Zinc transporter 3 modulates cell proliferation and neuronal differentiation in the adult hippocampus

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Funding information
National Research Foundation of Korea, Grant/Award Numbers:
NRF-2017M3C7A1028937, NRF-2019R1A2C4004912, NRF-2020R1A2C2008480

Abstract
The subgranular zone of the dentate gyrus is a subregion of the hippocampus that has two uniquely defining features; it is one of the most active sites of adult neurogenesis as well as the location where the highest concentrations of synaptic zinc are found, the mossy fiber terminals. Therefore, we sought to investigate the idea that vesicular zinc plays a role as a modulator of hippocampal adult neurogenesis. Here, we used ZnT3−/− mice, which are depleted of synaptic-vesicle zinc, to test the effect of targeted deletion of this transporter on adult neurogenesis. We found that this manipulation reduced progenitor cell turnover as well as led to a marked defect in the maturation of newborn cells that survive in the DG toward a neuronal phenotype. We also investigated the effects of zinc (ZnCl2), N-acetyl cysteine (NAC), and ZnCl2 plus 2NAC (ZN) supplement on adult hippocampal neurogenesis. Compared with ZnCl2 or NAC, administration of ZN resulted in an increase in proliferation of progenitor cells and neuroblast. ZN also rescued the ZnT3 loss-associated reduction of neurogenesis via elevation of insulin-like growth factor-1 and ERK/CREB activation. Together, these findings reveal that ZnT3 plays a highly important role in maintaining adult hippocampal neurogenesis and supplementation by ZN has a beneficial effect on hippocampal neurogenesis, as well as providing a therapeutic target for enhanced neuroprotection and repair after injury as demonstrated by its ability to prevent aging-dependent cognitive decline in ZnT3−/− mice. Therefore, the present study suggests that ZnT3 and vesicular zinc are essential for adult hippocampal neurogenesis.

Keywords
adult neurogenesis, hippocampus, N-acetyl cysteine, zinc, zinc transporter 3

1 INTRODUCTION

The divalent cationic zinc (Zn²⁺) is the second most abundant transition metal in the brain and spinal cord following iron and is a major player in numerous cellular and physiological processes. Most ionic zinc in the brain is tightly bound with proteins such as metallothionein or metalloproteins. Loosely bound or free-zinc is often found at increased concentrations in synaptic vesicles in brain regions where neurogenesis and

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neural migration actively occur in the adult brain, such as the hippocampal dentate gyrus (DG) and olfactory bulb.\(^3,4\) Zinc is a component of over 300 enzymes and highly involved in the regulation of cell division and DNA synthesis.\(^5\) Zinc deficiency leads to impaired DNA synthesis and growth retardation in animals.\(^6\) Zinc also influences hormonal regulation of cell division, such as that mediated by insulin-like growth factor-1 (IGF-1) or nerve growth factor.\(^7\) Several studies have reported the adverse effects of an insufficient supply of zinc on normal physiological function. Dietary zinc deprivation impairs performance in visual-attention and short-term-memory tasks.\(^8\) Taken together, the evidence suggests that zinc is an essential element for maintaining normal neuronal cell proliferation, development, and cognitive function.

The role of zinc transporter 3 (ZnT3) and vesicular zinc on cognitive function have been investigated by several labs. Adlard et al reported that ZnT3\(^{-/-}\) mice exhibit age-dependent deficits in learning and memory.\(^9\) Sindreu et al demonstrated that ZnT3\(^{-/-}\) mice have impaired contextual discrimination and spatial working memory.\(^10\) Martel et al showed that ZnT3\(^{-/-}\) mice performed normally at initial learning but showed difficulty finding a second platform location. These ZnT3\(^{-/-}\) mice display enhanced social interaction but were likewise strikingly poor at forming social and object recognition memory. Therefore, it appears that ZnT3 is involved in spatial memory and behavior that relies on the hippocampus and perirhinal cortex.\(^11\)

Although the above studies reported that ZnT3\(^{-/-}\) mice showed learning and memory deficits during the aging process, the precise mechanism is not clear. Thus, we hypothesized that deletion of ZnT3 gene negatively affects adult hippocampal neurogenesis. To determine whether synaptic zinc is an important factor for modulating hippocampal neurogenesis, the present study also used ZnT3\(^{-/-}\) mice. We demonstrate a severe reduction of progenitor cell proliferation and neuronal differentiation in the DG of ZnT3\(^{-/-}\) mice. We propose a cell-extrinsic mechanism of ZnT3 deficiency mediated by decreasing levels of IGF-1 and phosphorylation of both extracellular signal-regulated kinase (ERK) and cyclic AMP response element binding protein (CREB) in the hippocampus of ZnT3\(^{-/-}\) mice. The present study suggests that ZnT3\(^{-/-}\) mice display reduced hippocampal neurogenesis under physiological conditions even at 3 months of age. Our findings indicate that vesicular zinc plays an important role in adult hippocampal neurogenesis and have implications for cognitive health in aging and disease.

2 | MATERIALS AND METHODS

2.1 | Animals and ethics statement

ZnT3\(^{+/+}\) and ZnT3\(^{-/-}\) male mice (a kind gift from Dr Jae-Young Koh, Department of Neurology, University of Ulsan College of Medicine, Korea; background strains, C57BL/6 and Sv129 hybrid), aged 3 months (25-30 g) or 15-18 months (30-45 g), were bred and maintained in the facility of University of Hallym College of Medicine. The animals were housed in a temperature- and humidity-controlled environment (22°C ± 2°C, 55% ± 5% and a 12 hours light:12 hours dark cycle), supplied with Purina diet (Purina, Gyeonggi, Korea) and water ad libitum. Animal use and relevant experimental procedures were approved by the Institutional Animal Care and Use Committee, Hallym University (Protocol # Hallym 2017-16). This manuscript was written up in accordance with the ARRIVE (Animal Research: Reporting in vivo Experiments) guidelines.\(^12\)

2.2 | Experimental design and 5-bromo-2-deoxyuridine labeling

To test the role of vesicular zinc at different stages of adult neurogenesis, we conducted 4-phase studies using ZnT3\(^{-/-}\) mice and zinc supplementation. In phase 1, to assess the effects of eliminating vesicular zinc at the early or late phase of adult hippocampal neurogenesis, mice were divided into two groups in each phase: ZnT3\(^{+/+}\) (n = 7) and ZnT3\(^{-/-}\) (n = 9). The thymidine analog BrdU (50 mg/kg; Sigma Cat# B5002, St. Louis, Missouri) was intraperitoneally injected twice daily for four consecutive days and the brains were harvested at 5 days (early phase) or 6 weeks (late phase) after the initial BrdU injection. In phase 2, to evaluate the effects of zinc supplementation at the early or late phase of adult hippocampal neurogenesis, mice were divided into four groups in each phase: vehicle (Veh; early phase, n = 10; late phase, n = 6), zinc chloride (ZnCl\(_2\); early phase, n = 10; late phase, n = 8), N-acetyl cysteine (NAC; early phase, n = 11; late phase, n = 9), and ZnCl\(_2\) plus 2NAC (ZN; early phase, n = 10; late phase, n = 9). Mice were given ZnCl\(_2\) (4 mg/kg), NAC (20 mg/kg), or ZN intraperitoneally available once per day for 2 (early phase) or 8 weeks (late phase). BrdU was injected two times per day for four consecutive days starting 10 days after the initial ZN treatment, and then mice were sacrificed after the last dose of ZN. Control mice were injected with equal volumes of saline only (vehicle). In phase 3, zinc supplementation by ZN was initiated at the early or late phase of adult hippocampal neurogenesis and mice were divided into four groups in each phase: vehicle-treated ZnT3\(^{+/+}\) mice (early phase, n = 6; late phase, n = 7), vehicle-treated ZnT3\(^{-/-}\) mice (early phase, n = 6; late phase, n = 7), ZnCl\(_2\) plus 2NAC (ZN; early phase, n = 10; late phase, n = 9). Mice were sacrificed and the brains were harvested 5 days (early phase) or 6 weeks (late phase) after the last dose of ZN.
phase, n = 6), ZN-treated ZnT3+/+ mice (early phase, n = 9; late phase, n = 8), vehicle-treated ZnT3−/− mice (early phase, n = 6; late phase, n = 7), ZN-treated ZnT3+/− mice (early phase, n = 7; late phase, n = 6). Mice were given ZN intraperitoneally once per day for 2 or 8 weeks. BrdU was injected two times per day for four consecutive days starting 10 days after initial ZN treatment, then mice were sacrificed at 2 (early phase) or 8 weeks (late phase) after initial ZN treatment. In phase 4, to analyze the effects of zinc supplementation by ZN on cognitive abilities, mice were divided into four groups: (a) vehicle-treated aged ZnT3+/+ mice (n = 11), (b) vehicle-treated aged ZnT3−/− mice (n = 13), (c) ZN-treated aged ZnT3+/− mice (n = 12), and (d) ZN-treated aged ZnT3−/− mice (n = 14). ZN supplementation was performed once per day for 8 weeks. Morris water maze (MWM) test were conducted for six consecutive days starting 50 days after the initial ZN supplementation, then mice were sacrificed after the last dose of ZN.

2.3 | Quantitative analysis

To quantify BrdU-, Ki67-, DCX-, and pCREB-positive cells, every sixth coronal section spanning the septal hippocampus was collected. The number of BrdU+NeuN+ cells was estimated by multiplying the phenotype of the BrdU-positive cells, as mentioned previously, fluorochrome signals in the hippocampus (Figure 1B,B). We also stained brain sections of ZnT3+/+ and ZnT3−/− mice with the TSQ, zinc-specific fluorescent dye, to test whether vesicular zinc regulation is altered in ZnT3+/− mice. ZnT3−/− mice showed high intensity TSQ fluorescence signals in the hippocampal MF area. However, ZnT3−/− mice showed almost no fluorescence signals in the hippocampus (Figure 1D).

3 | RESULTS

3.1 | Vesicular zinc is eliminated in the hippocampus of ZnT3−/− mice

We determined mouse genotype by PCR. PCR analysis of genomic DNA shows loss of the ZnT3 band (650 bp) and presence of the NEO cassette (400 bp) in the ZnT3−/− mice (Figure 1A). We also stained brain sections of ZnT3+/+ and ZnT3−/− mice with the TSQ, zinc-specific fluorescent dye, to test whether vesicular zinc regulation is altered in ZnT3+/− mice. ZnT3−/− mice showed high intensity TSQ fluorescence signals in the hippocampal MF area. However, ZnT3−/− mice showed almost no fluorescence signals in the hippocampus (Figure 1B,B). After examining TSQ fluorescence, we evaluated ZnT3 immunoreactivity (IR) in both ZnT3+/+ and ZnT3−/− mice. ZnT3 localization on vesicle membranes was also lower in the hippocampus of ZnT3−/− mice. ZnT3-IR from ZnT3−/− mice was undetectable in the MFs of hippocampus (Figure 1C,C).

3.2 | Genetic deletion of ZnT3 reduces hippocampal progenitor cell proliferation

To assess whether endogenous ZnT3 affects DG progenitor cell proliferation, mice were injected with BrdU (50 mg/kg) twice per day for four consecutive days in both ZnT3+/+ and ZnT3−/− mice (Figure 1D). We found that the BrdU- and Ki67-immunopositive cells were distributed mainly in the SGZ/GCL. A significant decrease in the number of
BrdU (Figure 1E,F) and Ki67 (Figure 1G,H) labeled cells was seen in the SGZ/GCL of ZnT3−/− mice as compared to age-matched ZnT3+/+ mice at 5 days after first injection of BrdU. The ZnT3−/− mice had a 59.2% (BrdU) or 54.8% (Ki67) reduction of the hippocampal progenitor cell proliferation in comparison with controls.

3.3 | ZnT3−/− mice show reduced production of neuroblasts

To test whether ZnT3 influences the presence of immature neurons we performed immunostaining for DCX. Genetic deletion
of ZnT3 caused a 47.3% reduction in the number of DCX-immunopositive cells, the specific marker of immature neurons (neuroblast) (Figure 1I,J).

3.4 | Compromised adult neurogenesis in the hippocampus of ZnT3−/− mice

To investigate whether endogenous ZnT3 influences survival of DG newborn cells, we birthdated newly generated cells in ZnT3+/− and ZnT3−/− mice by injecting BrdU twice daily for four consecutive days and investigated their fate at 6 weeks after initial BrdU injection. A significant decrease in the number of BrdU-labeled cells was seen in the SGZ/GCL of ZnT3−/− mice (Figure 1K,L). The ZnT3−/− mice had a 60.6% decrease in survival of newly generated cells in comparison with controls. We also calculated the ratio of the number of BrdU+ cells in the SGZ/GCL at 6 weeks to the number of BrdU+ cells in the DG at 5 days after initial BrdU injection and found that the survival ratio of BrdU-labeled cells in ZnT3−/− mice (20.5%) was similar to that of controls (21.2%; data not shown).

Next, we determined the role of ZnT3 on neuronal maturation of cells that survived in the SGZ/GCL at 6 weeks after initial BrdU injection. When calculating the number of adult newborn neurons, genetic deletion of ZnT3 caused a significant decrease in the total number of BrdU+ cells double labeled for the specific marker of mature neurons (NeuN) in the SGZ/GCL (Figure 1M-Q). In addition, ZnT3−/− mice showed a 39.52% decrease in the percentage of BrdU+NeuN+ cells in BrdU+ cells (Figure 1R). These results show that deletion of ZnT3 decreases newborn mature neurons in the adult hippocampal DG.

3.5 | ZnT3 is expressed in newly generated neurons

Next, we investigated whether ZnT3 is expressed in newborn neurons in addition to mature neurons. To confirm the presence of ZnT3 in newborn neurons, the DG of ZnT3+/− mice at 6 weeks after initial BrdU injection were assayed via immunofluorescence staining for BrdU, ZnT3, and MAP2. We detected much stronger expression of ZnT3 and MAP2 in the MF of DG. We also found that ZnT3 protein colocalized with axons of BrdU+MAP2+ cells in the DG (Figure 1S-Y). This indicates that ZnT3 is expressed in the axonal terminal of newborn neurons.

3.6 | ZN increases adult hippocampal neurogenesis

To determine whether ZN supplementation enhances adult hippocampal neurogenesis, we analyzed the effects of ZN supplementation on the rate of cell proliferation and neuronal differentiation within the DG (Figure 2A). Compared with vehicle-treated mice, the BrdU- and Ki67-labeled cells were not significantly changed in ZnCl2-treated mice, whereas NAC- or ZN-treated mice exhibited a significant increase in the number of BrdU- (Figure 2B-F) and Ki67-labeled cells (Figure 2G-K) in the SGZ. In addition, we also observed a greatly increased number of DCX-immunopositive cells in ZN-treated mice compared with vehicle, ZnCl2 or NAC-treated mice (Figure 2L-P).

Furthermore, we also determined whether ZN supplementation increases levels of vesicular zinc in the hippocampal mossy fibers (MFs). We found that there were no differences in vesicular TSQ intensity between vehicle-treated and NAC-treated mice. However, vesicular TSQ intensity was increased in ZnCl2-treated mice compared with vehicle-treated mice and ZN-treated mice showed significantly increased vesicular TSQ intensity in the MFs compared with vehicle-, ZnCl2-, or NAC-treated mice (Figure 2Q,R).

We then explored whether ZN impacts the newly generated cell survival and neuronal maturation of newborn cells in the SGZ/GCL. ZN supplementation was performed once a day for 8 weeks. We injected BrdU twice per day for four consecutive days starting at 10 days after the initial ZN supplementation. The numbers of BrdU+ and BrdU+NeuN+ cells from mice treated with ZN were significantly higher than in sections from vehicle-, ZnCl2-, or NAC-treated mice (Figure 2S-V). In addition, ZN-treated mice showed a significant increase in the percentage of BrdU+NeuN+ cells among BrdU+ cells (Figure 2W).

3.7 | ZN treatment reversed adult hippocampal neurogenesis in ZnT3−/− mice

Here we sought to ascertain if the decline in progenitor cell proliferation and neuronal differentiation could be rescued by ZN. ZnT3+/− and ZnT3−/− mice were treated with ZN or vehicle (Figure 3A). We found that ZN treatment revealed an increase in the number of BrdU+, Ki67+ or DCX+ cells in the SGZ/GCL of both ZnT3+/− and ZnT3−/− mice compared with vehicle-treated mice, although ZnT3−/− mice treated with ZN showed a lower number of BrdU+, Ki67+ or DCX+ cells than ZnT3+/− mice treated with ZN (Figure 3B-G). These results show that ZN reverses the decrease in progenitor cell proliferation and neuroblast production, caused by genetic deletion of ZnT3.

We then explored whether ZN also impacts the survival of newly generated cells and the neuronal maturation of newborn cells that survive in the SGZ/GCL. ZN supplementation was performed once a day for 8 weeks. We injected BrdU twice per day for four consecutive days starting at 10 days after the initial ZN supplementation. The numbers of BrdU+ and BrdU+NeuN+ cells in ZnT3−/− mice were significantly decreased in both vehicle- and ZN-treated groups, as compared to ZnT3+/− mice. However, ZN treatment remarkably increased the numbers of BrdU+ and BrdU+NeuN+ cells in both ZnT3+/− and ZnT3−/− mice, as compared to vehicle-treated mice (Figure 3H-K). In addition, ZN treatment revealed an increase in the percentage of BrdU+NeuN+ cells among BrdU+ cells in both ZnT3+/− and ZnT3−/− mice compared with vehicle-treated mice, although...
ZnT3−/− mice treated with ZN or vehicle showed a lower percentage of BrdU+NeuN+ cells among BrdU+ cells than ZnT3+/+ mice treated with ZN or vehicle, respectively (Figure 3L). In contrast with results obtained after administration of ZN, no significant difference in the ratio of the number of surviving BrdU+ cells in the SGZ/GCL at 47 days to the number of BrdU+ cells in the DG at 5 days after initial BrdU injection in either ZnT3−/− and ZnT3−/− mice treated with ZN vs vehicle-treated mice, indicating that ZN does not regulate the
FIGURE 3  Administration of ZN in ZnT3−/− mice increases adult hippocampal neurogenesis. A, Timeline showing the experimental design. B, Representative images of the DG showing BrdU (green) staining from vehicle- or ZN-treated mice (either ZnT3+/+ or ZnT3−/−) at 2 weeks after initial ZN supplementation. Scale bar = 50 μm. C, Quantification of BrdU+ cells in the SGZ/GCL (mean ± SEM; n = 6-9 from each group). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test: F = 76.753, P < .001). D, Representative images of Ki67- (red) and DAPI- (blue) immunopositive cells as merged images from vehicle- or ZN-treated mice (either ZnT3+/+ or ZnT3−/−). Scale bar = 50 μm. E, Number of progenitor cells (Ki67+/DAPI+) in the SGZ/GCL (mean ± SEM; n = 6-9 from each group). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test: F = 92.234, P < .001). F, Representative images of DCX- (red) and DAPI- (blue) immunopositive cells as merged images from vehicle- or ZN-treated mice (either ZnT3+/+ or ZnT3−/−). Scale bar = 50 μm. G, Number of immature neurons (DCX+/DAPI+) in the SGZ/GCL (mean ± SEM; n = 6-9 from each group). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test: F = 34.601, P < .001). H, Representative images of the DG showing BrdU (green) staining to vehicle- or ZN-treated mice (either ZnT3+/+ or ZnT3−/−) at 8 weeks after initial ZN supplementation. Scale bar = 50 μm. I, Quantification of BrdU+ cells in the SGZ/GCL (mean ± SEM; n = 6-8). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test: F = 44.071, P < .001). J, Representative images of BrdU- (green) and NeuN- (red) immunopositive cells as merged images from vehicle- or ZN-treated mice (either ZnT3+/+ or ZnT3−/−). Scale bar = 50 μm. K,L, Bar graphs showing the number (K) and percentage (L) of cells that express the markers BrdU and NeuN (newborn neurons) in the SGZ/GCL (mean ± SEM; n = 6-8). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test; K: F = 60.390, P < .001; L: F = 24.074, P < .001).
survival ratio of newly generated cells. However, we noted that ZN significantly increased the number of BrdU+ and BrdU+NeuN+ cells in the SGZ/GCL of both ZnT3+/+ and ZnT3−/− mice compared with vehicle-treated mice. Thus, these results demonstrate that ZN reverses the reduction in adult hippocampal neurogenesis, caused by deletion of the ZnT3 gene.

**FIGURE 4** ZN increases IGF-1 expression and leads to ERK/CREB activation in the hippocampus. A, Western blotting analysis of IGF-1, pERK or pCREB in the hippocampus from either ZnT3+/+ or ZnT3−/− mice treated with vehicle or ZN. B–D, Quantification of IGF-1 (B), pERK (C), or pCREB (D) expression from the hippocampus (mean ± SEM; n = 5 from each group). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (Kruskal-Wallis test followed by Bonferroni post hoc test; B: Chi square = 9.134, df = 3, P = .028; C: Chi square = 14.634, df = 3, P = .002; D: Chi square = 14.429, df = 3, P = .002). E, DAPI- (blue), IGF-1- (green), and NeuN- (red) immunofluorescence in vehicle- and ZN-treated mice of either ZnT3+/+ or ZnT3−/− genotype. Scale bar = 50 μm. F, Quantification of IGF-1 immunoreactivity in the hippocampal CA1, CA3 and DG (mean ± SEM; n = 5 from each group). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (Kruskal-Wallis test followed by Bonferroni post hoc test; CA1: Chi square = 12.728, df = 3, P = .005; CA3: Chi square = 11.184, df = 3, P = .011; DG: Chi square = 12.482, df = 3, P = .006). G–J, Representative images showing pERK-immunopositive mossy fiber (MF) pathway to vehicle-treated ZnT3+/+ (G) and ZnT3−/− (H) mice or ZN-treated ZnT3+/+ (I) and ZnT3−/− (J) mice daily for 2 weeks. Scale bar = 50 μm. K, Quantification of pERK immunoreactivity in the hippocampal MF (mean ± SEM; vehicle-treated ZnT3+/+ mice, n = 7; ZN-treated ZnT3+/+ mice, n = 8; vehicle-treated ZnT3−/− mice, n = 9; ZN-treated ZnT3−/− mice, n = 7). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice. L–O, Representative images of the DG showing pCREB-immunopositive cells to vehicle-treated ZnT3+/+ (L) and ZnT3−/− (M) mice or ZN-treated ZnT3+/+ (N) and ZnT3−/− (O) mice daily for 2 weeks. Scale bar = 50 μm. P, Bar graph showing the number of pCREB immunopositive cells in the SGZ/GCL (mean ± SEM; vehicle-treated ZnT3+/+ mice, n = 7; ZN-treated ZnT3+/+ mice, n = 8; vehicle-treated ZnT3−/− mice, n = 9; ZN-treated ZnT3−/− mice, n = 7). *P < .05 vs ZnT3+/+ mice; #P < .05 vs vehicle-treated mice (one-way ANOVA followed by Bonferroni post hoc test: F = 22.439, P < .001). Q, DAPI- (blue), pCREB- (green), and DCX- (red) immunofluorescence in vehicle- and ZN-treated mice of either the ZnT3+/+ or ZnT3−/− genotype. Scale bar = 50 μm, P, phosphorylated.
3.8 | ZN Treatment increases IGF-1 expression and ERK/CREB activation in the hippocampus

The effects of ZnT3 gene deletion and ZN supplementation on the expression of IGF-1 and the activity of ERK/CREB pathways were investigated to elucidate the mechanisms underlying the effect of vesicular zinc on adult hippocampal neurogenesis. It has been known that IGF-1 acts to promote adult neurogenesis and that the ERK/CREB signaling pathway also plays an important role in maintaining normal levels of neurogenesis. We therefore examined IGF-1 expression in addition to measuring ERK and CREB phosphorylation (pERK and pCREB), which are the activated forms of these molecules. Western blot revealed a significant decrease in the level of IGF-1, pERK, and pCREB proteins in the hippocampus of ZnT3−/− mice as compared with the ZnT3+/+ mice. However, administration of ZN remarkably increased the protein level of IGF-1, pERK, and pCREB in both ZnT3+/+ and ZnT3−/− mice as compared with vehicle-treated mice, although ZnT3−/− mice showed lower IGF-1, pERK, and pCREB protein levels compared with the vehicle-treated mice, although ZnT3−/− mice treated with ZN showed lower IGF-1, pERK, and pCREB protein levels than ZnT3+/+ mice (Figure 4A-D). We also observed a significant reduction of IGF-1- and pERK-IR and the number of pCREB-positive cells in ZnT3−/− mice compared with ZnT3+/+ mice. However, administration of ZN remarkably increased IGF-1- and pERK-IR and pCREB+ cells in both ZnT3−/− mice treated with ZN showed lower IGF-1, pERK, and pCREB protein levels than ZnT3+/+ mice. However, administration of ZN remarkably increased IGF-1- and pERK-IR and pCREB+ cells in both ZnT3−/− mice as compared with vehicle-treated mice, although ZnT3−/− mice showed lower IGF-1, pERK, and pCREB protein levels compared with the vehicle-treated mice, although ZnT3−/− mice treated with ZN showed lower IGF-1, pERK, and pCREB protein levels than ZnT3+/+ mice (Figure 4A-D). We also observed a significant reduction of IGF-1- and pERK-IR and the number of pCREB-positive cells in ZnT3−/− mice compared with ZnT3+/+ mice. However, administration of ZN remarkably increased IGF-1- and pERK-IR and pCREB+ cells in both
ZnT3+/+ and ZnT3−/− mice, although ZnT3−/− mice treated with ZN showed a lower IGF-1- and pERK-IR or pCREB+ cells than ZnT3+/+ mice treated with ZN (Figure 4E-P).

Several studies have demonstrated that the pCREB has been found to be present in the majority of newborn immature neurons in the adult DG and thus CREB signaling is known to play an important role in neuronal activation and in the survival stages of neurogenesis. To confirm the relationship between immature neurons and pCREB-expressing cells in the SGZ, we performed immunofluorescence staining against pCREB and DCX. Most of the pCREB+ cells were colocalized with DCX+ neuroblasts in their nuclei. We observed a significant decrease of pCREB+DCX+ cells in ZnT3−/− mice compared with ZnT3+/+ mice. However, administration of ZN remarkably increased the number of pCREB+DCX+ cells in both ZnT3+/+ and ZnT3−/− mice (Figure 4Q). Together, these findings provide further evidence that ERK and CREB activation were both necessary for the effect of vesicular zinc on adult hippocampal neurogenesis.

To investigate whether ZN supplementation improves cognitive abilities in both aged ZnT3+/+ and ZnT3−/− mice, mice were subjected to the MWM test for six consecutive days starting 50 days after the initial ZN supplementation. At 24 hours after the last training session, their behavior was recorded for 60 seconds after removing the platform as a probe trial (Figure 5A). The escape latency decreased progressively during 5 training days. ZnT3−/− mice spent longer periods of time finding the platform, had a decreased number of platform crossings, and had reduced time spent in the target quadrant compared to ZnT3+/+ mice. However, ZN supplementation resulted in an improved cognitive ability in both ZnT3+/+ and ZnT3−/− mice, as proven by a significant reduction in escape latency (Figure 5B) and an increase in the number of platform crossings (Figure 5C) and time spent in the target quadrant (Figure 5D,E) compared to the vehicle-treated mice. These results demonstrate that ZN reverses aging-dependent cognitive decline in ZnT3−/− mice.

4 | DISCUSSION

The present study tested our hypothesis that vesicular zinc plays a central role in regulating adult neurogenesis occurring in the hippocampus. To test our hypothesis, we used mice in which vesicular zinc had been depleted by ZnT3 gene deletion. Here we found that ZnT3 gene deletion completely depleted vesicular zinc in the hippocampal MF tract and the number of progenitor cells, neuroblasts, and newborn neurons were remarkably reduced in the ZnT3−/− mice, even under physiological conditions. In addition, we also found that zinc supplementation with ZN reversed the reduction of adult neurogenesis in ZnT3−/− mice. This defective adult neurogenesis is due to a cell-extrinsic mechanism of ZnT3 deficiency and is accompanied by reduced levels of pERK and pCREB in the hippocampus of ZnT3−/− mice. The present study also demonstrated that IGF-1 expression is lower in the hippocampus of ZnT3−/− mice than wild type mice. Reduction of the level of IGF-1 is reversed by ZN supplementation in both ZnT3+/+ and ZnT3−/− mice, which suggests that ZnT3 is involved in IGF-dependent neurogenesis in the brain. Thus, these findings describe a central function for ZnT3 in maintaining adult hippocampal neurogenesis.

A possible relationship between zinc and hippocampal neurogenesis after brain injury has been reported by our previous studies. Our previous study suggests that decreased vesicular zinc content in the MF terminals of dentate granule neurons may cause impaired progenitor cell proliferation. We also reported that zinc chelation by clioquinol (5-chloro-7-iido-8-hydroxyquinoline; CQ) reduced hippocampal progenitor cell proliferation and neurogenesis after hypoglycemia, traumatic brain injury, and seizure.15,18,19 Supporting these findings, we recently demonstrated that enhancing hippocampal vesicular zinc via zinc supplementation with ZC increased hippocampal vesicular zinc concentration and thereby promotes adult neurogenesis under physiological20 or pathological conditions such as diabetes.21 These results support the notion that zinc is a critical regulator of adult neurogenesis.

Our previous studies demonstrated that zinc-deficient diet reduced zinc concentration in the presynaptic hippocampal vesicles in rats fed a zinc-deficient diet and that these mice showed reduced hippocampal neurogenesis.15 A separate group also reported that dietary zinc deprivation caused impaired cognitive function.5,22 However, the precise mechanism that leads to cognitive impairment in zinc-deficient animals is still unknown. Several laboratories have shown that ZnT3 gene deletion mice exhibit age-dependent deficits in learning and memory,9 impaired contextual discrimination, and spatial working memory10 and are deficient in social and object recognition memory.11 McAllister et al. demonstrated that that ZnT3−/− mice did not appear to readily develop the usual depression-like effects that occur after stress but however, were not completely free of stress-induced dysfunction,23 as fear memory generation was enhanced by stress in ZnT3−/− mice. Although the stress response is affected by ZnT3 gene deletion, hippocampal cellular proliferation was not significantly disrupted by stress in CD1 background mice. This group recently demonstrated that ZnT3−/− mice showed increased basal dendritic length in the cerebral cortex, which is not affected by housing condition.24 Thus, these studies suggest that ZnT3 and vesicular zinc are involved in certain types of spatial memory and behavior that are dependent on the background strain or on different stress conditions during development. We believe that further study will be needed to evaluate this difference as suggested.25,26 From the above studies, including our works, we hypothesized that a zinc deficient-induced cognitive impairment may be associated with a decline in hippocampal neurogenesis. Zinc-deficient diet not only affects hippocampal vesicular zinc concentration but also affects systemic zinc levels, including blood and cerebrospinal fluid (CSF) in the brain. For this reason, we cannot differentiate whether the dietary zinc-deficient-induced cognitive impairment is solely affected by...
decreased hippocampal neurogenesis. Thus, to test our hypothesis we used ZnT3−/− mice, which contain no vesicular zinc in the presynaptic terminal of hippocampus and cerebral cortex due to an inability to package zinc into synaptic vesicles.27,28 The basal level of BrdU-, Ki67-, and DCX-immunopositive cells was significantly diminished in ZnT3−/− mice compared with ZnT3+/+ mice. These results suggest that vesicular zinc originating from synaptic vesicles is necessary for progenitor cell proliferation and neuronal differentiation.

Why ZnT3−/− mice develop cognitive impairment during aging is not clear. Sindreu et al showed that ZnT3−/− mice have reduced activation of the ERK1/2 mitogen-activated protein kinase (MAPK) in hippocampal MF terminals and reduced inhibition of the zinc-sensitive tyrosine phosphatase MAPK in the setting of hippocampus-dependent learning.10 They suggested that a deficit of ERK signaling in ZnT3−/− mice caused impaired contextual discrimination, which is a possible mechanism of memory impairment of these mice. ERK signaling is also important for hippocampal neurogenesis and presynaptic pERK also modulates MF plasticity.29 Zinc can promote transcription of the BDNF gene and zinc supplementation increases the expression of BDNF mRNA in the adult rat brain.30 The zinc finger transcription factor CREB can stimulate transcription of BDNF.31 Currently, it is unknown how zinc induced BDNF expression occurs. One line of potential evidence to explain this phenomenon is the observation that zinc deficiency leads to a decreased phosphorylation of CREB in mice,32 which indicates that zinc could modulate CREB activity. ERK1/2 phosphorylates the kinases RSK and MSK, which can directly phosphorylate CREB.33 Zinc might stimulate CREB to increase the expression of BDNF via activation of ERK1/2, potentially forming a positive feedback loop to further activate local ERK1/2 signaling as an autocrine or paracrine factor. These results may explain why ZnT3−/− mice showed depressed progenitor cell proliferation and neurogenesis.

Studies using ZnT3−/− mice have produced mixed results with respect to how BDNF levels in the brain are affected by this manipulation. Several labs have reported that no difference exists between ZnT3+/+ and ZnT3−/− mice.9,24 On the other hand, other researchers have demonstrated that ZnT3−/− mice showed increased levels of hippocampal BDNF.34,35 Therefore, as an alternative line of investigation into whether neurotrophic factors are impacted by manipulation of zinc transporter function, the present study sought to explore IGF-1 levels in both ZnT3+/+ and ZnT3−/− mice treated with or without ZN supplementation. We found that IGF-1 levels in the hippocampus were reduced in ZnT3−/− mice, compared to ZnT3+/+ mice, which was reversed by ZN supplementation. Increased expression of IGF-1 and ERK phosphorylation during hippocampal neurogenesis has been suggested as potential mechanisms for these effects.36-40 However, subsequent studies are needed to elucidate how the observed decrease in ERK and CREB phosphorylation via IGF-1 arises from ZnT3 gene deletion.

One factor that can influence cellular differentiation is the redox potential. It is known to dynamically change as cells differentiate; for instance, it has been shown that the level of superoxide radicals increases as cells move from G1 to M phase.41 Additionally, concentrations of hydrogen peroxide and superoxide play a role in the choice to either enter or exit the cell cycle.42 In support of these observations, we previously demonstrated that newly generated neurons are exposed to oxidative stress at specific developmental timepoints, potentially explaining effects on hippocampal neurogenesis43 observed in these animals. To test this, we used ZN which is zinc chloride mixed with NAC in 1:2 M ratio to increase brain zinc and cytochrome levels. NAC, a glutathione precursor, is known to be a membrane-permeant cysteine prodrug with potent antioxidant, anti-inflammatory properties, and proneurogenesis. Strikingly, we observed increased pERK and pCREB expression in the hippocampus of both ZnT3+/+ and ZnT3−/− mice treated with ZN, thereby increasing adult hippocampal neurogenesis. This indicates that ZN supplementation has uniquely beneficial effects in that it can enhance de novo synthesis and also protect against the loss of newborn neurons.

The present study has potential limitations that need to be addressed in future studies. Here we found that zinc supplementation by ZN significantly increased not only hippocampal vesicular zinc levels but also neurogenesis. According to our results, the present study suggests that vesicular zinc is critical for adult hippocampal neurogenesis. However, ZN treatment did not affect vesicular zinc levels in the ZnT3−/− mice, which completely depleted vesicular zinc in the hippocampal MFs, suggesting the possibility that not only vesicular zinc but also extracellular zinc can promote neurogenesis. So, we cannot exclude the possibility that brain can use extracellular zinc arising from blood and CSF to maintain neurogenesis. Thus, exploring the effect of zinc supplementation by ZN on extracellular zinc concentrations could be a future direction for research that not only vesicular zinc but also extracellular zinc can promote adult hippocampal neurogenesis. The results presented here of defective adult neurogenesis in the DG of ZnT3−/− mice and decreased pERK and pCREB expression thus raise the intriguing possibility that this adult hippocampal ZnT3-related phenotype reflects aging-dependent cognitive decline in ZnT3−/− mice. However, future investigation will be surely be required to dissect the molecular and cellular events that underlie decreased ERK and CREB phosphorylation seen after genetic deletion of ZnT3.

5 | CONCLUSION

Together, the results from this study indicate that ZnT3 has a central function in maintaining adult hippocampal neurogenesis and zinc supplementation by ZN has a beneficial effect on hippocampal neurogenesis and may serve as a therapeutic target for enhanced neuroprotection and repair after injury. Therefore, the present study suggests that vesicular zinc is a critical regulator of adult hippocampal neurogenesis, under physiologic conditions.

ACKNOWLEDGMENTS

This study was supported by funding from the National Research Foundation of Korea (NRF) (NRF-2019R1A2C4004912) to B.Y.C. Additionally, this work was supported by the Brain Research Program through the NRF, funded by the Ministry of Science, Information and Communication Technology and Future Planning (NRF-2017M3C7A1028937 and 2020R1A2C2008480) to S.W.S.
CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
B.Y.C.: conception and design, financial support, collection, and assembly of data, data analysis and interpretation, manuscript writing; D.K.H., J.H.J., B.E.L.: collection and assembly of data; J.Y.K.: data analysis and interpretation, provision of study material or patients; S.W.S.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Choi BY, Hong DK, Jeong JH, Lee BE, Koh J-Y, Suh SW. Zinc transporter 3 modulates cell proliferation and neuronal differentiation in the adult hippocampus. *Stem Cells*. 2020;38:994–1006. [https://doi.org/10.1002/stem.3194](https://doi.org/10.1002/stem.3194)