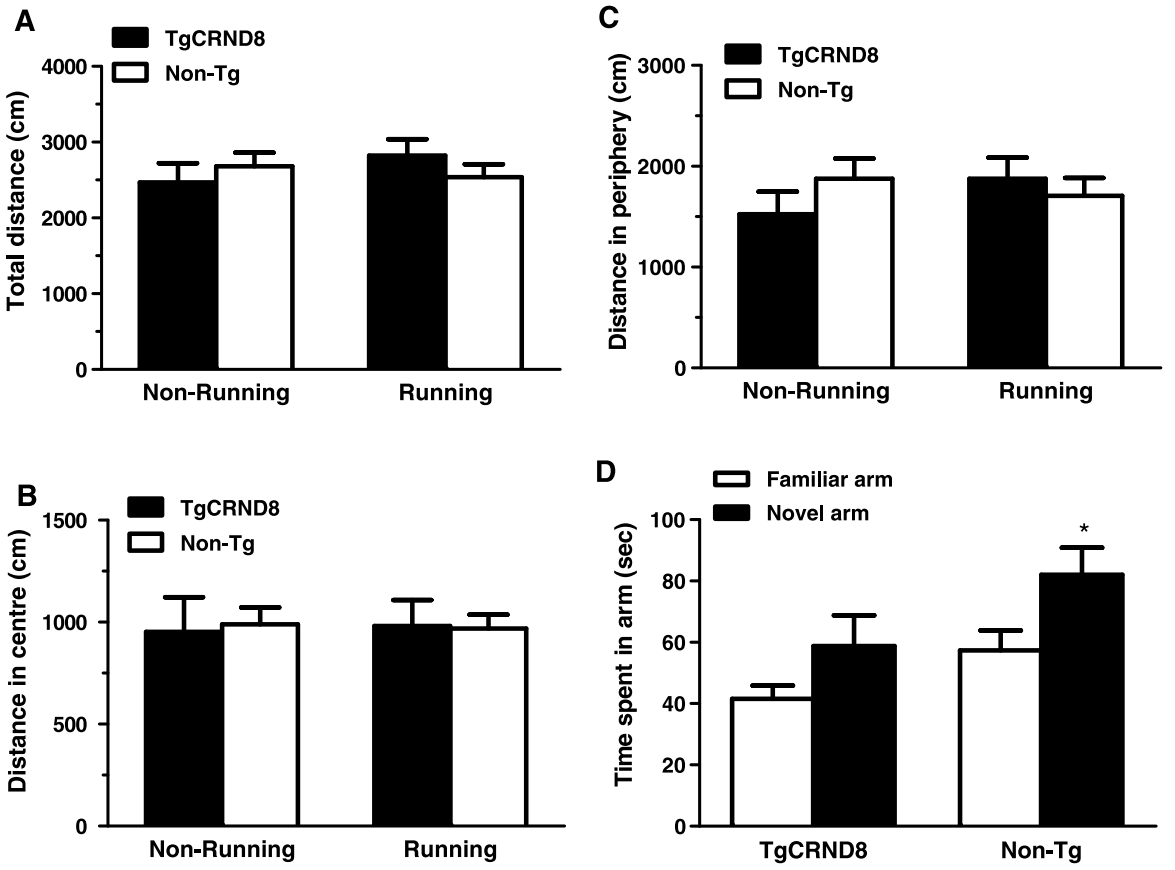


Fig. 2. Baseline behavioral assessment. A-C) Open field test in 4-month-old TgCRND8 mice and non-Tg littermates, running and non-running. A) Total distance covered in a novel environment by TgCRND8 and non-Tg mice. Distance covered in the center (B) and periphery (C) of the novel environment. D) In the Y-maze, 3-month-old non-Tg mice spent significantly more time in the novel arm (black bar) compared to familiar arms (white bar). In contrast, in age-matched TgCRND8 mice, the time spent in the novel arm was not significantly different than the time spent in the familiar arms. Statistics: 1-way ANOVA. Significance: $^{*}p < 0.05$. Data represent the mean ± SEM. N = 17 per group.

The impact of running is clear in Fig. 3, with running TgCRND8 mice being able to recognize the novel arm, as indicated by greater time spent in the novel arm compared to familiar arms of the maze (Fig. 3A, 4 months, $F_{(3,126)} = 4.4$, $^{*}p = 0.01$; 5 months, $F_{(3,126)} = 2.9$, $^{*}p = 0.03$). By contrast, TgCRND8 non-running mice failed to recognize the novel arms of the maze at 4 and 5 months of age (Fig. 3A, $F_{(3,126)} = 1.2$, $p = 0.5$; $F_{(3,126)} = 0.3$, $p = 0.9$, respectively). As expected, non-Tg mice spent more time in the novel arm compared to the familiar arms (Fig. 3A, 4 months running, $F_{(3,126)} = 2.2$, $^{*}p = 0.02$ and non-running, $F_{(3,126)} = 2.1$, $^{*}p = 0.01$; 5 months running, $F_{(3,126)} = 3.2$ $^{*}p = 0.04$ and non-running, $F_{(3,126)} = 3.6$, $^{**}p = 0.005$).

Data from the Y-maze can be used to evaluate whether spatial recognition memory (time spend in novel arm only) is different between groups. As the disease progresses, the performance of non-running TgCNRD8 declines (Fig. 3B, 5-month-old compared to 3-month-old TgCRND8 mice, indicated by the dotted line, $F_{(1,126)} = 1.8$, $^{\wedge}p = 0.04$). Non-Tg littermates at 4 and 5 months of age show no statistical difference in the Y-maze performance compared to 3-month-old non-transgenics (comparing Fig. 2D and 3B, 4 months, $F_{(1,126)} = 1.1$, $p = 0.2$, 5 months, $F_{(1,126)} = 0.2$, $p = 0.8$). Compared to their non-Tg littermates, non-running TgCRND8 mice exhibit deficits in the Y-maze test, spending less time in the novel arm (Fig. 3B, 4 months, $F_{(1,126)} = 2.6$, $^{+}p = 0.01$, 5 months, $F_{(1,126)} = 2.9$, $^{++}p = 0.008$). This impairment was abolished by running, as the time spent in the novel arm by running TgCRND8 mice and non-Tg running littermates was not statistically different (Fig. 3B, 4 months, $F_{(1,126)} = 1.0$, $p = 0.3$; 5 months, $F_{(1,126)} = 1.5$, $p = 0.2$). Further-

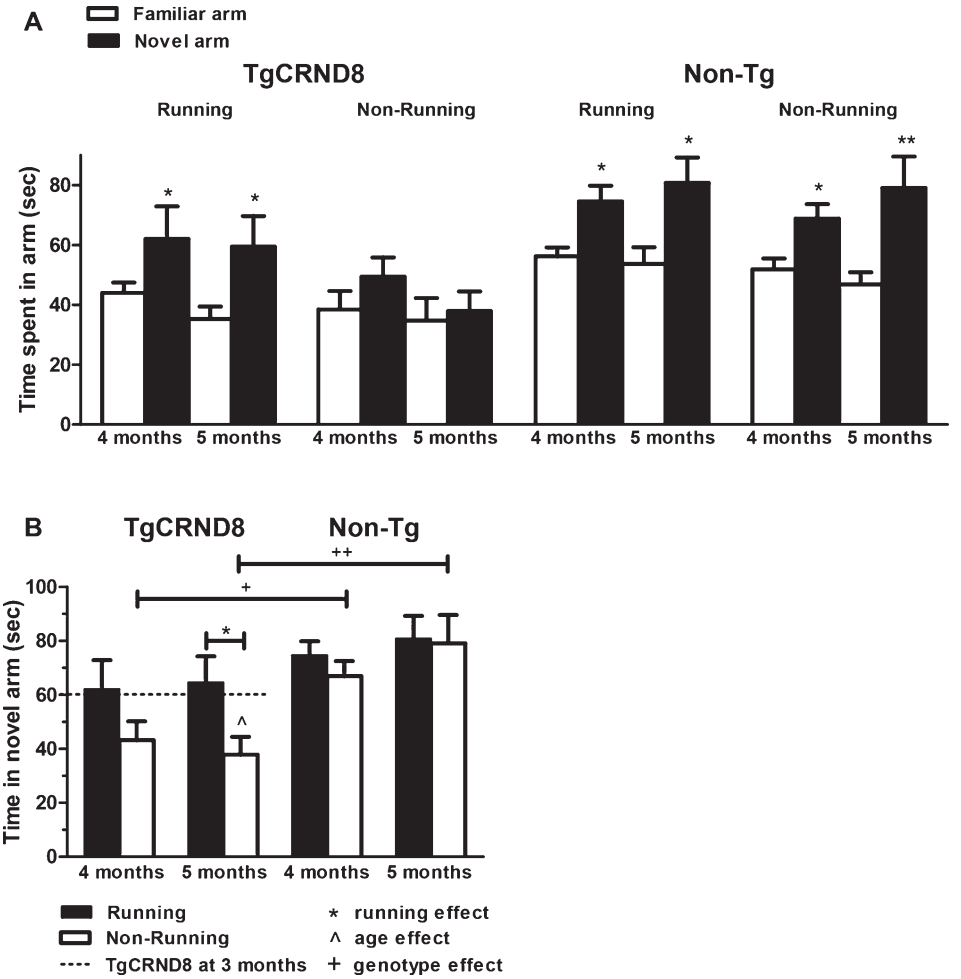


Fig. 3. Y-maze performance is improved with running in TgCRND8 mice. A,B) Mice were assessed with the Y-maze task for spatial working memory by quantifying time spent in the novel and familiar arms of the maze. A) Running TgCRND8 mice, at both 4 and 5 months of age, showed a significant preference for the novel arm compared to the familiar arms. In non-running TgCRND8 mice, no statistical difference was observed between the time spent in the novel compared to the familiar arms. Running and non-running 4- and 5-month-old non-Tg animals spent significantly more time in the novel arm compared to the familiar arms. B) Time spent in the novel arm in 4 and 5-month-old non-running and running mice was evaluated against time spent in novel arm by 3-month-old TgCRND8 mice (dotted line). Non-running TgCRND8 mice spent less time in the novel arm at 5 months of age, whereas running TgCRND8 mice maintained their performance on the Y-maze task. Statistics: (A) 1-way ANOVA, (B) 2-way ANOVA. Significance: $^{*,+,\wedge}p < 0.05$, $^{**,++}p < 0.01$. Data represent the mean ± SEM. N = 17 per group.

more, 5-month-old running TgCRND8 mice spent more time in the novel arm compared to non-running TgCRND8 mice (Fig. 3B, $F_{(1,126)} = 1.8$, $^{*}p = 0.03$).

Neurogenesis: Cell survival, differentiation, and maturation

Cells dividing in the dentate gyrus at 3 months of age incorporated BrdU and they were quantified at 4 and 5 months of age, as surviving proliferating cells (BrdU alone), and differentiating into neurons (BrdU/NeuN) or astrocytes (BrdU/GFAP) (Figs. 4 and 5).

BrdU

The levels of cell proliferation and survival in the dentate gyrus were first evaluated in all groups (Fig. 5A). The effects of running were significant at 5 months of age in TgCRND8 mice, with the number of BrdU-positive cells being greater compared to age-matched non-running TgCRND8 mice (Fig. 5A, $F_{(3,48)} = 7.7$, $^{*}p = 0.01$). This significant difference between the running and non-running groups was not observed in 4-month-old running TgCRND8 mice, most likely because of the increased number of BrdU-positive cells in 4-month-old

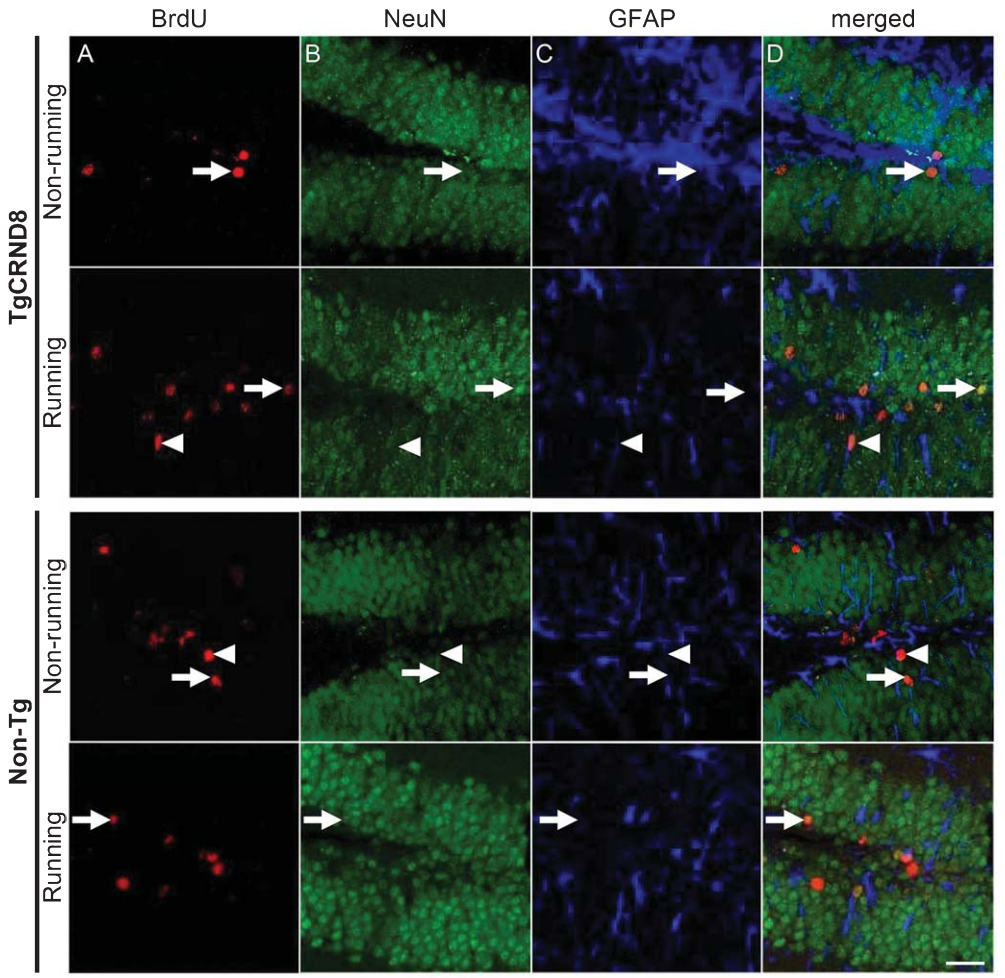


Fig. 4. Neurogenesis and astrogenesis in the dentate gyrus. A-C) Representative images of brain sections from TgCRND8 and non-Tg, 5-month-old mice, running and non-running, immunolabeled to detect (A) BrdU, (B) NeuN, and (C) GFAP. D) The merged images show colocalization of BrdU/NeuN markers (arrow) and BrdU/GFAP markers (arrowhead). Scale bar: 50 μm.

TgCRND8 mice compared to age-matched non-Tg mice (Fig. 5A, $F_{(3,48)} = 2.8$, $^{+}p = 0.02$). In non-Tg mice, running for 1 and 2 months increased the number of BrdU-positive cells (Fig. 5A, 4-month-old, $F_{(3,48)} = 4.0$, $^{*}p = 0.04$; 5-month-old, $F_{(3,48)} = 3.3$, $^{***}p = 0.0003$).

BrdU/NeuN

In TgCRND8 mice, the impact of running on neurogenesis was observed at 5 months of age, after 2 months of running, with greater number BrdU/NeuN-positive cells compared to age-matched non-running TgCRND8 mice (Fig. 5B, $F_{(3,48)} = 13.5$, $^{**}p = 0.003$). The deficit in BrdU/NeuN-positive cells found in 5-month-old non-running TgCRND8 mice compared to age-matched non-Tg mice (Fig. 5B, $F_{(3,48)} = 4.8$, $^{++}p = 0.003$) was abolished by running (Fig. 5B, $F_{(3,48)} = 0.6$, $p = 0.6$, running 5 month-old TgCRND8 mice compared to non-Tg littermates). Similarly, running also prevented the decline in NeuN/BrdU-positive cells observed with disease progression in TgCRND8 mice (Fig. 5B, $F_{(3,48)} = 2.0$, $^{\wedge}p = 0.02$; in contrast to running TgCRND8 mice at 4 and 5 months of age, $F_{(3,48)} = 1.2$, $p = 0.3$).

In 4- and 5-month-old non-Tg mice, the number of BrdU/NeuN-positive cells was greater in running cohorts compared to non-running age-matched mice (Fig. 5B, 4-month-old, $F_{(3,48)} = 5.1$, $^{***}p = 0.0002$; 5-month-old, $F_{(3,48)} = 5.7$, $^{***}p = 0.0002$).

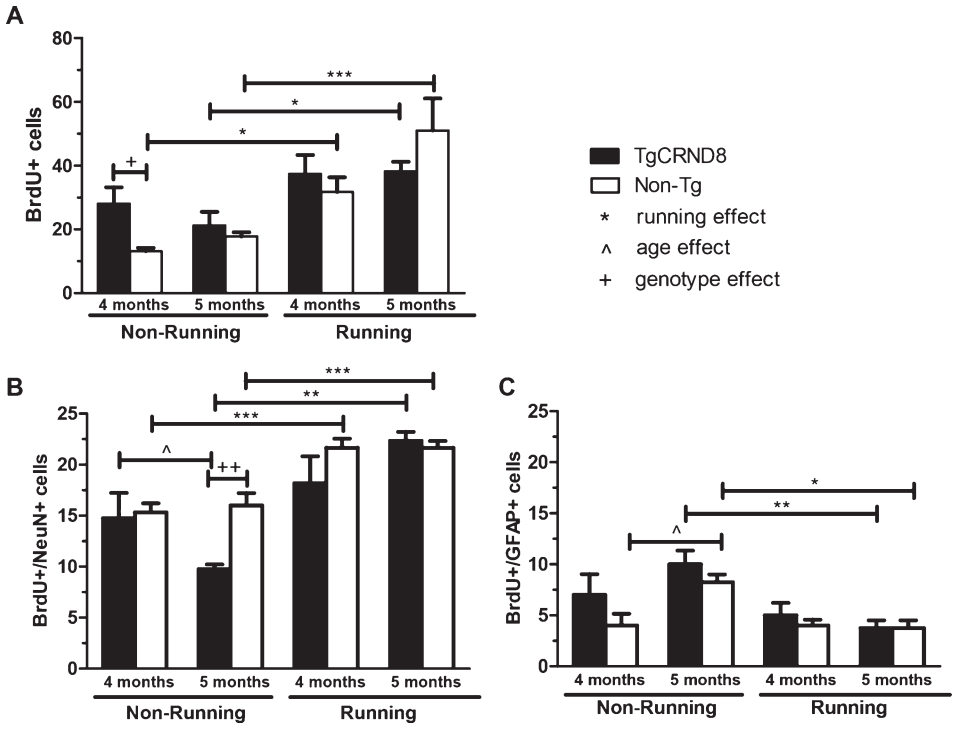


Fig. 5. The impact of running, age, and genotype on newborn cells of the dentate gyrus. A) Greater number of BrdU + cells was observed in the running groups, at 5 months in TgCRND8 mice and 4 and 5 months in non-Tg littermates, compared to age-matched non-running mice. Non-running TgCRND8 mice at 4 months of age had higher levels of BrdU + cells compared to non-Tg mice. B) The colocalization of BrdU and NeuN was used to quantify neurogenesis. At 5 months of age, neurogenesis was increased in running compared to non-running TgCRND8 and non-Tg mice. At 4 months of age, neurogenesis was increased in non-Tg mice running for 1 month compared to age-matched non-Tg mice. In non-running 5-month-old mice, neurogenesis was significantly greater in non-Tg compared to TgCRND8 animals. C) The colocalization of BrdU and GFAP was used to quantify astrogenesis. Running 5-month-old mice, TgCRND8 and non-Tg, had lower astrogenesis compared to their respective non-running groups. In non-running mice, astrogenesis was significantly greater in 5- compared to 4-month-old animals. Statistics: 2-way ANOVA. Significance: $^{*,\wedge,+}p<0.05$, $^{**,++}p<0.01$, and $^{***}p<0.001$. Data represent the mean ± SEM. N = 7 per group.

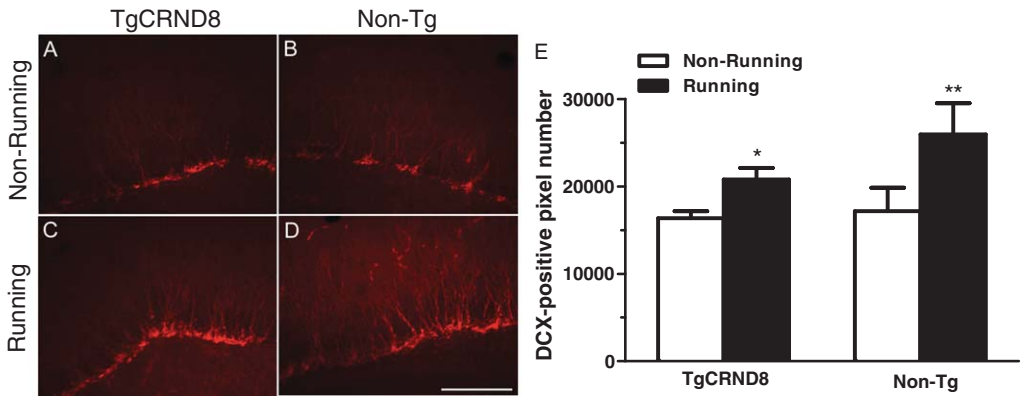


Fig. 6. Immature neurons in the dentate gyrus. Representative images of brain sections immunostained for DCX in the dentate gyrus of (A,C) TgCRND8 and (B,D) non-Tg mice. Scale bar: 100 μm. E) At 4 months of age, the number of DCX-positive pixels was significantly greater in running TgCRND8 and non-Tg mice compared to their respective non-running groups. Statistics: 2-way ANOVA. Significance: $^{*}p<0.05$, and $^{**}p<0.01$. Data represent the mean ± SEM. N = 7 per group.

BrdU/GFAP

In contrast to the BrdU/NeuN population in the dentate gyrus (Fig. 5B), a smaller number of newborn cells express BrdU and GFAP (Fig. 5C). The population of BrdU/GFAP-positive cells increased from 4 to 5 months of age in non-Tg mice (Fig. 5C, $F_{(3,48)} = 3.2$, $^{\wedge}p = 0.02$), with no statistical increase in TgCRND8 mice ($F_{(3,48)} = 1.2$, $p = 0.3$). Running from 3 to 5 months of age in TgCRND8 mice and their non-Tg littermates, maintained relatively low numbers of BrdU/GFAP-positive cells compared to non-running mice (Fig. 5C, TgCRND8, $F_{(3,48)} = 4.0$, $^{**}p = 0.008$; non-Tg, $F_{(3,48)} = 4.2$, $^{*}p = 0.04$).

DCX

Quantitative analysis of DCX immunostaining, labelling immature neurons, revealed an increased number of DCX-positive pixels in running compared to non-running mice at 4 months of age in TgCRND8 mice (Fig. 6A, C, and E, $F_{(1,24)} = 2.7$, $^{*}p = 0.01$) and non-Tg mice (Fig. 6B, D, and E, $F_{(1,24)} = 3.4$, $^{**}p = 0.009$). There was no difference in the number of DCX-positive pixels between running TgCRND8 and running non-Tg mice (Fig. 6A, B and E, $F_{(1,24)} = 1.7$, $p = 0.2$) and between non-running TgCRND8 and non-running non-Tg mice (Fig. 6C–E, $F_{(1,24)} = 0.4$, $p = 0.7$).

Exercise and Aβ plaque pathology

Aβ plaque pathology in TgCRND8 mice was quantified as Aβ plaque surface area (Fig. 7C), number (Fig. 7D), and mean size (Fig. 7E). Aβ plaque load was examined in two major regions of the hippocampal formation, namely the DG (Fig. 7A, yellow out line) and CA (Fig. 7B, red out line).

Running had a significant impact on plaque pathology in TgCRND8 mice exercising from 3 to 5 months of age. Specifically, running reduced the progression of Aβ pathology as measured by the surface area occupied by Aβ plaques (Fig. 7C; DG, $F_{(1,24)} = 3.4$, $^{*}p = 0.03$; CA, $F_{(1,24)} = 3.6$, $^{**}p = 0.004$), number of plaques (Fig. 7D; DG, $F_{(1,24)} = 4.8$, $^{**}p = 0.005$; $F_{(1,24)} = 3.5$, CA$^{*}p = 0.02$) and plaque size (Fig. 7E; $F_{(1,24)} = 2.8$, CA $^{*}p = 0.02$) compared to non-running in 5-month-old mice. Running for 1 month, from the age of 3 to 4 months, had no significant effect on the surface area, number, or size of Aβ plaques in the hippocampus (Fig. 7C–E).

As the disease progresses, Aβ plaque pathology increased significantly in the hippocampal formation (DG and CA) in both running and non-running mice (Fig. 7C–E). Specifically, comparing 4- and 5-month-old mice, significant increases were found regarding the surface of Aβ (Fig. 7C, $F_{(1,24)} = 8.5$, $^{\wedge\wedge\wedge}p < 0.001$), number of plaques (Fig. 7D, $F_{(1,24)} = 11.8$, $^{\wedge\wedge\wedge}p < 0.001$), and mean plaque size (Fig. 7E, $F_{(1,24)} = 14.1$, $^{\wedge}p < 0.05$).

Taken together, these data suggest that amyloid pathology accumulates in both running and non-running TgCRND8 mice. However, running considerably slows down the development of plaque pathology in TgCRND8 mice.

DISCUSSION

To further evaluate the potential of physical exercise as a treatment for AD, we introduced voluntary running as an intervention strategy in 3-month-old TgCRND8 mice, an age where cortical Aβ pathology and spatial memory deficits are present [23, 24]. In summary, we found that in TgCRND8 mice, running for 1 month (between 3 and 4 months of age) rescued spatial working memory and increased expression of immature neurons in the dentate gyrus. In comparison, 2 months of running (between 3 and 5 months of age) had a greater impact by maintaining improvements in memory, reducing hippocampal Aβ accumulation, and increasing neurogenesis as defined by the population of new mature neurons.

Neurogenesis in mouse models of AD has been reported as being increased or decreased depending on the model, disease stage, and methodology [39]. Several murine studies report increased cell proliferation and number of immature new neurons at early stages of Aβ pathology [40, 41]. Other studies report diminished number of newborn neurons reaching maturation [6, 7, 42–45], partly due to impaired survival of newly generated neurons [44]. Chen et al. [46] reported increased neurogenesis at early stages of neurodegeneration but not at late stages, suggesting that dynamic changes in neurogenesis were correlated with the severity of neuronal loss in DG, and perhaps serve as compensatory mechanism following neurodegeneration.

We compared the number of BrdU-positive cells, the status of immature neurons, and the number of new mature neurons at one and two months after BrdU pulse. Firstly, in non-running mice, a greater population of BrdU-positive cells was found in 4-

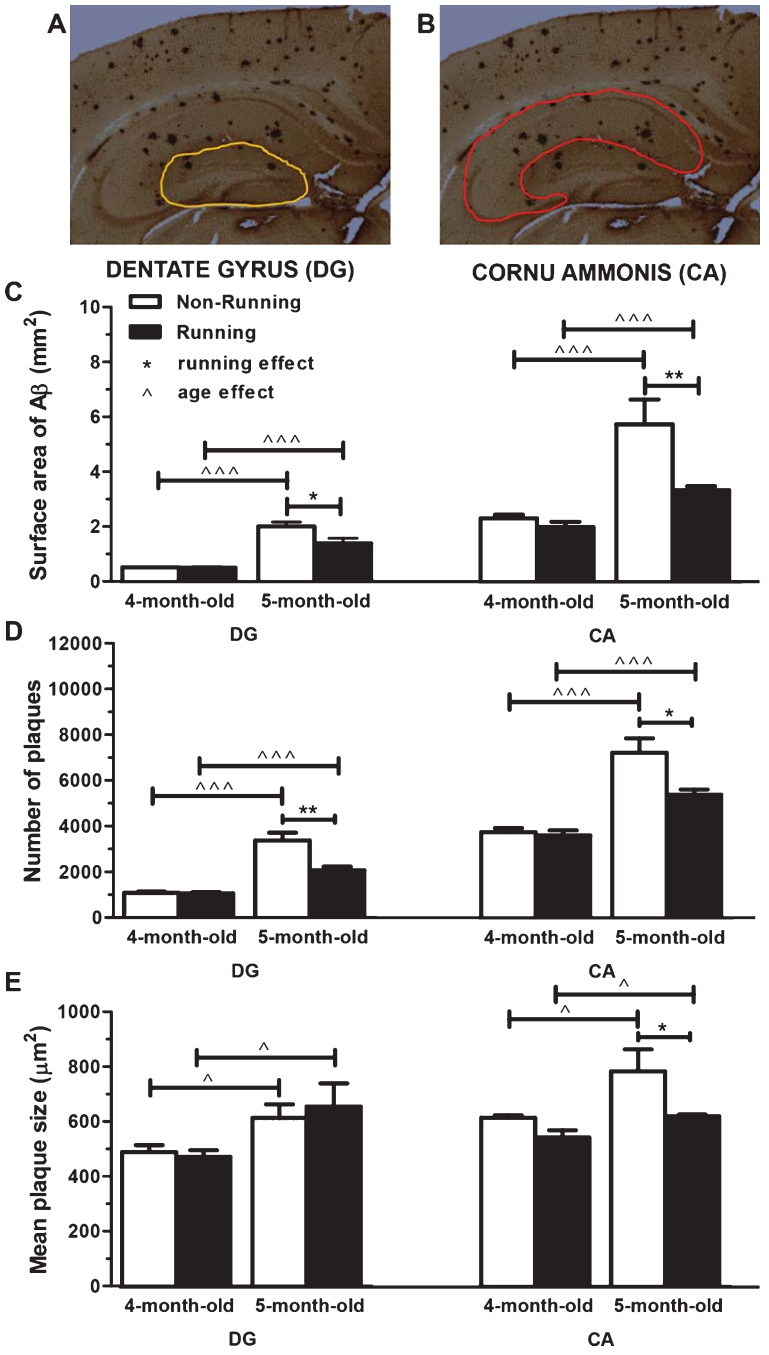


Fig. 7. The effects of running on Aβ plaque load. A,B) Representative images of Aβ immunohistochemistry on brain sections taken from a 5-month-old non-running TgCRND8. For Aβ quantification, the hippocampal formation was divided into two regions: (A) the dentate gyrus (DG, yellow out line) and (B) the Cornu Ammonis (CA, red out line). C-E) During disease progression, Aβ pathology increases from 4 to 5 months of age within the running and non-running groups, respectively. Aβ surface area (C) and the number of plaques (D) in the DG and CA, as well as mean plaque size (E) in the CA, were significantly lower in 5-month-old running compared to non-running TgCRND8 mice. Statistics: 2-way ANOVA. Significance: $^{*,\wedge}p<0.05$, $^{**}p<0.01$ and $^{\wedge\wedge\wedge}p<0.001$. Data represent the mean ± SEM. N = 7 per group.

month-old TgCRND8 compared to non-Tg mice. This increase in BrdU-positive cells may represent an initial compensatory mechanism to enhance cell proliferation in response to AβPP/Aβ exposure, as proposed by Chen and colleagues [46] and previously observed in TgCNRD8 mice at early stages

of the disease [47]. Our results indicate that this initial increase in proliferation does not promote neurogenesis, as no corresponding increase in DCX or BrdU/NeuN-positive cells is observed, possibly because of the toxic environment composed of Aβ [7, 8, 39]. Running significantly increased the number of BrdU-positive cells in 4-month-old non-Tg mice, thereby abolishing the difference previously observed with non-running TgCRND8 mice. Secondly, in running mice, the number of DCX-positive pixels significantly increased at 4 months of age (Fig. 6). Furthermore, the number of BrdU/NeuN positive cells in both TgCRND8 and non-Tg mice (with exception noted in 4-month-old TgCRND8) significantly increased with running (Fig. 4B).

Both NeuN (Fig. 4B) and DCX (Fig. 6) signals are prominent in 4- and 5-month-old mice, with the population of BrdU/NeuN cells being significantly smaller than the population of NeuN and DCX cells. This is expected as BrdU only incorporates into cells that are proliferating at the time of pulse. The colocalization of BrdU with NeuN identified cells that incorporated BrdU following its injection at 3 months of age (Fig. 1A), differentiated into neurons, and survived until 4 and 5 months of age (Fig. 4A–D). Taken together, this data indicates that physical exercise has a significant impact on the development of immature and mature neurons. Our findings of increased neurogenesis with physical exercise are in line with previous reports using adult and aged wild-type mice that found physical exercise augments the rate of hippocampal neuronal differentiation, maturation and survival [48–52]. Similarly, in the APP23 transgenic mice, physical exercise was able to increase the number of newborn granule cells in the dentate gyrus [21]. Our data demonstrate that the levels of neurogenesis in TgCRND8 running mice were not statistically different than in age-matched running non-Tg mice. In contrast, we observed a significantly lower number of newly-differentiated mature neurons in non-running TgCRND8 mice at 5 months of age, compared to 4 months of age (Fig. 5B), suggesting that the neuronal maturation and/or survival process is compromised as the pathology progresses. These data suggests that exercise can support the differentiation of newborn cells into mature neurons despite the presence of Aβ.

Recent reports propose a causative relationship between augmented adult neurogenesis and improved memory [53, 54]. For example, Rodríguez et al. [53] showed that the new hippocampal neurons are important in generating memory episodes, while cognitive stimuli are known to promote the survival of newborn cells. The relevance of DCX-positive neuroblasts in forming new memories is gaining support, as suggested by Vukovic and colleagues [55]. Indeed, the selective reduction of DCX positive cells in a knock-in mouse model impaired spatial memory acquisition in an active place avoidance test.

In our study, the relationship between memory function, as measured in the Y-maze, and levels of neurogenesis appears to be stronger in TgCRND8 mice than in non-transgenic mice. Indeed, in 4-month-old TgCRND8 mice, 1 month of running improved Y-maze performance and significantly increased DCX immunostaining. An increase in the number of BrdU/NeuN-positive cells in TgCRND8 mice was seen after 2 months of running. Our data in TgCNRD8 mice suggests that running first increases DCX levels and then the number of BrdU/NeuN-positive cells, at 4 and 5 months of age, respectively. Both immature (DCX-positive) and mature (BrdU/NeuN-positive) neurons have the potential to contribute to the memory improvements observed in TgCRND8 mice. In contrast, running in non-transgenic mice increased neurogenesis (DCX levels and the number of BrdU/NeuN-positive cells) without having a significant impact on Y-maze-related memory functions, compared to non-running mice. This finding is consistent with previous work in non-transgenic mice investigating the effect of physical exercise on Y-maze performance [56]. It remains to be established whether our exercise paradigm could improve memory on a more challenging spatial test such as the Morris water maze or Barnes maze in non-transgenic mice. Indeed, previous studies suggest that exercise-induced improvements in learning and memory can be correlated with enhanced hippocampal neurogenesis in non-transgenic mice (reviewed in [48]). Specifically, Marlatt et al. [57] showed that 15-month-old mice running for 6 months had significantly increased BrdU, DCX, and BrdU/NeuN cell populations compared to sedentary control animals. In addition, running mice performed significantly better on the Morris water maze task than non-running counterparts. In future studies, it would be of interest to establish which measurements of memory best correlate with exercise-induced neurogenesis in transgenic and non-transgenic mice. Furthermore, mechanisms other than neurogenesis are stimulated by physical exercise and can also contribute to improving cognition. Modulating vasculature, reducing neuroinflammation and preventing the loss of cholinergic neurotransmission may all play a role in circumventing AD-like pathologies, and may

directly or indirectly support increased neurogenesis and cognition [58–64].

As the disease progressed in TgCRND8 mice, running prevented the decrease in performance observed in the Y-maze (Fig. 5B, ^age effect, comparing the time spent in the novel arm at 3 and 5 months of age). Our findings are in contrast with results reported by Richter et al. [22] and may be due to several differences in experimental design. Firstly, we used the Y-maze while Richter and colleagues used the Barnes maze to evaluate spatial memory performance. Secondly, the running equipment used for the mice was different. We used low-resistance spinning disks, which resulted in increased compliance with only 1 mouse not using the spinning disk out of 48 mice in our study compared to 13 non-exercisers out of 54 mice in Richter and colleagues' study [22]. The low-resistance spinning disks that we used also allowed mice to run longer distances compared to standard metal wheels (9 km/day versus 1.4 km/day) [65]. These experimental differences may be of significance in the search of sufficient levels of physical activity translating into improved cognitive performance.

The effects of exercise on Aβ burden have been previously evaluated in AD mouse models. In Tg2576 and TgCRND8 mice, exercise lowered Aβ plaque pathology when it was introduced in a pre-plaque stage of disease progression [9–11] but not when animals began to exercise following Aβ plaque deposition (80-day old TgCRND8 for a period of 10 weeks) [33]. By contrast, our 2-month long exercise paradigm reduced Aβ load when initiated in 3-month-old TgCRND8 mice as intervention measure in the post-plaque stage of disease progression [15]. Two months of running reduced number of plaques and surface area of Aβ when compared to non-running mice. Contrastingly, no changes were detected after 1 month of running. In both running and non-running mice TgCRND8 mice, the plaque number, size, and surface area increased as animals aged from 4 to 5 months, with a reduced degree of increase observed with running. This suggests that physical exercise is capable of significantly attenuating hippocampal Aβ plaque burden and it can potentially be of significance in promoting neurogenesis through different mechanism in TgCRND8 mice, including decreased amyloidosis.

Physical exercise appears to be an effective multimodal intervention strategy against AD, as we were able to show in a mouse model of amyloidosis that running is able to promote an environment with reduced Aβ, enriched neuronal maturation, and improved cognitive performance. Physical voluntary exercise could serve as a lifestyle-modifying intervention to counteract AD pathologies and potentially improve the quality of life of AD patients.

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