

Original Research

# ZnT3–TMEM163 Mediates Zinc Homeostasis Imbalance Induced Neurodegeneration in Hippocampus

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## Abstract

**Background:** Vascular dementia (VaD) is a prevalent cognitive disorder associated with cerebrovascular pathologies, in which hippocampal dysfunction plays a critical role. Impaired zinc homeostasis, mediated by the zinc transporters 3 (ZnT3) and transmembrane protein 163 (TMEM163), has been implicated in neuronal damage. However, the underlying mechanisms remain unclear. The purpose of this study is to elucidate the molecular mechanisms by which these zinc transporters may contribute to VaD pathogenesis, and to determine whether these mechanisms are involved in the development of VaD. **Methods:** Rat primary hippocampal neurons were subjected to oxygen-glucose deprivation (OGD) to simulate ischemic conditions. To investigate the roles of ZnT3 and TMEM163, we employed siRNA-mediated silencing and plasmid-based overexpression. Neuronal viability was assessed using the methyl thiazolyl tetrazolium (MTT) assay, while apoptosis was quantified via TdT-mediated dUTP nick-end labeling (TUNEL) staining. Intracellular and extracellular zinc levels were measured using FluoZin-3 fluorescence and ELISA, respectively. Protein and mRNA expression levels were analyzed by western blot and real-time quantitative polymerase chain reaction (RT-qPCR). Protein-protein interactions were examined through co-immunoprecipitation, and subcellular localization was determined via cell surface biotinylation. **Results:** OGD induced significant extracellular zinc overload, neuronal apoptosis, and reduced cell viability, concomitant with upregulated expression of ZnT3 and TMEM163 (all  $p < 0.001$ ). Overexpression of these transporters exacerbated zinc efflux and neuronal damage under OGD, whereas their silencing attenuated zinc overload and neuronal degeneration ( $p < 0.001$ ). Co-immunoprecipitation confirmed a physical interaction between ZnT3 and TMEM163. Furthermore, OGD triggered the translocation of both proteins from the cell membrane to the cytoplasm ( $p < 0.001$ ), suggesting ischemia-induced dysregulation of zinc transport dynamics. These findings demonstrate that ZnT3 and TMEM163 cooperatively modulate zinc homeostasis and that their dysregulation during OGD contributes to neuronal injury. **Conclusion:** ZnT3 and TMEM163 are critical regulators of zinc homeostasis in hippocampal neurons. Their dysregulation under ischemic conditions promotes extracellular zinc overload and exacerbates neuronal damage, highlighting their potential therapeutic relevance in VaD.

**Keywords:** zinc transporter 3; TMEM163; zinc; hippocampus; vascular dementia

## 1. Introduction

Vascular dementia (VaD) represents a prevalent central nervous system disorder that arises from cerebrovascular pathologies and manifests as progressive cognitive impairment. Epidemiological studies have consistently demonstrated that VaD ranks as the second most common form of dementia after Alzheimer's disease (AD), accounting for approximately 15–30% of all dementia cases worldwide [1]. Clinically, VaD patients exhibit heterogeneous cognitive symptoms that vary depending on the specific brain regions affected by vascular lesions. Notably, deficits in short-term memory, working memory, attention, and executive functions have been strongly associated with ischemic damage and neuronal loss in the hippocampal formation [2].

Zinc, an essential trace element crucial for numerous biological processes including DNA function, protein catalysis, and neural signaling, has emerged as a key player in post-ischemic hippocampal dysfunction when its homeostasis becomes disrupted [3]. Under pathological conditions, excessive zinc accumulation within neurons triggers cell death pathways through activation of endoplasmic reticulum stress, particularly in vulnerable regions such as the ischemic penumbra and hippocampal CA1/CA3 subfields [4,5]. In the extracellular compartment, zinc overload contributes to blood-brain barrier disruption by promoting ferroptosis and accelerating amyloid-beta ( $A\beta$ ) aggregation through liquid-liquid phase separation mechanisms [6–8]. Furthermore, pathological zinc reuptake into cells can exacerbate oxidative stress, creating a positive feedback of neuronal damage [9].



The SLC30A gene family encodes zinc transporter (ZnT) proteins, which serve as primary regulators of cellular zinc homeostasis by controlling cytosolic zinc storage and efflux [9]. Among these transporters, ZnT3 stands out as the most abundantly expressed isoform in the hippocampus, where it plays a specialized role in zinc translocation into synaptic vesicles [10]. Clinical investigations have revealed significant correlations between cerebrospinal fluid ZnT3 levels and cognitive performance scores in AD patients [11]. This association may stem from hypoxia-induced ZnT3 dysregulation in the hippocampal CA1 region, where excessive zinc efflux leads to extracellular zinc accumulation and subsequent neuronal injury [12]. However, it is important to note that the role of ZnT3 in neurodegeneration appears to be complex and context-dependent. For instance, some studies have reported that ZnT3 knock-out can exacerbate cognitive deficits and synaptic dysfunction, suggesting a protective role for synaptic zinc under certain conditions [13–15].

Beyond ZnT3, recent research has identified transmembrane protein 163 (TMEM163) as a novel zinc efflux transporter localized to both cell membranes and lysosomes, where it appears to function synergistically with other zinc regulatory proteins in the human brain [16]. Genetic studies have linked specific TMEM163 variants to early-onset Parkinson's disease in Chinese populations, while experimental knockdown of TMEM163 in zebrafish models results in significant locomotor deficits and myelin abnormalities [17]. Intriguingly, elevated TMEM163 expression has been observed in specific microglial subpopulations even within non-lesional brain tissue from multiple sclerosis patients [18]. Structural analyses have further demonstrated that TMEM163 can form both homodimers and heterodimers with various ZnT family members, suggesting potential functional interactions [19]. These findings strongly imply that ZnT3 and TMEM163 may cooperate structurally and functionally to maintain normal neuronal physiology. However, whether dysregulation of these transporters and consequent zinc homeostasis disruption represents a critical mechanism underlying hippocampal neuronal damage and cognitive impairment in VaD remains an important unanswered question. In the present investigation, we systematically examined the roles of ZnT3 and TMEM163 in zinc regulation under both physiological conditions and oxygen-glucose deprivation (OGD) stress. Our study specifically focused on their effects on hippocampal neuronal function, subcellular distribution patterns, and potential protein-protein interactions. Through comprehensive experimental approaches, we aimed to elucidate the molecular mechanisms by which these zinc transporters may contribute to VaD pathogenesis.

## 2. Materials and Methods

### 2.1 Cell Culture and Stimulation

Primary hippocampal neurons (PHNs; CP-R107) were obtained from Pricella Biotechnology Co., Ltd. (Wuhan, Hubei, China) and cultured in complete medium at 37 °C under 5% CO<sub>2</sub> and saturated humidity. For subculturing, cells were dissociated with 1 mL of 0.25% trypsin for 1 min, neutralized with 5 mL of complete medium, gently pipetted to achieve a single-cell suspension, and returned to the incubator for further culture. A cellular model of oxygen-glucose deprivation (OGD) was constructed. PHNs were washed twice with phosphate-buffered saline (PBS), incubated in a glucose-free medium, and placed in a hypoxic chamber (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>) at 37 °C for 2–6 hours to simulate ischemia. The cell line was validated by STR profiling and tested negative for mycoplasma.

### 2.2 Cell Transfection

siRNA sequences targeting the coding regions of ZnT3 (NM\_001013243.1, Sangon, Shanghai, China) and TMEM163 (NM\_001110763.2, Sangon, Shanghai, China) were designed using the NCBI database (**Supplementary Table 1**). A scrambled siRNA (si-NC) served as the negative control. For transfection, 2 µL of siRNA duplexes and 10 µL of RNAiFit transfection reagent (HB-RF-1000, Hanbio, Shanghai, China) were separately diluted in 200 µL of Opti-MEM medium (31985088, Gibco, New York, USA), incubated at room temperature for 10 min to form complexes, and added to cell cultures. Cells were then incubated for 48 h to achieve gene silencing (si-ZnT3 and si-TMEM163).

The coding sequences (CDS) of ZnT3 and TMEM163 were cloned into the pcDNA3.1 (SYNBIO, Sunzhou, China) vector to generate overexpression plasmids (pcDNA3.1-ZnT3 and pcDNA3.1-TMEM163) (**Supplementary Table 2** and **Supplementary Fig. 1**). PHNs (80% confluent in 6-well plates) were transfected with 5 µg plasmid DNA mixed with 10 µL P3000 reagent (Thermo Fisher Scientific, MA, USA) and 3.75 µL Lipofectamine 3000 (ThermoFisher, MA, USA) in Opti-MEM. After 15 min of incubation, the mixture was added to cells for 24–48 h to induce overexpression (OE-ZnT3 and OE-TMEM163).

### 2.3 MTT Assay

PHNs (2000 cells/well in 96-well plates) were treated as per experimental groups (n = 6 replicates/group). After 6 h, 10 µL MTT (C0009S, Beyotime, Shanghai, China) (5 mg/mL) was added to each well, followed by 4 h of incubation. Formazan crystals were dissolved in DMSO, and absorbance at 570 nm was measured (Thermo Fisher Scientific, MA, USA).

## 2.4 TUNEL Assay

PHNs were fixed with 4% paraformaldehyde (30 min), permeabilized with 0.3% Triton X-100, and labeled using a TUNEL kit (Roche, Basel, Switzerland). Apoptotic nuclei (TUNEL-positive) and total nuclei (DAPI-stained, C1006, Beyotime, Shanghai, China) were counted under a fluorescence microscope (BZ-H4XD, Keyence, Osaka, Japan). The apoptosis rate was calculated as (TUNEL<sup>+</sup> cells/total cells) × 100%.

## 2.5 ELISA

Supernatants were centrifuged, and zinc levels were measured using a commercial ELISA kit (ab277419, Abcam, Cambridge, UK). A standard curve (0–5 nmol/well) was generated, and absorbance at 560 nm was recorded.

## 2.6 Immunofluorescence

PHNs were seeded in 6-well plates ( $2 \times 10^5$  per well) and serum-starved for 12 h before grouping. After culturing for 24 h, the medium was removed and PHNs were washed with PBS, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.25% Triton X-100 for 20 min. Following blocking with goat serum for 30 min, cells were incubated overnight at 4 °C with primary antibody NeuN (1:200, ab104225, Abcam). After washing with TBST, cells were incubated with secondary antibodies (1:200, ab96883, Abcam) for 1 h at room temperature in the dark. Nuclei were stained with DAPI (C1006, Beyotime, Shanghai, China) for 5 min. PHNs were observed and captured under a fluorescence microscope (BZ-H4XD, Keyence, Japan).

PHNs were loaded with FluoZin™-3 AM (F24195, Thermo Fisher Scientific, MA, USA) (5 μM, 30 min at 37 °C), washed, and incubated in PBS for 30 min. Fluorescence (ex/em: 494/525 nm) was quantified using a fluorescence microscope (BZ-H4XD, Keyence, Japan).

## 2.7 Western Blot (WB)

PHNs were washed with PBS, lysed with RIPA buffer, and centrifuged to collect supernatants for total protein extracts. Protein quantification was used the BCA assay with a standard curve generated by diluting standards. Samples were mixed with Loading Buffer and stored at –20 °C. SDS-PAGE was performed using 12% resolving and 5% stacking gels. Proteins were electrophoresed, transferred to PVDF membranes, and blocked. Then the membranes were incubated with primary antibodies ZnT3 (1:1000, 67262-1-Ig, Proteintech), TMEM163 (1:1000, PA5-114329, ThermoFisher), GAPDH (1:10000, ab70700, Abcam) at 4 °C overnight and incubated with Goat Anti-Rabbit IgG H&L/HRP (1:20000, ab205718, Abcam) or Goat Anti-Mouse IgG H&L/HRP secondary antibodies (1:10000, ab205719, Abcam) for 2 h. Chemiluminescent detection and image capture followed, with protein expression analyzed using ImageJ (v1.8.0, National Institutes of

Health, Bethesda, MD, USA).

## 2.8 RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from PHNs of logarithmic phase using the FastPure RNA kit (R0017M, Beyotime, Shanghai, China), following cell lysis and column purification with ethanol treatment. RNA concentration and purity were assessed using Nano 6000 (Nano-600, JIA-PENG, Shanghai, China). cDNA was synthesized from 500 ng RNA using HiScript III RT SuperMix with gDNA wiper. RT-qPCR was performed in a 20 μL reaction containing diluted cDNA, Nuclease-free water, Taq Pro Universal SYBR qPCR Master Mix, and gene-specific primers on the CFX96 Touch instrument (CFX96 Touch, Bio-rad, Shanghai, China). The results were calculated by the  $2^{-\Delta\Delta C_t}$  method. The sequences accessions of the primers are: mouse ZnT3 (5'-AGACCACCCGCCTAGTGAG-3' forward, 5'-CTGAGACACGCGTTCTTGT-3' reverse), mouse TMEM163 (5'-CCCAGTCGTCCACCATCAAA-3' forward, 5'-AAGCACCATAGGTGGCTTCC-3' reverse), and GAPDH (5'-CAATCCTGGGCGGTACAAC-3' forward, 5'-TACGGCCAAATCCGTTTACA-3' reverse). Gene mRNA levels were normalized to those of GAPDH. All samples were run in duplicate.

## 2.9 Co-Immunoprecipitation

PHNs were lysed in RIPA buffer with PMSF, and protein concentrations were determined using BCA assay. For co-immunoprecipitation (Co-IP), 500 μL cell lysates (200–1000 μg protein) were pre-cleared with Protein A/G Agarose at 4 °C for 1 h. Samples were incubated overnight with 2 μg primary antibodies (same antibodies as Western blot) or control IgG at 4 °C. Immune complexes were captured by adding Protein A/G Agarose, washed with lysis buffer and boiled in SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and detected by Western blot using specific antibodies. Protein bands were visualized using chemiluminescence reagents and quantified with Image J.

## 2.10 Cell Surface Biotinylation (CSB)

PHNs ( $1.5 \times 10^6$  cells/10 cm dish) were transfected with HA-tagged ZnT3 or DDK-tagged TMEM163 plasmids. Surface proteins were biotinylated (EZ-Link Sulfo-NHS-SS-Biotin), extracted with NeutrAvidin beads (Thermo Fisher Scientific, MA, USA), and detected by Western blot. Band intensities were quantified using ImageJ.

## 2.11 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism (v9.4.0, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD). A paired *t*-test was used to compare the difference between two groups. For

the multiple-group's comparisons, significance was tested with one-way ANOVA followed by Tukey's post hoc test. A  $p$ -value of less than 0.05 was considered statistically significant.

### 3. Results

#### 3.1 OGD Induces Neuronal Death Concomitant With Disruption of Zinc Homeostasis

We began by establishing an *in vitro* model of cerebral ischemia using oxygen-glucose deprivation (OGD) in primary hippocampal neurons (PHNs). Hypoxic-hypoglycemic conditions markedly reduced the fluorescence intensity of NeuN, a specific neuronal nuclear marker ( $p < 0.01$ ). This neuronal loss was concomitant with a significant increase in apoptosis ( $p < 0.001$ ) and a reduction in overall neuronal viability as measured by MTT assay ( $p < 0.001$ ) (Fig. 1A–C). In consideration of the role of zinc dyshomeostasis in exacerbating ischemic brain damage, we next asked whether our OGD model recapitulated this pathological feature. Indeed, using a fluorescent zinc indicator (FluoZin-3) and ELISA, we found that OGD triggered a profound efflux of zinc, resulting in a decrease in intracellular zinc (Fig. 1D) and a concomitant accumulation of zinc in the extracellular space (Fig. 1E). These data established a correlation between OGD-induced neuronal degeneration and pathological zinc efflux, prompting us to investigate the molecular mediators responsible for this zinc dysregulation.

#### 3.2 OGD Dysregulates ZnT3 and TMEM163 and Exacerbates Zinc Efflux

Having observed aberrant zinc efflux, we sought to identify the transporters responsible. We focused on ZnT3, given its predominant expression in hippocampal neurons and its critical role in synaptic zinc transport and its documented involvement in cerebral ischemia. We therefore hypothesized that ZnT3 and TMEM163 might act in concert to mediate the pathological zinc efflux observed in our OGD model. siRNA and the overexpression plasmid were designed for the manipulation of ZnT3 or TMEM163 expression in PHNs, respectively. The interference and overexpression efficiency were measured by Western blot and the results suggest that the protein expression of ZnT3 and TMEM163 were silenced by siRNA interference while the overexpression plasmid exhibited upregulation of ZnT3 and TMEM163 in PHNs (Fig. 2A). Since extracellular zinc level was significantly elevated by OGD, and ZnT3 is a high-expressed zinc transporter protein in neurons that already been confirmed to be colocalized with TMEM163, we hypothesis that both ZnT3 and TMEM163 and their expression may be affected with the changes of culture environment. Our experiment suggests that OGD significantly upregulated ZnT3 and TMEM163 at both protein and mRNA levels ( $p < 0.001$ ), mimicking the effects of overexpression (Fig. 2B). Conversely, siRNA silencing prevented

OGD-induced upregulation ( $p < 0.001$ ). Notably, OE-ZnT3/TMEM163 or OGD reduced intracellular zinc, while silencing restored normal levels ( $p < 0.001$ ), implicating both transporters in zinc efflux dysregulation (Fig. 2C). This demonstrated that both transporters are not merely correlative but are functionally required for the dysregulated zinc efflux under OGD.

#### 3.3 ZnT3 and TMEM163 Silencing Reverse the OGD-Induced PHNs Degeneration

Since manipulating ZnT3/TMEM163 expression directly affected zinc flux, we next asked whether this manipulation would correspondingly impact neuronal survival. It was evident that OGD and OE-ZnT3/TMEM163 exacerbated PHNs damage, as demonstrated by a reduction in NeuN-positive cells (indicating neuronal loss,  $p < 0.001$ ), an increase in apoptosis ( $p < 0.001$ ), and a decline in overall metabolic viability ( $p < 0.001$ ) (Fig. 3A–C). Conversely, silencing either transporter provided substantial protection against these degenerative events. This genetic evidence firmly establishes that the OGD-induced upregulation of ZnT3 and TMEM163 is a key pathogenic event driving neuronal death, likely through zinc efflux. Given their coordinated upregulation and parallel functional effects, we postulated that ZnT3 and TMEM163 might not act alone but could function as a complex. Co-immunoprecipitation assays confirmed a robust physical interaction between ZnT3 and TMEM163 under both normal and OGD conditions (Fig. 3D). This interaction provides a potential molecular basis for their observed functional synergy in regulating zinc homeostasis and neuronal survival.

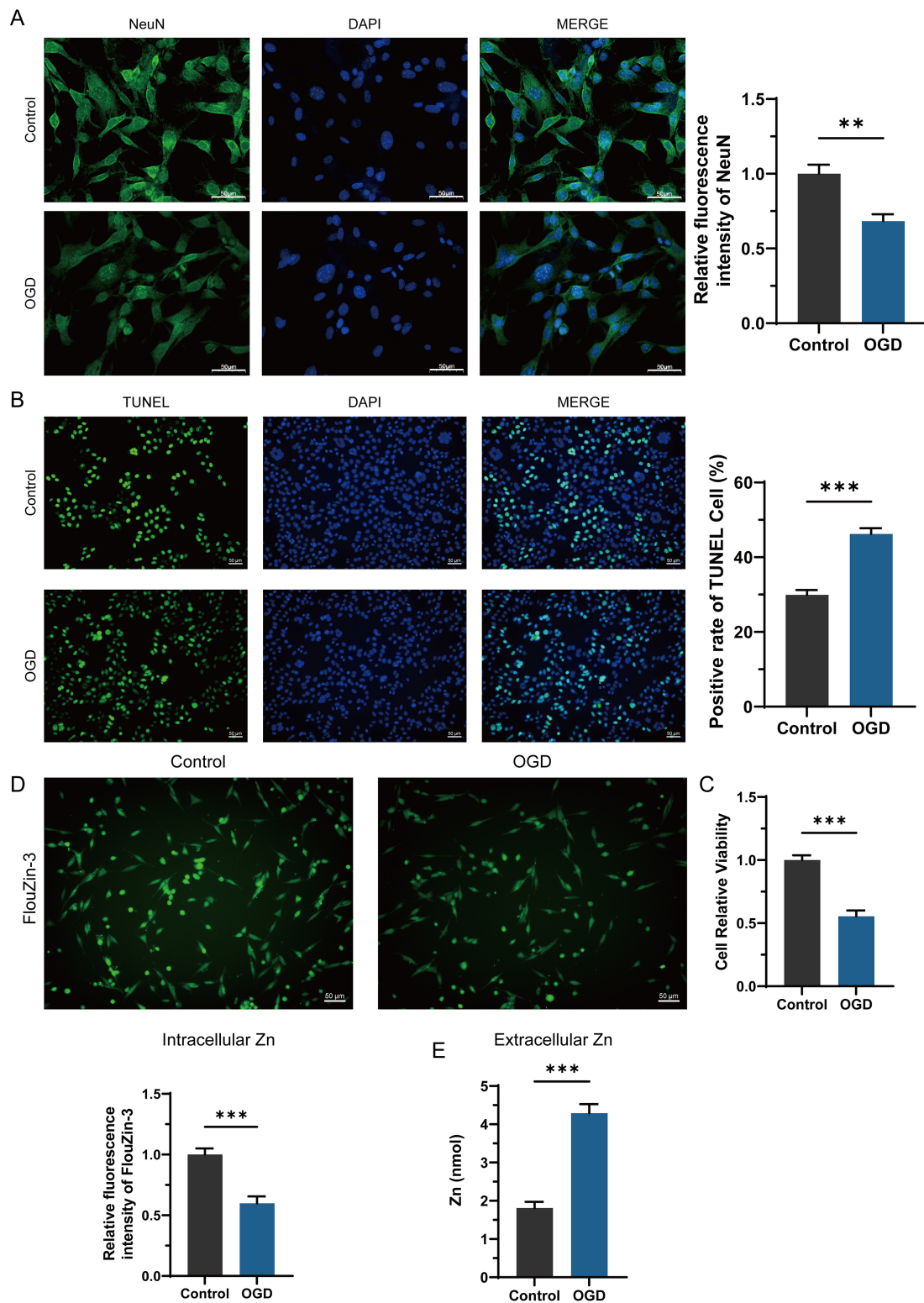
#### 3.4 OGD Alters the Subcellular Localization of ZnT3 and TMEM163

The discovery of a ZnT3-TMEM163 complex prompted us to investigate how OGD regulates its activity. Since the subcellular localization of membrane transporters is a critical regulatory mechanism for their function, we assessed whether OGD affects the distribution of ZnT3 and TMEM163 between the cell membrane and cytoplasm. Cell surface biotinylation assays revealed that OGD induced a significant translocation of both transporters from the plasma membrane to the cytoplasm (Fig. 4). This redistribution was prevented by siRNA knockdown. This OGD-induced relocation away from the membrane may represent a maladaptive response that disrupts precise zinc compartmentalization, ultimately contributing to the observed extracellular zinc overload and toxicity.

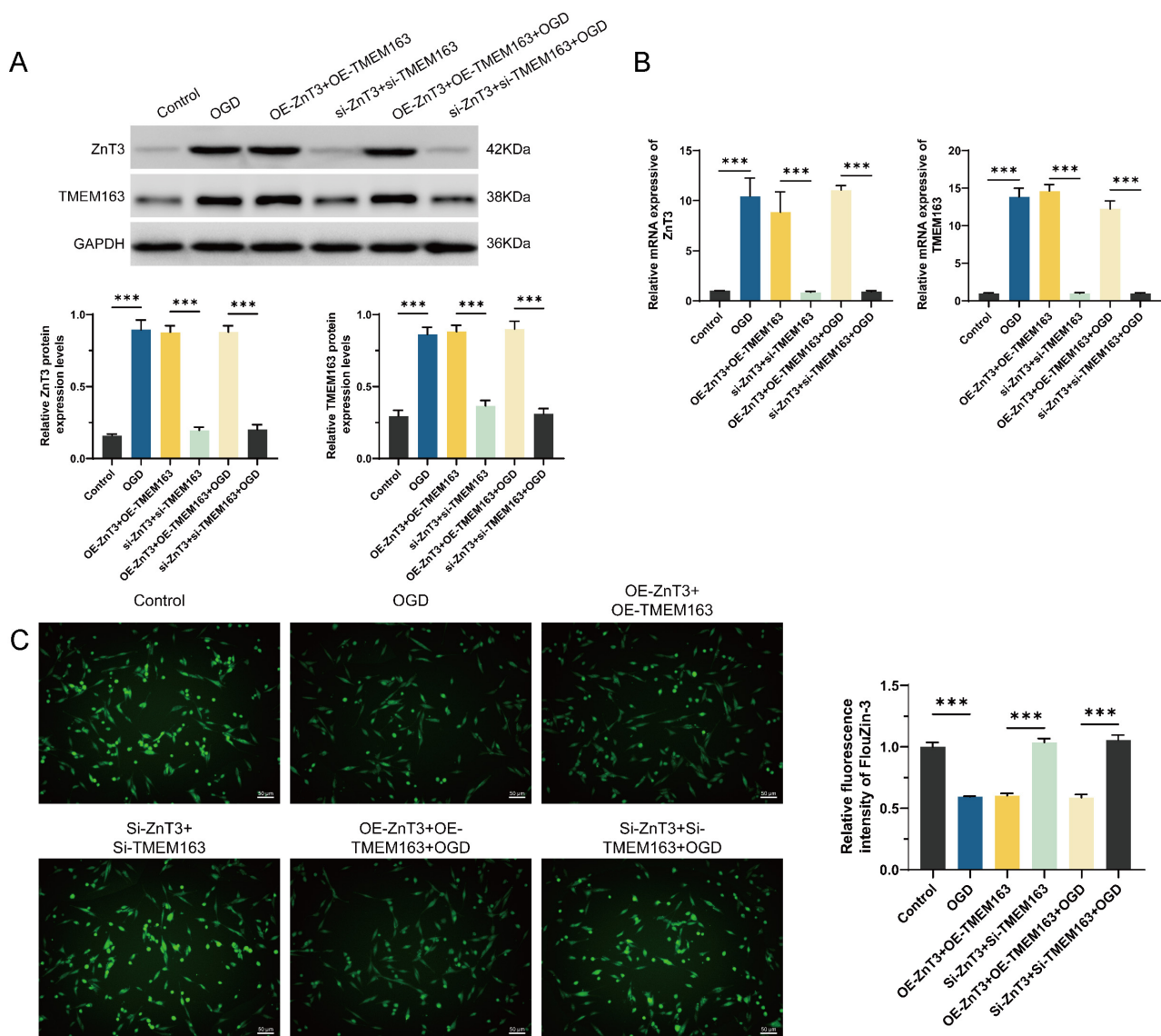
### 4. Discussion

Zinc transport in neurons represents a complex physiological process that requires precise regulation by a series of transporter proteins, including ZnT family members and TMEM163 [9,16]. Our comprehensive study has demonstrated that intracellular and extracellular zinc levels





**Fig. 1. OGD induces neuronal loss and zinc efflux in PHNs.** (A) NeuN fluorescence intensity was significantly reduced in PHNs after OGD, scale bar = 50  $\mu$ m. (B) OGD increased the apoptosis rate of PHNs, scale bar = 50  $\mu$ m. (C) Neuronal viability declined under OGD conditions. (D) Decreased intracellular zinc levels, measured by FluoZin-3 fluorescence, were elevated post-OGD, scale bar = 50  $\mu$ m. (E) Extracellular zinc efflux, measured by ELISA. Data represent mean  $\pm$  SD (n = 6). \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 vs. control. OGD, oxygen-glucose deprivation; PHNs, primary hippocampal neurons; DAPI, 4',6-diamidino-2-phenylindole; TUNEL, TdT-mediated dUTP nick-end labeling.

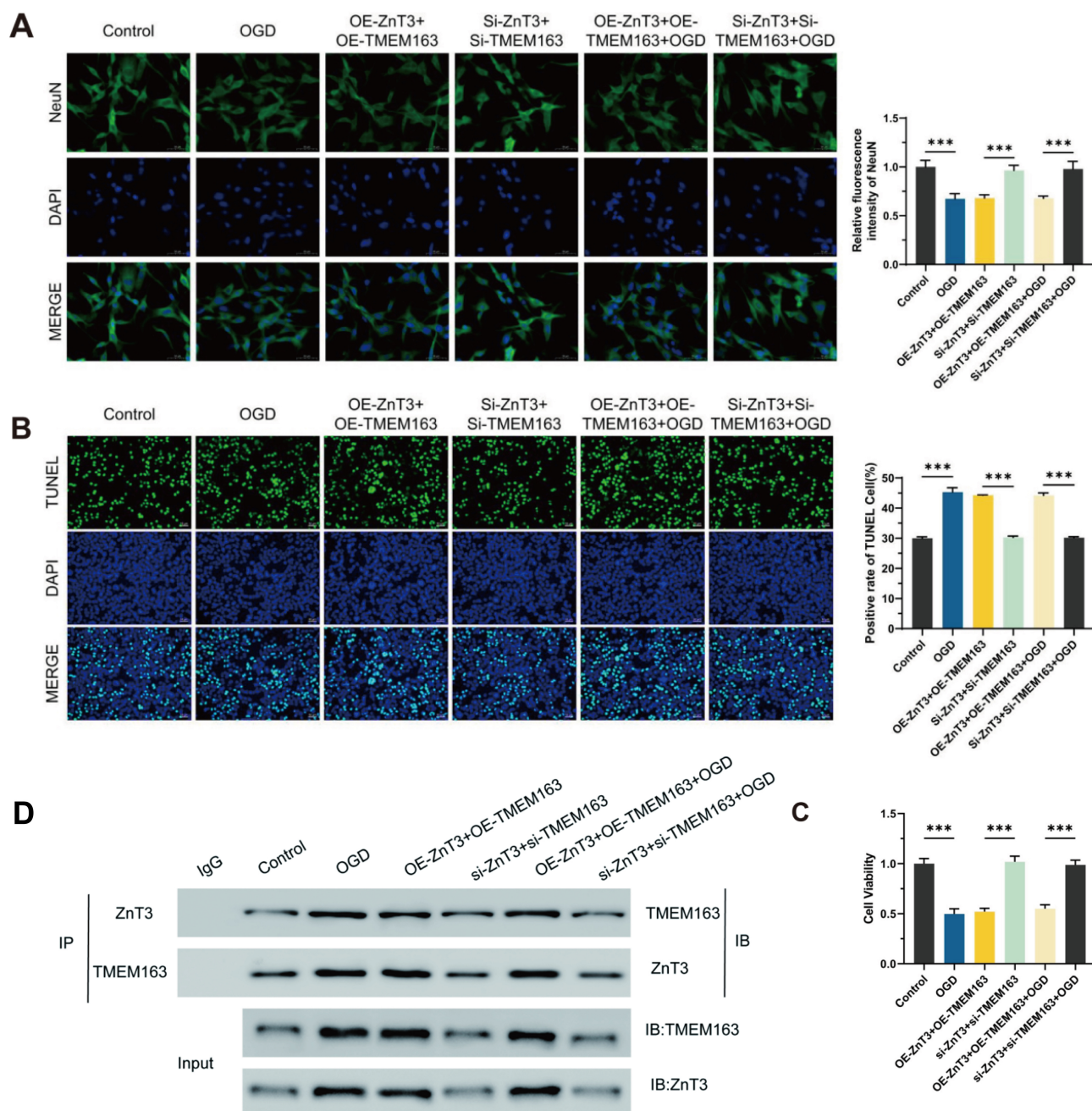


**Fig. 2. OGD alters ZnT3 and TMEM163 expression and zinc distribution in PHNs.** (A) Protein levels of ZnT3 and TMEM163 increased under OGD and overexpression (OE) conditions but decreased after siRNA silencing. (B) Corresponding mRNA expression changes. (C) Intracellular zinc levels decreased after OGD or OE but were restored by siRNA-mediated knockdown, scale bar = 50  $\mu$ m. Data represent mean  $\pm$  SD (n = 6). \*\*\* $p$  < 0.001 vs. control. ZnT3, zinc transporters 3; TMEM163, transmembrane protein 163.

in hippocampal neurons are dynamically regulated through the coordinated actions of ZnT3 and TMEM163 proteins. Importantly, we found that OGD can induce significant abnormalities in both the distribution patterns and functional states of ZnT3-TMEM163 complexes on neuronal cell membranes and within the cytoplasm. These pathological changes ultimately lead to extracellular zinc overload and subsequent neuronal degeneration (Fig. 5). Notably, our experimental results show that targeted modulation of ZnT3-TMEM163 expression can effectively reverse OGD-induced structural and functional damage to neurons. The current findings provide a solid theoretical foundation for future research focusing on VaD animal models, while also

highlighting the crucial importance of zinc-centered therapeutic targets for VaD treatment.

Synaptic zinc serves as a pivotal element for normal neural transmission and neurogenesis processes. The disruption of zinc homeostasis has been well documented in various neurological disorders including AD, multiple sclerosis, epilepsy, and traumatic brain injury [3,20–23]. In chronic cerebral ischemia models, elevated levels of extracellular zinc coupled with ZnT3 overexpression have been shown to significantly exacerbate blood-brain barrier damage [24]. This pathological phenomenon was similarly observed in acute ischemic cell and rat models, where zinc leakage into microvasculature activates superoxide produc-



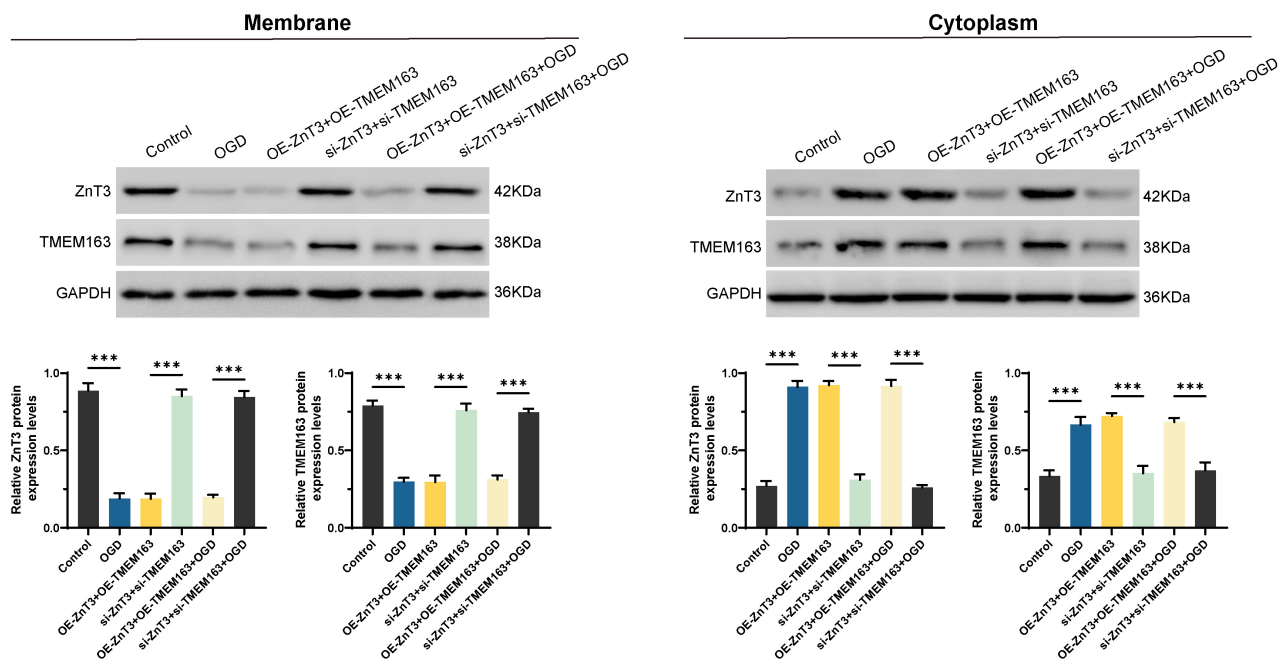
**Fig. 3. ZnT3 and TMEM163 contribute to OGD-induced PHNs degeneration.** (A) NeuN expression declined after OGD or OE but was preserved by siRNA, scale bar = 50  $\mu$ m. (B) Apoptosis increased under OGD/OE but decreased with silencing, scale bar = 50  $\mu$ m. (C) Neuronal viability followed similar trends. (D) Co-immunoprecipitation confirms ZnT3-TMEM163 interaction. Immunoprecipitation assays detected ZnT3-TMEM163 complexes in all experimental groups, regardless of expression levels. Data represent mean  $\pm$  SD (n = 6). \*\*\* $p$  < 0.001 vs. control.

tion and increases metalloproteinase 2/9 activity, ultimately resulting in the loss of tight junction proteins and endothelial cell death [25]. Our study extends these important findings by demonstrating that zinc efflux and extracellular overload are associated not only with ZnT3 overexpression but also with increased TMEM163 expression levels. Most remarkably, even under severe OGD conditions, simultaneous gene silencing of both ZnT3 and TMEM163

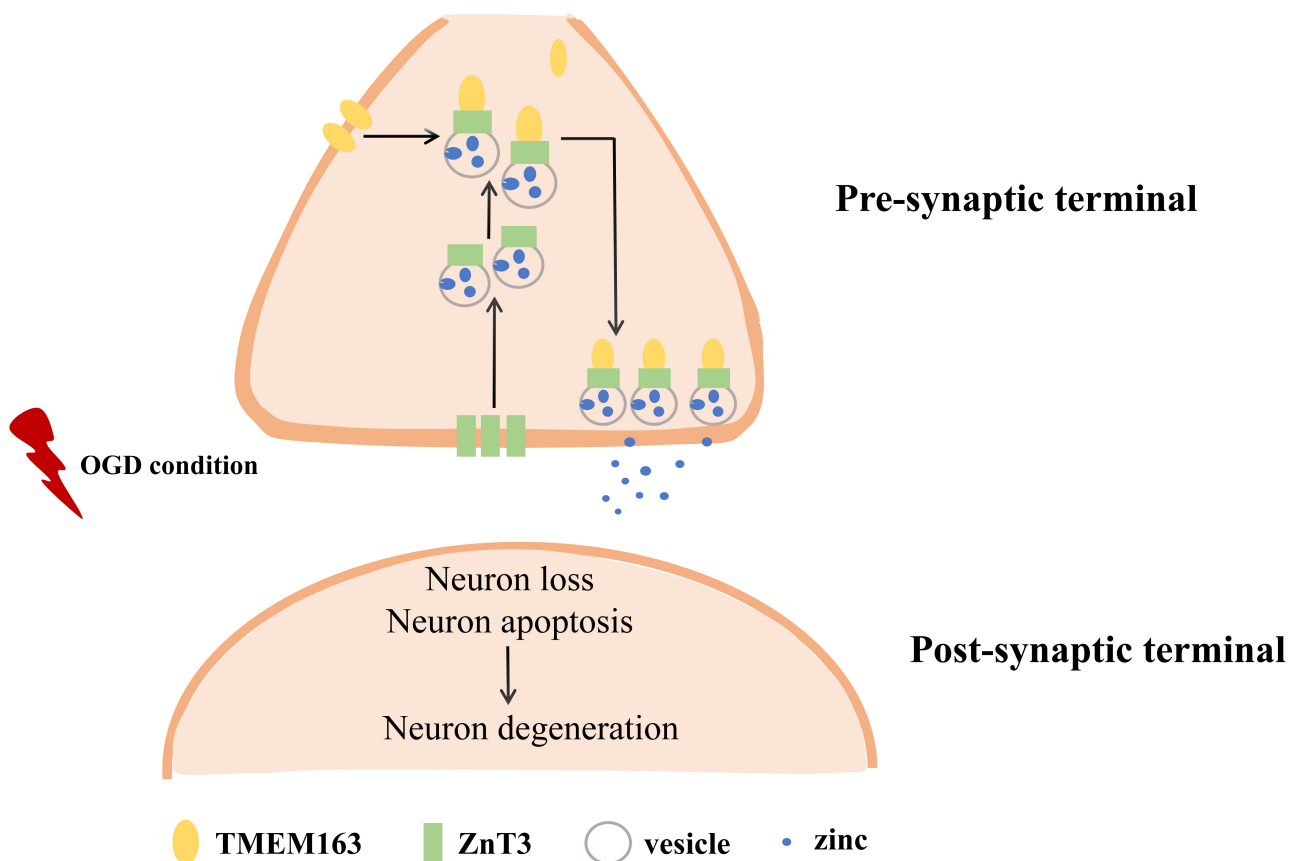
could effectively reverse zinc-mediated neuronal damage. These groundbreaking results strongly suggest a previously unrecognized role of TMEM163 in zinc-related pathological mechanisms during cerebral ischemia.

Previous studies have established that TMEM163 can physically co-localize and functionally interact with TRPML1 and P2X receptors [26,27]. A particularly relevant recent study conducted in HEK293 cells observed





**Fig. 4. OGD promotes cytoplasmic translocation of ZnT3 and TMEM163.** Under OGD, membrane-localized ZnT3/TMEM163 translocated to the cytoplasm. Silencing reduced this translocation, while overexpression did not further enhance it. Data represent mean  $\pm$  SD (n = 6). \*\*\* $p$  < 0.001 vs. control.



**Fig. 5. Schematic illustration of ZnT3-TMEM163 on zinc efflux and neuronal degeneration in OGD condition.**



a strong functional correlation between TMEM163 and ZnT proteins, providing direct evidence that TMEM163 can form heterodimers with ZnT3 [19]. Intriguingly, these TMEM163-ZnT3 heterodimers exhibited similar zinc efflux capabilities as TMEM163 homodimers. These important findings suggested the intriguing possibility that disruption of the structural and functional synergy between TMEM163 and ZnT3 might represent a fundamental mechanism underlying neural injury during hypoxic conditions. In our current study, we systematically tested this hypothesis and made several key discoveries: First, we confirmed that TMEM163 and ZnT3 do indeed form immunoprecipitable complexes in neurons. Second, we found that this co-expression was significantly elevated under OGD conditions. Third, we observed that the interaction showed synchronous changes with both overexpression and silencing of the proteins. Furthermore, given the established expression synergy between ZnT3 and TMEM163 in regulating zinc homeostasis, we conducted detailed subcellular localization studies. Our results revealed a fascinating redistribution pattern: under pathological conditions, both proteins showed decreased membrane localization but increased cytoplasmic accumulation, suggesting their active translocation to facilitate zinc transport. Most importantly, when both proteins were overexpressed, we observed dramatically increased zinc outflow and corresponding neurotoxic effects. These comprehensive findings support a novel conceptual framework in which the combined dysfunction of ZnT3 and TMEM163 represents a central pathological mechanism driving zinc overload, neuronal damage, and consequent cognitive impairment in OGD model of cerebral ischemia.

Further investigation is required to elucidate the downstream signalling pathways that bridge ZnT3/TMEM163-mediated zinc efflux to neuronal apoptosis. The present study demonstrates a clear sequence of events: OGD, upregulation and dysregulation of ZnT3/TMEM163, extracellular zinc overload, neuronal apoptosis. However, the precise molecular machineries transducing zinc toxicity into activation of the apoptotic cascade are not fully delineated here. Based on existing literature, several plausible mechanisms can be proposed. Firstly, zinc overload is a potent inducer of mitochondrial dysfunction. Excessive zinc can permeate into mitochondria, leading to loss of mitochondrial membrane potential, cytochrome c release, and activation of the intrinsic (mitochondrial) apoptotic pathway [28–30]. Secondly, disruptions in zinc homeostasis are closely linked to Endoplasmic Reticulum (ER) stress. Zinc deficiency within the ER lumen, potentially exacerbated by efflux transporters like ZnT3 and TMEM163, can impair the function of ER-resident zinc-dependent enzymes, leading to the accumulation of misfolded proteins and triggering the Unfolded Protein Response (UPR), which can ultimately culminate in apoptosis [31,32]. Furthermore, the interplay

between zinc and oxidative stress is well-established. The disruption of zinc homeostasis can foster the generation of reactive oxygen species (ROS), which in turn can damage lipids, proteins, and DNA, activating stress kinases and apoptotic signaling [33,34]. Intriguingly, our co-immunoprecipitation data confirms a physical interaction between ZnT3 and TMEM163. It is tempting to speculate that this complex may not only regulate zinc flux but also serve as a scaffold to recruit downstream signaling partners, thereby directly influencing cell death pathways. Future studies employing transcriptomic and proteomic approaches in our model will be essential to map the entire signaling network from zinc efflux to apoptosis, with a specific focus on mitochondrial integrity, ER stress markers, and oxidative stress endpoints.

It is imperative to reconcile our findings with reports that present an apparently contradictory role for ZnT3. On the one hand, in present study and others, upregulation of ZnT3 contributes to zinc efflux and neuronal injury in conditions of ischemia [12,24]. On the other hand, as demonstrated by the preceding study, in chronic neurodegenerative models such as AD, loss of ZnT3 and vesicular zinc have been shown to accelerate amyloid plaque pathology and exacerbate cognitive decline [35,36]. Additionally, ZnT3 plays a crucial role in maintaining adult hippocampal neurogenesis by regulating cell proliferation and neuronal differentiation in hippocampal cells [10]. The temporal and spatial dynamics of zinc release are critical. In conditions of acute ischemia, a substantial and unregulated efflux of zinc may be triggered by the upregulation of ZnT3/TMEM163, resulting in rapid excitotoxic and metabolic injury. Conversely, in chronic conditions, the physiological, activity-dependent release of zinc from synapses via ZnT3 may serve a regulatory function in modulating neuronal circuits and preventing pathological protein aggregation. Consequently, the net effect of ZnT3 activity is likely to be determined by the disease context, the magnitude and duration of zinc dyshomeostasis, and the specific cellular compartments involved.

While our study provides significant insights into the role of ZnT3-TMEM163 in OGD-induced injury, it has certain limitations that point to future research directions. First, it must be noted that a large number of zinc transporter proteins participate in the complex regulation of zinc homeostasis. Beyond ZnT3, other family members such as ZnT1 have been implicated in amyloid plaque accumulation in AD mouse models, with elevated hippocampal ZnT1 levels observed in early-stage AD patients [37]. Similarly, ZnT6 expression in the hippocampus has been shown to increase significantly in subjects with amnesic mild cognitive impairment [38]. Therefore, the specific functional contributions of individual ZnT family members require systematic investigation in future studies. Second, while our *in vitro* cell culture system provided excellent control over experimental variables by minimizing confounding humoral fac-

tors, the critical findings of this work clearly need validation in appropriate VaD mouse models to provide *in vivo* evidence for the roles of ZnT3 and TMEM163 in VaD pathogenesis. Most importantly, as noted in the preceding discussion, the specific downstream signaling cascades that link ZnT3/TMEM163-mediated zinc dyshomeostasis to the execution of neuronal apoptosis are not fully elucidated here. The mechanisms we proposed, including mitochondrial dysfunction, ER stress, and oxidative stress, require direct experimental confirmation in future studies. Unraveling these detailed pathways will be crucial for identifying the most tractable molecular targets for therapeutic intervention downstream of zinc overload.

## 5. Conclusion

In conclusion, our study provides compelling evidence that ZnT3 and TMEM163 engage in a sophisticated functional partnership to maintain zinc homeostasis under both physiological and hypoxic conditions. The OGD-induced degeneration of primary hippocampal neurons, characterized by significant structural damage, decreased cell viability, and increased apoptosis, appears to be mechanistically linked to extracellular zinc overload mediated by ZnT3-TMEM163 dysregulation. Most importantly, our demonstration that targeted inhibition of ZnT3 and TMEM163 can effectively mitigate neuronal degeneration highlights their strong potential as novel therapeutic targets for treating VaD and other cerebral ischemia-related cognitive disorders. These findings open new avenues for developing zinc-centered neuroprotective strategies against vascular cognitive impairment.

## Availability of Data and Materials

All data reported in this paper will be shared by the correspondence author upon reasonable request.

## Author Contributions

Study design: WY, ZG and CZ; Conceptualization: ZG and CZ; Data curation: SQ and JX; Formal analysis: JW and NL; Investigation: ZG and CZ; Writing — original draft: WY, ZG and CZ; Writing — review and editing: WY. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

## Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation and revision of this work, the authors used ChatGPT and DeepSeek in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL45588>.

## References

- [1] van der Flier WM, Skoog I, Schneider JA, Pantoni L, Mok V, Chen CLH, *et al.* Vascular cognitive impairment. *Nature Reviews. Disease Primers*. 2018; 4: 18003. <https://doi.org/10.1038/nrdp.2018.3>.
- [2] Gorelick PB, Scuteri A, Black SE, Decarli C, Greenberg SM, Iadecola C, *et al.* Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the american heart association/american stroke association. *Stroke*. 2011; 42: 2672–2713. <https://doi.org/10.1161/STR.0b013e3182299496>.
- [3] Wang B, Fang T, Chen H. Zinc and Central Nervous System Disorders. *Nutrients*. 2023; 15: 2140. <https://doi.org/10.3390/nu15092140>.
- [4] Zhao Y, Ding M, Yang N, Huang Y, Sun C, Shi W. Zinc Accumulation Aggravates Cerebral Ischemia/Reperfusion Injury Through Inducing Endoplasmic Reticulum Stress. *Neurochemical Research*. 2022; 47: 1419–1428. <https://doi.org/10.1007/s11064-022-03536-w>.
- [5] Xiao P, Huang H, Zhao H, Liu R, Sun Z, Liu Y, *et al.* Edaravone dextro-neo protects against cerebral ischemia/reperfusion-induced blood-brain barrier damage by inhibiting ferroptosis via activation of nrf-2/HO-1/GPX4 signaling. *Free Radical Biology & Medicine*. 2024; 217: 116–125. <https://doi.org/10.1016/j.free-radbiomed.2024.03.019>.
- [6] Sensi SL, Granzotto A, Siotto M, Squitti R. Copper and Zinc Dysregulation in Alzheimer's Disease. *Trends in Pharmacological Sciences*. 2018; 39: 1049–1063. <https://doi.org/10.1016/j.tips.2018.10.001>.
- [7] Singh V, Xu L, Boyko S, Surewicz K, Surewicz WK. Zinc promotes liquid-liquid phase separation of tau protein. *The Journal of Biological Chemistry*. 2020; 295: 5850–5856. <https://doi.org/10.1074/jbc.AC120.013166>.
- [8] Gao YY, Zhong T, Wang LQ, Zhang N, Zeng Y, Hu JY, *et al.* Zinc enhances liquid-liquid phase separation of Tau protein and aggravates mitochondrial damages in cells. *International Journal of Biological Macromolecules*. 2022; 209: 703–715. <https://doi.org/10.1016/j.ijbiomac.2022.04.034>.
- [9] McAllister BB, Dyck RH. Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function. *Neuroscience and Biobehavioral Reviews*. 2017; 80: 329–350. <https://doi.org/10.1016/j.neubiorev.2017.06.006>.

- [10] Choi BY, Hong DK, Jeong JH, Lee BE, Koh JY, Suh SW. Zinc transporter 3 modulates cell proliferation and neuronal differentiation in the adult hippocampus. *Stem Cells* (Dayton, Ohio). 2020; 38: 994–1006. <https://doi.org/10.1002/stem.3194>.
- [11] Whitfield DR, Vallortigara J, Alghamdi A, Howlett D, Hortobágyi T, Johnson M, *et al.* Assessment of ZnT3 and PSD95 protein levels in Lewy body dementias and Alzheimer's disease: association with cognitive impairment. *Neurobiology of Aging*. 2014; 35: 2836–2844. <https://doi.org/10.1016/j.neurobiolaging.2014.06.015>.
- [12] Olesen RH, Hyde TM, Kleinman JE, Smidt K, Rungby J, Larsen A. Obesity and age-related alterations in the gene expression of zinc-transporter proteins in the human brain. *Translational Psychiatry*. 2016; 6: e838. <https://doi.org/10.1038/tp.2016.83>.
- [13] Adlard PA, Parncutt JM, Finkelstein DI, Bush AI. Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience*. 2010; 30: 1631–1636. <https://doi.org/10.1523/JNEUROSCI.5255-09.2010>.
- [14] Grabrucker S, Jannetti L, Eckert M, Gaub S, Chhabra R, Pfaender S, *et al.* Zinc deficiency dysregulates the synaptic ProSAP/Shank scaffold and might contribute to autism spectrum disorders. *Brain: a Journal of Neurology*. 2014; 137: 137–152. <https://doi.org/10.1093/brain/awt303>.
- [15] Zong R, Zhang X, Dong X, Liu G, Zhang J, Gao Y, *et al.* Genetic deletion of zinc transporter ZnT<sub>3</sub> induces progressive cognitive deficits in mice by impairing dendritic spine plasticity and glucose metabolism. *Frontiers in Molecular Neuroscience*. 2024; 17: 1375925. <https://doi.org/10.3389/fnmol.2024.1375925>.
- [16] Sanchez VB, Ali S, Escobar A, Cuajungco MP. Transmembrane 163 (TMEM163) protein effluxes zinc. *Archives of Biochemistry and Biophysics*. 2019; 677: 108166. <https://doi.org/10.1016/j.abb.2019.108166>.
- [17] do Rosario MC, Bey GR, Nmezi B, Liu F, Oranburg T, Cohen ASA, *et al.* Variants in the zinc transporter TMEM163 cause a hypomyelinating leukodystrophy. *Brain: a Journal of Neurology*. 2022; 145: 4202–4209. <https://doi.org/10.1093/brain/awac295>.
- [18] Lam M, Lee D, Kosater I, Khairallah A, Taga M, Zhang Y, *et al.* Human disease-specific cell signatures in non-lesional tissue in Multiple Sclerosis detected by single-cell and spatial transcriptomics. *bioRxiv*. 2023. <https://doi.org/10.1101/2023.12.20.572491>. (preprint)
- [19] Escobar A, Styrpejko DJ, Ali S, Cuajungco MP. Transmembrane 163 (TMEM163) protein interacts with specific mammalian SLC30 zinc efflux transporter family members. *Biochemistry and Biophysics Reports*. 2022; 32: 101362. <https://doi.org/10.1016/j.bbrep.2022.101362>.
- [20] Wang J, Huang Y, Bei C, Yang H, Lin Z, Xu L. Causal associations of antioxidants with Alzheimer's disease and cognitive function: a Mendelian randomisation study. *Journal of Epidemiology and Community Health*. 2024; 78: 424–430. <https://doi.org/10.1136/jech-2023-221184>.
- [21] Luo D, Liu Y, Li J, Liu X, Zhang R, Liu X, *et al.* Systematic Analysis of the Relationship Between Elevated Zinc and Epilepsy. *Journal of Molecular Neuroscience: MN*. 2024; 74: 39. <https://doi.org/10.1007/s12031-024-02213-7>.
- [22] Portbury SD, Adlard PA. Traumatic Brain Injury, Chronic Traumatic Encephalopathy, and Alzheimer's Disease: Common Pathologies Potentiated by Altered Zinc Homeostasis. *Journal of Alzheimer's Disease: JAD*. 2015; 46: 297–311. <https://doi.org/10.3233/JAD-143048>.
- [23] Tardy AL, Pouteau E, Marquez D, Yilmaz C, Scholey A. Vitamins and Minerals for Energy, Fatigue and Cognition: A Narrative Review of the Biochemical and Clinical Evidence. *Nutrients*. 2020; 12: 228. <https://doi.org/10.3390/nu12010228>.
- [24] Qi Z, Zhou X, Dong W, Timmins GS, Pan R, Shi W, *et al.* Neuronal Zinc Transporter ZnT3 Modulates Cerebral Ischemia-Induced Blood-Brain Barrier Disruption. *Aging and Disease*. 2023; 15: 2727–2741. <https://doi.org/10.14336/AD.2023.1011>.
- [25] Qi Z, Liang J, Pan R, Dong W, Shen J, Yang Y, *et al.* Zinc contributes to acute cerebral ischemia-induced blood-brain barrier disruption. *Neurobiology of Disease*. 2016; 95: 12–21. <https://doi.org/10.1016/j.nbd.2016.07.003>.
- [26] Cuajungco MP, Basilio LC, Silva J, Hart T, Tringali J, Chen CC, *et al.* Cellular zinc levels are modulated by TRPML1-TMEM163 interaction. *Traffic* (Copenhagen, Denmark). 2014; 15: 1247–1265. <https://doi.org/10.1111/tra.12205>.
- [27] Salm EJ, Dunn PJ, Shan L, Yamasaki M, Malewicz NM, Miyazaki T, *et al.* TMEM163 Regulates ATP-Gated P2X Receptor and Behavior. *Cell Reports*. 2020; 31: 107704. <https://doi.org/10.1016/j.celrep.2020.107704>.
- [28] Liu HY, Gale JR, Reynolds IJ, Weiss JH, Aizenman E. The Multifaceted Roles of Zinc in Neuronal Mitochondrial Dysfunction. *Biomedicines*. 2021; 9: 489. <https://doi.org/10.3390/biomedicines9050489>.
- [29] Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. *Nature Reviews. Neuroscience*. 2009; 10: 780–791. <https://doi.org/10.1038/nrn2734>.
- [30] Ji SG, Medvedeva YV, Wang HL, Yin HZ, Weiss JH. Mitochondrial Zn<sup>2+</sup> Accumulation: A Potential Trigger of Hippocampal Ischemic Injury. *The Neuroscientist: a Review Journal Bringing Neurobiology, Neurology and Psychiatry*. 2019; 25: 126–138. <https://doi.org/10.1177/1073858418772548>.
- [31] Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. *Nature Reviews. Molecular Cell Biology*. 2020; 21: 421–438. <https://doi.org/10.1038/s41580-020-0250-z>.
- [32] Guo X, Mutch M, Torres AY, Nano M, Rauth N, Harwood J, *et al.* The Zn<sup>2+</sup> transporter ZIP7 enhances endoplasmic-reticulum-associated protein degradation and prevents neurodegeneration in *Drosophila*. *Developmental Cell*. 2024; 59: 1655–1667.e6. <https://doi.org/10.1016/j.devcel.2024.04.003>.
- [33] Kiouri DP, Tsoupra E, Peana M, Perlepes SP, Stefanidou ME, Chasapis CT. Multifunctional role of zinc in human health: an update. *EXCLI Journal*. 2023; 22: 809–827. <https://doi.org/10.17179/excli2023-6335>.
- [34] Plum LM, Rink L, Haase H. The essential toxin: impact of zinc on human health. *International Journal of Environmental Research and Public Health*. 2010; 7: 1342–1365. <https://doi.org/10.3390/ijerph7041342>.
- [35] Zhang LH, Wang X, Stoltenberg M, Danscher G, Huang L, Wang ZY. Abundant expression of zinc transporters in the amyloid plaques of Alzheimer's disease brain. *Brain Research Bulletin*. 2008; 77: 55–60. <https://doi.org/10.1016/j.brainresbull.2008.03.014>.
- [36] Hancock SM, Portbury SD, Gunn AP, Roberts BR, Bush AI, Adlard PA. Zinc Transporter-3 Knockout Mice Demonstrate Age-Dependent Alterations in the Metalloproteome. *International Journal of Molecular Sciences*. 2020; 21: 839. <https://doi.org/10.3390/ijms21030839>.
- [37] Lovell MA, Smith JL, Xiong S, Markesbery WR. Alterations in zinc transporter protein-1 (ZnT-1) in the brain of subjects with mild cognitive impairment, early, and late-stage Alzheimer's disease. *Neurotoxicity Research*. 2005; 7: 265–271. <https://doi.org/10.1007/BF03033884>.
- [38] Lovell MA, Smith JL, Markesbery WR. Elevated zinc transporter-6 in mild cognitive impairment, Alzheimer disease, and pick disease. *Journal of Neuropathology and Experimental Neurology*. 2006; 65: 489–498. <https://doi.org/10.1097/01.jnen.0000229237.98124.91>.