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ORIGINAL ARTICLE

proBDNF Attenuates Hippocampal Neurogenesis and Induces Learning and Memory Deficits in Aged Mice

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Abstract Mature brain-derived neurotrophic factor has shown promotive effect on neural cells in rodents, including neural proliferation, differentiation, survival, and synaptic formation. Conversely, the precursor of brain-derived neurotrophic factor (proBDNF) has been emerging as a differing protein against its mature form, for its critical role in aging process and neurodegenerative diseases. In the present study, we investigated the role of proBDNF in neurogenesis in the hippocampal dentate gyrus of aged mice and examined the changes in mice learning and memory functions. The results showed that the newborn cells in the hippocampus revealed a significant decline in proBDNF-treated group compared with bovine serum albumin group, but an elevated level in anti-proBDNF group. During the maturation period, no significant change was observed in the proportions of phenotype of the newborn cells among the three groups. In water maze, proBDNF-treated mice had poorer scores in place navigation test and probe test, compared with those from any other group. Thus, we conclude that proBDNF attenuates neurogenesis in the hippocampus and induces the deficits in learning and memory functions of aged mice.

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Keywords proBDNF · Neurogenesis · Cognitive impairment · Neurodegenerative disease · Morris water maze

Abbreviations

AD	Alzheimer's disease
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2′-deoxyuridine
BSA	Bovine serum albumin
CNS	Central nervous system
DCX	Doublecortin
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
LTD	Long-term depression
LTP	Long-term potentiation
mBDNF	Mature BDNF
MBP	Myelin basic protein
NSCs	Neural stem cells
p75NTR	p75 neurotrophin receptor
proBDNF	BDNF precursor
SGZ	Subgranular zone
SVZ	Subventricular zone
TrkR	Tropomyosin-related kinase receptor

Introduction

Age-related neurodegenerative diseases, such as Alzheimer's disease (AD), are torturing the aged people with the memory loss and cognitive decline, which always lead to severe dementia in the end (Qiu et al. 2007; Bishop et al. 2010). Neurons degenerate and lose with age in central nervous system (CNS), specifically in the brains of patients

with AD and other neurodegenerative diseases (Schliebs and Arendt 2011). Nonetheless, insufficiency of neurogenesis in aged brain, especially in the hippocampus, has come to be associated with exacerbated learning and memory impairment in these neurodegenerative diseases (Sahay et al. 2011; Winner et al. 2011). Neurotrophins have been involved in neuronal survival, differentiation, neurogenesis, and synaptic plasticity (Thoenen 1995; Bartrup et al. 1997; Lu et al. 2005; Park and Poo 2013), as well as structural and functional integrity of the brain, specifically the hippocampus that links to learning and memory (Tyler et al. 2002). Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, plays a crucial role in CNS, with underlying signal paths mediated by two receptors coming from distinct families, the tropomyosin-related kinase receptor (TrkR) and p75 neurotrophin receptor (p75NTR) (Reichardt 2006; Park and Poo 2013). BDNF is first synthesized as a precursor (proBDNF) and subsequently cleaves either intracellularly by prohormone convertases and furin (Mowla et al. 2001) or extracellularly by plasmin and matrix metalloproteases (Lee et al. 2001), to produce a mature form (mBDNF). Unlike mBDNF, proBDNF has displayed largely opposing effects against its mature counterpart (Lu et al. 2005; Greenberg et al. 2009; Deinhardt and Chao 2014). Those opposing effects include, but not limited to, promoting cell death (Teng et al. 2005), inhibiting neuronal migration (Xu et al. 2011), collapsing neurite outgrowth (Sun et al. 2012), and suppressing the growth of glioma cells (Xiong et al. 2013). proBDNF is also involved in the induction of long-term depression (LTD), which is proposed to exert a critical effect in synaptic plasticity and cognitive function (Rösch et al. 2005; Woo et al. 2005). However, the role of proBDNF in neurogenesis in aged brain and in cognitive functions has not been well characterized. We in the present study seek to explore evidence of the effects of proBDNF on hippocampal neurogenesis and on learning and memory functions of aged mice.

Materials and Methods

Animals

Eighteen-month-old female C57BL/6J mice ($n = 60$) were randomly divided into bovine serum albumin (BSA) group and proBDNF group. All animals had access to water and food ad libitum and were housed 5–10 per cage under a 12-h light/dark cycle at room temperature. The mice were supplied by Animal Center of Daping Hospital, Third Military Medical University, China. The experiment was approved by Daping Hospital Ethics Committee. Every effort was made to minimize animal suffering.

Drug Delivery and BrdU Labeling

Chemical (BSA, cleavage-resistant proBDNF or anti-proBDNF antibody) was stereotactically and continuously micro-injected into mice hippocampus by a micro-osmotic pump (Alzet 1002, CA) for 6 days according to a modified method (Rogove and Tsirka 1998). Briefly, mouse was anesthetized in a chamber with 3 % isoflurane and ventilated with 2 % isoflurane in a gas mixture (30 % O_2, 70 % N_2), and then fixed in a stereotaxic frame (Stoelting, USA). A craniotomy was carried out using a dentist's micro-drill to drill through the right parietal bone. The injection coordinate point was taken from bregma following the Stereotaxic coordinates: anteroposterior, −2.5 mm; lateral, 0.5 mm; and ventral, 1.6 mm. The skin was sutured, with minipump implanted subcutaneously. Chemicals were micro-pumped into the hippocampus (1 μg/μl and 2 μl/h for BSA and proBDNF or anti-proBDNF, respectively) for 6 consecutive days. Correct location in the hippocampus was verified by postmortem for each mouse.

5-Bromo-2-deoxyuridine (BrdU, Sigma, USA) was injected intraperitoneally at 50 mg/kg (in sterile 0.9 % saline with 0.007 N NaOH), which was incorporated as thymidine analog into the DNA of dividing cells during the S phase of cell division, twice a day for 6 consecutive days. BrdU-labeled cells in the hippocampus were examined at day 7 and day 28 by an approach of immunohistochemistry.

Sections and Multiple Immunostaining

BrdU-injected mice were humanely sacrificed at day 7 and day 28, respectively. Brain samples were dissected and fixed in 4 % paraformaldehyde for 24 h at 4 °C, then equilibrated in 30 % sucrose overnight. Coronal 40-μm sections were cut throughout the dorsal and ventral hippocampus with a freezing microtome (CM1900, Leica). For staining of BrdU-positive cells, sections were incubated with primary antibody (mouse anti-BrdU, 1:5000, Sigma) and then biotin-conjugated secondary antibody (goat anti-mouse IgG, 1:500, Sigma). In order to examine the phenotype of the newborn cells in the hippocampus, sections were multi-stained by incubation with primary antibodies (rabbit anti-DCX at 1:1000, or mouse anti-Brdu at 1:200, or goat anti-MBP at 1:1000; rabbit anti-MAP2 at 1:200 or mouse anti-Brdu at 1:200, or goat anti-GFAP at 1:1000, Abcam) and then secondary antibodies (568-donkey anti-rabbit IgG at 1:1000, 647-donkey anti-mouse IgG at 1:1000, 488-donkey anti-goat IgG at 1:1000 from Invitrogen). Images were taken by Olympus BX51 Microscope or Leica TCX SP5 Confocal Microscope.

Water Maze Test

Morris water maze test (Morris 1984) was performed to investigate the alteration of mice learning and memory function. A video tracking system (ANZ-video tracking system, Stoelting, USA) was used to record and analyze the movement in the maze. Briefly, the test started at day 22 postoperatively, including place navigation test for 5 days and spatial probe test for the last day. For place navigation test, each mouse was given three trials per day for 5 consecutive days. Mice were placed in one of the four start quadrants (southwest, northwest, northeast, and southeast) in a randomized manner, of which the time was limited to 60 s. Animal that failed to find the submarine platform within 60 s would be replaced on the platform for 30 s before being sent back into water. At day 28, the platform was removed and spatial probe test was conducted. The percentage of swimming time in target quadrant was recorded. The time interval to find the platform (latency) was the primary dependent variable for assessment of cognitive performance. Swimming speed was also recorded to ensure that cognitive performance was not confounded by motor deficits.

Statistical Analysis

The data were expressed as mean ± SD. One-way ANOVA and LSD were used to analyze the data from multiple groups while using Student's t test for the statistical difference between two groups. p value less than 0.05 was considered to be statistically significant. All statistical analyses of the experimental data were performed by SPSS for Windows 18.0 (SPSS Inc).

Results

Decreased BrdU-Positive Cells in proBDNF Group

To examine neurogenesis in the hippocampus of aged mice, newly born cells were labeled by BrdU. BrdU-positive cells were mainly located in granule cell layer (GCL) of hippocampal dentate gyrus (Fig. 1). BrdU-positive cells were decreased in proBDNF group, compared with BSA group with a significant difference (day 7: 21.79 ± 2.41 for proBDNF group vs. 37.87 ± 1.13 for BSA group, $p < 0.05$; day 28: 10.06 ± 1.05 for proBDNF group vs. 19.88 ± 2.23 for BSA group, $p < 0.05$). However, an increased number of BrdU-positive cells was observed in anti-proBDNF group both at day 7 (67.25 ± 6.37) and day 28 (39.75 ± 5.83). Thus, proBDNF inhibited the neurogenesis in the hippocampus.

Phenotype of Newly Born Cells

To further investigate the role of proBDNF in cell differentiation during neurogenesis, phenotype of newborn cells was identified. Co-labeling with BrdU and DCX at day 7 revealed that the majority of BrdU-positive cells were DCX-labeled immature neurons ($BrdU^+/DCX^+$: 75.4 % in BSA group; 77.8 % in proBDNF group; 76.1 % in anti-proBDNF group, Fig. 2a, b). Similarly, newly matured cells visualized by co-labeling with BrdU and MAP2 at day 28 presented a constitution like immature cells ($BrdU^+/MAP2^+$: 80.9 % in BSA group; 78.4 % in proBDNF group; 78.9 % in anti-proBDNF group, Fig. 2c, d). Although there was a generally decreased total amount of BrdU-positive cells at day 28, the proportion of neuron remained a major part.

proBDNF Inhibits Learning and Memory

Since the deficit of neurogenesis in the hippocampus had been implicated in cognitive function, we herein examined the alteration of mice learning and memory function by an approach of Morris water maze (Fig. 3a for time line). We first guaranteed a minimized confounding factor caused by swimming speed (Fig. 3b). With an adaption of training for 5 consecutive days, mice gained a shorter latency to reach the goal platform, which meant a learning process in the experiment (Fig. 3c). However, mice from proBDNF group performed a limited reduction of latency compared with any other group during the test. In probe test, proBDNF-treated mice spent less time in target quadrant compared with those from any other groups and showed an aimless movement. Thus, aged mice from proBDNF group had a poor performance in cognitive function, which indicated deficit in memory and learning ability.

Discussion

The hypothesis whether new neurons are continuously added in adult or aged brain and help recovering neural functions has been concerned for decades (Eriksson et al. 1998; Gross 2000; Deng et al. 2010). Much evidence of brain neurogenesis throughout lifespan was provided in many species, including humans (Shetty et al. 2013; Ernst et al. 2014). Moreover, studies have demonstrated that cortical or hippocampal neurogenesis can be regulated by several factors, such as environmental enrichment and voluntary exercise (van Praag et al. 1999; Bruel-Jungerman et al. 2005; Olson et al. 2006). As an intrinsic and natural change, aging attenuates neurogenesis in hippocampus and has been proposed to impair maintenance of cognitive function in numerous dementia-related neurodegenerative

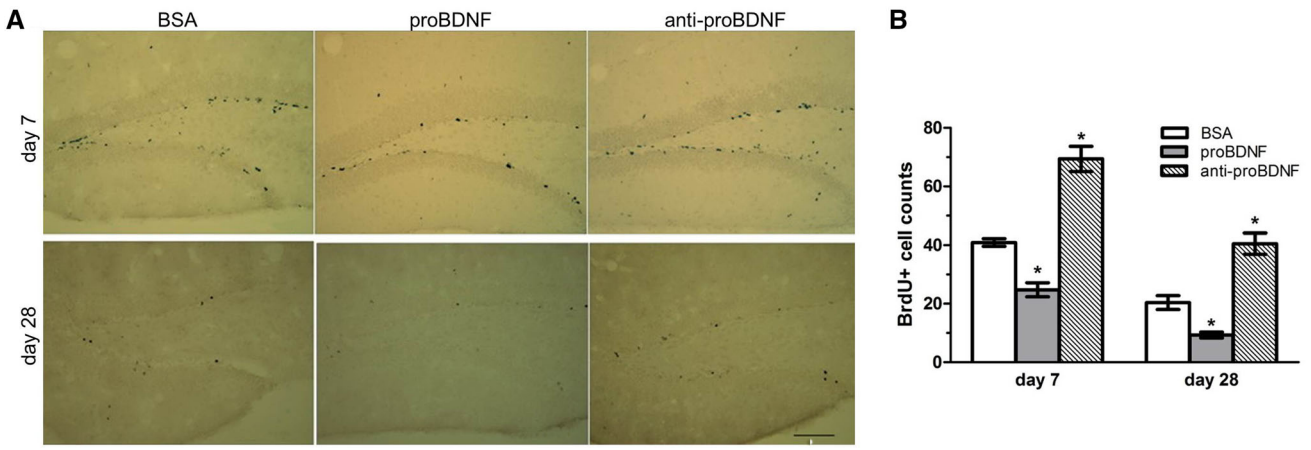

Fig. 1 BrdU-positive cells residing in the hippocampal dentate gyrus (DG) at day 7 and day 28. **a** Images of BrdU-positive cells in DG. Immunohistochemistry showed BrdU-positive cells in granule cell layer (GCL) of DG in all three groups. BrdU-positive cells at day 28 showed a significant reduction in different group compared with the corresponding group at day 7. For proBDNF group, the BrdU-positive cells in DG were less than other groups both at day 7 and day 28. *Scale bar* = 200 μm. **b** *Bar graph* of the amount of BrdU-positive cells in different groups and at different time. Statistical result showed a significant reduction at day 28 in all three groups ($p < 0.05$, *t* test, compared with that at day 7, correspondingly) and significant differences between proBDNF group and any other group, both at day 7 and day 28 ($p < 0.05$, *t* test). * denotes $p < 0.05$

diseases (Lazarov et al. 2010; Villeda et al. 2011). In our present study, we showed the proBDNF-related changes in neurogenesis in the hippocampal dentate gyrus (DG) of aged mice and examined the alteration of hippocampus-dependent learning and memory functions by an approach of Morris water maze.

Neurons can be continuously generated in adult brain. Newly born cells had been observed in two regions of the adult/aged brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal DG (Fuentealba et al. 2012). While SVZ-born neuronal cells integrating into the olfactory bulb, the cells born in the SGZ migrate and integrate into GCL of the DG (Shetty et al. 2013). Neuronal cells are continuously generated in DG, but only part of these newly generated cells can survive during the maturation period. Although recent studies in rat models have demonstrated that the attenuated neurogenesis in the SGZ is due to an increased quiescence of neural stem cells (NSCs) rather than loss of them (Spalding et al. 2013), the fates of neurons that already generated by non-quiescent NSCs are under the control of various factors. Aging, which is responsible for facilitating neuronal reduction in relation to cell proliferation with different intensity in different age stages, leads to less "new neurons for old brain" (Ben Abdallah et al. 2010; Winner et al. 2011). With the increasing loss of brain neurons either in aged people or in dementia-related neurodegenerative diseases, especially in the hippocampus, newborn neurons are highly expected to regenerate and integrate into the neural network, which might help retard the cognitive impairment or recover its normal functions (Marlatt and Lucassen 2010; Arellano 2011). Using a nucleotide analog of BrdU in aged mice, we showed that the newly generated cells were locating in the GCL of the hippocampal DG. As expected, the number of these BrdU-labeled cells was significantly reduced after the maturation period. This finding was in line with the hypothesis of "new neurons for old brain."

Neurotrophins are crucial in central neural system. As a member of neurotrophin family, BDNF is released as a mix of preform and mature form, and the ratio of pre-form/mature form is regulated by proteolytic enzymes extracellularly. As the addition of exogenous cleavage-resistant proBDNF makes it a dominant level against its mature form, proBDNF in vivo tends to exert a negative effect (Deinhardt and Chao 2014). Our result showed a further decrease of newborn cells in GCL of DG when treated with proBDNF, but an attenuated reduction when treated with anti-proBDNF antibody, compared with BSA group. The result suggests that both exogenous and endogenous proBDNFs exert a negative effect on neurogenesis in aged hippocampus. Newly born cells that survive are about to differentiate into either neurons or glial cells during normal maturation period. As the effect of proBDNF on the differentiation of neural cells had been less characterized, we herein examined the phenotype of the newborn cells that might be affected by proBDNF. With an approach of immunofluorescent staining with specific markers, neurons and glial cells were visualized. The result showed that there was no change in proportions of newborn cell phenotype in different groups. Thus, we concluded that proBDNF could inhibit proliferation of

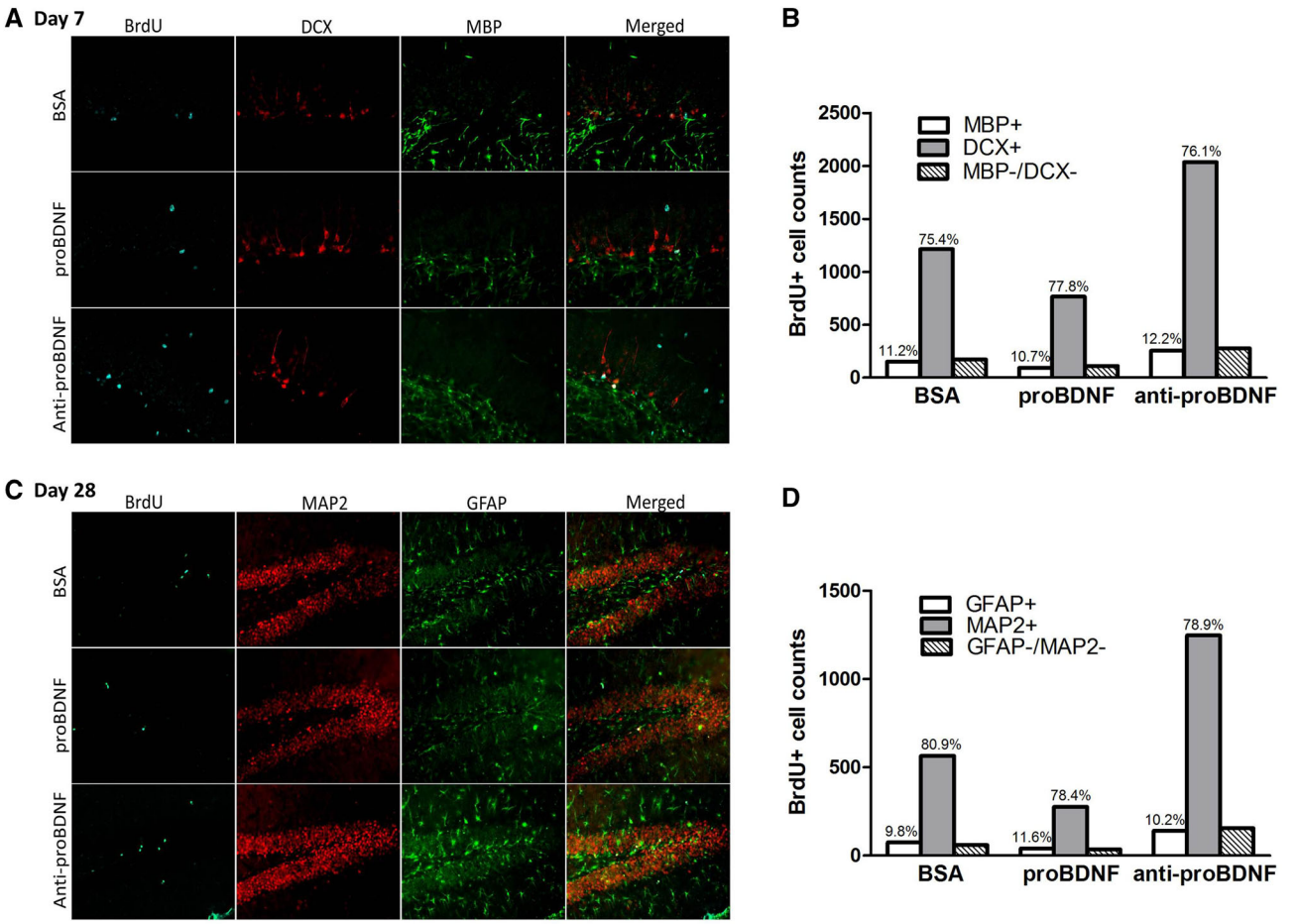

Fig. 2 Phenotype of BrdU-labeled cells in dentate gyrus (DG). **a** Images of phenotype of newly born cells in DG at day 7 by immunofluorescent staining. The newborn BrdU-positive neurons were stained with DCX antibody, and the glial cells with MBP antibody. **b** *Bar graph* of the percentage of labeled cells at day 7. **c** Images of phenotypes of mature BrdU-positive cells in DG at day 28. The mature BrdU-positive neurons were stained with MAP2 antibody, and the glial cells with GFAP antibody. **d** *Bar graph* of the percentage of labeled cells at day 28. *Scale bar* = 50 μm. The amount of BrdU-positive cells at day 28 was obviously less than that at day 7, but no significant change in phenotype ($p > 0.05$, Chi-square test, compared with the corresponding group)

newborn cells in the hippocampus, but had no effect on their differentiation.

In the brain of adult mice, new neurons could still generate and integrate into the neural network as a supplementation, and this neurogenesis probably contributes to cognitive performance as suggested in prior studies (Dupret et al. 2008; Jessberger et al. 2009). Therefore, we conducted water maze test to examine the changes in memory and learning function of the aged mice. In place navigation test, the mice showed similar swimming speeds, which meant limited bias caused by any physically disabled mice. As the training carried on, it suggested a reduced latency in all mice groups, which represented an elevated ability in spatial learning function. However, mice from proBDNF-treated group turned out to show poor scores in the test, compared with those from the control group. Intriguingly, the mice treated with anti-proBDNF antibody gave the best performance among the three groups. This result indicated that proBDNF played a role in mice spatial learning function as a suppressive factor. In the following probe test, proBDNF-treated mice, as expected, appeared to take more time and seemed more difficult to find the target quadrant comparing with those of the control. Similar to that in the place navigation test, mice treated with anti-proBDNF gained the best scores than those from any other groups, which represented an applausive memory function of aged mice. The cognitive performances correlated with hippocampal neurogenesis in our study, which was in line with those studies mentioned above (Dupret et al. 2008; Jessberger et al. 2009). Evidences had also been shown in some disease models such as stroke. In the model of ischemia-induced cognitive deficits, spatial cognitive impairment improved with enhanced hippocampal neurogenesis when treated with fluoxetine or minocycline in a

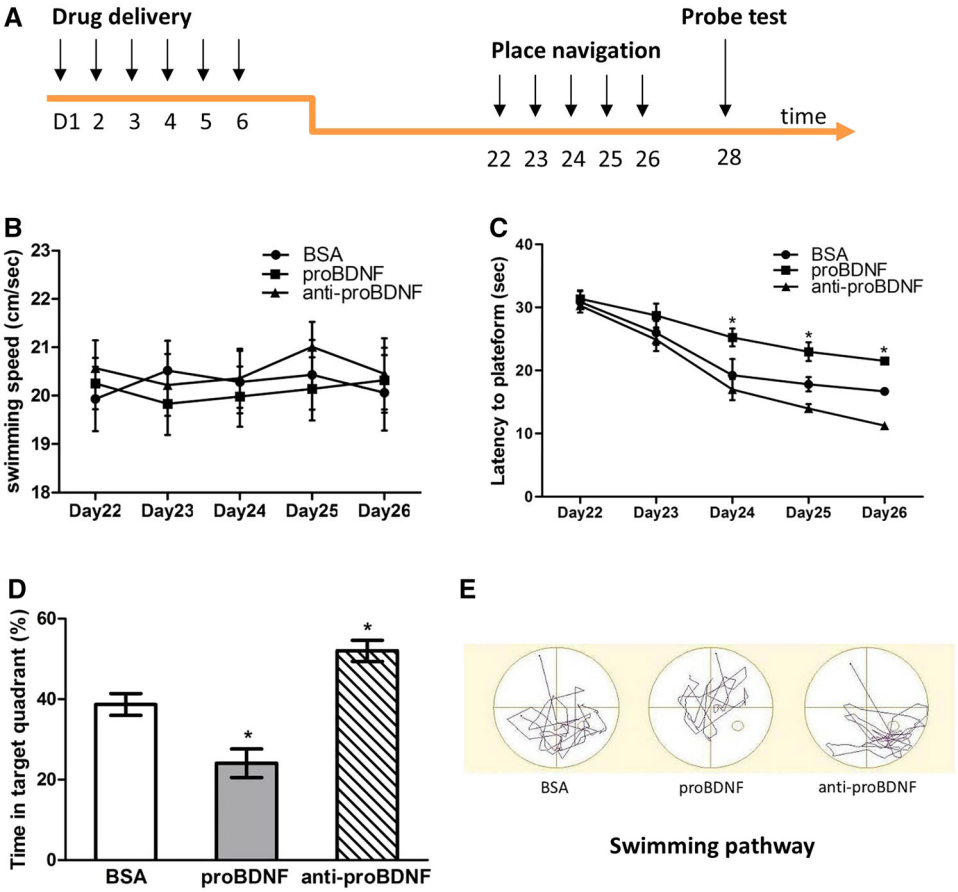

Fig. 3 ProBDNF-treated mice performed poorly in the test of Morris water maze. **a** Flow diagram of the water maze test. **b** Similar swimming speeds in all three groups as a negligible confounding factor ($p > 0.05$, one-way ANOVA). **c** All mice had a decreased latency due to the adaption of training; however, the mice from proBDNF group took a longer latency to reach the hidden platform compared with those from any other groups ($p < 0.05$, t test). **d** The mice from proBDNF group spent less time in the target quadrant ($p < 0.05$, t test, compared with any other group). **e** Swimming path of mice in spatial probe test. Thus, proBDNF-treated mice gained the poorest score in the test. In contrast, the mice from anti-proBDNF group performed best in the test

chronic manner (Liu et al. 2007; Li et al. 2009). These studies consolidate the relationship between neurogenesis and cognition improvement.

With regard to proBDNF, it has been also involved in synaptic plasticity, which is considered to be related with cognitive functions. Studies had shown that proBDNF facilitated LTD through an interaction with p75 neurotrophic factor receptor (p75NTR) in the hippocampus or contributed to the late phase of long-term potentiation (LTP) while converting into its mature form (Burke and Barnes 2006; Greenberg et al. 2009). These induced LTD and LTP were important for synaptic plasticity which underlay cognitive functions. Thus, questions whether and how the development and function of synapses of the new neurons are affected by proBDNF, and the role of proBDNF in neurodegenerative diseases, need to be answered. Moreover, the underlying molecular mechanism of the effect of proBDNF on neurogenesis and cognitive functions needs to be further explored.

Taken together, our experiment revealed that proBDNF could inhibit neural proliferation in the hippocampal dentate gyrus of the aged mice, which correlated with the performances in the test of Morris water maze. We herein propose that proBDNF exerts a suppressive effect on hippocampal neurogenesis and contributes to cognitive deficit of aged mice.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest involved.

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