Invited Review Article

# Neurotoxicity of Zinc : The Involvement of Calcium Homeostasis and Carnosine

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**Abstract**

Zinc is an essential trace element that is abundantly present in the brain. In spite of its importance for normal brain functions, it is widely recognized that excess zinc is neurotoxic. Numerous studies have indicated that zinc is crucial for neuronal injury after transient global ischemia and is linked with the pathogenesis of vascular type of dementia. We have investigated the molecular mechanisms of zinc-induced neurotoxicity *in vitro* and have explored substances that protect zinc-induced neurotoxicity. Pharmacological evidence based on results of our own and numerous other studies has indicated the significance of $Ca^{2+}$ dyshomeostasis in the mechanism of zinc-induced neuronal injury. The introduction of zinc into neurons is reportedly mediated through several types of $Ca^{2+}$-permeable channels. $Ca^{2+}$ channel blockers attenuate zinc-induced neurotoxicity. Furthermore, calcium overload attenuates zinc neurotoxicity, and *vice versa*. In this paper, we review the routes of zinc entry and mechanisms of zinc-induced neuronal death in relation with calcium homeostasis. The possible role of carnosine ($\beta$-alanyl histidine), a dipeptide that is present in the brain, as an endogenous protective substance for neuronal injury is also discussed.

Keywords : Calcium homeostasis, vascular type of dementia, ischemia, excite toxicity

## Introduction

Zinc is essential for most organisms. Zinc plays important roles in various physiological functions such as mitotic cell division, protein synthesis, and DNA and RNA synthesis as a co-factor of more than 300 enzymes or metalloproteins[1]. The human body contains approximately 2 g of zinc, mostly in testes, muscle, liver, and brain tissues. In the brain, zinc is accumulated in the hippocampus, amygdala, cerebral cortex, thalamus, and olfactory cortex. The total zinc content in the hippocampus is estimated as 70-90 ppm (dry weight)[2]. Although some zinc in the brain binds firmly to metalloproteins or enzymes, a substantial fraction of zinc (approximately 10% or more) forms free zinc ions ($Zn^{2+}$) or is loosely bound and is histochemically detectable by the staining using chelating reagents[3]. The chelatable zinc is stored in the presynaptic vesicles of particular excitatory neurons ; it is secreted from vesicles to synaptic clefts with excitatory neurotransmitter glutamate during the neuronal excitation. Its concentration is estimated as approximately 300 $\mu$M[4]. Although the physiological role of synaptically released zinc has not yet been defined precisely, there is increasing evidence suggesting that zinc is necessary for learning and memory[5].

Nonetheless, despite its importance, the disruption of zinc homeostasis has been implicated in several neurodegenerative diseases including Alzheimer's disease[6,7], prion disease[8], amyotrophic lateral sclerosis (ALS)[9], and Wilson's disease[10]. In particular, excess zinc can be neurotoxic, and is suspected to have a causative role in neuronal injury after transient global ischemia, ultimately leading to vascular-type of dementia[11,12]. We have inves-

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tigated the mechanism of zinc-induced neurotoxicity *in vitro* using GT1-7 cells (immortalized hypothalamic neurons[13-17]. Our pharmacological studies have implicated calcium homeostasis is implicated in the routes of zinc entry and its neurotoxicity.

A substance that protects against zinc-induced neuronal death can be a candidate for prevention or treatment of neurodegeneration after ischemia, and ultimately provide a clue to the drugs that can treat vascular-type of senile dementia[18]. After exploring such substances, we have found that carnosine (ß-alanyl histidine) protects GT1-7 cells against zinc-induced neurotoxicity[19]. Carnosine is localized in neurons of the olfactory bulb and in glial cells[20], where it is not vulnerable to ischemic neuronal injuries. In this paper, we review the possible mechanism of zinc neurotoxicity and its relation with $Ca^{2+}$ dyshomeostasis based on the results of our own and other studies. The possible role of carnosine as an endogenous protective substance for neuronal injuries is also discussed.

## Zinc and delayed neuronal death in ischemia

After transient global ischemia, the interruption of blood flow and the consequent oxygen-glucose deprivation causes the delayed neuronal death in the hippocampus or in the cerebral cortex. Neurons in CA1 or CA3 regions in the hippocampus, which exhibit the accumulation of zinc, are most vulnerable. Neuronal death and consequent cognitive dysfunction are believed to be based on pathogenesis of vascular-type of dementia in elderly people[21]. In response to ischemia, an excitatory neurotransmitter - glutamate - is released from nerve terminals and accumulates in synaptic clefts. Excess glutamate causes over-stimulation of its receptors. It then induces the entry of large quantities of $Ca^{2+}$ to responding neurons through N-methyl-D-aspartate (NMDA)-type glutamate receptors or voltage-gated $Ca^{2+}$ channels. It is widely believed that the increased intracellular $Ca^{2+}$ triggers various pathways of apoptotic neuronal death after ischemia[22].

Recent studies have suggested that zinc plays essential roles in the glutamate-induced neuronal death after ischemia[23]. As described above, a submillimolar level of zinc is co-released with glutamate to synaptic clefts by membrane depolarization in the ischemic condition. Choi and co-workers reported that zinc caused apoptotic death of primary cultured cortical neurons[24]. They also revealed that zinc is accumulated in the cell bodies of degenerating neurons after transient global ischemia[25]. The movement of chelatable zinc from presynaptic terminals into postsynaptic neuronal cell bodies, namely, 'zinc translocation' is suggested to contribute to the mechanism of zinc accumulation and neuronal injury. Zinc translocation occurred in vulnerable neurons in the hippocampus prior to the onset of the delayed neuronal death after transient global ischemia[26]. Administration of calcium EDTA (Ca EDTA), a membrane-impermeable chelator that chelates cations except for calcium, blocked translocation of zinc, protected the hippocampal neurons after transient global ischemia, and reduced the infarct volume[27]. These results firmly indicate zinc as a key factor in delayed neuronal death after transient global ischemia, which might be involved in the pathogenesis of vascular-type of dementia. The accumulation of zinc has also been observed after head trauma[28] and seizures[29] implying that zinc neurotoxicity might underlie the pathological mechanisms of various neuronal injuries.

## GT1-7 cells : a model system for investigating zinc neurotoxicity *in vitro*

To elucidate the role of zinc in neuronal injuries, many researchers have investigated the characteristics and the mechanism of zinc neurotoxicity *in vitro*, mainly using primary cultured neurons of the rat cerebral cortex[24] or PC-12 cells[30]. However, we found that GT1-7 cells (immortalized hypothalamic neurons) are much more sensitive to zinc than are other neuronal cells[15], and have investigated the mechanism of zinc-induced neurotoxicity using the GT1-7 cells. Figure 1 shows the viability of GT 1-7 cells, PC-12 cells, B-50 cells (neuroblastoma cell line), primary cultured neurons of the rat cerebral cortex, and primary cultured neurons of the rat hippocampus after the exposure to identical concentrations of zinc. Among these neuronal cells, GT1-7 cells exhibited the lowest viability after zinc exposure. Furthermore, zinc caused apoptotic death of GT1-7 cells in a dose-dependent and time-dependent manner[13,14]. The degenerated GT1-7 cells were terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) positive and exhibited the appearance of DNA fragmentation.

The GT1-7 cells were developed by Mellon *et al.* by genetically targeting tumorigenesis of mouse hypothalamic neurons[31]. The cells possess neuronal characteristics such as the extension of neuritis, the secretion of gonadotropin-releasing hormone (GnRH), and the expression of neuron-specific proteins or receptors including microtubule-associated protein 2 (MAP2), tau protein,

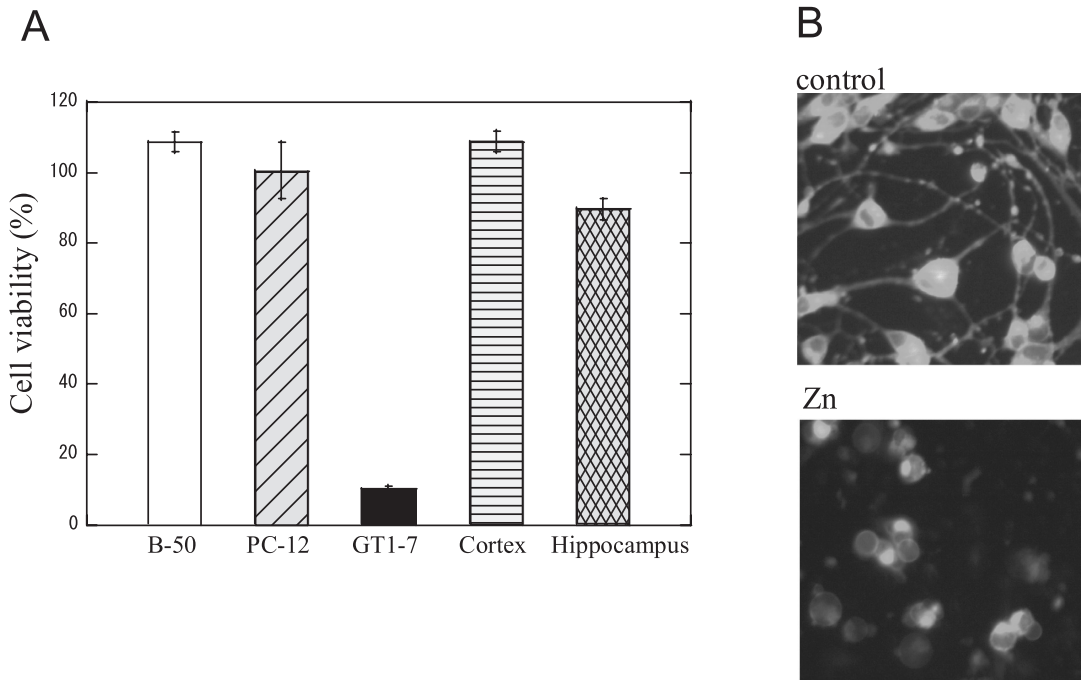


**Fig. 1** Zinc-induced death of GT1-7 cells

A : Zinc-induced neurotoxicity of GT1-7 cells and other neuronal cells

$ZnCl_2$ (50 $\mu$M) was administered to B-50 cells, PC-12 cells, GT1-7 cells, and primary cultured neurons of rat cerebral cortex (Cortex), or hippocampus (Hippocampus). After 24 h exposure, viability was measured using WST-1 method. Data are means ± S.E.M., $n$=6. Results are modified from Ref. No. 15.

B : Images of GT1-7 cells with or without zinc exposure

GT1-7 cells were observed with C18-rhodamine staining by fluorescent microscopy. GT1-7 cells could extend neuronal processes under a serum-free condition (control). However, after 24 h of exposure to $ZnCl_2$ (50 $\mu$M), the shrinkage of the processes and the cell bodies was observed (Zn).

neurofilament, synaptophysine, GABAA receptor, glutamate receptor, dopamine receptor, and L-type $Ca^{2+}$ channels. Meanwhile, the GT1-7 cells lack or possess low levels of ionotropic glutamate receptor and did not exhibit glutamate toxicity[32]. Glutamate and zinc are both neurotoxic. Therefore, it is difficult to distinguish the effects of zinc and glutamate using other neuronal cells. These properties imply that the GT1-7 cell line is as an excellent model system for investigation of zinc-induced neurotoxicity.

## Mechanism of zinc-induced neurotoxicity

Numerous studies have been undertaken to elucidate the mechanism of zinc-induced cell death. Considerable accumulated evidence indicates that zinc causes the failure in energy production in mitochondoria and produces reactive oxygen species (ROS)[33-35]. An imaging study using a zinc-sensitive fluorescent dye and a mitochondrial marker revealed that zinc is localized in mitochondria[36]. Zinc is reported to inhibit various mitochondrial enzymes such as mitochondrial complex I, aconitase, cytochrome c oxidase, $\alpha$-ketogulutarate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and monoamine oxidase. Zinc also inhibits the intracellular trafficking of mitochondria[37]. We have demonstrated that the administration of sodium pyruvate, an energy substrate, significantly inhibited zinc-induced death of GT1-7 cells[13]. Shelline and his collegues have reported that zinc exposure caused the decreased levels of $NAD^+$ and ATP of cultured cortical neurons, and that pyruvate restored the $NAD^+$ level[33,34]. Pyruvate was also reported to attenuate zinc-induced death of oligodendrocyte progenitor cells[38] or retinal cells[39]. Furthermore, the administration of pyruvate attenuated the neuronal death after ischemia *in vivo*[40]. Therefore, it is possible that energy failure and the inhibition of glycolysis in mitochondria are based on the mechanism of zinc neurotoxicity.

It is also reported that zinc produced ROS and caused the oxidative damages as a result of mitochondrial impairments[35]. However, considering that zinc is required

in Cu, Zn-super oxide dismutase (SOD) and that it protects neurons from oxidative damage, the role of zinc in oxidative stress remains controversial.

The most established effect of zinc in the central nervous system is the inhibition of NMDA-type glutamate receptor[41]. Meanwhile, zinc does not affect or increases the responses mediated by amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. Although it is widely believed that zinc regulates the excitability of glutamatergic neurons, involvement of glutamate receptors in zinc neurotoxicity in cultured cortical neurons has also been suggested. Agonists of glutamate receptors, such as NMDA or AMPA, enhance zinc-induced neurotoxicity in cultured cortical neurons[24),42]. However, our findings in GT1-7 cells, which lack such glutamate receptors, are inconsistent ; antagonists or agonists of excitatory neurotransmitters (D-APV, glutamate, CNQX), or those of inhibitory neurotransmitters (bicuculline, muscimol, baclofen, GABA) did not attenuate the viability of GT1-7 cells after zinc exposure[13-17]. Therefore, it is possible that NMDA or AMPA contributes to processes of zinc entry into neurons, but is not related the intracellular apoptotic pathways.

To evaluate the involvement of other metal ions in zinc neurotoxicity, we have observed the viability of GT 1-7 cells with or without various metal ions after exposure to zinc (Fig. 2), and found that equimolar addition of $Al^{3+}$ and $Gd^{3+}$ markedly inhibited zinc-induced neurotoxicity[16]. Moreover, overloading of $Ca^{2+}$ and $Mg^{2+}$ also blocked zinc-induced death of GT1-7 cells ; zinc prevented GT1-7 cells from neurotoxicity induced by calcium overload *vice versa*. These results suggest that dyshomeostasis of calcium might be involved in the zinc neurotoxicity mechanism.

## Routes of zinc entry through $Ca^{2+}$ permeable channels

It is widely believed that the entry of zinc into the target neurons and the increase of intracellular $Zn^{2+}$ ($[Zn^{2+}]_i$) is the primary event of the pathway of zinc-induced

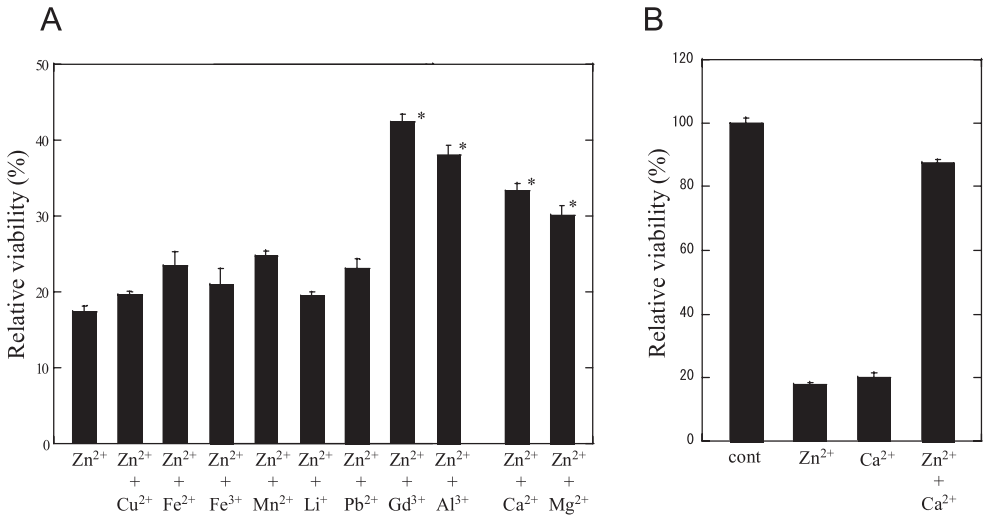


**Fig. 2** Metal-metal interactions in zinc-induced neurotoxicity

A : Effects of various metals on zinc neurotoxicity to GT1-7 cells

Various metal solutions including 50 $\mu$M of $CuCl_2$ ($Cu^{2+}$), $FeCl_2$ ($Fe^{2+}$), $FeCl_3$ ($Fe^{3+}$), $MnCl_2$ ($Mn^{2+}$), $LiCl_3$ ($Li^{+}$), $PbCl_2$ ($Pb^{2+}$), $GdCl_3$ ($Gd^{3+}$), $AlCl_3$ ($Al^{3+}$), or 2 mM of $CaCl_2$ ($Ca^{2+}$), $MgCl_2$ ($Mg^{2+}$) were preadministered to GT1-7 cells prior to the exposure to ZnCl2 (50 $\mu$M). After 24 h, the viability was measured using the WST-1 method. For the compensation of endogenous toxicity of the metal, the difference was calculated between the viability of the metal alone and viability of zinc and metal, and was described as the relative viability. Data are means ± S.E.M., $n$=6. * $p<0.01$. Results are modified from Ref. No. 16.

B : Effects of calcium overload

The viability of GT1-7 cells was compared among control (culture media contains 1.8 mM of $Ca^{2+}$), $Zn^{2+}$ (50 $\mu$M of $ZnCl_2$ was preadministered), $Ca^{2+}$ (5 mM of $CaCl_2$ was preadministered), and $Zn^{2+}$+ $Ca^{2+}$ (50$\mu$M of $ZnCl_2$ and 5 mM of $CaCl_2$ were co-administered). Both $Zn^{2+}$ and $Ca^{2+}$ both caused marked death of GT1-7 cells, but co-administration of $Zn^{2+}$ and $Ca^{2+}$ protected GT1-7 cells. Data are means ± S.E.M., $n$=6. * $p<0.01$.

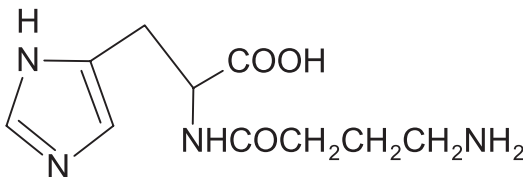


**Fig. 3** Structure of carnosine

apoptosis. Sensi *et al.* observed a temporal change of $[Zn^{2+}]_i$ in cultured cortical neurons using a zinc-sensitive fluorescent dye, Mag fura-5 ; those results revealed that $[Zn^{2+}]_i$ is increased after 15 s of exposure to zinc under a depolarization condition by high $K^+$ [43]. The $[Zn^{2+}]_i$ increase was attenuated by blockers of the voltage-gated $Ca^{2+}$ channel, including $Gd^{3+}$, verapamil, and nimodipine. Results also showed that the application of NMDA or kainite in the presence of extracellular zinc increased $[Zn^{2+}]_i$. Activation of these glutamate-related channels also causes the entry of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels. Therefore at least three major routes of $Zn^{2+}$ entry have been identified ; voltage-gated $Ca^{2+}$ channels, NMDA-type glutamate receptors, and AMPA/kainite-type glutamate receptors[23]. Although the NMDA-type glutamate receptors are present in most neurons and gate highly $Ca^{2+}$-permeable channels, the permeablity of $Zn^{2+}$ through AMPA/kainate channels is greater than NMDA-receptor channels. In a normal condition, most hippocampal neurons express AMPA receptors with subunit GluR2, which are poorly permeable to divalent cations including $Ca^{2+}$ and $Zn^{2+}$. However, after ischemia, the acute reduction in the expression of GluR2 subunit occurs, and neurons possess specific type of AMPA receptors which channels are directly $Ca^{2+}$ permeable (Ca-AMPA/kainate channels)[44]. The appearance of Ca-AMPA/kainate channels causes the increased permeability of $Ca^{2+}$ and enhances the toxicity. Furthermore, intracerebral administration of 1-naphtyl acetyl spermine, a blocker of Ca-AMPA/kainate channel, protected hippocampal neurons from ischemia-induced neurodegeneration and the accumulation of zinc in vulnerable neurons[46]. Therefore, the expression of $Zn^{2+}$-permeable Ca-AMPA/kainite channels and the entry of $Ca^{2+}$ and/or $Zn^{2+}$ through the channels are mediators of the delayed neuronal death after ischemia[46]. Considering that Ca EDTA, a zinc chelator, attenuates the ischemia-induced downregulation of GluR2 gene[27], zinc is also implicated in the transcriptional regulation in Ca-AMPA/kainite channels.

These data are consistent with our pharmacological results. We have demonstrated that $Gd^{3+}$, a blocker of voltage-gated $Ca^{2+}$channel, and $Al^{3+}$, reportedly inhibits various types of $Ca^{2+}$ channels, prevented zinc-induced apoptosis of GT1-7 cells. Furthermore, Kim *et al.* reported that zinc neurotoxicity in PC-12 cells was blocked by an L-type $Ca^{2+}$ channel blocker, nimodipine, and enhanced by the L-type $Ca^{2+}$ channel opener, S(-)-Bay K 8644[30].

Our results, which indicate that $Zn^{2+}$ inhibits $Ca^{2+}$ toxicity, and *vice versa*, might be explained by the competition with $Zn^{2+}$ and $Ca^{2+}$ through the above $Ca^{2+}$-permeable channels. However, the zinc's effects might be complex considering that low concentrations of $Zn^{2+}$ inhibit voltage-gated $Ca^{2+}$ channels.

## Zinc influx and efflux through zinc transporters

Zinc-specific membrane transporter proteins (zinc transporters) also play a role in the influx and efflux of zinc[47]. Zinc transporters mainly control zinc homeostasis ; they facilitate zinc influx in deficiency and efflux during zinc excess. Recently, several putative zinc transporters were identified and characterized. One of them, zinc transporter 1 (ZnT-1) is a membrane protein with six transmembrane domains. It is widely distributed in mammalian cells. In the brain, the distribution of ZnT-1 is parallel to chelatable zinc. ZnT-1 is activated by excess zinc and the expression of ZnT-1 is induced after transient global ischemia[48]. On the contrary, dietary zinc deficiency decreases expression of ZnT-1. Consequently, it is provable that ZnT-1 plays a pivotal role in efflux of zinc and in protection from zinc toxicity[49]. Another important zinc transporter in the brain is ZnT-3, which localizes in the membranes of presynaptic vesicles, transports zinc into synaptic vesicles, and maintains high zinc concentrations in the vesicles[50]. However, the physiological role of ZnT-3 and vesicular zinc remain elusive considering recent results obtained from ZnT-3 knock out mice[51,52].

## Carnosine as an endogenous protective substance for zinc neurotoxicity

The implication of zinc in transient global ischemia suggests that substances that inhibit zinc neurotoxicity might be candidates for drugs for prevention or treatment of brain ischemia, and finally for vascular-type of dementia. We have developed a convenient and sensitive assay system for screening such substances using GT1-7 cells, which are highly vulnerable to zinc, and have examined inhibitory effects of various agricultural products[18]. Among those tested, we found that carnosine ($\beta$-alanyl histidine) significantly inhibited zinc-induced neu-

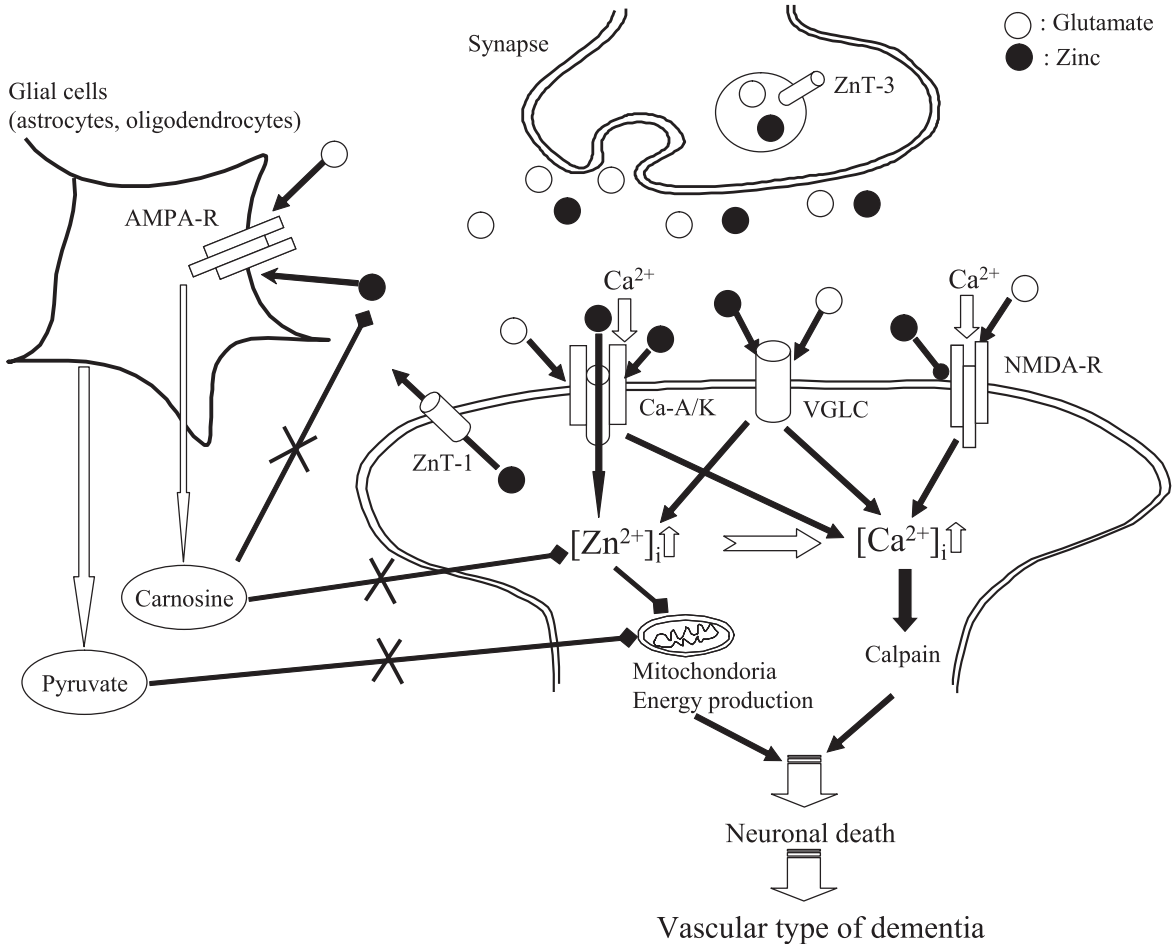


**Fig. 4** Hypothetical scheme of zinc neurotoxicity

Zinc (Zn : closed circle) coexists with glutamate (Glu : open circle) in presynaptic vesicles and is secreted with neuronal excitation. In normal conditions, secreted zinc binds to NMDA type glutamate receptor (NMDA-R) and modulates postsynaptic excitability. Carnosine is synthesized in the glial cells and is secreted with the stimulus by glutamate and zinc ; it protects neurons from glutamate-zinc neurotoxicity. The feedback pathway contributes to zinc homeostasis. Concentrations of intracellular zinc ($[Zn^{2+}]_i$) are also maintained by ZnT-1. Pyruvate is also released from glial cells and protects neurons from mitochondrial energy deficit caused by zinc.

However, in pathological conditions such as transient global ischemia, great amounts of both glutamate and zinc are released into synaptic clefts. Zinc potentiates the expression of $Ca^{2+}$-permeable AMPA/kainate-type glutamate receptor (Ca-A/K) channels. Zinc is translocated into postsynaptic target neurons through Ca-A/K channels or other pathways such as voltage-gated L-type $Ca^{2+}$ channel (VGLC) or NMDA receptors (NMDA-R). Increased $[Zn^{2+}]_i$ inhibits numerous enzymes including mitochondria respiratory enzymes, and causes energy depletion. Meanwhile, excess glutamate also increases $[Ca^{2+}]_i$. The increase of Ca-A/K channels and the increased $[Zn^{2+}]_i$ contribute to the increase of $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ consequently triggers various apoptotic pathways including the activation of calpain. Carnosine is decreased in aged bodies. Therefore, the protective effects of carnosine in the hippocampus or cerebral cortex might be insufficient in cases of pathological conditions in the aged brain.

Dyshomeostasis of zinc and calcium eventually causes delayed neuronal death after transient global ischemia ; it ultimately leads to the pathogenesis of vascular-type of dementia.

rotoxicity[19]. Carnosine is a small dipeptide that is abundant in muscles of fishes, chickens, and mammals[53]. Although its physiological roles remain elusive, it is suggested that carnosine plays important functions in pH balance in muscles after exercise. It is also suggested that carnosine has antioxidant activity[54] and chelating ability of metals including $Zn^{2+}$ and $Cu^{2+}$ [55]. In the brain, carnosine exists in neurons of the olfactory bulb ; it is se-

creted to synaptic clefts with excitatory neurotransmitter glutamate during neuronal excitation[20]. It is interesting that olfactory bulb neurons are less sensitive to damages after ischemia than are hippocampal neurons despite the accumulation of zinc. Moreover, carnosine is synthesized and stored in glial cells, such as astrocytes and oligodendrocytes. Bakardjiev reported that glutamate caused the release of carnosine from oligodendrocytes[56]. The response was mediated through AMPA-type glutamate receptors and was enhanced by zinc. Considering this evidence correctively, carnosine might serve as an endogenous protector from neuronal injury. Furthermore, carnosine content is varied during development and it decreases in muscles of aged animals[57]. Therefore, dietary supplementation of carnosine might be effective for prevention or treatment of neurodegeneration after ischemia.

## Conclusion

Considering the evidence presented in this paper collectively, we have inferred a scheme for zinc neurotoxicity and the role of carnosine (Fig. 4). In the normal condition, neuronal excitation causes the release of glutamate and zinc. However, zinc regulates the postsynaptic excitability by the binding to NMDA-type glutamate receptor. Zinc in the synaptic clefts is re-uptaken or binds to carnosine released from glial cells by the stimuli of glutamate and zinc. This feedback pathway of carnosine-zinc protects neurons from glutamate toxicity and zinc toxicity. However, in the pathological conditions such as ischemia, oxygen-glucose deprivation induces the release of excess glutamate as well as zinc in the synaptic clefts. Excess zinc enhances the expression of Ca-AMPA/kainite channels, and is translocated through the Ca-AMPA /kainite channels or through other pathways into the target neuron, where zinc inhibits various enzymes, inhibits mitochondria respiration, causes energy depletion, and produces ROS. Excess glutamate induces elevation of intracellular $Ca^{2+}$ level of the target neuron. Elevated levels of intracellular $Ca^{2+}$ trigger various apoptotic pathways such as activation of calpain, the activation of caspases or other enzymatic pathways related to apoptosis ; ultimately it leads to neuronal death. Zinc also influences intracellular $Ca^{2+}$ levels and enhances effects of glutamate.

Zinc might play a role like that of Janus, an ancient Roman god of doorways with two different faces, in the brain : both zinc depletion and excess zinc cause severe damage to neurons[12]. Further research about the role of zinc in neuronal injury and the significance of zinc homeostasis might give rise to the development of new treatments for neurodegenerative diseases.

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