Serial Review

Copper Biology in Health and Disease
Guest Editor: Hirokazu Hara

Crosstalk of copper and zinc in the pathogenesis of vascular dementia

Masahiro Kawahara,* Ken-ichiro Tanaka, and Midori Kato-Negishi

Department of Bio-Analytical Chemistry, Faculty of Pharmacy, Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan

(Received 1 April, 2022; Accepted 12 May, 2022; Released online in J-STAGE 1 July, 2022)

Copper and zinc are essential for normal brain functions. Both are localized in presynaptic vesicles and are secreted into synaptic clefts during neuronal excitation. Despite their significance, excesses of copper and zinc are neurotoxic. In particular, excess zinc after transient global ischemia plays a central role in the ischemia-induced neurodegeneration and pathogenesis of vascular type senile dementia. We previously found that sub-lethal concentrations of copper remarkably exacerbated zinc-induced neurotoxicity, and we investigated the molecular pathways of copper-enhanced zinc-induced neurotoxicity. The endoplasmic reticulum stress pathway, the stress-activated protein kinases/c-Jun amino-terminal kinases pathway, and mitochondrial energy production failure were revealed to be involved in the neurodegenerative processes. Regarding the upstream factors of these pathways, we focused on copper-derived reactive oxygen species and the disruption of calcium homeostasis. Because excess copper and zinc may be present in the synaptic clefts during ischemia, it is possible that secreted copper and copper-induced reactive oxygen species may enhance zinc neurotoxicity and eventually contribute to the pathogenesis of vascular type senile dementia.

***Key Words*:** **reactive oxygen species (ROS), neurotoxicity, senile dementia, calcium homeostasis, Alzheimer's disease, prion diseases**

Copper (Cu) and zinc (Zn) are both essential elements and play critical roles in normal brain functions.[1,2] They participate in neurotransmitter synthesis, myelination, and prevention of reactive oxygen species (ROS) formation as cofactors of various enzymes or functional proteins. Furthermore, both Cu and Zn are localized in presynaptic vesicles, are secreted into synaptic clefts during neuronal excitation, and modulate neuronal information processing.[3,4] Thus, deficiencies of Cu and Zn lead to neurological disorders or memory disorders in infants.

Despite their significance, excesses of Cu and Zn are neurotoxic. Moreover, increasing evidence suggests that disruptions of Cu and/or Zn homeostasis are involved in the pathogenesis of various neurodegenerative diseases including Alzheimer's disease (AD), prion diseases, Parkinson's disease (PD), dementia with Lewy bodies (DLB), and amyotrophic lateral sclerosis (ALS).[5–8] We focus here on the involvement of Cu and Zn in the pathogenesis of vascular dementia associated with senile dementia (VD).[9,10] It is widely believed that excess Zn, which is secreted into the synaptic clefts after transient global ischemia, plays crucial roles in ischemia-induced neurodegeneration and eventually the pathogenesis of VD. We previously investigated the molecular pathways of Zn-induced neurotoxicity and found that sub-lethal concentrations of Cu remarkably exacerbated Zn-induced neurotoxicity.[11,12] On the basis of our results and those of numerous other studies, we review the interactions of Cu and Zn in the synapse in pathophysiological conditions and the synergistic role of Cu and Zn in the pathogenesis of VD.

Roles of Cu and Zn in the Synapse

Several trace elements, such as iron (Fe), Zn, Cu, and manganese (Mn), exist in the brain at different levels and with various distributions.[13] These trace elements, termed "neurometals", play essential roles in the maintenance of brain structures and normal functions.[14]

Among these neurometals, Zn is the second most abundant trace element after Fe in the brain. Zn accumulates in the hippocampus, amygdala, cerebral cortex, thalamus, and olfactory cortex and is involved in more than 300 important biological functions as a cofactor of various enzymes or metalloproteins.[15] Thus, Zn deficiency in children results in dwarfism, delayed mental and physical development, immune dysfunction, and learning disabilities.[16] Zn deficiency also causes smell and taste disorders in adults. Although a portion of Zn binds firmly to metalloproteins or enzymes, a substantial fraction of Zn either forms free Zn ions (Zn^{2+}) or is loosely bound and is histochemically detectable by chelating agent staining.[4] This chelatable Zn^{2+} is mainly stored in the presynaptic vesicles of specific excitatory glutamatergic neurons and is secreted into synaptic clefts along with glutamate during neuronal excitation. Secreted Zn^{2+} can bind to various receptors such as *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, γ-amino butyric acid (GABA) receptors, glycine receptors, and P2-type purinergic receptors.[17–19] Morebito *et al.*[20] demonstrated that secreted Zn^{2+} modulates dendritic functions via NMDA-type glutamate receptors in an activity-dependent manner. Zn^{2+} also inhibits AMPA-type glutamate receptors.[21] Ueno *et al.*[22] revealed that secreted Zn^{2+} diffuses into heterogenic synapses, modulates spatio-temporal neural information, and mediates synaptic plasticity. Zn in the hippocampus is reported to be

*To whom correspondence should be addressed.
E-mail: makawa@musashino-u.ac.jp

doi: 10.3164/jcbn.22-40

essential for the induction of long-term potentiation, a form of synaptic information storage that is a well-known paradigm for the mechanisms underlying memory formation.[23]

Cu is the third most abundant trace element in the brain and is localized in the thalamus, substantia nigra, striatum, and hippocampus. Cu acts as a cofactor of various enzymes, including cytochrome C, lysyl oxidase, uricase, dopamine hydroxylase, tyrosinase, and Cu/Zn superoxide dismutase, and plays central roles in the synthesis of neurotransmitters, myelination, and neuroprotection against ROS.[1] Cu is also involved in Fe homeostasis because ceruloplasmin, a Cu-binding protein, acts as a ferroxidase that converts Fe^{2+} to Fe^{3+}. Cu is a redox-active metal and exists both as oxidized Cu^{2+} and reduced Cu^{+}. Similar to Zn, Cu is also localized in the synapse and secreted into synaptic clefts during neuronal firing.[3] Secreted Cu regulates neuronal excitability by binding to NMDA-type and AMPA-type glutamate receptors and GABA receptors.[24,25] It is possible that synaptic Cu loosely binds to small molecular compounds such as organic acids and ATP, since an excess of free Cu ions is toxic.

Because Cu and Zn are important in neuronal functions, their levels are strictly regulated under normal conditions. The cerebrospinal fluid (CSF) levels of Cu and Zn in healthy individuals are less than 1 μM.[26] However, the synaptic cleft is a small compartment that is conceptualized as a cylinder with a 120-nm radius and height of 20 nm, and the total volume of synaptic clefts is estimated to account for approximately 1% of the extracellular space of the brain, which is similar to the CSF volume.[27] Therefore, it is possible that the Cu and Zn concentrations in synapses are much higher than those in the CSF. Indeed, the concentration of glutamate in the synaptic cleft is estimated to reach the mM range after neuronal depolarization. Although the level of Zn increases to approximately 10 nM in the CSF under ischemic conditions, the Zn concentration in synapses is estimated to be 1–100 μM.[28] Zhang *et al.*[29] observed a spontaneous and synchronous "Zn spike" in cultured hippocampal neurons along with calcium (Ca) spikes. In such situations, the Zn concentration in synapses may become much higher. The Cu level in the synapse is also controversial. Using atomic absorption spectroscopy, Kardos *et al.*[30] found that the concentration of Cu released into the synaptic cleft is approximately 100 μM. However, a study using a Cu-sensitive fluorescent probe demonstrated that the concentration of Cu released into the synaptic cleft is approximately 3 μM.[31]

There are several factors that regulate Cu and Zn homeostasis in the brain. Zn homeostasis is mainly regulated by Zn transporters and metallothioneins.[32,33] Two types of Zn transporters exist in mammals: ZnT transporters and Zrt-, Irt-like protein (ZIP) transporters. ZnT transporters facilitate Zn^{2+} influx when Zn is deficient, and ZIP transporters facilitate Zn^{2+} efflux when it is in excess. Among the 14 types of ZnT transporters in mammals, ZnT-1, a membrane protein with six transmembrane domains, plays a pivotal role in Zn^{2+} efflux and protection from excess Zn^{2+} in the brain. Moreover, ZnT-1 is localized in postsynaptic membranes, binds to NMDA-type glutamate receptors and voltage dependent Ca^{2+} channels, and controls their functions.[34,35] ZnT-3 is localized in presynaptic vesicle membranes and maintains high Zn^{2+} concentrations in synaptic vesicles.[36] ZIP transporters are localized in cellular membranes or in Golgi apparatus or endoplasmic reticulum (ER) membranes and control Zn^{2+} influx into subcellular organelles. Among the 13 ZIP transporters, ZIP4 is mainly present in cellular membranes and is also localized at synapses.[37]

Metallothioneins, which are ubiquitous metal binding proteins with 68 amino acids that bind seven metal atoms (including Zn, Cu, and cadmium) via 20 cysteine residues, also regulate Cu and/or Zn homeostasis. There are three types of metallothioneins: MT-1, MT-2, and MT-3. MT-1 and MT-2 are ubiquitously expressed throughout the body, whereas MT-3 is primarily localized in the central nervous system. MT-3 is present in the synapse and is secreted from neurons or glial cells.[38] Uchida *et al.*[39] found that neuronal growth inhibitory factor (GIF), which inhibits neurite extension and prevents neuronal death, was decreased in the brains of AD patients and determined that GIF is equivalent to MT-3. Therefore, MT-3 (GIF) has been implicated in AD-associated neuronal death. It is possible that carnosine (β-alanyl histidine) is involved in the regulation of metal homeostasis because carnosine can bind to Cu and Zn. Carnosine is synthesized in glial cells and secreted into the synaptic cleft.[40]

Cu homeostasis is regulated by copper-transporting ATPase (ATP7A and ATP7B). A deficiency or excess of Cu caused by genetic disorders associated with these transporters can cause neurodegenerative diseases such as Menkes disease and Wilson's disease.[41] ATP7A is mainly localized in the Golgi apparatus and plays an essential role in Cu maintenance in the synapse.[42] ATP7A is also involved in axonal targeting and synaptogenesis.[43] Divalent metal transporter 1 (DMT1) and Cu transporter 1 (Ctr1) also contribute to Cu homeostasis. Han *et al.*[44] reported that loss of DMT1 caused the accumulation of Cu in the brain and promotes impulsivity-like behavior. Ctr1 transports Cu^{+}, and is reportedly enriched in the synaptic area.[45]

Additionally, several metal-binding proteins are present in the synapse, such as amyloid precursor protein (APP), prion protein (PrP), and α-synuclein.[46] Interestingly, these metal-binding proteins are involved in the pathogenesis of neurodegenerative diseases including AD, prion diseases, and Lewy body diseases (PD and DLB). These proteins or their fragment peptides exhibit similarities in the formation of fibril-like oligomers with β-pleated sheet structures (amyloid fibrils), accumulation in the brains of patients with neurodegenerative diseases, and induction of neurotoxicity.

APP is a precursor protein of β-amyloid protein (AβP), which accumulates in the brains of AD patients. AβP accumulation and its neurotoxicity are believed to play critical roles in the pathogenesis of AD. APP possesses two Zn^{2+} and/or Cu^{2+} binding domains at its N-terminal. Both Zn^{2+} and Cu^{2+} are involved in the dimerization, trafficking, and expression of APP and the production of AβP. APP converts oxidized Cu^{2+} into the reduced Cu^{+} form and is primarily localized in the presynaptic membrane.[47,48] Furthermore, APP regulates Fe^{2+} efflux from cells by binding to ferroportin, an iron transporter.[49]

PrP, a 30–35-kDa cell surface glycoprotein, is widely distributed in the brain.[50] A conformational change of normal prion protein (PrP^{C}) to the abnormal scrapie-type isoform of PrP (PrP^{Sc}) is involved in the pathogenesis of prion diseases including scrapie in sheep, bovine spongiform encephalopathy in cows, and Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and Kuru in humans. PrP^{C} binds four Cu atoms in the octarepeat domain (-PHGGGWGQ-) at its N-terminal, and an additional two Cu atoms bind to His^{96} and His^{111}. PrP^{C} is localized in postsynaptic membranes, binds to AMPA-type and/or NMDA-type glutamate receptors, and affects their function in a Cu-dependent manner.[51] Because the sequence of PrP^{C} is evolutionarily related to that of ZIP-type Zn transporters, PrP^{C} enhances cellular uptake of Zn^{2+}, acting as a Zn^{2+} sensor in the synapse by binding to AMPA-type glutamate receptors.[52] Meanwhile, Cu and Zn participate in the conformational change of PrP^{C} into PrP^{Sc}. PrP^{C} is also involved in Fe homeostasis because it functions as a ferrireductase that converts Fe^{3+} to Fe^{2+}.[53]

α-Synuclein is a 140-amino acid protein that is abundantly localized at presynaptic terminals and plays critical roles in synaptic functions and the maintenance of synaptic plasticity.[54] The fragment peptide of α-synuclein, termed non-amyloid component (NAC), was shown to co-accumulate with AβP in

doi: 10.3164/jcbn.22-40

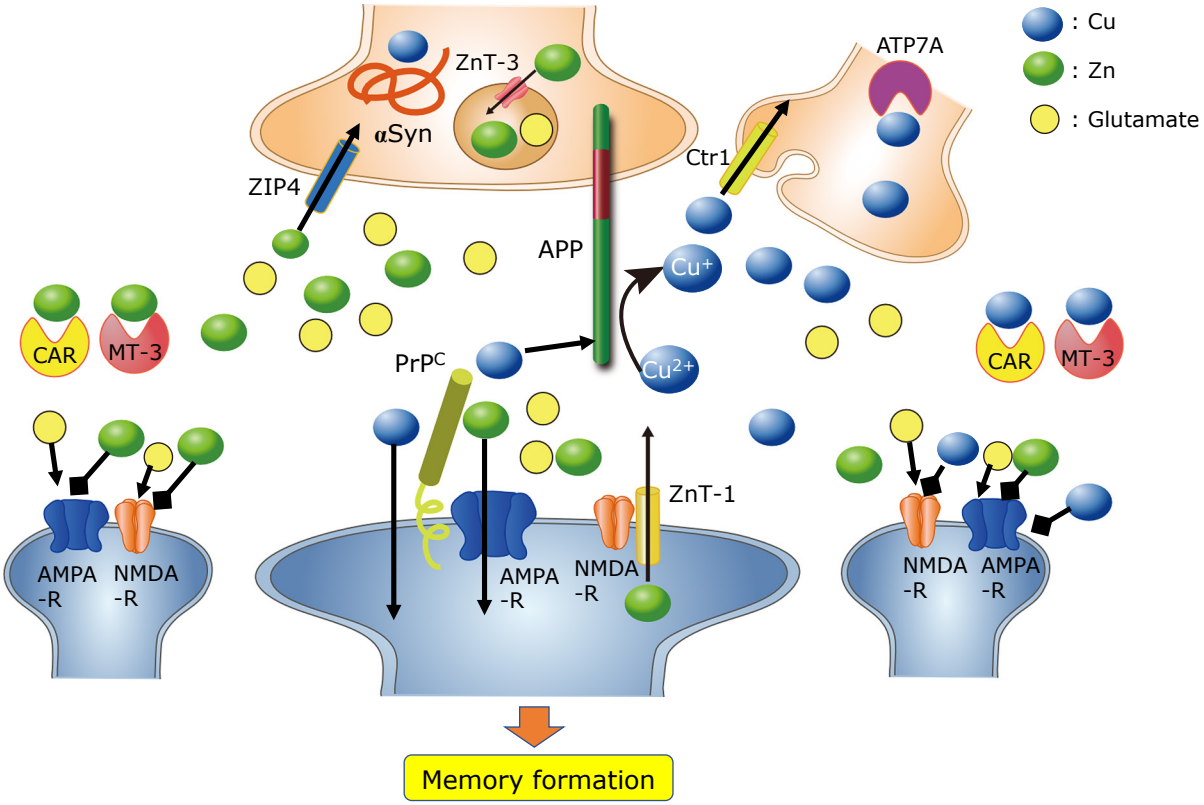

Fig. 1. Roles of copper (Cu) and zinc (Zn) in synapses under normal conditions. Cu and Zn are stored in presynaptic vesicles, released with neurotransmitters such as glutamate, inhibit *N*-methyl-D-aspartate-type (NMDA-R) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPA-R), and regulate excitability. ZnT-3, a Zn transporter, and copper-transporting ATPase (ATP7A) are involved in the accumulation of Zn and Cu in the synapse, respectively. Cu and Zn may spill over into neighboring synapses and modulate excitability and are involved in the maintenance of synaptic plasticity and memory formation. Zinc transporters (ZnT-1, ZIP4) are present in the synapse and regulate the Zn levels. Several amyloidogenic proteins including amyloid precursor protein (APP), normal prion protein (PrP^C), and α-synuclein (α-Syn) participate in the maintenance of Cu, Zn, and other metal ions in the synapse. Metallothionein-3 (MT-3) and carnosine (Car) also control the levels of these ions.

senile plaques in AD patients. The oligomerization of α-synuclein contributes to the pathogenesis of Lewy body diseases including PD, DLB, and multiple system atrophy. α-Synuclein binds Cu^{2+}, Zn^{2+} and other metal ions such as Mn^{2+} at its N and C-terminal domains. Moreover, α-synuclein is a ferrireductase that converts Fe^{3+} to Fe^{2+}, similar to PrP^C.[55]

On the basis of these findings, we constructed a figure depicting the possible roles of Cu and Zn in the synapse under normal conditions (Fig. 1). Both Cu and Zn are secreted into the synaptic cleft in an activity-dependent manner, inhibit glutamate receptors, and regulate neuronal excitability. It is possible that the secreted Cu and Zn can diffuse across the synaptic cleft, spill over into neighboring synapses, and modulate the activity of those neighboring synapses. The differing concentrations of excitatory signals (glutamate) and inhibitory signals (Cu and/or Zn) in adjacent synapses transmit spatio-temporal information about neuronal firing and facilitate the precise modulation of neuronal activity. This modulation of neuronal activity at adjacent synapses generates contrasting signals that may enable lateral inhibition, which is the capacity of an excited neuron to reduce the activity of neighboring neurons, creating a contrast in sensory stimulation that allows increased sensory perception.[56] Lateral inhibition serves as the basis for synaptic plasticity. Thus, it is possible that Cu signaling and Zn signaling coordinate at the synapse and precisely modulate neuronal information.

To maintain Cu and Zn levels at synaptic clefts, Cu and Zn undergo rapid reuptake into presynaptic neurons via ZIP4 and Ctr1. Zn accumulation in synaptic vesicles is facilitated by ZnT-3. α-Synuclein binds to Cu and Zn, and regulates its levels in presynaptic terminals. Secreted Cu and Zn can be transported into postsynaptic neurons through several pathways including voltage-gated Ca^{2+} channels (VGCC) and NMDA-type glutamate channels, causing an increase in intracellular Cu and Zn levels. PrP^C acts as a Zn sensor at synapses and controls Zn influx into postsynaptic neurons. Considering the narrow width (about 20 nm) between synaptic clefts, it is possible that APP and PrP^C interact each other, and PrP^C may transfer Cu to APP, which then reduces Cu^{2+} to Cu^+ and transports it to Ctr1. Excess intracellular Zn can be extruded by ZnT-1, which inhibits Ca^{2+} channels that pass Zn^{2+}. MT-3 and carnosine also bind to Cu and Zn and regulate their levels in synapses. Because of these regulatory factors, the Cu and Zn levels are maintained, resulting in normal brain functions.

Contributions of Zn to Ischemia-induced Neuronal Death and the Pathogenesis of VD

As noted previously, the Cu and Zn levels in the synapse are strictly controlled by regulatory factors. Thus, disruption of their homeostasis by regulatory factor disorders may trigger the pathogenesis of neurodegenerative diseases including AD, prion diseases, and Lewy body diseases. Here, we focus on the involvement of Cu and Zn in the pathogenesis of VD. Senile dementia is characterized by profound memory loss and the inability to form new memories in older adults, and its prevalence increases with age. The number of patients in Japan was

estimated to be more than 700 million in 2020. VD accounts for approximately one-third of senile dementia cases. The risk factors of VD include age, male sex, diabetes, and high blood pressure.[57] The most common type of VD is caused by a series of small strokes or ischemia.[58] Following transient global ischemia or stroke, the interruption of blood flow and the resulting oxygen-glucose deprivation induce long-lasting membrane depolarization and excessive release of glutamate into synaptic clefts.[59] Thereafter, the excess glutamate causes over-stimulation of its receptors, and the entry of large quantities of Ca^{2+} into glutamate-responsive neurons triggers delayed death of neurons in the hippocampus or cerebral cortex. Thereafter, the development of an infarct and subsequent cognitive dysfunction mark the pathogenesis of VD in elderly people. Approximately 30% of stroke patients show symptoms of dementia within 3 months of the initial stroke.

Under ischemic conditions, excess Zn is secreted into synaptic clefts along with glutamate. The increase in intracellular Zn^{2+} levels ($[Zn^{2+}]_i$) caused by movement of chelatable Zn from presynaptic terminals to postsynaptic cell bodies, namely Zn translocation, is observed in vulnerable neurons in the CA1 or CA3 regions of the hippocampus prior to the onset of delayed neuronal death.[60] This Zn translocation has been reported to enhance the appearance of infarcts. 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.[61] Under normal physiological conditions, hippocampal neurons typically express AMPA receptors with GluR2 subunits, which are poorly permeable to divalent cations including Ca^{2+} and Zn^{2+}. However, following ischemia, an acute reduction in GluR2 subunit expression occurs, and neurons possess specific types of AMPA receptors with channels that are directly Ca^{2+}- and Zn^{2+}-permeable (Ca-AMPA/kainate channels).[62] The appearance of Ca-AMPA/kainate channels results in increased Ca^{2+} permeability, thereby enhancing toxicity. 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 that follows ischemia. Koh *et al.*[63] demonstrated that Zn accumulates in degenerated hippocampal neurons after ischemia. The administration of Ca ethylenediaminetetraacetic acid (Ca-EDTA), a membrane-impermeable chelator of Zn^{2+}, has been shown to block the translocation of Zn, protect hippocampal neurons after transient global ischemia, and reduce infarct volume.[64] Ca-EDTA also attenuates ischemia-induced downregulation of the GluR2 gene. These results strongly implicate Zn as a key player in delayed neuronal death after transient global ischemia and in the pathogenesis of VD.

Thus, we have investigated the molecular mechanism of Zn-induced neurotoxicity. For this task, we employed an *in vitro* model system using GT1-7 cells (immortalized hypothalamic neurons). GT1-7 cells were developed from murine hypothalamic neurons by site-directed mutagenesis and have been used as a model for neuroendocrine system studies because they maintain neuron-like characteristics such as neurite extension, expression of neuron-specific proteins, and the ability to secrete gonadotropin-releasing hormone.[65] We found that Zn causes the apoptotic death of GT1-7 cells in a dose-dependent and time-dependent manner.[66] Furthermore, GT1-7 cells were more vulnerable to Zn neurotoxicity than other neuronal cells such as primary cultured rat hippocampal neurons and PC12 cells.[67] In the ischemic condition, hypothalamus can be damaged as well as other brain areas including hippocampus or cerebral cortex. It is widely believed that hypothalamic-pituitary-adrenal (HPA) axis plays a key role in neurohormonal disorders following brain ischemia which exacerbate brain damages.[68] GT1-7 cells lack or express low levels of glutamate receptors,[69] and therefore, glutamate does not cause death of GT1-7 cells,[9] even though glutamate is neurotoxic in other neuronal cells. These characteristics make the GT1-7 cell line an excellent model system for the investigation of Zn-induced neurotoxicity.

First, we examined the effects of various pharmacological agents prior to Zn treatment of GT1-7 cells. Neither antagonists nor agonists of excitatory neurotransmitters (D-APV, glutamate, and CNQX) nor those of inhibitory neurotransmitters (bicuculline, muscimol, baclofen, and GABA) influenced Zn-induced neurotoxicity in GT1-7 cells. We demonstrated that the administration of sodium pyruvate, an energy substrate, significantly inhibited Zn-induced death in GT1-7 cells.[66] Sheline *et al.*[70] reported that Zn exposure decreased the levels of NAD^+ and ATP in cultured cortical neurons and that treatment with pyruvate restored the NAD^+ level. Administration of pyruvate attenuated neuronal death after ischemia also *in vivo*.[71] Moreover, Zn has been reported to inhibit various mitochondrial enzymes, such as mitochondrial complex I, aconitase, cytochrome *c* oxidase, α-ketoglutarate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and monoamine oxidase.[72] Thus, the energy failure pathway in mitochondria is suggested to be involved in the process of Zn-induced neurotoxicity.

To identify substances that protect against Zn-induced neurotoxicity, we examined the extracts of various fruits, vegetables, and fish. Among the tested substances, we found that extracts from eel and round herring possessed marked protective activity.[73,74] After isolation and determination of the active compounds, we found that carnosine and histidine protect against Zn-induced neurotoxicity. Anserine (1-methyl carnosine), an analog of carnosine, also attenuated Zn-induced neurotoxicity.[75] We also demonstrated that D-histidine and L-histidine protected against Zn-induced neuronal death.[73] Because carnosine possesses various beneficial characteristics such as anti-oxidation, anti-glycation, anti-crosslinking, and metal chelation, we propose carnosine may become a candidate for prevention/treatment of VD.[9] Our DNA microarray analysis revealed that Zn exposure causes upregulation of several genes, including metal-related and ER stress-related genes. According to reverse transcription polymerase chain reaction (RT-PCR) analysis, carnosine significantly inhibited Zn-induced upregulation of ER stress-related genes.[74,75] Anserine and histidine exhibited similar results. Thus, we further determined that the ER stress pathway is involved in the molecular mechanism of Zn-induced neurotoxicity.

Effects of Cu on Zn-induced Neurotoxicity

During the investigation of molecular pathways involved in Zn-induced neurodegeneration, we evaluated the involvement of other metal ions. Among the investigated ions, equimolar addition of aluminum ions (Al^{3+}) and gadolinium ions (Gd^{3+}) significantly inhibited Zn-induced neurotoxicity.[67,76] Al^{3+} is widely known to inhibit various types of Ca^{2+} channels,[77] and Gd^{3+} is a blocker of voltage-gated Ca^{2+}channels.[78] Exposure to Zn caused an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in GT1-7 cells, and pretreatment with Al^{3+} significantly inhibited the increase in $[Ca^{2+}]_i$.[67] Furthermore, Kim *et al.*[79] reported that Zn neurotoxicity was attenuated in PC-12 cells by nimodipine, an L-type Ca^{2+} channel blocker, and enhanced by S(−)-Bay K 8644, an L-type Ca^{2+} channel activator. These results suggest that Ca^{2+} dyshomeostasis is involved in the mechanism of Zn-induced neurotoxicity.

Furthermore, we found that sub-lethal concentrations of Cu^{2+} and nickel ions (Ni^{2+}) significantly exacerbated Zn-induced neurotoxicity in GT1-7 cells.[12] Figure 2A shows the cell viability after Zn exposure with or without addition of 5–20 μM Cu. Zn alone caused death in GT1-7 cells in a dose-dependent manner. The viability of GT1-7 cells exposed to 30 μM Zn was 67.4 ± 4.5%. Meanwhile, administration of Cu alone (less than

doi: 10.3164/jcbn.22-40

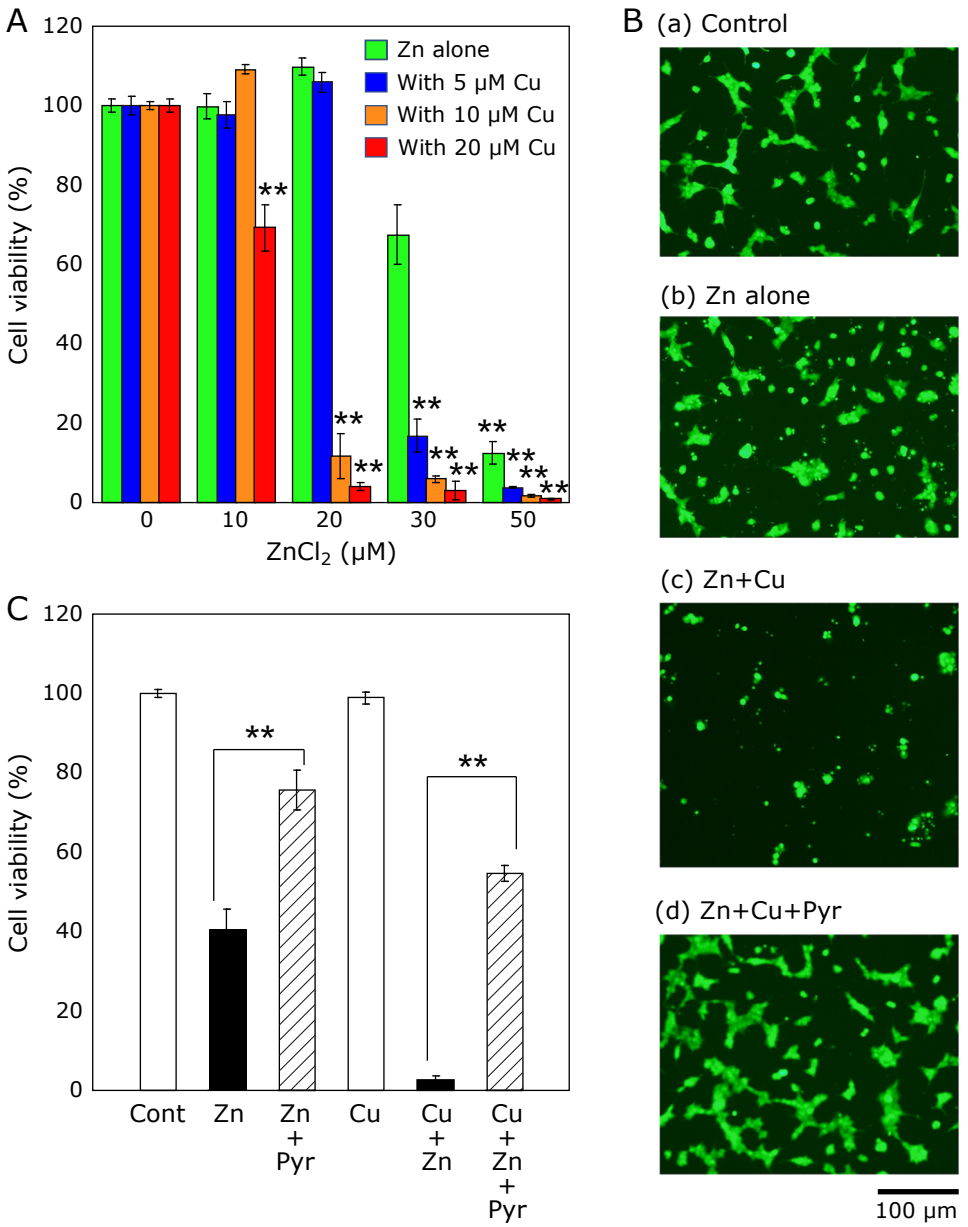

Fig. 2. Copper (Cu) enhanced zinc (Zn)-induced neurotoxicity. (A) The cell viability of GT1-7 cells after exposure to Zn with or without Cu. GT1-7 cells were exposed to various concentrations of $ZnCl_2$ with or without 5–20 μM $CuCl_2$. After 24 h, the cell viability was measured using a CellTiter Glow® assay. Data represent the mean ± SEM. $n = 6$. $^{**}p<0.01$ (vs Zn alone). (B) Morphological changes of GT1-7 cells after exposure to Zn with or without Cu. GT1-7 cells were exposed to 30 μM $ZnCl_2$ with or without 20 μM $CuCl_2$. After 24 h, cells were stained with fluorescein diacetate, which indicates living cells, and were observed with a fluorescent microscope. (a) control, (b) Zn alone, (c) Zn with Cu, (d) pretreatment with sodium pyruvate with Zn and Cu. The bar represents 100 μm. (C) Effects of sodium pyruvate on Zn-induced neurotoxicity and Cu/Zn neurotoxicity. GT1-7 cells were exposed to 30 μM $ZnCl_2$ or 20 μM $CuCl_2$ with or without 1 mM sodium pyruvate. After 24 h, cell viability was measured using a CellTiter Glow® assay. Data represent the mean ± SEM. $n = 6$. $^{**}p<0.01$.

200 μM) did not cause cell death. The viability of cells exposed to 30 μM Zn and 5 μM Cu (molar ratio of Zn:Cu = 6:1) was significantly decreased to 16.8 ± 4.1%, and that of cells exposed to 30 μM Zn with 10 μM Cu (molar ratio of Zn:Cu = 6:1) was 5.8 ± 0.94%. Figure 2B exhibits the morphological changes of GT1-7 cells after exposure to Zn and/or Cu. After 24 h of exposure to 30 μM Zn, neurite extension decreased and the cells degenerated. After exposure to 30 μM Zn and 20 μM Cu, most cells were degenerated. Pretreatment with sodium pyruvate recovered the degenerative changes.

In general, Cu and Zn have antagonistic functions because they share similar chemical characteristics and bind to the same sites of many metal-binding proteins. An excess of Zn can cause Cu deficiency and *vice versa*. Thus, we focused the cooperative action of Cu-enhanced Zn-induced neurotoxicity (Cu/Zn neurotoxicity) and investigated the mechanism.

We have demonstrated that sodium pyruvate attenuates Cu/Zn neurotoxicity and Zn-induced neurotoxicity,[80] as shown in Fig. 2C and Fig. 2B(d). Citrate and isocitrate also attenuate Cu/Zn neurotoxicity but not Zn neurotoxicity. Although these organic acids can bind to Zn^{2+} and/or Cu^{2+}, the co-existence of pyruvate and citrate did not influence the intracellular concentrations of Zn^{2+} and Cu^{2+} or the increase in metallothionein mRNA in GT1-7 cells. Therefore, it is unlikely that pyruvate and citrate attenuate

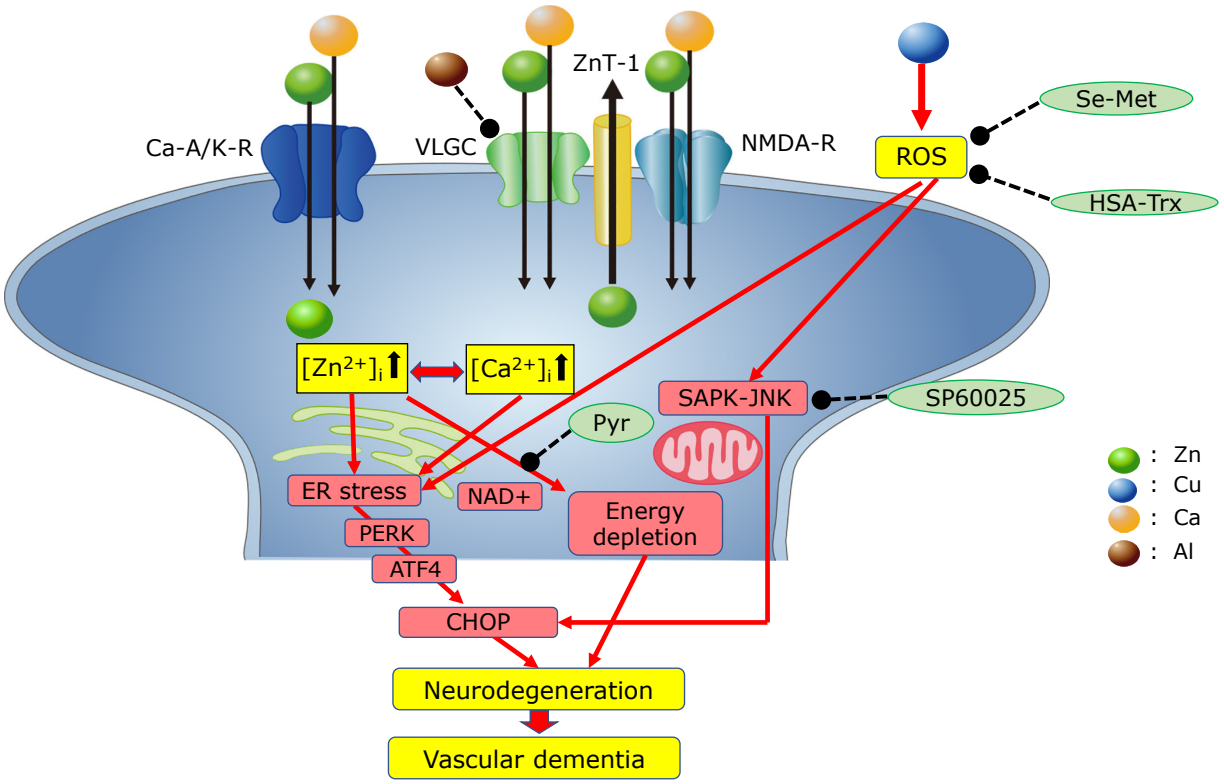

Fig. 3. Hypothetical scheme of copper (Cu)/zinc (Zn)-induced neurotoxicity. Under pathological conditions such as transient global ischemia, excess Cu^{2+} and Zn^{2+} are secreted into the synaptic cleft and co-exist in the same synapse. Zn^{2+} can be translocated through calcium (Ca)-permeable amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-type glutamate receptors (Ca-A/K-R), *N*-methyl-D-aspartate-type glutamate receptors (NMDA-R), and voltage gated Ca^{2+} channels (VGCC). In general, ZnT-1 acts to maintain the intracellular Zn concentration ($[Zn^{2+}]_i$) by facilitating Zn^{2+} efflux and inhibiting voltage-gated Ca^{2+} channels and NMDA-R. However, excess Zn^{2+} can cause the elevation of both $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$ and trigger endoplasmic reticulum (ER) stress pathways, which inhibits NAD^+, causes energy depletion in mitochondria, and induces neurodegeneration. Aluminum (Al^{3+}) inhibits voltage-gated Ca^{2+} channels and attenuates Zn neurotoxicity. The addition of Cu^{2+} produces ROS, which upregulate the ER stress and stress-activated protein kinases/c-Jun amino-terminal kinases (SAPK/JNK) pathways, exacerbate neuronal death, and eventually induce the pathogenesis of vascular dementia. SP600125, an inhibitor of the SAPK/JNK signaling pathway, attenuates Cu/Zn neurotoxicity. Selenomethionine (Se-Met) and a conjugated protein consisting of thioredoxin and human serum albumin (HSA-Trx) suppress ROS production and attenuate Cu/Zn neurotoxicity. Colored circles represent Zn, Cu, Ca, and Al.

Cu/Zn neurotoxicity by chelation of Cu^{2+} and/or Zn^{2+}.

In a comprehensive analysis using a DNA microarray, we found that several genes were upregulated after co-exposure to Cu and Zn compared with Zn alone or Cu alone. Thus, we used RT-PCR to investigate the detailed expression of these genes, including metal-related genes [zinc transporter 1 (*ZnT-1*), metallothionein 1 (*MT1*), and metallothionein 2 (*MT2*)], ER stress-related genes [CCAAT-enhancer-binding protein homologous protein (*CHOP*), growth-arrest- and DNA-damage-inducible gene 34 (*GADD34*), activating transcription factor 4 (*ATF4*), immunoglobulin binding protein (*Bip*), ER degradation-enhancing α-mannosidase-like protein (*EDEM*), spliced X-box binding protein-1 (*sXBP1*), glucose-regulated protein 94 (*GRP94*), and protein disulfide isomerase (*PDI*)], and Ca^{2+}-related genes [activity-related cytoskeleton protein (*Arc*)].[12] After exposure to Zn alone, *ZnT-1*, *MT1*, and *MT2* expression, increased. In addition, enhanced expression levels were observed for the *Arc*, *CHOP*, *GADD34*, and *ATF4 genes*. In contrast, other ER stress-related genes including *Bip*, *EDEM*, *sXBP1*, *GRP94*, and *PDI* did not exhibit significant changes. Exposure of cells to Cu^{2+} alone did not induce significant changes in these genes. However, a synergistic increase in the gene expression levels of *Arc*, *CHOP*, and *GADD34* was observed in cells co-exposed to Cu and Zn. We also used Western blotting analysis to measure the amount of CHOP protein, which is responsible for initiating an apoptotic cascade,[81] and found that CHOP protein was significantly increased after co-administration of Cu and Zn compared with administration of Zn alone. These results revealed that the ER stress pathway participates in Cu/Zn neurotoxicity. The ER stress pathway, which impairs ER function and leads to accumulation of unfolded or misfolded proteins, has been implicated in many neurodegenerative diseases, including AD, PD, and cerebral ischemia.[82] ER stress is mediated by three sensors at the ER membrane: PKR-like endoplasmic reticulum eIF2a kinase (PERK), inositol requiring 1 (IRE1), and ATF6.[83] In the PERK branch, ATF4 induces CHOP, which triggers an intrinsic apoptotic pathway, including caspase cascades, and thereafter CHOP induces GADD34 (protein phosphatase 1 regulatory subunit 15A). We demonstrated that genes related to the PERK branch (*ATF4*, *CHOP*, and *GADD34*) were upregulated because of Cu/Zn neurotoxicity. Therefore, it is possible that the ATF4-CHOP-GADD34 axis is responsible for the apoptosis resulting from Cu/Zn neurotoxicity. We also found that the ER stress pathway is involved in Ni^{2+}-enhanced, Zn-induced neurotoxicity of GT1-7 cells.[84]

Furthermore, our DNA microarray analysis suggested that the stress-activated protein kinases/c-Jun amino-terminal kinases (SAPK/JNK) pathway is upregulated by co-exposure to Cu and Zn. SAPK/JNK is a member of the mitogen-activated protein kinase (MAPK) family, and the SAPK/JNK signaling pathway plays an important role in apoptotic cell death, necroptosis, and autophagy.[85] Upon activation of this pathway by various

doi: 10.3164/jcbn.22-40

stressors, MAPK kinase 4 (MKK4) or MKK7 phosphorylates and activates SAPK/JNK.[86] Then, c-Jun and ATF2, major downstream factors of SAPK/JNK, are phosphorylated and activated by SAPK/JNK. Ultimately, phosphorylated forms of c-Jun and ATF2 induce downstream factors related to cell death. We demonstrated that treatment of GT1-7 cells with Cu and Zn increased the expression of phospho-SAPK/JNK and the downstream factors of SAPK/JNK, specifically c-Jun and ATF2.[87] Moreover, an inhibitor of the SAPK/JNK signaling pathway (SP600125) significantly attenuated Cu/Zn neurotoxicity.

Considering the upstream factors that underlie the Cu/Zn-induced ER stress pathway and SAPK/JNK pathway, we focused on two possible upstream pathways: Ca^{2+} homeostasis and ROS production. As noted, Al^{3+} protected GT1-7 cells from Zn-induced neurotoxicity by blocking the Zn-induced Ca^{2+} increase. The expression of a Ca^{2+}-related gene (*Arc*) was increased by Cu/Zn neurotoxicity and Zn-induced neurotoxicity. Increased Ca^{2+} levels can induce ER stress. Thus, it is possible that Ca^{2+} dyshomeostasis may underlie Cu/Zn-related neurodegenerative pathways.

Increasing evidence suggests that ROS induce the ER stress pathway,[88] the SAPK/JNK pathway,[89] and numerous other adverse effects. Cu is a redox-active metal that exists as oxidized Cu^{2+} and reduced Cu^{+}, while Zn exists only as Zn^{2+} and is not directly involved in the redox pathway. We found that the addition of Cu^{2+} produced ROS in GT1-7 cells, while addition of Zn^{2+} alone did not produce ROS or influence Cu^{2+}-induced ROS production.[90] Moreover, several antioxidants attenuated Cu/Zn neurotoxicity. A conjugated protein consisting of thioredoxin and human serum albumin (HSA-Trx) suppressed activation of the SAPK/JNK signaling pathway, inhibited ROS production, and attenuated Cu/Zn-induced neuronal death in GT1-7 cells.[90] Additionally, selenomethionine (Se-Met), an endogenous selenium (Se)-containing amino acid, induced glutathione peroxidase and blocked ROS production.[91] Pretreatment with Se-Met significantly suppressed the induction of CHOP and attenuated Cu/Zn neurotoxicity.

On the basis of these results, we have developed a hypothetical scheme regarding the involvement of Cu and/or Zn in the pathogenesis of VD (Fig. 3).

Under pathological conditions, such as transient global ischemia, sustained excitation of neurons occurs for prolonged periods in broad areas of the brain, and excess Zn and Cu are simultaneously secreted into the same synaptic clefts for a long period of time. In general, ZnT-1 maintains the $[Zn^{2+}]_i$ level by facilitating Zn^{2+} efflux and by inhibiting voltage-gated Ca^{2+} channels and NMDA receptors. However, an excess of Zn^{2+} can cause elevation of both $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$, induce ER stress pathways, inhibit NAD^{+}, causing energy depletion in mitochondria, and trigger neuronal death in postsynaptic neurons. Cu produces ROS, which subsequently trigger the ER stress and SAPK-JNK pathways, exacerbating Zn-induced neurotoxicity and eventually contributing to the pathogenesis of VD.

Conclusion

We have reviewed the cooperative actions of Cu and Zn in the neurodegenerative processes on the basis of our studies and other evidence. Because Cu has been found to be a risk factor for stroke in several epidemiological studies,[92–94] it is possible that Cu plays a crucial role in the pathogenesis of VD, but further research is necessary. This working hypothesis may contribute to a more precise understanding of the pathogenesis of VD and may aid in the development of treatments or prevention of VD. Furthermore, investigations of substances that attenuate Cu/Zn neurotoxicity may lead to strategies for the prevention or treatment of VD.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (JSPS Kakennhi Grant No. 18K06669, 17H03197, and 21H01786). We thank Ms. Shimoda M for technical assistance. We thank Lisa Kreiner, PhD, from Edanz (https://www.jp.edanz.com/ac) for editing a draft of this manuscript.

Abbreviations

AβP	Alzheimer's β-amyloid protein
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATF4	activating transcription factor 4
ATP7A	copper-transporting ATPase 7A
Arc	activity-related cytoskeleton protein
CHOP	CCAAT-enhancer-binding protein homologous protein
CSF	cerebrospinal fluid
Ctr1	copper transporter 1
D-APV	2-amino-5-phosphonovalerate
DLB	dementia with Lewy bodies
DMT1	divalent metal transporter 1
ER	endoplasmic reticulum
GABA	γ-aminobutyric acid
GADD34	growth-arrest and DNA-damage-inducible gene 34
MT	metallothionein
NAC	non-amyloid component
NMDA	*N*-methyl-D-aspartate
PD	Parkinson's disease
PrP	prion protein
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SAPK/JNK	stress-activated protein kinases/c-Jun amino-terminal kinases
VD	vascular dementia
VGCC	voltage-gated Ca^{2+} channel

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Scheiber IF, Mercer JF, Dringen R. Metabolism and functions of copper in brain. *Prog Neurobiol* 2014; **116**: 33–57.
2 Black MM. Zinc deficiency and child development. *Am J Clin Nutr* 1998; **68 (2 Suppl)**: 464S–469S.
3 D'Ambrosi N, Rossi L. Copper at synapse: release, binding and modulation of neurotransmission. *Neurochem Int* 2015; **90**: 36–45.
4 Frederickson CJ, Suh SW, Silva D, Frederickson CJ, Thompson RB. Importance of zinc in the central nervous system: the zinc-containing neuron. *J Nutr* 2000; **130 (5S Suppl)**: 1471S–1483S.
5 Sensi SL, Granzotto A, Siotto M, Squitti R. Copper and zinc dysregulation in Alzheimer's disease. *Trends Pharmacol Sci* 2018; **39**: 1049–1063.
6 Acevedo K, Masaldan S, Opazo CM, Bush AI. Redox active metals in neurodegenerative diseases. *J Biol Inorg Chem* 2019; **24**: 1141–1157.
7 Gil-Bea FJ, Aldanondo G, Lasa-Fernández H, López de Munain A,

Vallejo-Illarramendi A. Insights into the mechanisms of copper dyshomeostasis in amyotrophic lateral sclerosis. *Expert Rev Mol Med* 2017; **19**: e7.
8 Gromadzka G, Tarnacka B, Flaga A, Adamczyk A. Copper dyshomeostasis in neurodegenerative diseases-therapeutic implications. *Int J Mol Sci* 2020; **21**: 9259.
9 Kawahara M, Sadakane Y, Mizuno K, Kato-Negishi M, Tanaka KI. Carnosine as a possible drug for zinc-induced neurotoxicity and vascular dementia. *Int J Mol Sci* 2020; **21**: 2570.
10 Shuttleworth CW, Weiss JH. Zinc: new clues to diverse roles in brain ischemia. *Trends Pharmacol Sci* 2011; **32**: 480–486.
11 Kawahara M, Mizuno D, Koyama H, Konoha K, Ohkawara S, Sadakane Y. Disruption of zinc homeostasis and the pathogenesis of senile dementia. *Metallomics* 2014; **6**: 209–219.
12 Tanaka KI, Kawahara M. Copper enhances zinc-induced neurotoxicity and the endoplasmic reticulum stress response in a neuronal model of vascular dementia. *Front Neurosci* 2017; **11**: 58.
13 Becker JS, Matusch A, Palm C, Salber D, Morton KA, Becker JS. Bioimaging of metals in brain tissue by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and metallomics. *Metallomics* 2010; **2**: 104–111.
14 Kawahara M, Kato-Negishi M, Tanaka K. Cross talk between neurometals and amyloidogenic proteins at the synapse and the pathogenesis of neurodegenerative diseases. *Metallomics* 2017; **9**: 619–633.
15 Takeda A. Zinc homeostasis and functions of zinc in the brain. *Biometals* 2001; **14**: 343–351.
16 Prasad AS. Lessons learned from experimental human model of zinc deficiency. *J Immunol Res* 2020; **2020**: 9207279.
17 Pochwat B, Nowak G, Szewczyk B. Relationship between Zinc (Zn^{2+}) and glutamate receptors in the processes underlying neurodegeneration. *Neural Plast* 2015; **2015**: 591563.
18 Chuang SH, Reddy DS. Zinc reduces antiseizure activity of neurosteroids by selective blockade of extrasynaptic GABA-A receptor-mediated tonic inhibition in the hippocampus. *Neuropharmacology* 2019; **148**: 244–256.
19 Richards-Williams C, Contreras JL, Berecek KH, Schwiebert EM. Extracellular ATP and zinc are co-secreted with insulin and activate multiple P2X purinergic receptor channels expressed by islet beta-cells to potentiate insulin secretion. *Purinergic Signal* 2008; **4**: 393–405.
20 Morabito A, Zerlaut Y, Serraz B, Sala R, Paoletti P, Rebola N. Activity-dependent modulation of NMDA receptors by endogenous zinc shapes dendritic function in cortical neurons. *Cell Rep* 2022; **38**: 110415.
21 Carrillo E, Bhatia NK, Akimzhanov AM, Jayaraman V. Activity dependent inhibition of AMPA receptors by Zn^{2+}. *J Neurosci* 2020; **40**: 8629–8636.
22 Ueno S, Tsukamoto M, Hirano T, *et al*. Mossy fiber Zn^{2+} spillover modulates heterosynaptic *N*-methyl-D-aspartate receptor activity in hippocampal CA3 circuits. *J Cell Biol* 2002; **158**: 215–220.
23 Sullivan JA, Zhang XL, Sullivan AP, *et al*. Zinc enhances hippocampal long-term potentiation at CA1 synapses through NR2B containing NMDA receptors. *PLoS One* 2018; **13**: e0205907.
24 Kardos J, Héja L, Simon Á, Jablonkai I, Kovács R, Jemnitz K. Copper signalling: causes and consequences. *Cell Commun Signal* 2018; **16**: 71.
25 Squitti R, Malosio ML, Rongioletti MCA, Tecchio F. Copper involvement in glutamatergic transmission in physiology and disease as revealed by magnetoencephalography/electroencephalography (MEG/EEG) studies. *Aging Clin Exp Res* 2021; **33**: 2023–2026.
26 Roos PM, Vesterberg O, Syversen T, Flaten TP, Nordberg M. Metal concentrations in cerebrospinal fluid and blood plasma from patients with amyotrophic lateral sclerosis. *Biol Trace Elem Res* 2013; **151**: 159–170.
27 Schikorski T, Stevens CF. Quantitative ultrastructural analysis of hippocampal excitatory synapses. *J Neurosci* 1997; **17**: 5858–5867.
28 Vogt K, Mellor J, Tong G, Nicoll R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. *Neuron* 2000; **26**: 187–196.
29 Zhang C, Maslar D, Minckley TF, LeJeune KD, Qin Y. Spontaneous, synchronous zinc spikes oscillate with neural excitability and calcium spikes in primary hippocampal neuron culture. *J Neurochem* 2021; **157**: 1838–1849.
30 Kardos J, Kovács I, Hajós F, Kálmán M, Simonyi M. Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. *Neurosci Lett* 1989; **103**: 139–144.
31 Hopt A, Korte S, Fink H, *et al*. Methods for studying synaptosomal copper release. *J Neurosci Methods* 2003; **128**: 159–172.
32 Hara T, Yoshigai E, Ohashi T, Fukada T. Zinc transporters as potential therapeutic targets: an updated review. *J Pharmacol Sci* 2022; **148**: 221–228.
33 Subramanian Vignesh K, Deepe GS Jr. Metallothioneins: emerging modulators in immunity and infection. *Int J Mol Sci* 2017; **18**: 2197.
34 Mellone M, Pelucchi S, Alberti L, Genazzani AA, Di Luca M, Gardoni F. Zinc transporter-1: a novel NMDA receptor-binding protein at the postsynaptic density. *J Neurochem* 2015; **132**: 159–168.
35 Levy S, Beharier O, Etzion Y, *et al*. Molecular basis for zinc transporter 1 action as an endogenous inhibitor of L-type calcium channels. *J Biol Chem* 2009; **284**: 32434–32443.
36 Sindreu C, Palmiter RD, Storm DR. Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. *Proc Natl Acad Sci U S A* 2011; **108**: 3366–3370.
37 De Benedictis CA, Haffke C, Hagmeyer S, Sauer AK, Grabrucker AM. Expression analysis of zinc transporters in nervous tissue cells reveals neuronal and synaptic localization of ZIP4. *Int J Mol Sci* 2021; **22**: 4511.
38 Koh JY, Lee SJ. Metallothionein-3 as a multifunctional player in the control of cellular processes and diseases. *Mol Brain* 2020; **13**: 116.
39 Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M. The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron* 1991; **7**: 337–347.
40 Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev* 2013; **93**: 1803–1845.
41 Kodama H, Fujisawa C, Bhadhprasit W. Inherited copper transport disorders: biochemical mechanisms, diagnosis, and treatment. *Curr Drug Metab* 2012; **13**: 237–250.
42 Hartwig C, Méndez GM, Bhattacharjee S, *et al*. Golgi-dependent copper homeostasis sustains synaptic development and mitochondrial content. *J Neurosci* 2021; **41**: 215–233.
43 El Meskini R, Crabtree KL, Cline LB, Mains RE, Eipper BA, Ronnett GV. ATP7A (Menkes protein) functions in axonal targeting and synaptogenesis. *Mol Cell Neurosci* 2007; **34**: 409–421.
44 Han M, Chang J, Kim J. Loss of divalent metal transporter 1 function promotes brain copper accumulation and increases impulsivity. *J Neurochem* 2016; **138**: 918–928.
45 Fransson AE, Kisiel M, Pirttilä K, Pettersson C, Videhult Pierre P, Laurell GFE. Hydrogen inhalation protects against ototoxicity induced by intravenous cisplatin in the guinea pig. *Front Cell Neurosci* 2017; **11**: 280.
46 Kawahara M, Kato-Negishi M, Tanaka KI. Amyloids: regulators of metal homeostasis in the synapse. *Molecules* 2020; **25**: 1441.
47 Multhaup G, Schlicksupp A, Hesse L, *et al*. The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I). *Science* 1996; **271**: 1406–1409.
48 Del Prete D, Lombino F, Liu X, D'Adamio L. APP is cleaved by Bace1 in pre-synaptic vesicles and establishes a pre-synaptic interactome, via its intracellular domain, with molecular complexes that regulate pre-synaptic vesicles functions. *PLoS One* 2014; **9**: e108576.
49 Wong BX, Tsatsanis A, Lim LQ, Adlard PA, Bush AI, Duce JA. β-Amyloid precursor protein does not possess ferroxidase activity but does stabilize the cell surface ferrous iron exporter ferroportin. *PLoS One* 2014; **9**: e114174.
50 Kawahara M, Kato-Negishi M, Tanaka KI. Neurometals in the pathogenesis of prion diseases. *Int J Mol Sci* 2021; **22**: 1267.
51 Black SA, Stys PK, Zamponi GW, Tsutsui S. Cellular prion protein and NMDA receptor modulation: protecting against excitotoxicity. *Front Cell Dev Biol* 2014; **2**: 45.
52 Watt NT, Griffiths HH, Hooper NM. Neuronal zinc regulation and the prion protein. *Prion* 2013; **7**: 203–208.
53 Singh A, Haldar S, Horback K, *et al*. Prion protein regulates iron transport by functioning as a ferrireductase. *J Alzheimers Dis* 2013; **35**: 541–552.
54 Moons R, Konijnenberg A, Mensch C, *et al*. Metal ions shape α-synuclein. *Sci Rep* 2020; **10**: 16293.
55 Davies P, Moualla D, Brown DR. Alpha-synuclein is a cellular ferrireductase. *PLoS One* 2011; **6**: e15814.
56 Macknik SL. Visual masking approaches to visual awareness. *Review Prog Brain Res* 2006; **155**: 177–215.
57 Román GC. Vascular dementia prevention: a risk factor analysis. *Cerebrovasc Dis* 2005; **20 Suppl 2**: 91–100.
58 de Haan EH, Nys GM, Van Zandvoort MJ. Cognitive function following stroke and vascular cognitive impairment. *Curr Opin Neurol* 2006; **19**: 559–564.
59 Lee JM, Grabb MC, Zipfel GJ, Choi DW. Brain tissue responses to ischemia.

doi: 10.3164/jcbn.22-40

J Clin Invest 2000; **106**: 723–731.
60 Weiss JH, Sensi SL, Koh JY. Zn^{2+}: a novel ionic mediator of neural injury in brain disease. *Trends Pharmacol Sci* 2000; **21**: 395–401.
61 Sensi SL, Canzoniero LM, Yu SP, *et al*. Measurement of intracellular free zinc in living cortical neurons: routes of entry. *J Neurosci* 1997; **17**: 9554–9564.
62 Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS. The GluR2 (GluR-B) hypothesis: Ca^{2+}-permeable AMPA receptors in neurological disorders. *Trends Neurosci* 1997; **20**: 464–470.
63 Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW. The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science* 1996; **272**: 1013–1016.
64 Calderone A, Jover T, Mashiko T, *et al*. Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death. *J Neurosci* 2004; **24**: 9903–9913.
65 Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 1990; **5**: 1–10.
66 Kawahara M, Kato-Negishi M, Kuroda Y. Pyruvate blocks zinc-induced neurotoxicity in immortalized hypothalamic neurons. *Cell Mol Neurobiol* 2002; **22**: 87–93.
67 Koyama H, Konoha K, Sadakane Y, Ohkawara S, Kawahara M. Zinc neurotoxicity and the pathogenesis of vascular-type dementia: involvement of calcium dyshomeostasis and carnosine. *J Clin Toxicol* **2011**; S3-002.
68 Radak D, Resanovic I, Isenovic ER. Changes in hypothalamus-pituitary-adrenal axis following transient ischemic attack. *Angiology* 2014; **65**: 723–732.
69 Mahesh VB, Zamorano P, De Sevilla L, Lewis D, Brann DW. Characterization of ionotropic glutamate receptors in rat hypothalamus, pituitary and immortalized gonadotropin-releasing hormone (GnRH) neurons (GT1-7 cells). *Neuroendocrinology* 1999; **69**: 397–407.
70 Sheline CT, Behrens MM, Choi DW. Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. *J Neurosci* 2000; **20**: 3139–3154.
71 Lee JY, Kim YH, Koh JY. Protection by pyruvate against transient forebrain ischemia in rats. *J Neurosci* 2001; **21**: RC171.
72 Tang J, Zhuo Y, Li Y. Effects of iron and zinc on mitochondria: potential mechanisms of glaucomatous injury. *Front Cell Dev Biol* 2021; **9**: 720288.
73 Kawahara M, Konoha K, Nagata T, Sadakane Y. Protective substances against zinc-induced neuronal death after ischemia: carnosine a target for drug of vascular type of dementia. *Recent Pat CNS Drug Discov* 2007; **2**: 145–149.
74 Kawahara M, Sadakane Y, Koyama H, Konoha K, Ohkawara S. D-Histidine and L-histidine attenuate zinc-induced neuronal death in GT1-7 cells. *Metallomics* 2013; **5**: 453–460.
75 Mizuno D, Konoha-Mizuno K, Mori M, *et al*. Protective activity of carnosine and anserine against zinc-induced neurotoxicity: a possible treatment for vascular dementia. *Metallomics* 2015; **7**: 1233–1239.
76 Konoha K, Sadakane Y, Kawahara M. Effects of gadolinium and other metal on the neurotoxicity of immortalized hypothalamic neurons induced by zinc. *Biomed Res Trace Elements* 2004; **15**: 275–277.
77 Platt B, Büsselberg D. Combined actions of Pb^{2+}, Zn^{2+}, and Al^{3+} on voltage-activated calcium channel currents. *Cell Mol Neurobiol* 1994; **14**: 831–840.
78 Gulati P, Muthuraman A, Jaggi AS, Singh N. Neuroprotective effect of gadolinium: a stretch-activated calcium channel blocker in mouse model of ischemia-reperfusion injury. *Naunyn Schmiedebergs Arch Pharmacol* 2013; **386**: 255–264.
79 Kim AH, Sheline CT, Tian M, *et al*. L-type Ca^{2+} channel-mediated Zn^{2+} toxicity and modulation by ZnT-1 in PC12 cells. *Brain Res* 2000; **886**: 99–107.
80 Tanaka KI, Shimoda M, Kawahara M. Pyruvic acid prevents Cu^{2+}/Zn^{2+}-induced neurotoxicity by suppressing mitochondrial injury. *Biochem Biophys Res Commun* 2018; **495**: 1335–1341.
81 Hu H, Tian M, Ding C, Yu S. The C/EBP homologous protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. *Front Immunol* 2019; **9**: 3083.
82 Koksal AR, Verne GN, Zhou Q. Endoplasmic reticulum stress in biological processing and disease. *J Investig Med* 2021; **69**: 309–315.
83 Sano R, Reed JC. ER stress-induced cell death mechanisms. *Biochim Biophys Acta* 2013; **1833**: 3460–3470.
84 Tanaka KI, Kasai M, Shimoda M, Shimizu A, Kubota M, Kawahara M. Nickel enhances zinc-induced neuronal cell death by priming the endoplasmic reticulum stress response. *Oxid Med Cell Longev* 2019; **2019**: 9693726.
85 Kyosseva SV. Mitogen-activated protein kinase signaling. *Int Rev Neurobiol* 2004; **59**: 201–220.
86 Dhanasekaran DN, Reddy EP. JNK-signaling: a multiplexing hub in programmed cell death. *Genes Cancer* 2017; **8**: 682–694.
87 Tanaka KI, Shimoda M, Kasai M, Ikeda M, Ishima Y, Kawahara M. Involvement of SAPK/JNK signaling pathway in copper enhanced zinc-induced neuronal cell death. *Toxicol Sci* 2019; **169**: 293–302.
88 Konno T, Melo EP, Chambers JE, Avezov E. Intracellular sources of ROS/H_2O_2 in health and neurodegeneration: spotlight on endoplasmic reticulum. *Cells* 2021; **10**: 233.
89 Chang CC, Kuan CP, Lin JY, Lai JS, Ho TF. Tanshinone IIA facilitates TRAIL sensitization by up-regulating DR5 through the ROS-JNK-CHOP signaling axis in human ovarian carcinoma cell lines. *Chem Res Toxicol* 2015; **28**: 1574–1583.
90 Tanaka KI, Shimoda M, Chuang VTG, *et al*. Thioredoxin-albumin fusion protein prevents copper enhanced zinc-induced neurotoxicity via its anti-oxidative activity. *Int J Pharm* 2018; **535**: 140–147.
91 Nakano Y, Shimoda M, Okudomi S, Kawaraya S, Kawahara M, Tanaka KI. Seleno-L-methionine suppresses copper-enhanced zinc-induced neuronal cell death via induction of glutathione peroxidase. *Metallomics* 2020; **12**: 1693–1701.
92 Xu J, Xu G, Fang J. Association between serum copper and stroke risk factors in adults: evidence from the National Health and Nutrition Examination Survey, 2011–2016. *Biol Trace Elem Res* 2022; **200**: 1089–1094.
93 Hu L, Bi C, Lin T, *et al*. Association between plasma copper levels and first stroke: a community-based nested case-control study. *Nutr Neurosci* 2021; 1–10.
94 Zhang M, Li W, Wang Y, Wang T, Ma M, Tian C. Association between the change of serum copper and ischemic stroke: a systematic review and meta-analysis. *J Mol Neurosci* 2020; **70**: 475–480.