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Restored presynaptic synaptophysin and cholinergic inputs contribute to the protective effects of physical running on spatial memory in aged mice

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ABSTRACT

The effects of prolonged physical training on memory performance and underlying presynaptic mechanisms were investigated in old C57BL/6 mice. Training *via* voluntary running wheels was initiated at 16 months of age and continued for 5 months (1 h per day, 5 days per week), followed by testing of learning and memory functions and counting of presynaptic puncta and cholinergic inputs in the hippocampus. Trained old mice were compared to their age-matched sedentary controls and adult controls. This training strategy improved hippocampal-dependent spatial memory function tested *via* a novel location task, and enhanced memory was accompanied by restored presynaptic puncta and cholinergic fibers in area CA1 and DG of the hippocampus in old mice. Particularly, the training selectively affected presynaptic vesicle protein synaptophysin but not growth associated protein GAP-43, and the increased number of synaptophysin puncta positively correlates with improved memory performance. To better understand the neurochemical mechanisms by which prolonged physical training protects against aging-related memory deficits, the cholinergic inputs to the hippocampus were compared among the three groups of mice and correlated with memory performance. While the running prevented age-related loss of cholinergic inputs, it has limited impact on the projection source cells in the medial septum-diagonal band (MS-DB). Importantly, cholinergic fibers in area CA1 and DG positively correlated with spatial memory function. These data suggest that the preservation of presynaptic inputs, particularly those involved in the integrity of memory performance, contributes critically to the beneficial effects of physical running initiated at an older age.

1. Introduction

The beneficial effects of physical exercise on cognitive functions have been well documented in the studies of both rodents and humans (for review see Mandolesi et al., 2018). During normal aging, cognitive functions that rely on the integrity of neuronal circuits in the hippocampus, such as spatial learning and memory, show considerable age-related decline. It is therefore not surprising that synaptic contacts in this brain region also seem to be particularly vulnerable during the aging process. Physical exercise initiated at an older age has been shown to improve spatial learning and memory in old animals (van Praag et al., 2005; Siette et al., 2013; Snigdha et al., 2014) and in animal models of neurodegenerative diseases (*e.g.*, Nithianantharajah and Hannan, 2006; Choi et al., 2018), as well as in elderly people including those suffering from cognitive impairment and dementia (*e.g.*, Larson et al., 2006; Scarmeas et al., 2011; Hötting and Röder, 2013; Niemann et al., 2014). The mechanisms that underlie these benefits may include increased neurogenesis and cell proliferation (Eadie et al., 2005; van Praag et al., 2005; Kronenberg et al., 2006), reserved synaptic contacts and facilitated neuroplasticity (Farmer et al., 2004; Christie et al., 2008; Stern, 2012; Siette et al., 2013), reinstated expression of genes that promote neurogenesis, angiogenesis and synaptic plasticity (Intlekofer and Cotman, 2013; Snigdha et al., 2014), and enhanced blood circulation *via* an increased vascularization in the hippocampus (Thomas et al., 2012; Maass et al., 2015). We have recently reported that prolonged wheel running (5 months) prevents loss of dendritic spines on

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specific dendritic segments of CA1 pyramidal cells in aged mice, which impressively contributes to the memory performance of these animals (Xu et al., 2017). In the hippocampus, dendritic spines are key components of a variety of microcircuits and receive input innervations from distinct presynaptic cellular sources, including the intrinsic hippocampal cells and external cholinergic cells in the medial septum-diagonal band (MS-DB) complex (Takács et al., 2012), and thus contribute to specific profiles of learning and memory functions (Nakashiba et al., 2008; Brun et al., 2002, 2008; Remondes and Schuman, 2004; Suh et al., 2011). While enhanced memory functions *via* physical running have been linked to the conservation of postsynaptic spines in old mice, it is unknown whether external presynaptic inputs that make synaptic contact with these spines are also affected by physical running.

The presynaptic inputs received by the hippocampal cells are derived from several extrinsic sources (Takács et al., 2012), which can be visualized *via* presynaptic proteins such as growth-associated protein 43 (GAP-43) and synaptophysin. These proteins are richly expressed in the axonal terminals or presynaptic vesicles (*e.g.*, Calhoun et al., 1996; Nemes et al., 2017). Although GAP-43 is first verified in neuronal growth cones during development, it has been implicated in both axonal growth and synaptic plasticity. Importantly, GAP-43 has revealed a close relationship between its expression and action-dependent neuroplastic processes, such as long-term potentiation (LTP) and memory formation (Denny, 2006; Holahan, 2017). Synaptophysin is a synaptic vesicle glycoprotein, which is the most abundant integral membrane protein constituent of synaptic vesicles of neurons (McMahon et al., 1996). The immunoreactivity of synaptophysin is present in a punctate pattern in the hippocampus and has been used as a presynaptic marker for quantification of synapses (Calhoun et al., 1996). During the aging process, decreased expressions of GAP-43 and synaptophysin at protein or mRNA level have been reported in the hippocampus (Chao et al., 1992; Casoli et al., 1996), suggesting the loss of presynaptic inputs.

Recent data from monosynaptic rabies tracing studies have revealed that CA1 pyramidal cells receive a large amount of cholinergic inputs directly from the cells in the MS-DB (Sun et al., 2014). Functionally, the MS-DB cholinergic inputs are important for hippocampal network oscillations (Buzsáki, 2002), and acetylcholine plays a crucial role in structural and functional remodeling of synaptic contacts, thus contributing to cognitive functions (Kaufman et al., 2012; Solari and Hangya, 2018). In aged animals, loss and/or atrophy of cholinergic cells in MS-DB have been reported in numerous studies (Fischer et al., 1987, 1989; Gilad et al., 1987; Mesulam et al., 1987; Altavista et al., 1990; Stroessner-Johnson et al., 1992 Monkey; Schliebs and Arendt, 2011), while no difference in the cholinergic cells in the septum has been reported in aged rats when compared to young adult rats (Ypsilanti et al., 2008). Importantly, loss of cholinergic cells is most pronounced in aged subjects with severe memory deficits, and cholinergic dysfunction has been correlated with memory loss in normal aging as well as in Alzheimer's disease (Bartus et al., 1982; Fischer et al., 1989; Härtig et al., 2002; Schliebs and Arendt, 2006, 2011; Douchamps and Mathis, 2017).

In the current studies, we report that prolonged and voluntary physical training initiated at an older age (16 months old) can prevent aging-related selective loss of presynaptic protein synaptophysin and cholinergic inputs in the hippocampus. Particularly, the number of synaptophysin-immunoreactive (ir) puncta and the density of cholinergic fibers in the hippocampus correlates with memory performance in a hippocampal-dependent spatial memory task. While running protects against age-related loss of cholinergic fibers in the hippocampus, the cholinergic cells in the MS-DB, the cellular source of these cholinergic innervations in the hippocampus, are less affected by the running.

2. Materials and methods

2.1. Animals

C57BL/6J male mice were group-housed in standard Plexiglas cages in a quiet facility and maintained on a 12-h light/dark cycle (lights on at 7 am), with *ad libitum* access to food and water. All experiment procedures were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Bioethics Committees of the Yangtze University and Guangzhou Medical University. The mice were subjected to a 5 months voluntary wheel running when they were 16 months of age and compared with their age-matched sedentary controls and adult controls. Therefore, three groups of animals were included in the study and they were adult control, old sedentary control, and old runner. At the end of training and behavioral testing, the adult controls and old animals (runners and sedentary controls) were 16 and 22 months of age, respectively. Total two cohorts of mice were employed. Cohort 1 was subjected to short-term memory testing (2 h after training session), and cohort 2 was subjected to long-term memory testing (24 h after training session). In cohort 1, the heart-to-body weight ratios were 5.04 ± 0.38, 4.62 ± 0.21, and 5.06 ± 0.28 (mg per g body weight, mean ± SD) in aged adult controls, old sedentary controls, and old runners, respectively. Differences were found among the groups (one-way ANOVA, $F_{[2,34]} = 7.45$, $P = .002$) with a higher ratio in the runners compared with age-matched controls (*post hoc*, $P < .01$). In cohort 2, the ratios were 4.98 ± 0.42, 4.72 ± 0.19, and 5.09 ± 0.24 in aged adults, old sedentary controls, and old runners, respectively. The runners have a higher ratio (one-way ANOVA, $F_{[2,32]} = 3.87$, $P = .03$; *post hoc*, $P < .05$).

2.2. Physical training and running

The mice were individually placed in vertically revolving activity wheels (16 cm in diameter and 5 cm in depth) (Xu et al., 2017). Animals were trained for running 10 min on the first day, and running time progressively increased 10 min per day until they were running for 1 h per day. The training at the first 2 weeks was performed between 6 and 10 pm. After 2 weeks of training, the mice that had learned to run voluntarily in wheels were chosen and assigned to sedentary and trained groups. The mice in trained group continued to run for 1 h per day during the dark cycle (7–10 pm), 5 days per week, and a total of 5 months. Wheel rotations were automatically recorded, and deviations in daily scores were observed as an indication of animal's health and running status. The sedentary controls were also placed in individual running wheels for 1 h per day, but the wheels were immobile.

At the end of running, animals were subjected to behavioral tasks, followed by histology, acetylcholinesterase (AChE) enzyme-staining, and immunohistochemistry. Because wheel running is a voluntary activity in contrast to forced exercise models in mice, we set the criterion for effective training in the trained mice such that the heart-to-body weight ratio is at least 2 standard deviation (SD) above the mean in age-matched group (Mean + 2 SD) (Xu et al., 2017). All mice were weighed weekly throughout the protocol. Adrenal glands were weighed as measures of stress at the time of sacrifice, and no significant difference was found among the groups (data not shown). When the brain tissues were harvested, adult controls and old animals (sedentary control and runner) were 16- and 22-month-old, respectively, as described above.

2.3. Object location memory test

At the end of 5-month running, the runners were subjected to object location test and compared with their age-matched old controls and aged adult controls. Object location test was performed as described previously (Chen et al., 2016; Xu et al., 2018). Prior to training, mice were handled 2 min for 1 week and then habituated to the experimental

apparatus for 10 min per day for 5 days in the absence of objects. In the training session, two identical objects were presented for exploration for 10 min. In the testing session, 2 or 24 h later (two cohorts of animals, one for 2 h short-term memory testing and another for 24 h long-term memory testing), object exploration was scored for 5 min. Exploration was scored when a mouse's head was oriented toward the object within 1 cm or when the nose was touching the object. Exploration times of object in familiar and novel locations were recorded and expressed as a ratio (novel/familiar). Total exploration times were calculated, and the novel/familiar ratio was compared among the groups. Two mice that explored both objects for < 20 s in total during training were removed from further analysis (probably due to poor vision because of cloudy corneal). A video tracking system (EthoVision, Noldus, the Netherlands) was used to record both training and testing phases. The training and test were performed without knowledge of group, meaning 'blindly'.

2.4. Tissue handling and AChE histochemistry

When the 2 h short-term memory testing was finished, the mice were anesthetized with sodium pentobarbital (40 mg/kg) and perfused *via* the aorta initially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C). Basing on the standard of effective training described above, 10 of the 14 runners (~71%) were used in the experiments. The brains of effective trained mice and their controls (adult and old) were postfixed in the same fixative for 4–6 h. Brain tissue blocks were cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB, 4 °C) and then sectioned coronally (20 μm) throughout the forebrain and the hippocampus using a Leica cryostat. Series sections (1 in 6, 10–11 sections per animal) from the dorsal hippocampus (Bregma: −1.22 mm ~ −2.54 mm) were subjected to AChE histochemistry.

AChE-positive fibers were stained with the protocol recommended by Hedreen et al. (1985). Briefly, after washing with 0.1 M acetate buffer (pH 6.0) (2 × 5 min), the sections were incubated in 0.04% acetylthiocholine iodide (Sigma) for 30 min at room temperature. The sections were washed with 0.1 M acetate buffer (5 × 3 min), and then incubated in 1% ammonium sulfide for 2 min. After washing in 0.1 M sodium nitrate (5 × 3 min), the sections were treated with 0.1% silver nitrate (1 min), followed by wash in 0.1 M sodium nitrate (5 × 3 min). In negative control test, acetylthiocholine iodide was not added to the incubation solution. The specificity of the staining was also tested using eserine sulfate (0.1 mM), a reversible cholinesterase inhibitor. No staining was found in these control sections. The laminar pattern of the AChE-positive fibers in the hippocampus was visualized by counterstaining adjacent sections with 0.5% cresyl violet (Bioenno Tech LLC, Santa Ana, CA).

2.5. Immunohistochemistry (IHC) and fluorescent immunostaining

For GAP-43 and synaptophysin IHC, fixed brain tissue blocks from 24 h long-term memory testing were embedded in paraffin. Along with the 2 SD effective training criterion, 9 of the 13 runners (~69%) were selected for analysis, therefore, $n = 10$, 12, and 9 mice in the group of adult control, old sedentary control, and old runner, respectively. The paraffin-embeded tissue was sectioned coronally at 7 μm throughout the dorsal hippocampus and collected onto gelatin coated slides. Corresponding sections from the hippocampus of three groups were mounted on one slide and every eighteenth section was used. For IHC, the mounted sections were first deparaffinized in xylene and then hydrated. Sections were treated in 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity, followed by blockade of nonspecific binding with 5% normal horse serum in PBS-T for 30 min. The sections were incubated with monoclonal anti-GAP-43 (1:5000, Boehringer Mannheim) or anti-synaptophysin (1:5000, Sigma) for 3 days at 4 °C. The sections were then incubated in biotinylated horse-anti-mouse IgG (1:400, Vector) for 2 h, and avidin-biotin-peroxidase complex solution (ABC, 1:200, Vector) for 3 h. The reaction product was visualized by incubating the sections for 10 min in 3,3′-diaminobenzidine (DAB) containing H_2O_2 (Bioenno Tech LLC, Santa Ana, CA). The specificity of the immunoreaction was tested by omitting the primary antibody during the first incubation. No immunoreactivity was found in these sections. The laminar pattern of cells was counterstained with neutral red.

For fluorescent immunostaining of GAP-43 and synaptophysin, fixed tissue blocks from 2 h short-term memory testing were cryoprotected and sectioned coronally (20 μm). Every sixth section from the dorsal hippocampus (Bregma: −1.22 mm ~ −2.54 mm) was collected and subjected to free-floating fluorescent immunostaining (Wang et al., 2011; Xu et al., 2018). Briefly, after washing for 10–15 min with PBS containing 0.3% Triton X-100 (PBS-T, pH 7.4), sections were treated with 0.3% H_2O_2/PBS-T for 30 min and then blocked with 5% normal goat serum for 1 h to prevent non-specific binding. After rinsing in PBS-T for 10–15 min, sections were incubated at 4 °C with primary antibodies in PBS-T containing 1% BSA. The antibodies included mouse monoclonal anti-GAP-43 (1:5000, Boehringer Mannheim) or anti-synaptophysin (1:5000, Sigma) for 3 days at 4 °C. After washing in PBS-T (3 × 5 min), antibody binding was visualized with anti-mouse IgG conjugated to Alexa Fluor 488 (1:200, Molecular Probes).

For choline acetyltransferase (ChAT) IHC, fixed tissue blocks from 2 h short-term memory testing were cryoprotected in 30% sucrose in 0.1 M PB and sectioned coronally (20 μm). Every sixth section from the MS-DB was harvested and used for standard avidin-biotin complex method (Wang et al., 2011; Xu et al., 2018). Free-floating sections from three groups were incubated with monoclonal anti-ChAT (1:5000, MA1–25629, clone 7E3-1B8, Affinity BioReagents, Golden, CO) in a parallel manner, followed by incubation in biotinylated anti-mouse IgG (1:400, Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complex solution (ABC, 1:200, Vector). The reaction product was visualized by incubating the sections for 10 min in DAB containing H_2O_2 (Bioenno Tech LLC, Santa Ana, CA).

2.6. Evaluations of AChE-positive fibers and ChAT-immunoreactive (ir) neurons

AChE-positive fibers in areas CA1 and DG of the hippocampus were analyzed. Systematic series of sections (1 in 6) throughout the entire anteroposterior extent of these areas was collected, resulting in 10–11 sections per animal (100 μm interval to each other). AChE fibers in the different laminar layers of these areas were captured based on unbiased stereological principles (West, 1999). Images were taken using a Nikon E400 microscope equipped with an Apochromat 63× oil objective (numeric aperture = 1.40), a focus drive motor, and a Nikon digital sight camera system (DS-Fi3). With the aid of a square lattice system (Lewis, 1991), the density of AChE fibers in each layer was expressed as the number of intersects in a 40,000 μm^2 real area, which was calculated based on the average value from two fields from each section. Image capture and section analysis were performed without knowledge of group treatment.

Total numbers of ChAT-ir cells in the MS-DB were estimated based on unbiased stereological principles (Gundersen et al., 1988; West, 1999). Unbiased determination of cells was performed on systematic series of sections (1 in 6, 9–10 sections per animal) throughout the MS-DB. Cholinergic cell bodies were strongly immunoreactive for ChAT (see Fig. 7), leading the nuclei of these cells to be identified easily. Cells with clearly visible nuclei were counted using the "optical disector" technique (West, 1999; Xu et al., 2018). ChAT-ir cells in each of the MS-DB subdivisions were inspected under 10×, 20× objectives, and the counting of cells was performed under 63×/1.4 objective. To accurately define the MS-DB subdivisions, adjacent sections of those used for quantification were counterstained with 0.5% cresyl violet.

2.7. Quantitative analysis of presynaptic immunoreactive puncta

GAP-43 and synaptophysin immunoreactive products were analyzed with two independent approaches. (1) Sections derived from standard avidin-biotin-peroxidase immunohistochemistry were subjected to the analysis of the optical density (OD) of immunoreactivity. The OD was calculated based on the average value from three fields containing layers of interest in areas CA1 and DG using ImageJ (v2). 9–10 sections (~120 μm interval each other) per animal were counted. The OD of the corpus callosum on the same section was measured and served as background. All measurements were done under the same optical and lighting conditions. (2) Sections subjected to fluorescent immunostaining were employed for counting the number of individual presynaptic puncta in the area of interest based on unbiased stereological principles (West, 1999). 8–9 sections per animal were used and z-stack images were taken from strata oriens, radiatum, lacunosum-moleculare at 63×/1.4 using a Zeiss 510 confocal microscope or a Leica microscope (DM6000). The layers were first defined using a 10× objective. We used a counting frame of 25 × 25 μm, a sampling grid of 200 × 200 μm, a guard zone of 10 μm and a disector height of 5 μm. The precision of the study was estimated by calculating the coefficient of error (CE) (Gundersen et al., 1999). The CE value for each individual animal ranged between 0.02 and 0.05. Three-dimensional image stacks were deconvolved for 10 iterations using AutoQuant (v2) or processed for iterative deconvolution at 99% confidence (Volocity 6.3). Counts (per 22,500 μm^3) of labeled puncta in each layer from each section were averaged or grouped to obtain a value for each brain and analyzed as described (Xu et al., 2018). Sections from three groups were processes concurrently and analyzed without knowledge of treatment group.

2.8. Statistical analysis

Data were analyzed using SPSS 15.0 (SPSS, Chicago, IL, USA) or Prism 6 (GraphPad, Prism, San Diego, CA, USA). A one-sample *t*-test was performed to distinguish whether exploration times of objects were different from those predicted by chance. One-way ANOVA was employed to compare the heart-to-body weight ratios, exploration times, and novel/familiar ratios among three groups. Cell and synapse data were analyzed by two-way analysis of variance (ANOVA) with group and subregion/size as factors, followed by Tukey's or Bonferroni's *post hoc* test. Pearson test was used for the correlation analysis. Data were expressed as mean ± SEM, significance was set at 95% confidence.

3. Results

3.1. Physical running for 5 months enhances hippocampal-dependent memory function in old mice

The effects of physical running on memory function were evaluated in two cohorts of animals by employing a relatively stress-free and hippocampal-dependent object location task (Buzsáki and Moser, 2013; Xu et al., 2018). The memory function was tested at 2 and 24 h after training. As shown in Fig. 1, memory decline was apparent in old sedentary mice (22-month-old) compared with aged adults (16-month-old) (Fig. 1B,C), which was consistent with published data of mice (von Bohlen und Halbach et al., 2006; Xu et al., 2017). However, running for 5 months improved both short-term and long-term memory functions, tested at 2 and 24 h after training, respectively. Two hours after the training, old runners (22-month-old) preferentially explored a novel-location object compared to a familiar-location object (one-sample *t*-test, $t_9 = 11.16$, $P < .0001$), whereas old sedentary controls spent almost equal time exploring the two objects (one-sample *t*-test, $t_{11} = 0.79$, $P = .45$). One-way ANOVA indicated that old runners spent more time exploring the novel-location object compared with old sedentary controls (one-way ANOVA, $F_{[2,30]} = 42.83$, $P < .0001$; *post hoc* test, $^{*}P < .01$) (Fig. 1B), but no difference was found between the runners and aged adults (*post hoc*, $P > .05$). In another cohort of animals, 24 h after the training, old runners preferentially explored the novel-location object ($t_8 = 9.95$, $P < .0001$), but sedentary controls explored the two objects equally ($t_{11} = 1.76$, $P = .11$). In this cohort, the novel/familiar ratio of old runners was significantly higher than that of old sedentary mice (one-way ANOVA, $F_{[2,28]} = 38.75$, $P < .0001$; *post hoc* test, $^{*}P < .01$) (Fig. 1C), but similar with aged adults (*post hoc*, $P > .05$). No differences were observed in total exploration time in both training and testing (all $P > .05$), indicating that the reduced ability to remember the location of an object was not a result of residual effects of the age on general features of behavior.

3.2. Running selectively prevents age-related degeneration of presynaptic proteins in old mice

Considering that long-term running may enhance synaptogenesis, thus protecting age-related deficits of memory function, we first focused on the expressions of GAP-43 in the hippocampus, because GAP-43 contributes to synaptic plasticity and growth of presynaptic terminal, which has been linked to memory formation (Denny, 2006; Holahan, 2017). As shown in Fig. 2A, in area CA1 of aged adults, GAP-43-ir puncta were primarily localized in the dendritic field of CA1 pyramidal cells, including strata oriens, radiatum and lacunosum-moleculare, with different staining intensities. When the optical density (OD) of the GAP-43 signals was quantified, age-related decrease was apparent in several measured layers in old sedentary mice compared to aged adults (two-way ANOVA, $F_{[2,84]} = 7.214$, $P = .0013$; Tukey's *post hoc* test, $^{*}P < .05$). However, no significant difference was found in the measurements between old runners and old sedentary controls (*post hoc* test, $P > .05$). Two-way ANOVA suggested no group-by-layer interaction ($F_{[4,84]} = 0.196$, $P = .94$). In the dentate gyrus (DG), GAP-43 was less expressed in the hilus, but intensely presented in the inner molecular layer and lightly in the outer molecular layer (Fig. 2A). Age-related decrease of OD was found in the inner molecular layer (two-way ANOVA, $F_{[2,84]} = 3.260$, $P = .0433$; *post hoc* test, $^{*}P < .05$). Running had limited effects on GAP-43 in the DG, and group-by-layer interaction was not found ($F_{[4,84]} = 0.782$, $P = .54$). Furthermore, the number of individual GAP-43-ir puncta was quantified *via* another method on three-dimensional (3D) image stacks in a different cohort of animals (Fig. 2C–E). In strata of oriens and radiatum of CA1 as well as in the inner molecular layer of DG, a decreased number of GAP-43 puncta was apparent in old mice compared to aged adults (two-way ANOVA in CA1: $F_{[2,90]} = 18.70$, $P < .0001$; in DG: $F_{[2,90]} = 4.48$, $P = .014$; Tukey's *post hoc* test, $^{**}P < .01$), but running had limited effects on the age-related decrements (*post hoc* test, $P > .05$). Group-by-layer interaction was not found ($P = .52$ in CA1 and 0.26 in DG).

To explore the presynaptic mechanisms by which long-term running protects against age-related deficits of memory function, we then measured the expressions of synaptophysin, one commonly recognized marker of presynaptic vesicle protein in the hippocampus (Masliah et al., 1990; Calhoun et al., 1996). Synaptophysin-ir puncta were primarily localized in the dendritic field of CA1 pyramidal cells and DG granular cells with different staining intensities. In the hilus, synaptophysin-ir puncta were seen in larger size and intensity. No changes were observed in the laminar pattern of immunostaining among the three groups (Fig. 3A–C). Reduced ODs of synaptophysin immunoreactivity were found in area CA1 (Fig. 3D) (two-way ANOVA, $F_{[2,84]} = 31.47$, $P < .0001$) and DG (Fig. 3E) (two-way ANOVA, $F_{[2,84]} = 22.57$, $P < .0001$) when comparing old sedentary mice to adult controls (Tukey's *post hoc* test, $^{*}P < .05$, $^{**}P < .01$). Interestingly, increased density of synaptophysin was found in old runners compared with old sedentary controls (Tukey's *post hoc* test, $^{*}P < .05$, $^{**}P < .01$). Group-by-layer interaction was not found in CA1 ($F_{[4,84]} = 2.214$, $P = .07$) and DG ($F_{[4,84]} = 1.824$, $P = .13$). Remarkably, prevented loss of synaptophysin in CA1 was confined to the dendritic fields in strata radiatum and oriens of area CA1, in keeping with the effects of running on

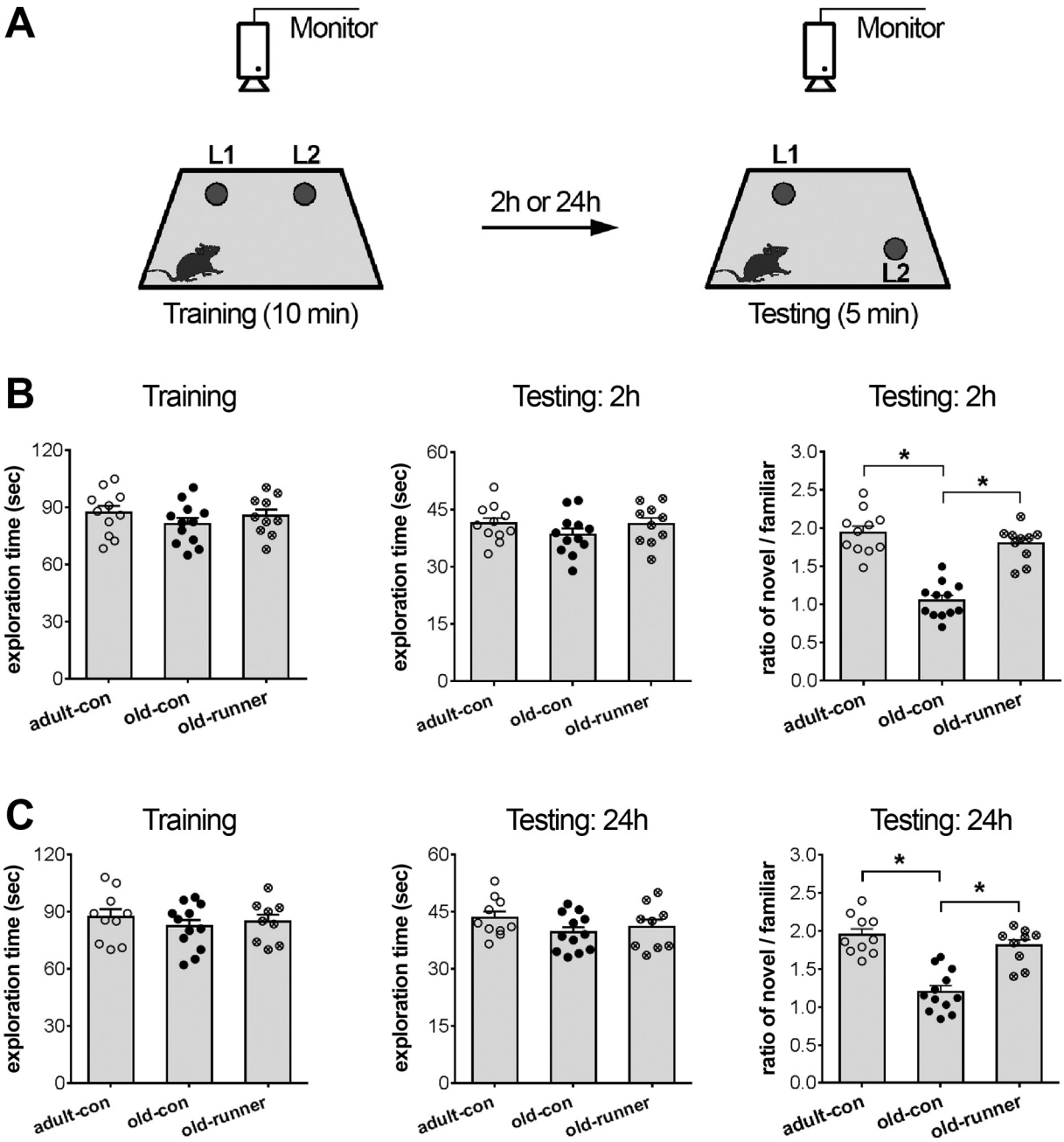

Fig. 1. Long-term running enhances spatial memory in aged mice. (A) Spatial memory function was detected *via* a novel object location task during which C57 BL/6J mice received 10 min of training with two identical objects located at L1 and L2, followed by 5 min of testing at 2 h and 24 h later. During the testing, same two identical objects were used, but one object was moved to a novel location (L2, moved), and the discrimination ratios (novel *vs.* familiar) were used to represent the short- (2 h) and long-term (24 h) memory function. (B, C) Impaired memory function was apparent in old sedentary mice (old-con) compared with aged adults (adult-con) and old runners 2 h and 24 h after the training phases as indicated by the novel/familiar ratio (right panels). The ratio of old controls was significantly lower than that of adult controls and old runners 2 h (one-way ANOVA, $F_{[2,30]} = 42.83$, $P < .0001$; Bonferroni's *post hoc* test, $^{*}P < .01$) and 24 h after the training (one-way ANOVA, $F_{[2,28]} = 38.75$, $P < .0001$; Bonferroni's *post hoc* test, $^{*}P < .01$). There were no significant differences in total exploration time among the three groups during training (left panels, 2 h: $F_{[2,30]} = 0.86$, $P = .43$; 24 h: $F_{[2,28]} = 0.46$, $P = .63$) and testing (middle panels, 2 h: $F_{[2,30]} = 1.09$, $P = .35$; 24 h: $F_{[2,28]} = 0.96$, $P = .39$).

postsynaptic spines in this area (Xu et al., 2017).

The immunoreactive puncta of synaptophysin correspond to labeled synaptic vesicles or boutons and may represent the individual synapses in the hippocampus (Masliah et al., 1990; Calhoun et al., 1996). We then measured the number of individual synaptophysin-ir puncta in the dendritic field of pyramidal cells and granular cells *via* quantification on 3D image stacks in another cohort of animals (Fig. 3F–J). In area CA1, age-related decrease of synaptophysin puncta was apparent in all measured regions (two-way ANOVA, $F_{[2,90]} = 30.16$, $P < .0001$; Tukey's *post hoc* test, $^{*}P < .05$, $^{**}P < .01$). However, running prevented the age-related loss of synaptophysin in strata oriens and radiatum (*post hoc* test, $^{**}P < .01$). In the DG, aging-related loss of synaptophysin was mainly detected in the inner molecular layer (two-way ANOVA, $F_{[2,90]} = 13.10$, $P < .0001$; *post hoc* test, $^{**}P < .01$), and old runners had a higher number of synaptophysin compared with old sedentary controls (*post hoc* test, $^{**}P < .01$) (Fig. 3I,J). No group-by-layer interaction was found in CA1 ($F_{[4,90]} = 1.956$, $P = .11$) and DG ($F_{[4,90]} = 1.709$, $P = .15$). Taken together, the results from two

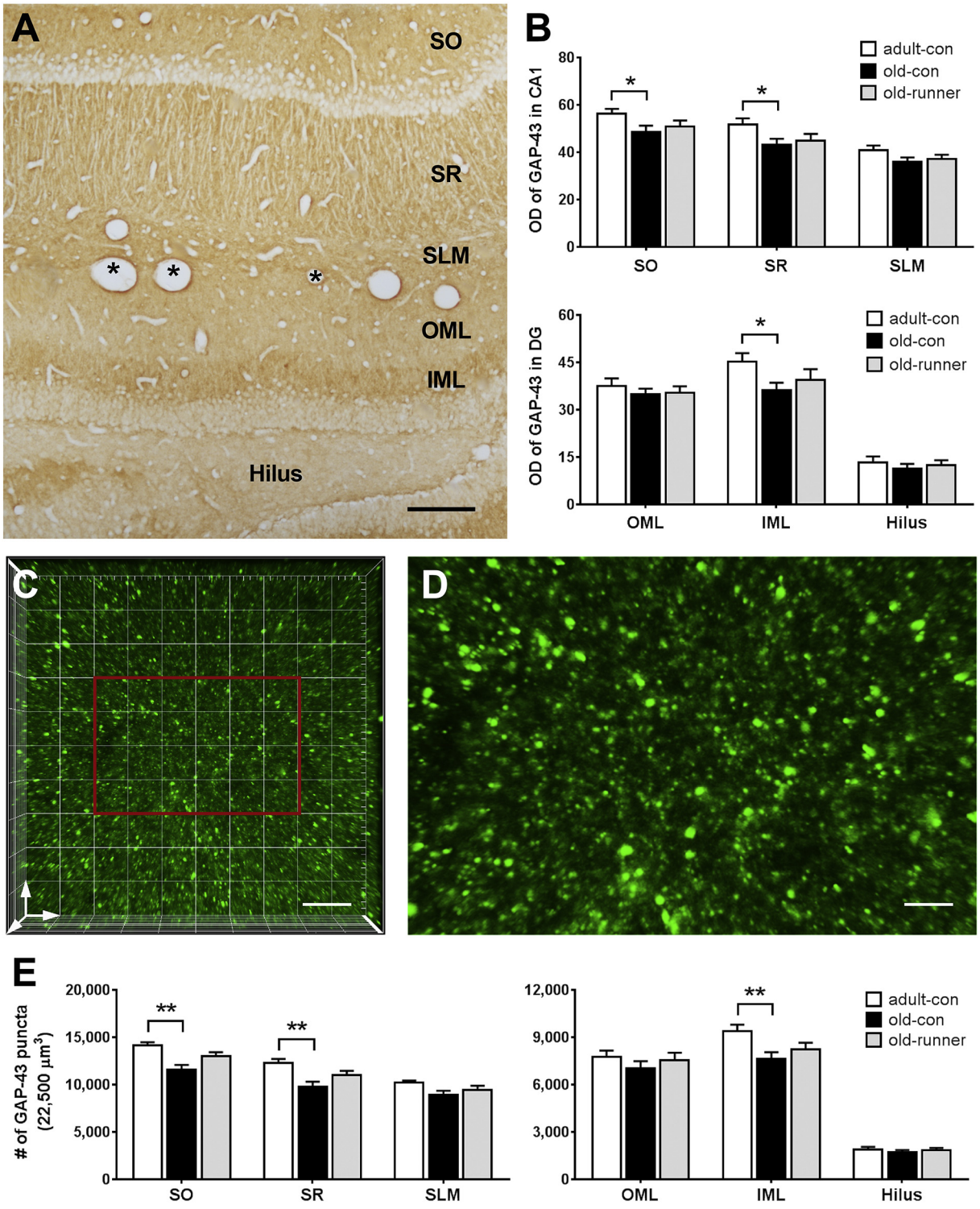

Fig. 2. Physical running has limited effect on the expression of presynaptic protein GAP-43 in the hippocampus. (A) Representative image from an aged adult mouse to show the GAP-43 immunostaining in the hippocampus. The optical densities (ODs) of GAP-43 immunoreactivity in strata oriens (SO), radiatum (SR) and lacunosum-moleculare (SLM) in area CA1 as well as in the outer molecular layer (OML), inner molecular layer (IML) and hilus of DG were measured and compared with old runners and old sedentary controls. (B) In area CA1 (up panel), the OD values were higher in the adult controls compared with old sedentary and trained mice, especially in SO and SR (two-way ANOVA, $F_{[2,84]} = 7.214$, $P = .0013$; Tukey's *post hoc* test, $^{*}P < .05$). No difference was found between old sedentary controls and runners (*post hoc* test, $P > .05$). In the DG (down), decreased OD value was found in the inner molecular layer in old sedentary animals compared with aged adults (two-way ANOVA, $F_{[2,84]} = 3.26$, $P = .0433$; *post hoc* test, $^{*}P < .05$), but no difference was found with old runners ($P > .05$). (C, D) Representative 3D deconvolution images (9° perspective) showing numerous GAP-43-ir puncta in the radiatum of an adult mouse. Boxed area in C was magnified (D) to show presynaptic puncta with distinct size. (E) Age-related loss of individual GAP-43-ir puncta was found in area CA1 (left, two-way ANOVA, $F_{[2,90]} = 18.70$, $P < .0001$; *post hoc* test, $^{**}P < .01$) and DG (right, two-way ANOVA, $F_{[2,90]} = 4.48$, $P = .014$; *post hoc* test, $^{**}P < .01$). However, 5-month running had limited effect on the number of GAP-43-ir puncta (*post hoc* test, $^{*}P > .05$). Bars: 150 μm in A, 20 μm in C and 7 μm in D.

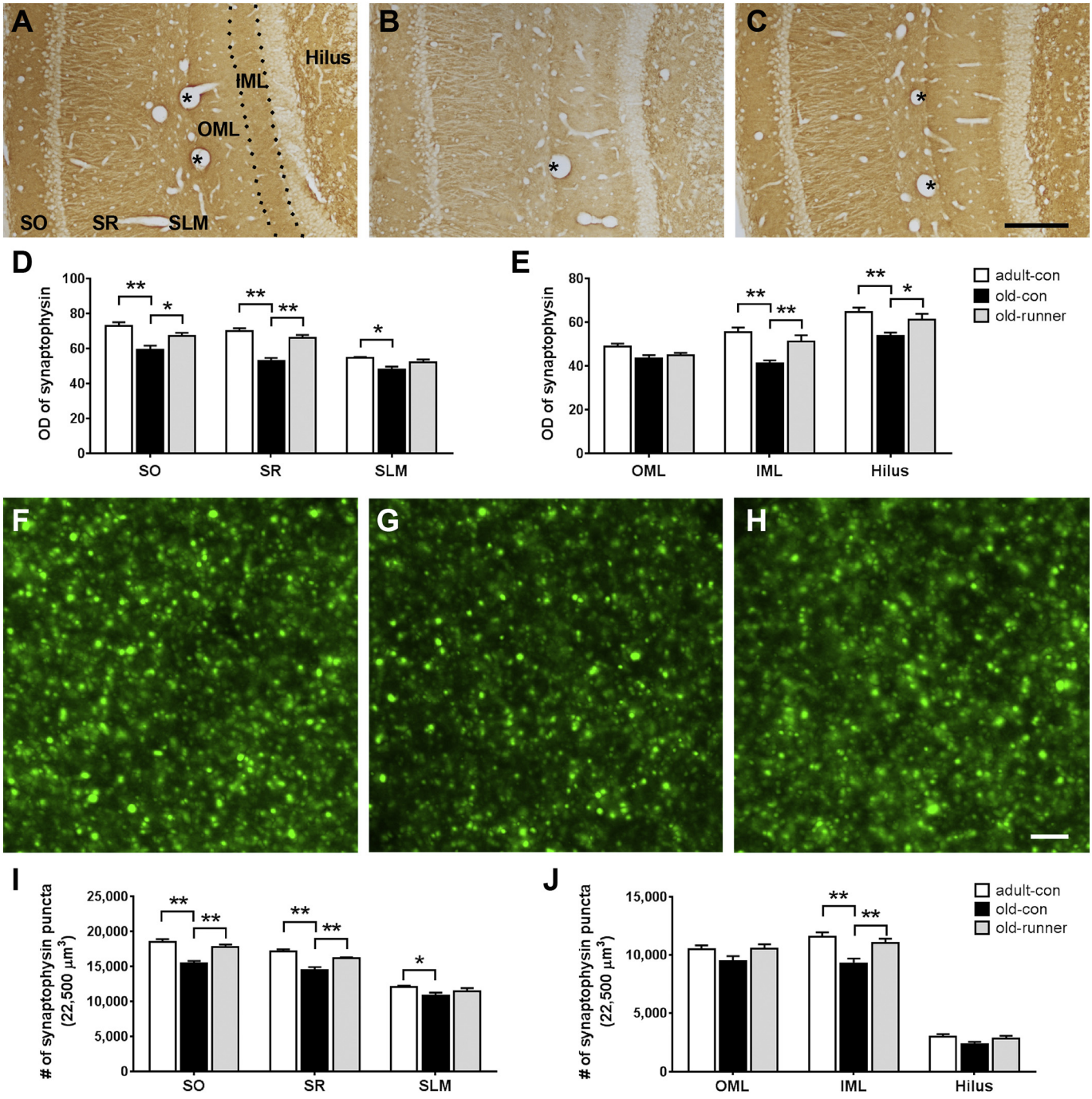

Fig. 3. Physical running prevents age-related loss of presynaptic protein synaptophysin in the hippocampus. (A–C) Representative images of synaptophysin immunostaining in the hippocampus of adult control (A), old control (B), and old runner (C). (D, E) Age-related loss of synaptophysin was found in area CA1 (D, two-way ANOVA, $F_{[2,84]} = 31.47$, $P < .0001$; Tukey's *post hoc* test, $^{**}P < .01$) and DG (E, $F_{[2,84]} = 22.57$, $P < .0001$; *post hoc* test, $^{**}P < .01$). The runners had higher OD values compared with old sedentary controls (*post hoc* test, $^{*}P < .05$, $^{**}P < .01$), but no difference from adult controls ($P > .05$). (F—H) 3D images represented at 1° perspective to show synaptophysin-ir puncta in the radiatum of three groups of mice. (I, J) The number of individual synaptophysin-ir puncta were determined. In area CA1 (I), loss of synaptophysin puncta was detected in old sedentary animals compared with aged adults, and an increased number of synaptophysin puncta was apparent in the old runners compared with age-matched controls ($F_{[2,90]} = 30.16$, $P < .0001$; *post hoc* test, $^{*}P < .05$, $^{**}P < .01$). In the DG (J), two-way ANOVA analysis (group × region) showed a significant group effect ($F_{[2,90]} = 13.10$, $P < .0001$) with no group-by-region interaction ($F_{[4,90]} = 1.709$, $P = .155$). Old sedentary mice had a significantly lower number of puncta in the inner molecular layer compared with old runners and adult controls (*post hoc*, $^{**}P < .01$). No difference was observed between runners and adult controls ($P > .05$). SO: oriens; SR: radiatum; SLM: lacunosum-moleculare (SLM); OML: outer molecular layer; IML: inner molecular layer. Bars: 200 µm in A-C and 5 µm in F—H.

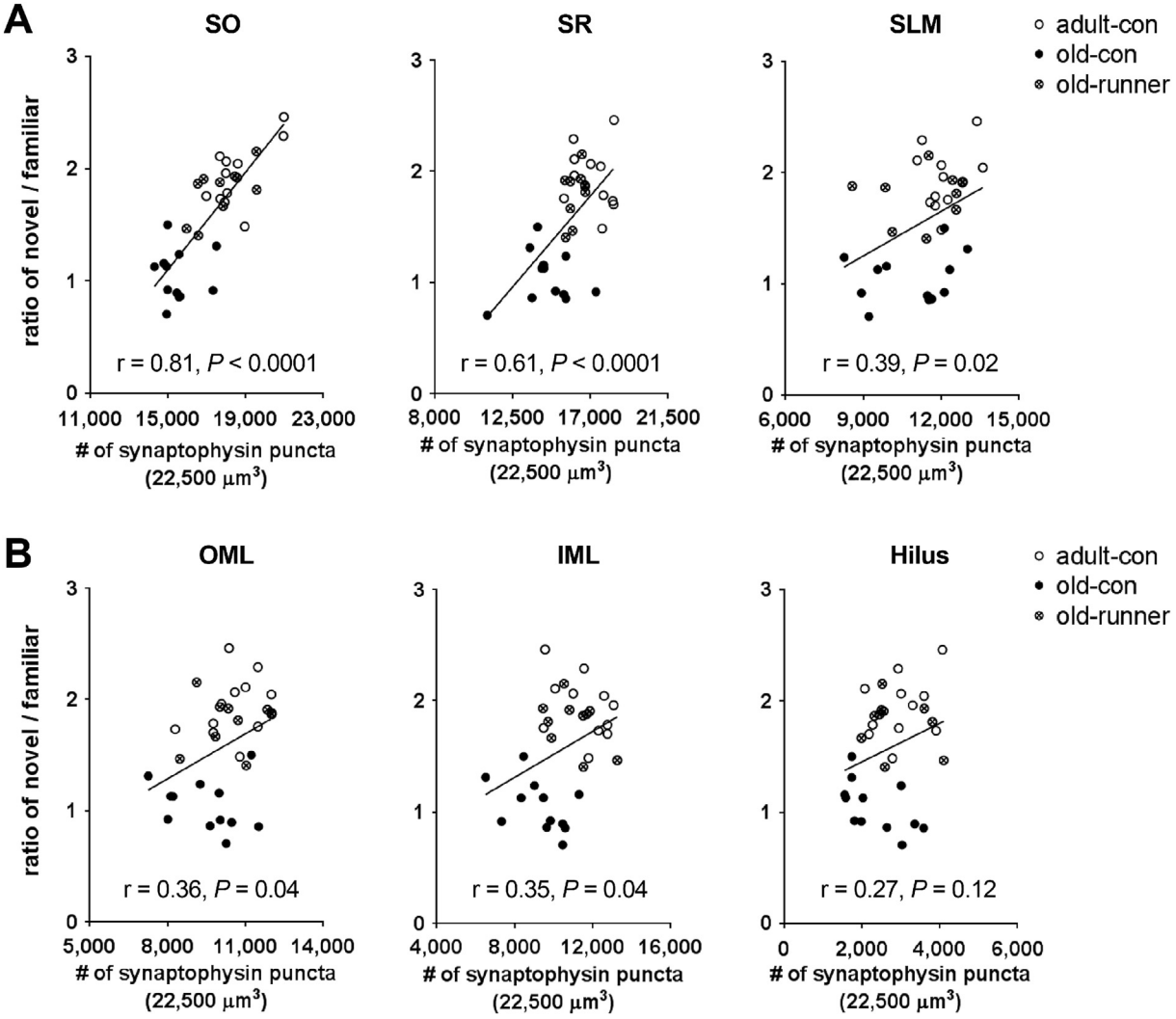

Fig. 4. Correlation between the number of synaptophysin-ir puncta in the hippocampus and memory performance in the novel object location (NOL) task. (A) The novel/familiar ratio in a NOL task positively correlated with the number of synaptophysin-ir puncta in strata oriens and radiatum (Pearson $r = 0.81$ and 0.61, respectively, $P < .0001$) as well as in SLM (Pearson $r = 0.39$, $P = .02$) in area CA1. (B) The novel/familiar ratio also correlated with the number of synaptophysin-ir puncta in OML and IML (Pearson $r = 0.36$ and 0.35, respectively, $P = .04$), but not with the density of puncta in the hilus ($r = 0.27$, $P = .12$) in the DG.

different measurements support the conclusion that prolonged running prevents age-related loss of synaptophysin labeled synapses.

3.3. Correlation between spatial memory performance and the density of synaptophysin

Given the critical role of presynaptic proteins in synaptic plasticity and learning processes, we examined whether the density and/or number of synaptophysin-ir puncta contribute to memory performance. The correlation analyses were performed on mice that were subjected to a novel object location task, followed by fluorescent immunostaining and counting of individual puncta/synapses (Fig. 4). In area CA1 (Fig. 4A), the novel/familiar ratio obtained at 2 h after training in the task positively correlated with the number of synaptophysin-ir puncta in strata oriens and radiatum (Pearson $r = 0.81$ and 0.61, respectively, $P < .0001$) as well as in SLM (Pearson $r = 0.39$, $P = .02$). In the DG (Fig. 4B), the novel/familiar ratio also correlated with the number of synaptophysin-ir puncta in OML and IML (Pearson $r = 0.36$ and 0.35, respectively, $P = .04$), but not with the density of puncta in the hilus ($r = 0.27$, $P = .12$), further revealing the importance of presynaptic terminals in memory performance.

3.4. The effects of wheel running on cholinergic fibers in the hippocampus of old mice

Because presynaptic inputs terminated on the hippocampal cells contained several neurotransmitters, we focused on the cholinergic inputs in the hippocampus to better elucidate the neurochemical mechanisms by which long-term running protects against age-related memory deficits. The cholinergic fibers were revealed *via* AChE enzyme histochemistry. As shown in Fig. 5, AChE-positive fibers were thoroughly distributed in the hippocampus with a laminar pattern. These fibers were particularly dense in the dendritic fields including strata oriens, radiatum, and lacunosum-moleculare of CA1 as well as in the molecular layer and hilus of DG. No difference was found in the distribution pattern and morphology of AChE fibers in examined regions among the three groups (Fig. 5A–F). In area CA1, the density of AChE fibers in old sedentary mice decreased by 23.58%, 25.12%, and 10.65% in strata oriens, radiatum, and lacunosum-moleculare, respectively, when compared to aged adults. The average levels of reduction were ~20% in this area. While aging-related loss of AChE fibers was apparent, running for 5 months prevented the reduction of AChE fibers in CA1 (two-way ANOVA, $F_{[2,90]} = 25.22$, $P < .0001$), particularly in the radiatum and oriens (Tukey's *post hoc* test, $^{**}P < .01$). In the DG, the

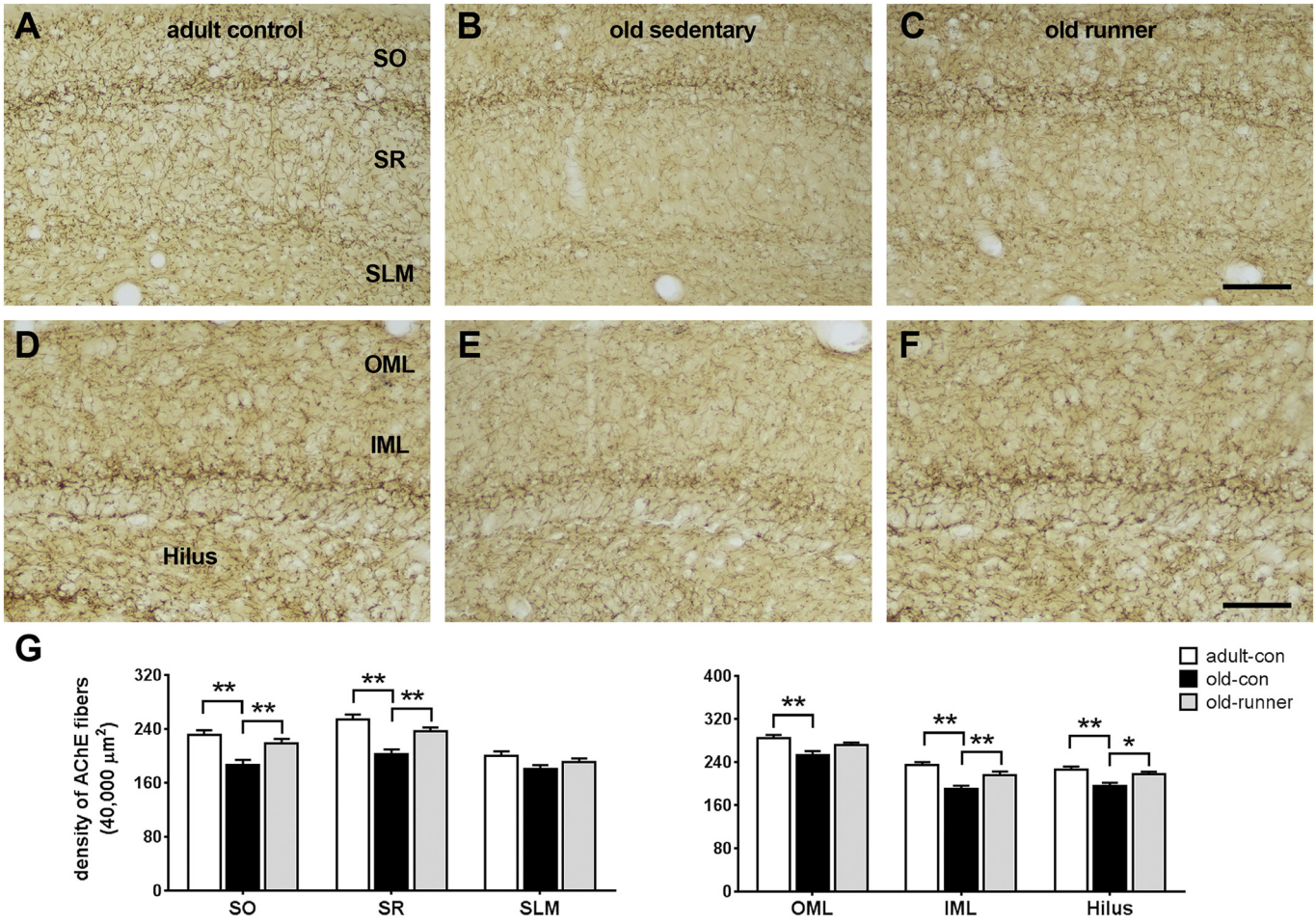

Fig. 5. Running prevents selective loss of cholinergic fibers in aged hippocampus. (A–F) Cholinergic fibers in area CA1 (A–C) and dentate gyrus (D–F) labeled with AChE enzyme-histochemistry. SO: stratum oriens, SR: stratum radiatum, SLM: stratum lacunosum-moleculare. OML: outer molecular layer, IML: inner molecular layer. Bars: 100 μm in A-C and 75 μm in D–F. (G) The quantitative analyses on the density (number of intersections/40,000 μm^2) of AChE fibers. In area CA1 (left), the analyses revealed an aging-related loss of fibers ($F_{[2,90]} = 25.22$, $P < .0001$) and region-dependent difference ($F_{[2,90]} = 26.74$, $P < .0001$), but no interaction ($F_{[4,90]} = 1.74$, $P = .1489$). Loss of fibers was apparent in the radiatum and oriens in old sedentary mice compared with aged adults and old runners (*post hoc* test, $^{**}P < .01$). The runners had a higher density of AChE fibers in the radiatum and oriens compared with old sedentary controls (*post hoc* test, $^{**}P < .01$). In DG (right), the density of fibers was reduced in old sedentary mice compared with adult controls and old runners ($F_{[2,90]} = 24.90$, $P < .0001$; *post hoc* test, $^{**}P < .01$). Running for 5 months prevented the reduction of AChE fibers in IML and hilus ($^{*}P < .05$, $^{**}P < .01$).

density of AChE fibers in old sedentary mice decreased by 12.15%, 22.84%, and 15.63% in OML, IML, and hilus, respectively, compared to aged adults. The average levels of reduction were ~17% in the dentate gyrus. While aging-related loss of AChE fibers was apparent, running for 5 months prevented the reduction of AChE fibers in DG (two-way ANOVA, $F_{[2,90]} = 24.90$, $P < .0001$), particularly in IML and hilus (*post hoc* test, $^{*}P < .05$, $^{**}P < .01$) (Fig. 5G). Group-by-layer interaction was not found in CA1 ($F_{[4,90]} = 1.736$, $P = .15$) and DG ($F_{[4,90]} = 0.393$, $P = .81$).

Correlation between the density of AChE fibers and spatial memory performance was further analyzed in the cohort of mice that was subjected to a novel object location task (Fig. 6). All novel/familiar ratios were obtained at 2 h after training in the task. In area CA1 (Fig. 6A), the novel/familiar ratio positively correlated with the density of AChE in strata oriens and radiatum (Pearson $r = 0.59$ and 0.58, respectively, $P = .0003$) as well as in SLM ($r = 0.37$, $P = .03$). In the DG (Fig. 6B), the novel/familiar ratio also correlated with the density of AChE fibers in OML (Pearson $r = 0.47$, $P = .006$), IML ($r = 0.61$, $P = .0001$), and in the hilus (r = 0.47, $P = .005$), supporting the critical role of cholinergic inputs in hippocampal-dependent memory function.

3.5. The effects of wheel running on ChAT-ir cells in the MS-DB of old mice

Cholinergic cells were not found in the hippocampus (Fig. 7E). The cholinergic fibers in the hippocampus are believed to be exclusively derived from the MS-DB (Blusztajn and Rinnofner, 2016), so we tested whether aging-related loss of hippocampal cholinergic fibers is caused by degeneration of cholinergic resource cells (Stroessner-Johnson et al., 1992), and explored the effects of running on these resource cells in the MS-DB. Numerous ChAT-immunoreactive cells were found in the MS-DB. The general staining pattern and cellular characteristics of these cells in the three groups were similar (Fig. 7A–C) and in line with published data of rats (Ypsilanti et al., 2008).

The total number of ChAT-ir cells in the MS and DB was estimated based on optical dissector and unbiased stereological principles (Gundersen et al., 1988; West, 1999). As shown in Fig. 7D, the total number of ChAT cells in the MS and DB of old sedentary mice was slightly lower than that of aged adult controls (old *vs.* adult in MS: 2442 ± 53 *vs.* 2578 ± 46; in DB: 4580 ± 89 *vs.* 4728 ± 75), but the difference was not significant (*post hoc* test, $P > .05$). No difference was found between the runners and age-matched controls (*post hoc* test, $P > .05$). Two-way ANOVA suggested no group effect ($F_{[2,60]} = 2.29$,

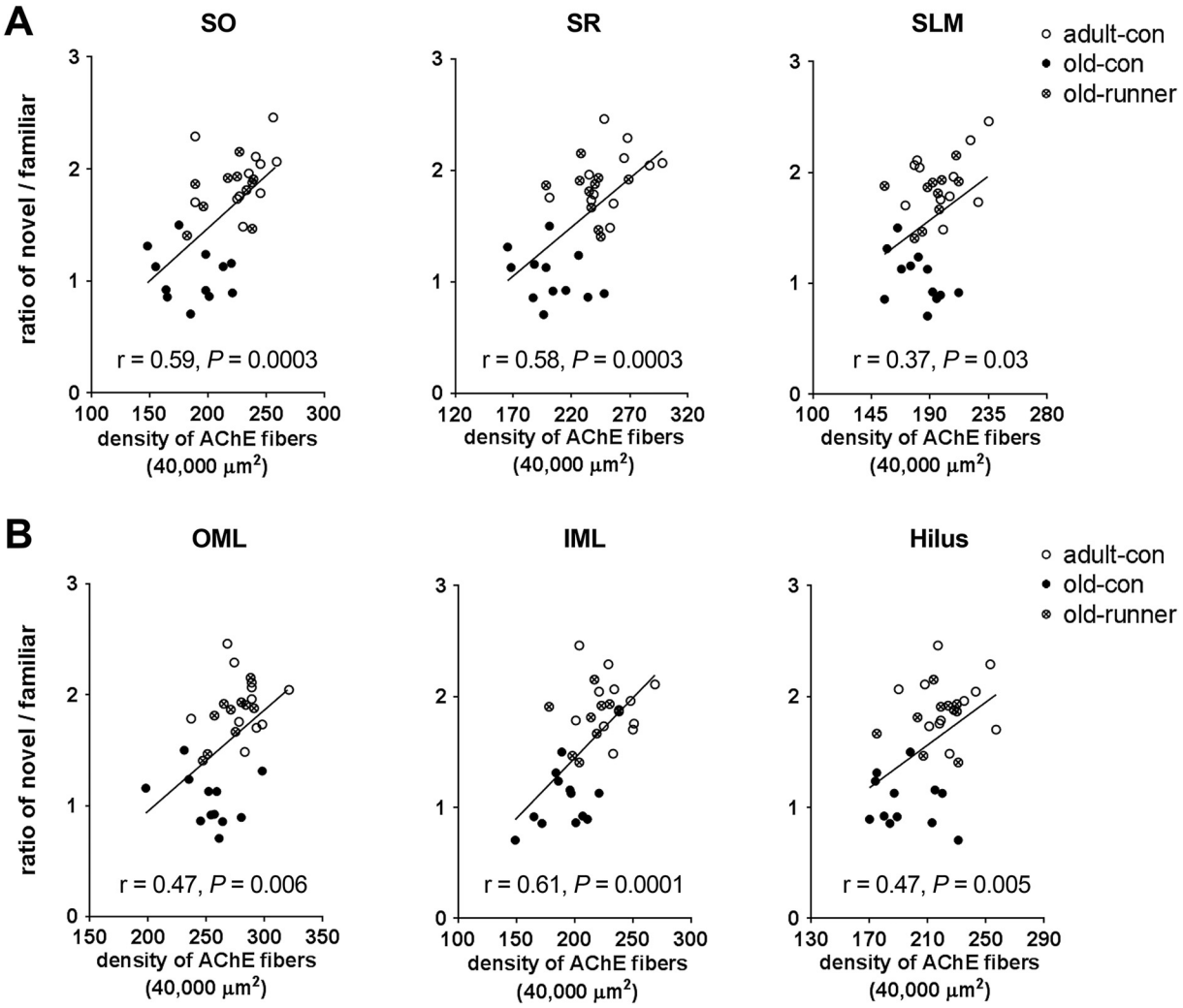

Fig. 6. Correlation between the density of AChE fibers in the hippocampus and spatial memory performance in the novel object location (NOL) task. (A) In area CA1, the novel/familiar ratio in a NOL task positively correlated with the density of AChE in strata oriens and radiatum (Pearson $r = 0.59$ and 0.58, respectively, $P = .0003$) as well as in SLM ($r = 0.37$, $P = .03$). (B) In the DG, the novel/familiar ratio correlated with the density of AChE fibers in OML (Pearson $r = 0.47$, $P = .006$), IML ($r = 0.61$, $P = .0001$), and in the hilus (r = 0.47, $P = .005$).

$P = .11$) and no group-by-region interaction ($F_{[2,60]} = 0.09$, $P = .92$). Because age-related loss of cholinergic cells in the MS has been reported to be regionally selective (Stroessner-Johnson et al., 1992), series sections through the MS-DB were further grouped into anterior (Bregma: 1.42–0.98 mm), middle (Bregma: 0.96–0.52 mm), and posterior (Bregma: 0.50–0.06 mm) subdivisions, and the average numbers of ChAT cells at these subdivisions were analyzed. No regional-dependent difference was found among the three groups (data not shown).

4. Discussion

Physical training is beneficial to cognitive function and cellular survival (Fucà et al., 2017; Stefanko et al., 2017; Xu et al., 2017). In the current study, we investigated the effect of prolonged and voluntary physical running initiated at an older age on aging-related deficits of spatial memory and its underlying presynaptic mechanisms. Voluntary wheel running initiated at 16 months of age can improve hippocampal-dependent spatial memory in C57 mice. Daily running (1 h per day) for 5 months selectively prevents age-related loss of presynaptic vesicle protein synaptophysin and cholinergic innervation in the hippocampus. Particularly, running-induced enhancement of memory function correlates with the restoration of both presynaptic protein synaptophysin and cholinergic inputs in the hippocampus. While training in the form of a moderate-pace running wheel can modify aging-related loss of cholinergic fibers in the hippocampus that contribute to memory performance, the running has limited impacts on the cholinergic cells in the medial septal nucleus and diagonal band area, the source cells of the hippocampal cholinergic innervation.

The function of encoding and retrieval of spatial memory largely relies on the integrity of structural components of synaptic contacts in the hippocampus. It is generally believed that target cells are important in regulating the survival of afferent neurons. Thus, loss of postsynaptic targets may result in degeneration of presynaptic elements. In recent studies, we have reported age-related loss of postsynaptic elements, the spines on specific dendritic segments of hippocampal CA1 pyramidal cells (Xu et al., 2017, 2018). Although studies have linked the loss of dendritic spines to aging-related memory deficits, the role of presynaptic elements in cognition is far from clear. Interestingly, evidence from clinical studies has suggested a specific association between presynaptic proteins and cognitive function (Honer et al., 2012; Ramos-Miguel et al., 2017). By focusing on complexin-I and complexin-II, the presynaptic proteins enriched in GABAergic terminals and glutamatergic terminals, respectively, it has been found that disruption of inhibitory synaptic terminals may trigger early cognitive impairment, while disruption of excitatory terminals contributes relatively more to later cognitive impairment (Ramos-Miguel et al., 2017). However, it is

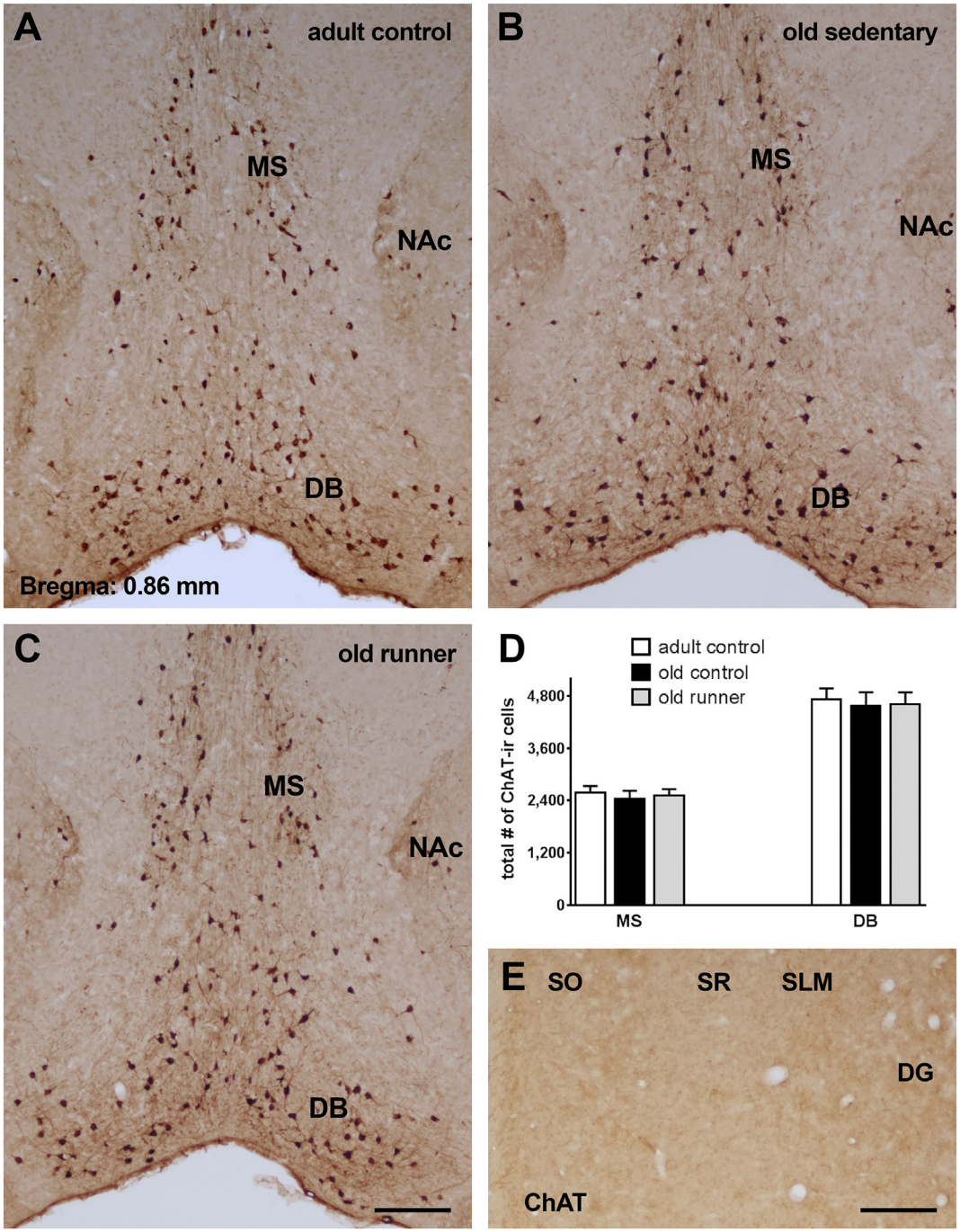

Fig. 7. Effects of running on age-related alteration of ChAT-ir cells in the medial septal nucleus (MS) and diagonal band area (DB). (A–C) Representative images of ChAT immunostaining in the MS-DB of adult control, old control, and old runner taken at the same level of Bregma. (D) Total numbers of ChAT-ir cells were estimated in the MS and DB *via* unbiased stereology. No difference was found among the three groups (Two-way ANOVA, $F_{[2,60]} = 2.29$, $P = .11$). (E) ChAT immunostaining in the hippocampus, no ChAT-ir cells found in area CA1 and dentate gyrus (DG). SO: stratum oriens, SR: stratum radiatum, SLM: stratum lacunosum-moleculare. Bars: 200 μm in A–C and 75 μm in E.

unclear whether normal aging differentially affects the presynaptic terminals, thus contributing to specific profiles of learning and memory performance (Nakashiba et al., 2008; Brun et al., 2002, 2008; Remondes and Schuman, 2004; Suh et al., 2011). Furthermore, it is unknown whether physical running affects external presynaptic inputs that contacted these spines. Therefore, we labeled presynaptic puncta using presynaptic protein markers, GAP-43 and synaptophysin, to explore the impact of running on presynaptic inputs and its correlation to memory performance in older animals. GAP-43 is a presynaptic membrane phosphoprotein, contributing to plasticity and growth of the presynaptic terminal that may occur during the formation of memory (Aigner et al., 1995; Skene, 1989). During the aging process, decreased expression of GAP-43 has been observed in the hippocampus (Chao et al., 1992; Casoli et al., 1996). In the current study, age-related loss of individual GAP-43-ir puncta was found in area CA1 and DG. Because GAP-43 is primarily expressed in excitatory synapses (Nemes et al., 2017), loss of GAP-43-ir puncta found here suggests the loss of excitatory presynaptic inputs. Interestingly, 5-month running had limited effect on the number of GAP-43-ir puncta. To understand the presynaptic mechanisms by which long-term running protects against age-

related deficits of memory function, we then measured the expressions of presynaptic vesicle protein synaptophysin, a commonly recognized presynaptic protein marker in the hippocampus (Navone et al., 1986; Thomas et al., 1988; Wiedenmann and Franke, 1985). Synaptophysin immunoreactivity matches with the synaptic profile distribution completely, and increased expression of synaptophysin is accompanied by the formation of synapses (Masliah et al., 1990; Calhoun et al., 1996). Consistent with the loss of postsynaptic targets, reduced number of synaptophysin-ir puncta is apparent in all measured regions including strata radiatum, oriens, and lacunosum-moleculare of area CA1. Particularly, running prevented the age-related loss of synaptophysin in strata oriens and radiatum. In the dentate gyrus, aging-related loss of synaptophysin was mainly detected at the inner molecular layer. However, daily voluntary running prevents aging-related loss of synaptophysin in this area. We have further examined synaptophysin puncta from behaviorally characterized aged sedentary and trained mice. The correlation data indicated that memory performance positively correlated with the number of synaptophysin-ir puncta in area CA1 and DG. Taken together, these data suggest that prolonged running prevents aging-related loss of presynaptic vesicle protein synaptophysin but has less effect on GAP-43 that is mainly detected in glutamatergic excitatory synapses (Nemes et al., 2017).

In the current study, decreased expressions of GAP-43 and synaptophysin have been found in the aged hippocampus. Age-related decrease was apparent in several dendritic fields in old sedentary mice compared to adult controls. However, physical training lasting for 5 months selectively prevented the age-related loss of synaptophysin, but not GAP-43. The differential effect might be related to the different nature and function of the presynaptic proteins analyzed. GAP-43 contributes to the growth of presynaptic terminals during development (Denny, 2006; Holahan, 2017). It is generally believed that increased expression of GAP-43 may represent enhanced synaptogenesis (Holahan, 2017; Nemes et al., 2017). The vesicle protein synaptophysin is found in all types of synapses and important for synaptic integrity (Wiedenmann and Franke, 1985; Calhoun et al., 1996; McMahon et al., 1996). Alternatively, each presynaptic protein may not be equally affected by aging or physical training. For example, previous studies from patients with Alzheimer's disease (AD) have suggested that synaptophysin is more vulnerable than are other synaptic proteins, such as syntaxin or SNAP-25 (Minger et al., 2001), and loss of synaptophysin is an early event that correlates with initial cognitive impairment (Masliah et al., 2001). Therefore, limited effect of running on GAP-43 suggested that physical training initiated at a late stage of life (16 months of age in mouse) might not enhance synaptogenesis in aged hippocampus. Instead, restoration of synaptophysin largely contributed to the protective effect of running on memory function as indicated by the correlation analyses.

In the hippocampus, the pyramidal cells and interneurons receive differential innervations arriving from several extrinsic sources (Huh et al., 2010; Takács et al., 2012). One of the key sources necessary for memory processes is the cholinergic inputs from the MS-DB (Sarter and Parikh, 2005; Teles-Grilo Ruivo and Mellor, 2013). Cholinergic fibers from the MS-DB are thoroughly distributed in the hippocampus with a laminar pattern. Dense axon terminals in the cellular layers in area CA1 and DG suggests that the somata of glutamatergic principle cells are innervated by the MS-DB cholinergic projections. In the dendritic fields of the hippocampus, cholinergic fibers are particularly dense in strata oriens, radiatum, and lacunosum-moleculare of CA1 as well as in the molecular layer and hilus of DG. Loss of cholinergic fibers and abnormal cholinergic neurotransmission in the hippocampus contribute to the memory deficits seen in aging (Blusztajn and Rinnofner, 2016). Degeneration and/or malfunction of the septo-hippocampal cholinergic system have also been recorded in animal models of AD (Savonenko et al., 2005; Mufson et al., 2008; Machová et al., 2010; Nikolajsen et al., 2011; Burke et al., 2013; Mellott et al., 2014; Chen et al., 2015) as well as in patients with AD (Whitehouse et al., 1982; Bowen et al., 1983; Mufson et al., 2008; Grothe et al., 2013). Therefore, it has been postulated that abnormal cholinergic neurotransmission, due to dysfunction and/or degeneration of MS-DB, contributes to the memory deficits seen in advanced age and in AD (Mufson et al., 2008; Grothe et al., 2013; Haense et al., 2012). In the current study, aging-related loss of cholinergic fibers was apparent in the radiatum and oriens in old sedentary mice when compared with aged adults. The density of AChE fibers in old sedentary mice decreased by 23.58% and 25.12% in strata oriens and radiatum, respectively, compared with aged adults. While aging-related loss of AChE fibers was apparent, running for 5 months prevented the loss of AChE fibers in area CA1, particularly in the radiatum and oriens, the areas of dendritic profiles of pyramidal cells. Similarly, while aging-related loss of AChE fibers was by 17% in the dentate gyrus (DG), prolonged running at a moderate level prevented the reduction of AChE fibers in DG, particularly in IML and hilus. Importantly, improvement of the cholinergic innervation in area CA1 and DG significantly contributes to spatial memory performance.

Given the importance of hippocampal cholinergic fibers in the beneficial effects of physical running in memory performance, the cholinergic cells that project to the hippocampus were investigated. Consistent with studies on the expression of ChAT at protein and mRNA levels (Satoh et al., 1983; Ichikawa and Hirata, 1986; Oh et al., 1992; Lauterborn et al., 1993), we did not detect any ChAT-ir cells in the hippocampus, supporting the notion that there are no intrinsic cholinergic cells in the hippocampus (Blusztajn and Rinnofner, 2016). Similarly, studies on VAChT expression did not show cellular staining in the hippocampus (Gilmor et al., 1996; Roghani et al., 1998; Schäfer et al., 1998; Ichikawa et al., 1997). Therefore, it is accepted that the cholinergic fibers in the hippocampus were mainly derived from the cholinergic cells in the MS-DB, and the loss of hippocampal cholinergic fibers may indicate the degeneration and/or loss of cholinergic cells in the MS-DB. Since the cholinergic inputs to the hippocampus derive primarily from the MS-DB (Amaral and Cowan, 1980; Mesulam et al., 1983), we examined the aging-related changes of MS-DB cells and the effects of running. Although we found a robust loss of cholinergic fibers in the hippocampus, the degeneration of cells in the MS-DB was limited, consistent with previous studies in which it has been reported that ablation of target neurons has limited effects on cholinergic neurons in the basal forebrain (Sofroniew et al., 1990). It has also been reported that neuronal cell loss is found predominantly in pathological aging, such as AD, while normal aging is accompanied by dendritic, synaptic, and axonal degeneration with nearly no cell loss (Burke and Barnes, 2006; Ypsilanti et al., 2008). These findings suggest that functional decline associated with aging across species does not primarily result from cell loss, but rather, other mechanisms such as decreases in gene expression, impairments in intracellular signaling, and cytoskeletal transport that may mediate cholinergic cell atrophy leading to age-related functional decline in the brain (Schliebs and Arendt, 2011; Craig et al., 2011). Early studies have also shown that fimbria fornix axotomy does not lead to the degeneration or loss of the cholinergic neurons in the septum (Panni et al., 1999; Lazo et al., 2010) because the septal cholinergic neurons may sprout and project to the hippocampus *via* the supracallosal pathway (Lewis and Shute, 1967; Gaykema et al., 1990) or another ventral pathway (ansa lenticularis) (Gage et al., 1984; Milner and Amaral, 1984).

Here, we have reported that restored cholinergic inputs in area CA1 and DG *via* physical running contribute to spatial memory performance in aged mice, supporting the importance of cholinergic circuits in the hippocampal cognitive function. Retardation of cholinergic fibers and loss of presynaptic puncta were apparent in aged hippocampus, which is correlated with memory performance. However, the correlation between the cholinergic fibers and presynaptic puncta is unclear and requires further study. Data from *in vitro* studies have shown that cholinesterase can promote cellular proliferation and neurite outgrowth during early development (Layer et al., 1993; Jones et al., 1995; Small et al., 1995; Sternfeld et al., 1998), likely by modulating the adhesion

capacity of neurites (Sternfeld et al., 1998). It is unknown whether the enzyme can prevent aging-related loss of presynaptic inputs in the hippocampus. Considering that cholinesterase inhibitors including donepezil, rivastigmine, and galantamine, have proven to be beneficial in improving cognitive function in patients with AD and dementia with Lewy bodies (*e.g.*, Trinh et al., 2003; Anand and Singh, 2013; Colović et al., 2013; Andrieu et al., 2015), it would be interest to explore the potential effects of cholinesterase inhibitors on age-driven decay of synaptic inputs and cognitive function during normal aging.

In summary, we find that voluntary running for 5 months selectively protects against aging-related loss of presynaptic protein synaptophysin and cholinergic inputs in the hippocampus. The density of presynaptic protein and amount of cholinergic fibers in area CA1 and the dentate gyrus positively correlate with spatial memory performance. The data further highlights the importance of physical exercise and training initiated at an old age for memory performance.

Declaration of competing interests

No potential conflicts of interest exist.

Acknowledgements

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References

Aigner, L., Arber, S., Kapfhammer, J.P., Laux, T., Schneider, C., Botteri, F., Brenner, H.R., Caroni, P., 1995. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell 83 (2), 269–278.

Altavista, M.C., Rossi, P., Bentivoglio, A.R., Crociani, P., Albanese, A., 1990. Ageing is associated with diffuse loss of forebrain cholinergic neurons. Brain Res. 508 (1), 51–59.

Amaral, D.G., Cowan, W.M., 1980. Subcortical afferents to the hippocampal formation in the monkey. J. Comp. Neurol. 189 (4), 573–591.

Anand, P., Singh, B., 2013. A review on cholinesterase inhibitors for Alzheimer's disease. Arch. Pharm. Res. 36 (4), 375–399.

Andrieu, S., Coley, N., Lovestone, S., Aisen, P.S., Vellas, B., 2015. Prevention of sporadic Alzheimer's disease: lessons learned from clinical trials and future directions. Lancet Neurol. 14 (9), 926–944.

Bartus, R.T., Dean 3rd, R.L., Beer, B., Lippa, A.S., 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408–414.

Blusztajn, J.K., Rinnofner, J., 2016. Intrinsic cholinergic neurons in the hippocampus: fact or artifact? Front. Synaptic. Neurosci. 8, 6.

Bowen, D.M., Allen, S.J., Benton, J.S., Goodhardt, M.J., Haan, E.A., Palmer, A.M., Sims, N.R., Smith, C.C., Spillane, J.A., Esiri, M.M., Neary, D., Snowdon, J.S., Wilcock, G.K., Davison, A.N., 1983. Biochemical assessment of serotonergic and cholinergic dysfunction and cerebral atrophy in Alzheimer's disease. J. Neurochem. 41 (1), 266–272.

Brun, V.H., Otnass, M.K., Molden, S., Steffenach, H.A., Witter, M.P., Moser, M.B., Moser, E.I., 2002. Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. Science 296, 2243–2246.

Brun, V.H., Leutgeb, S., Wu, H.Q., Schwarcz, R., Witter, M.P., Moser, E.I., Moser, M.B., 2008. Impaired spatial representation in CA1 after lesion of direct input from entorhinal cortex. Neuron 57 (2), 290–302.

Burke, S.N., Barnes, C.A., 2006. Neural plasticity in the ageing brain. Nat. Rev. Neurosci. 7 (1), 30–40.

Burke, R.M., Norman, T.A., Haydar, T.F., Slack, B.E., Leeman, S.E., Blusztajn, J.K., Mellott, T.J., 2013. BMP9 ameliorates amyloidosis and the cholinergic defect in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 110 (48), 19567–19572.

Buzsáki, G., 2002. Theta oscillations in the hippocampus. Neuron 33 (3), 325–340.

Buzsáki, G., Moser, E.I., 2013. Memory, navigation and theta rhythm in the hippocampal-entorhinal system. Nat. Neurosci. 16 (2), 130–138.

Calhoun, M.E., Jucker, M., Martin, L.J., Thinakaran, G., Price, D.L., Mouton, P.R., 1996. Comparative evaluation of synaptophysin-based methods for quantification of synapses. J. Neurocytol. 25 (12), 821–828.

Casoli, T., Spagna, C., Fattoretti, P., Gesuita, R., Bertoni-Freddari, C., 1996. Neuronal plasticity in aging: a quantitative immunohistochemical study of GAP-43 distribution in discrete regions of the rat brain. Brain Res. 714 (1–2), 111–117.

Chao, H.M., Spencer, R.L., Sakai, R.R., McEwen, B.S., 1992. The expression of growth-associated protein GAP-43 mRNA in the rat hippocampus in response to adrenalectomy and aging. Mol. Cell. Neurosci. 3 (6), 529–535.

Chen, Y., Pan, C., Xuan, A., Xu, L., Bao, G., Liu, F., Fang, J., Long, D., 2015. Treatment efficacy of NGF nanoparticles combining neural stem cell transplantation on Alzheimer's disease model rats. Med. Sci. Monit. 21, 3608–3615.

Chen, Y., Molet, J., Lauterborn, J.C., Trieu, B.H., Bolton, J.L., Patterson, K.P., Gall, C.M., Lynch, G., Baram, T.Z., 2016. Converging, synergistic actions of multiple stress hormones mediate enduring memory impairments after acute simultaneous stresses. J. Neurosci. 36 (44), 11295–11307.

Choi, S.H., Bylykbashi, E., Chatila, Z.K., Lee, S.W., Pulli, B., Clemenson, G.D., Kim, E., Rompala, A., Oram, M.K., Asselin, C., Aronson, J., Zhang, C., Miller, S.J., Lesinski, A., Chen, J.W., Kim, D.Y., van Praag, H., Spiegelman, B.M., Gage, F.H., Tanzi, R.E., 2018. Combined adult neurogenesis and BDNF mimic exercise effects on cognition in an Alzheimer's mouse model. Science 361 (6406). https://doi.org/10.1126/science.aan8821.

Christie, B.R., Eadie, B.D., Kannangara, T.S., Robillard, J.M., Shin, J., Titterness, A.K., 2008. Exercising our brains: how physical activity impacts synaptic plasticity in the dentate gyrus. NeuroMolecular Med. 10 (2), 47–58.

Colović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., Vasić, V.M., 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr. Neuropharmacol. 11 (3), 315–335.

Craig, L.A., Hong, N.S., McDonald, R.J., 2011. Revisiting the cholinergic hypothesis in the development of Alzheimer's disease. Neurosci. Biobehav. Rev. 35 (6), 1397–1409.

Denny, J.B., 2006. Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43. Curr. Neuropharmacol. 4 (4), 293–304.

Douchamps, V., Mathis, C., 2017. A second wind for the cholinergic system in Alzheimer's therapy. Behav. Pharmacol. 28 (2 and 3-Spec Issue), 112–123.

Eadie, B.D., Redila, V.A., Christie, B.R., 2005. Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density. J. Comp. Neurol. 486 (1), 39–47.

Farmer, J., Zhao, X., van Praag, H., Wodtke, K., Gage, F.H., Christie, B.R., 2004. Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague–Dawley rats in vivo. Neuroscience 124 (1), 71–79.

Fischer, W., Wictorin, K., Björklund, A., Williams, L.R., Varon, S., Gage, F.H., 1987. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329, 65–68.

Fischer, W., Gage, F.H., Björklund, A., 1989. Degenerative changes in forebrain cholinergic nuclei correlate with cognitive impairments in aged rats. Eur. J. Neurosci. 1 (1), 34–45.

Fucà, E., Guglielmotto, M., Boda, E., Rossi, F., Leto, K., Buffo, A., 2017. Preventive motor training but not progenitor grafting ameliorates cerebellar ataxia and deregulated autophagy in tambaleante mice. Neurobiol. Dis. 102, 49–59.

Gage, F.H., Björklund, A., Stenevi, U., 1984. Cells of origin of the ventral cholinergic septohippocampal pathway undergoing compensatory collateral sprouting following fimbria-fornix transection. Neurosci. Lett. 44 (2), 211–216.

Gaykema, R.P., Luiten, P.G., Nyakas, C., Traber, J., 1990. Cortical projection patterns of the medial septum-diagonal band complex. J. Comp. Neurol. 293 (1), 103–124.

Gilad, G.M., Rabey, J.M., Tizabi, Y., Gilad, V.H., 1987. Age-dependent loss and compensatory changes of septohippocampal cholinergic neurons in two rat strains differing in longevity and response to stress. Brain Res. 436 (2), 311–322.

Gilmor, M.L., Nash, N.R., Roghani, A., Edwards, R.H., Yi, H., Hersch, S.M., Levey, A.I., 1996. Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. J. Neurosci. 16 (7), 2179–2190.

Grothe, M., Heinsen, H., Teipel, S., 2013. Longitudinal measures of cholinergic forebrain atrophy in the transition from healthy aging to Alzheimer's disease. Neurobiol. Aging 34 (4), 1210–1220.

Gundersen, H.J., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Srensen, F.B., Vesterby, A., West, M.J., 1988. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96, 857–881.

Gundersen, H.J., Jensen, E.B., Kiêu, K., Nielsen, J., 1999. The efficiency of systematic sampling in stereology – reconsidered. J. Microsc. 193 (Pt 3), 199–211.

Haense, C., Kalbe, E., Herholz, K., Hohmann, C., Neumaier, B., Krais, R., Heiss, W.D., 2012. Cholinergic system function and cognition in mild cognitive impairment. Neurobiol. Aging 33 (5), 867–877.

Härtig, W., Bauer, A., Brauer, K., Grosche, J., Hortobágyi, T., Penke, B., Schliebs, R., Harkany, T., 2002. Functional recovery of cholinergic basal forebrain neurons under disease conditions: old problems, new solutions? Rev. Neurosci. 13 (2), 95–165.

Hedreen, J.C., Bacon, S.J., Price, D.L., 1985. A modified histochemical technique to visualize acetylcholinesterase-containing axons. J. Histochem. Cytochem. 33 (2), 134–140.

Holahan, M.R., 2017. A shift from a pivotal to supporting role for the growth-associated protein (GAP-43) in the coordination of axonal structural and functional plasticity. Front. Cell. Neurosci. 11, 266.

Honer, W.G., Barr, A.M., Sawada, K., Thornton, A.E., Morris, M.C., Leurgans, S.E., Schneider, J.A., Bennett, D.A., 2012. Cognitive reserve, presynaptic proteins and dementia in the elderly. Transl. Psychiatry 2, e114.

Hötting, K., Röder, B., 2013. Beneficial effects of physical exercise on neuroplasticity and cognition. Neurosci. Biobehav. Rev. 37 (9 Pt B), 2243–2257.

Huh, C.Y., Goutagny, R., Williams, S., 2010. Glutamatergic neurons of the mouse medial septum and diagonal band of Broca synaptically drive hippocampal pyramidal cells: relevance for hippocampal theta rhythm. J. Neurosci. 30 (47), 15951–15961.

Ichikawa, T., Hirata, Y., 1986. Organization of choline acetyltransferase-containing structures in the forebrain of the rat. J. Neurosci. 6 (1), 281–292.

Ichikawa, T., Ajiki, K., Matsuura, J., Misawa, H., 1997. Localization of two cholinergic

markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: in situ hybridization histochemistry and immunohistochemistry. J. Chem. Neuroanat. 13 (1), 23–39.
Intlekofer, K.A., Cotman, C.W., 2013. Exercise counteracts declining hippocampal function in aging and Alzheimer's disease. Neurobiol. Dis. 57, 47–55.
Jones, S.A., Holmes, C., Budd, T.C., Greenfield, S.A., 1995. The effect of acetylcholinesterase on outgrowth of dopaminergic neurons in organotypic slice culture of rat mid-brain. Cell Tissue Res. 279 (2), 323–330.
Kaufman, M., Corner, M.A., Ziv, N.E., 2012. Long-term relationships between cholinergic tone, synchronous bursting and synaptic remodeling. PLoS One 7 (7), e40980.
Kronenberg, G., Bick-Sander, A., Bunk, E., Wolf, C., Ehninger, D., Kempermann, G., 2006. Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus. Neurobiol. Aging 27 (10), 1505–1513.
Larson, E.B., Wang, L., Bowen, J.D., McCormick, W.C., Teri, L., Crane, P., Kukull, W., 2006. Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older. Ann. Intern. Med. 144, 73–81.
Lauterborn, J.C., Isackson, P.J., Montalvo, R., Gall, C.M., 1993. In situ hybridization localization of choline acetyltransferase mRNA in adult rat brain and spinal cord. Mol. Brain Res. 17 (1–2), 59–69.
Layer, P.G., Weikert, T., Alber, R., 1993. Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res. 273 (2), 219–226.
Lazo, O.M., Mauna, J.C., Pissani, C.A., Inestrosa, N.C., Bronfman, F.C., 2010. Axotomy-induced neurotrophic withdrawal causes the loss of phenotypic differentiation and downregulation of NGF signalling, but not death of septal cholinergic neurons. Mol. Neurodegener. 5, 5.
Lewis, D.A., 1991. Distribution of choline acetyltransferase-immunoreactive axons in monkey frontal cortex. Neuroscience 40 (2), 363–374.
Lewis, P.R., Shute, C.C., 1967. The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. Brain 90 (3), 521–540.
Maass, A., Düzel, S., Goerke, M., Becke, A., Sobieray, U., Neumann, K., Lövden, M., Lindenberger, U., Bäckman, L., Braun-Dullaeus, R., Ahrens, D., Heinze, H.J., Müller, N.G., Düzel, E., 2015. Vascular hippocampal plasticity after aerobic exercise in older adults. Mol. Psychiatry 20 (5), 585–593.
Machová, E., Rudajev, V., Smycková, H., Koivisto, H., Tanila, H., Dolezal, V., 2010. Functional cholinergic damage develops with amyloid accumulation in young adult APPswe/PS1dE9 transgenic mice. Neurobiol. Dis. 38 (1), 27–35.
Mandolesi, L., Polverino, A., Montuori, S., Foti, F., Ferraioli, G., Sorrentino, P., Sorrentino, G., 2018. Effects of physical exercise on cognitive functioning and well-being: biological and psychological benefits. Front. Psychol. 9, 509.
Masliah, E., Terry, R.D., Alford, M., DeTeresa, R., 1990. Quantitative immunohistochemistry of synaptophysin in human neocortex: an alternative method to estimate density of presynaptic terminals in paraffin sections. J. Histochem. Cytochem. 38 (6), 837–844.
Masliah, E., Mallory, M., Alford, M., DeTeresa, R., Hansen, L.A., McKeel Jr., D.W., Morris, J.C., 2001. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. Neurology 56 (1), 127–129.
McMahon, H.T., Bolshakov, V.Y., Janz, R., Hammer, R.E., Siegelbaum, S.A., Südhof, T.C., 1996. Synaptophysin, a major synaptic vesicle protein, is not essential for neurotransmitter release. Proc. Natl. Acad. Sci. U. S. A. 93 (10), 4760–4764.
Mellott, T.J., Pender, S.M., Burke, R.M., Langley, E.A., Blusztajn, J.K., 2014. IGF2 ameliorates amyloidosis, increases cholinergic marker expression and raises BMP9 and neurotrophin levels in the hippocampus of the APPswePS1dE9 Alzheimer's disease model mice. PLoS One 9 (4), e94287.
Mesulam, M.M., Mufson, E.J., Levey, A.I., Wainer, B.H., 1983. Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata), and hypothalamus in the rhesus monkey. J. Comp. Neurol. 214 (2), 170–197.
Mesulam, M.M., Mufson, E.J., Rogers, J., 1987. Age-related shrinkage of cortically projecting cholinergic neurons: a selective effect. Ann. Neurol. 22 (1), 31–36.
Milner, T.A., Amaral, D.G., 1984. Evidence for a ventral septal projection to the hippocampal formation of the rat. Exp. Brain Res. 55 (3), 579–585.
Minger, S.L., Honer, W.G., Esiri, M.M., McDonald, B., Keene, J., Nicoll, J.A., Carter, J., Hope, T., Francis, P.T., 2001. Synaptic pathology in prefrontal cortex is present only with severe dementia in Alzheimer disease. J. Neuropathol. Exp. Neurol. 60 (10), 929–936.
Mufson, E.J., Counts, S.E., Perez, S.E., Ginsberg, S.D., 2008. Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. Expert. Rev. Neurother. 8 (11), 1703–1718.
Nakashiba, T., Young, J.Z., McHugh, T.J., Buhl, D.L., Tonegawa, S., 2008. Transgenic inhibition of synaptic transmission reveals role of CA3 output in hippocampal learning. Science 319, 1260–1264.
Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P., De Camilli, P., 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J. Cell Biol. 103 (6 Pt 1), 2511–2527.
Nemes, A.D., Ayasoufi, K., Ying, Z., Zhou, Q.G., Suh, H., Najm, I.M., 2017. Growth associated protein 43 (GAP-43) as a novel target for the diagnosis, treatment and prevention of epileptogenesis. Sci. Rep. 7 (1), 17702.
Niemann, C., Godde, B., Voelcker-Rehage, C., 2014. Not only cardiovascular, but also coordinative exercise increases hippocampal volume in older adults. Front. Aging Neurosci. 6, 170.
Nikolajsen, G.N., Jensen, M.S., West, M.J., 2011. Cholinergic axon length reduced by 300 meters in the brain of an Alzheimer mouse model. Neurobiol. Aging 32 (11), 1927–1931.
Nithianantharajah, J., Hannan, A.J., 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat. Rev. Neurosci. 7 (9), 697–709.
Oh, J.D., Woolf, N.J., Roghani, A., Edwards, R.H., Butcher, L.L., 1992. Cholinergic neurons in the rat central nervous system demonstrated by in situ hybridization of choline acetyltransferase mRNA. Neuroscience 47 (4), 807–822.
Panni, M.K., Atkinson, J., Sofroniew, M.V., 1999. Leukaemia inhibitory factor prevents loss of p75-nerve growth factor receptor immunoreactivity in medial septal neurons following fimbria-fornix lesions. Neuroscience 89 (4), 1113–1121.
Ramos-Miguel, A., Sawada, K., Jones, A.A., Thornton, A.E., Barr, A.M., Leurgans, S.E., Schneider, J.A., Bennett, D.A., Honer, W.G., 2017. Presynaptic proteins complexin-I and complexin-II differentially influence cognitive function in early and late stages of Alzheimer's disease. Acta Neuropathol. 133 (3), 395–407.
Remondes, M., Schuman, E.M., 2004. Role for a cortical input to hippocampal area CA1 in the consolidation of a long-term memory. Nature 431, 699–703.
Roghani, A., Shirzadi, A., Butcher, L.L., Edwards, R.H., 1998. Distribution of the vesicular transporter for acetylcholine in the rat central nervous system. Neuroscience 82 (4), 1195–1212.
Sarter, M., Parikh, V., 2005. Choline transporters, cholinergic transmission and cognition. Nat. Rev. Neurosci. 6 (1), 48–56.
Satoh, K., Armstrong, D.M., Fibiger, H.C., 1983. A comparison of the distribution of central cholinergic neurons as demonstrated by acetylcholinesterase pharmacohistochemistry and choline acetyltransferase immunohistochemistry. Brain Res. Bull. 11 (6), 693–720.
Savonenko, A., Xu, G.M., Melnikova, T., Morton, J.L., Gonzales, V., Wong, M.P., Price, D.L., Tang, F., Markowska, A.L., Borchelt, D.R., 2005. Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitter abnormalities. Neurobiol. Dis. 18 (3), 602–617.
Scarmeas, N., Luchsinger, J.A., Brickman, A.M., Cosentino, S., Schupf, N., Xin-Tang, M., Gu, Y., Stern, Y., 2011. Physical activity and Alzheimer disease course. Am. J. Geriatr. Psychiatry 19 (5), 471–481.
Schäfer, M.K., Eiden, L.E., Weihe, E., 1998. Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. I. Central nervous system. Neuroscience 84 (2), 331–359.
Schliebs, R., Arendt, T., 2006. The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J. Neural Transm. 113 (11), 1625–1644 (Vienna).
Schliebs, R., Arendt, T., 2011. The cholinergic system in aging and neuronal degeneration. Behav. Brain Res. 221 (2), 555–563.
Siette, J., Westbrook, R.F., Cotman, C., Sidhu, K., Zhu, W., Sachdev, P., Valenzuela, M.J., 2013. Age-specific effects of voluntary exercise on memory and the older brain. Biol. Psychiatry 73 (5), 435–442.
Skene, J.H., 1989. Axonal growth-associated proteins. Annu. Rev. Neurosci. 12, 127–156.
Small, D.H., Reed, G., Whitefield, B., Nurcombe, V., 1995. Cholinergic regulation of neurite outgrowth from isolated chick sympathetic neurons in culture. J. Neurosci. 15, 144–151.
Snigdha, S., de Rivera, C., Milgram, N.W., Cotman, C.W., 2014. Exercise enhances memory consolidation in the aging brain. Front. Aging Neurosci. 6, 3.
Sofroniew, M.V., Galletly, N.P., Isacson, O., Svendsen, C.N., 1990. Survival of adult basal forebrain cholinergic neurons after loss of target neurons. Science 247, 338–342.
Solari, N., Hangya, B., 2018. Cholinergic modulation of spatial learning, memory and navigation. Eur. J. Neurosci. 48 (5), 2199–2230.
Stefanko, D.P., Shah, V.D., Yamasaki, W.K., Petzinger, G.M., Jakowec, M.W., 2017. Treadmill exercise delays the onset of non-motor behaviors and striatal pathology in the CAG140 knock-in mouse model of Huntington's disease. Neurobiol. Dis. 105, 15–32.
Stern, Y., 2012. Cognitive reserve in ageing and Alzheimer's disease. Lancet Neurol. 11, 1006–1012.
Sternfeld, M., Ming, G., Song, H., Sela, K., Timberg, R., Poo, M., Soreq, H., 1998. Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable C termini. J. Neurosci. 18 (4), 1240–1249.
Stroessner-Johnson, H.M., Rapp, P.R., Amaral, D.G., 1992. Cholinergic cell loss and hypertrophy in the medial septal nucleus of the behaviorally characterized aged rhesus monkey. J. Neurosci. 12 (5), 1936–1944.
Suh, J., Rivest, A.J., Nakashiba, T., Tominaga, T., Tonegawa, S., 2011. Entorhinal cortex layer III input to the hippocampus is crucial for temporal association memory. Science 334, 1415–1420.
Sun, Y., Nguyen, A.Q., Nguyen, J.P., Le, L., Saur, D., Choi, J., Callaway, E.M., Xu, X., 2014. Cell-type-specific circuit connectivity of hippocampal CA1 revealed through Cre-dependent rabies tracing. Cell Rep. 7 (1), 269–280.
Takács, V.T., Klausberger, T., Somogyi, P., Freund, T.F., Gulyás, A.I., 2012. Extrinsic and local glutamatergic inputs of the rat hippocampal CA1 area differentially innervate pyramidal cells and interneurons. Hippocampus 22, 1379–1391.
Teles-Grilo Ruivo, L.M., Mellor, J.R., 2013. Cholinergic modulation of hippocampal network function. Front. Synaptic Neurosci. 5, 2.
Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W.W., Betz, H., 1988. Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. Science 242, 1050–1053.
Thomas, A.G., Dennis, A., Bandettini, P.A., Johansen-Berg, H., 2012. The effects of aerobic activity on brain structure. Front. Psychol. 3, 86.
Trinh, N.H., Hoblyn, J., Mohanty, S., Yaffe, K., 2003. Efficacy of cholinesterase inhibitors in the treatment of neuropsychiatric symptoms and functional impairment in Alzheimer disease: a meta-analysis. JAMA 289 (2), 210–216.
van Praag, H., Shubert, T., Zhao, C., Gage, F.H., 2005. Exercise enhances learning and hippocampal neurogenesis in aged mice. J. Neurosci. 25 (38), 8680–8685.
von Bohlen und Halbach, O., Zacher, C., Gass, P., Unsicker, K., 2006. Age-related

alterations in hippocampal spines and deficiencies in spatial memory in mice. J. Neurosci. Res. 83 (4), 525–531.
Wang, X.D., Chen, Y., Wolf, M., Wagner, K.V., Liebl, C., Scharf, S.H., Harbich, D., Mayer, B., Wurst, W., Holsboer, F., Deussing, J.M., Baram, T.Z., Müller, M.B., Schmidt, M.V., 2011. Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. Neurobiol. Dis. 42, 300–310.
West, M.J., 1999. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends Neurosci. 22 (2), 51–61.
Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., Delon, M.R., 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215, 1237–1239.
Wiedenmann, B., Franke, W.W., 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. Cell 41 (3), 1017–1028.
Xu, B., Sun, A., He, Y., Qian, F., Liu, L., Chen, Y., Luo, H., 2017. Running-induced memory enhancement correlates with the preservation of thin spines in the hippocampal area CA1 of old C57BL/6 mice. Neurobiol. Aging 52, 106–116.
Xu, B., Sun, A., He, Y., Qian, F., Xi, S., Long, D., Chen, Y., 2018. Loss of thin spines and small synapses contributes to defective hippocampal function in aged mice. Neurobiol. Aging 71, 91–104.
Ypsilanti, A.R., Girão da Cruz, M.T., Burgess, A., Aubert, I., 2008. The length of hippocampal cholinergic fibers is reduced in the aging brain. Neurobiol. Aging 29 (11), 1666–1679.