New Insights into Neurozinc and Metallothioneins

Physiological Roles of Metallothioneins in Central Nervous System Diseases

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Metallothioneins (MTs) are small-molecular weight metal-binding proteins involved in the maintenance of tissue structure, efficient metal metabolism, and metal detoxification and have an antioxidative effect. Moreover, MTs are expressed as four isoforms, and there are no known patterns in their localization with various effects. According to recent studies, MTs affect central nervous system (CNS) diseases, and many reports suggest that each isoform of MT has a protective effect against disease. Notably, MTs are involved in regions of diseases related to unmet medical needs, and MTs affect intractable neurological diseases, such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). This review specifically focuses on MT-related ocular diseases, cerebral ischemia, psychological disorders, ALS, and SMA. Each of these diseases has a separate cause, but the conditions are related to MTs. To understand the physiological roles of MTs in CNS diseases, we reviewed the current literature on the complex interactions between each MT, pathological conditions, and perspectives. We also discuss current evidence on the expression and function of MTs for diagnosis and new therapeutic strategies.

1. INTRODUCTION

Metallothioneins (MTs) are small-molecular weight (6–7 kDa) proteins with free radical scavenging properties, and are expressed as four isoforms. The most represented, MT-1 and -2, are ubiquitous in the body, particularly the kidney and liver. These MTs have important roles in metal homeostasis and detoxification. In contrast, MT-3 shows a characteristic six-amino acid insertion in the α-domain and an additional threonine in the β-domain, was originally believed brain-specific but has since been detected in other tissues. The latest isoform discovered is MT-4, with variety compared to MT-1, -2 and -3, and is expressed in squamous epithelia.

The roles of MT-4 in central nervous system (CNS) diseases are not fully known, so we review previous reports associated with MT-1, -2 and -3 in the CNS, such as in the eye, brain, and spinal cord. We focus on CNS disorders without definitive therapy, ocular diseases (age-related macular disease [AMD], diabetic retinopathy [DR], and glaucoma), stroke, psychiatric disorders (schizophrenia and depression), and motor neuron diseases (spinal muscular atrophy [SMA] and amyotrophic lateral sclerosis [ALS]). These diseases may interfere with homeostasis of zinc and/or copper. The affinity of MTs to metal ions is thought to cause their bioactivity and protective roles such as in neuronal and vascular cells. Many studies have reported using transgenic mice with suppressed or overexpressed MTs, and the biological functions of MTs in the CNS are becoming increasingly clear. The involvement of MTs in physiological and pathophysiological processes, such as angiogenesis, apoptosis and the detoxification of heavy metals, suggests their participation in various CNS diseases. For example, it has been reported that MT-1/2 play a role in suppression of oxidative stress, inflammation, and apoptosis in the CNS.

2. ROLES OF MTs IN OCULAR DISEASES

2.1. Age-Related Macular Degeneration (AMD) AMD is the world’s leading cause of severe vision loss in people aged over 50. The prevalence of AMD will continue to increase due to aging societies worldwide. AMD in the early phase is characterized by drusenoid deposits and pigmentation in the macula. Late AMD develops into either an exudative type choroidal neovascularization (CNV) into the subretinal space or a geographic atrophy type. In either case, late AMD leads to severe central vision loss. Unfortunately, details of the pathogenic and progression mechanisms of AMD remain unknown. At present, known risk factors include aging, smoking, and genetic mutation. Among them, aging and smoking cause oxidative damage throughout the body including in the eyes. The retina’s exposure to light in daily life makes it particularly susceptible to oxidative damage. Oxidation is involved in the pathogenic mechanisms of CNV, which causes retinal pigment epithelium (RPE) detachment, sub- or intra-retinal hemorrhage, and fibrovascular scarring, resulting in vision loss. Previous reports suggest that an antioxidant diet and drugs delay the pathogenesis and progression of AMD. Our laboratory reported that a free radical scavenger protects against laser-induced CNV in mice.

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and common marmosets. In this way, oxidation is one of the key factors forming AMD pathogenesis.

MT is a known scavenger of free radicals, and the absence of nitric oxide (NO)-mediated increase in labile Zn^{2+} in MLEC from MT-1 and -2 knockout (KO) mice infers the critical role of MT in the regulation of Zn^{2+} homeostasis by NO. Based on these reports, we examined the role of MT-1/2 in CNV using a laser-induced CNV model with MT-1/2 KO mice. Our data suggest that CNV is decreased in MT-1/2 KO mice. On the other hand, MT3, which is expressed at high levels in the neurosensory retina, is involved in both CNV itself and vascular leakage from CNV. MT-1, -2, and -3 might help suppress the pathogenesis and progression of AMD via the mechanism of CNV. Also, MT-3 is known to protect the outer nuclear layer and photoreceptor cells related to non-exudative AMD, so MT-3 reduction is involved in AMD onset.

### 2.2. Diabetic Retinopathy (DR)

Diabetic retinopathy (DR) is the most common microvascular complication induced by diabetes and occurs in approximately one third of diabetes patients. Recently, it was reported that the prevalence of DR has decreased gradually due to improvements in the systemic control of diabetes in the U.S.A. However, the worldwide data are grim regarding vision loss. Indeed, DR-related visual impairment and blindness have increased by 64% and 27%, respectively.

The onset and progression of DR are caused by numerous factors. For example, hyperglycemia leads to the development of retinal microvascular abnormalities, such as retinal neovascularization and edema. Diabetic macular edema results in microvascular hyperpermeability by disrupting the blood–retinal barrier (BRB). These microvascular abnormalities also cause retinal capillary occlusion with ischemia, triggering an expansion in vascular endothelial growth factor (VEGF) levels. As a result, retinal ischemia promotes abnormal neovascularization via VEGF signaling.

In this section, we will discuss the relation between cytokines including VEGF and MT. It has been previously indicated that MT-1/2 KO mice decreased expression of the growth factors tumor growth factor-β1 (TGF-β1), NT-3, basic fibroblast growth factor (b-FGF), and VEGF. Furthermore, we first reported the involvement of MT-1/2 in retinal neovascularization (Fig. 1). In this study, we confirmed
that hypoxia-inducible factor (HIF)-1α was stabilized in the nuclear fraction, but MT-1/2 was increased in the cytosol fraction under hypoxia, and that knockdown of MT-1 or MT-2 increased HIF-1α polyubiquitination[53] (Fig. 1). The concentrations of MT-1/2 and VEGF were significantly higher in the vitreous fluid of proliferative diabetic retinopathy (PDR) patients than in that of the macular hole (control group).[52] Clinical data showed the participation in DR pathogenesis of MT-1/2 as well as basic research using mice. Our study is the first report that MT-1/2 could be new molecular markers for retinal neovascularization including DR via HIF-1α degradation. On the other hand, some previous reports suggest that MT prevented diabetes-related impairments by inactivation of GSK-3β in cardiac energy metabolism, inflammation, and remodeling.[54] and MT was effective against these reactive oxygen species (ROS) and reduced apoptosis and necrosis produced by NO exposure.[55] Taken together, MTs are important in diabetes in various capacities. Because DR is one complication generated by diabetes, the radical therapeutics of DR need to be part of comprehensive treatments.

2.3. Glaucoma Glaucoma is characterized as an optic neuropathy. Progressive degeneration of retinal ganglion cells (RGCs) leads to characteristic patterns of visual field loss. By the year 2020, it is estimated that the number of glaucoma patients worldwide will approach 80 million and 11.2 million will be bilaterally blind from glaucoma.[56] It is well known that intraocular pressure (IOP) is related to retinal ganglion cell death. However, there are many normal-pressure glaucoma patients, so the pathogenesis of glaucoma is not fully understood.[57] Without elevating IOP, the apoptotic mechanisms of RGC death are induced by the impaired microcirculation, oxygenation, and high glutamate.[58,59] MT binds to metal ions such as Zn²⁺ and Cu²⁺ to regulate detoxification in various tissues including the CNS.[60,61] In addition, the decrease in free Zn²⁺ or Mg²⁺ could activate N-methyl-D-aspartic acid (NMDA) receptors, resulting in an increased intracellular Ca²⁺ level.[62] Suemori et al.[53] reported that MT-1/2 knockdown attenuated RGCs impairments using a murine glaucoma model induced by NMDA intravitreal injection. These results support the hypothesis that MT might have a protective effect against neurological disorders including glaucoma. However, there are many unknown factors about the differences of each isoform (MT-1, -2, -3, and -4). Detailed studies of each MT in the retina will help to elucidate the roles of MT isoforms in ocular diseases.

3. CENTRAL NERVOUS SYSTEM DISEASE

3.1. Ischemic Stroke Stroke is a brain ischemic condition in which poor blood flow causes neuronal cell death. Reasons for ischemic stroke are thrombosis, embolism, and systemic hypoperfusion. Unfortunately, despite stroke being the second leading cause of death worldwide, the only approved treatment is thrombolysis at present. In addition, the time window of thrombolysis is very limited from the onset. After stroke, in the central ischemic area, called the ‘core,’ the disruption of oxygen and nutrition causes immediate cell death. By contrast, in the neighboring region of the core, called the ‘penumbra,’ neuronal cells remain alive. Therefore the penumbra could be the ideal target for neuroprotective strategies aiming to reduce brain ischemic damage.[64] Previous reports indicate that MT has a protective effect against ischemia stroke using middle cerebral artery occlusion (MCAO) model mice. For example, there was a report that intraperitoneal treatment of MT-2 resulted in a significant reduction in infarct volumes and neurological deficit after mild MCAO (30min MCAO/72 h reperfusion).[65] Eidizadeh et al.[66] discussed that MT-2 had a protective effect via the reduction of pro-inflammatory tumor necrosis factor (TNF)-α mRNA induction after MCAO, which is in agreement with the known immunomodulatory actions of MT-2. Moreover, we reported that after a 2-h MCAO and a 22-h reperfusion, cerebral infarction in MT-3 KO mice was aggravated compared with wild-type mice; the fatal rate of the MT-3 KO mice increased from 3d after MCAO and neurological deficits at 5 and 7d after MCAO of the MT-3 KO mice were worse than those of the wild-type.[66] The main mechanism of ischemic/reperfusion (I/R) injury is the production of ROS. It has been reported that MT-3 could remove the superoxide anion in vitro,[67] and 8-hydroxy-deoxyguanosine (8-OHdG, an oxidative stress marker)-positive cells were higher in MT-3 KO mice than in wild-type mice in vivo.[68] Therefore the contention that MT-3 affects I/R injury seems reasonable. There are many other mechanisms that cause neuronal cell death induced by transient brain ischemia, such as influx of calcium and toxic metals, which cause DNA fragmentation. MTs may be effective against mild ischemic stroke, and induction of zinc, which is a known action of MT, may be effective as a treatment to prevent secondary cell death in the penumbra area after stroke.

3.2. Psychiatric Disorders Among MT isoforms, MT-1 and -2 are present in many organs, and MT-3 and -4 are expressed in the CNS, specifically in the prefrontal cortex (PFC).[66,68] PFC injuries are symptomatically similar to schizophrenia, depression, dementia, and autism.[59–72] Schizophrenia is a chronic disease that affects approximately 1% of the population.[73] Symptoms usually remain from early adulthood until later life, despite active treatment.[74–76] Such a prolonged course of illness leads to personal disability, psychological distress, and financial burden in terms of nursing care expenses.[77] Furthermore, patients with depression may commit suicide due to the terrible symptoms; the number of such cases is increasing annually and becoming a social problem all over the world. Interestingly, it has been reported that schizophrenia and depression are related to maintaining zinc levels.[78,79] Pettrilli et al.[79] explained the hypothesis that increasing zinc through dietary supplementation may be a route to inhibiting NMDA receptors, decreasing glutamate-mediated excitotoxicity and thus normalizing glutamatergic transmission in the PFC. As mentioned above, MT can bind free zinc, which activates NMDA receptors.[82] Current research shows that MTs are involved in the redox regulatory mechanism of Zn–S interaction and the coupling of zinc,[80,81] and the structure of Zn-MT may result in this moiety becoming an electron donor for the controlled release of iron.[82] When considering molecular mechanisms, postsynaptic density (PSD) proteins might be involved in many psychiatric diseases, including depression and schizophrenia. Both antipsychotics and antidepressants modulate PSD molecules to play roles in behavioral response.[83–90] Among them, PSD95 is a member of the synapse-associated protein family of scaffolding molecules that modulates the organization and function of synapses.[90] Interestingly, Hirabayashi et al.[90] purified
a PSD-95 fusion protein coupled to three tandem repeats of MT (PSD95-3MT) from COS7 cells grown in the presence of Cd²⁺. There is no detailed report on the action mechanisms related to psychological disorders and PSD95-3MT; therefore functional analysis in future studies might also be beneficial. In addition, there are some reports on psychological stress-induced zinc accumulation and MT upregulation in the liver,⁹⁵ and MT gene expression mediated by zinc was upregulated in children with autism spectrum disorder.⁹⁴ Furthermore, our laboratory reported that reduction of MT-3 might be induced by abnormal behaviors such as psychiatric disorders using MT-3 KO mice.⁹⁵ We confirmed in the social interaction test that mean duration per contact of MT-3 KO mice was significantly shorter than that of wild-type mice, and in the prepulse-inhibition (PPI) test, MT-3 KO mice showed diminished Ppi.⁹⁵ In general, less social interaction and a PPI deficit mimics the negative symptoms of schizophrenia by PFC dysfunction. This study indicated that MT-3 KO mice were useful for modeling psychological disorders such as schizophrenia and autism. Taken together, MT-3 and/or -4, present in the CNS, are likely involved with schizophrenia and depression via regulating expression of zinc.

### 3.3. Motor Neuron Diseases: Spinal Muscular Atrophy and Amyotrophic Lateral Sclerosis

SMA is a genetic disease related to voluntary muscle movement, involves the loss of nerve cells called motor neurons in the spinal cord, and is classified as a motor neuron disease, affecting approximately 1 person in 6000–10000 births.⁹⁶ SMA is an autosomal recessive disease due to a loss-of-function mutation of the survival motor neuron 1 (SMN1) gene, resulting in muscular atrophy, loss of motor neurons, neuromuscular junction degeneration, and eventually leading to paralysis and respiratory failure.⁹⁶–⁹⁸ A second, duplicated gene, SMN2, has been identified as a sufficient functional protein for survival.⁹⁹ Based on these findings, therapeutic strategies to increase SMNs are under development, and some clinical trials are currently in progress.¹⁰⁰ However, it is very difficult for SMN-targeting therapy to control the long-term maintenance of neuromuscular and other functions in SMA patients. Bowerman et al.¹⁰⁰ mentioned the potential for developing combination therapeutic approaches for SMA via SMN-dependent and -independent processes. With advancements in our knowledge of both SMA pathology and skeletal muscle biology, skeletal muscle has emerged as a therapeutic target for SMA. In some muscle atrophy studies with experimental animals, MT was identified as an oxidative-related factor,¹⁰¹,¹⁰² and MT gene expression increases in humans following muscle limb immobilization and spinal cord injury.¹⁰³,¹⁰⁴ Importantly, from a previous report of spinal cord transection, MT-1/2 shortage resulted in dysfunction and increase of lipid peroxidation in skeletal muscle.¹⁰⁵ Although future research is required to investigate the mechanisms in detail, remedies to increase MT-1/2 could be a candidate therapy to improve the QOL for SMA patients.

ALS is a neurodegenerative disease characterized by progressive loss of structure or function of upper and lower motor neurons in the brain and spinal cord. The majority (more than 90%) of ALS is sporadic, whereas a minor percentage (approximately 5–10%) is familial. Recent genetic studies have identified genes related to ALS pathogenesis. In its current state, Riluzole only could extend the life span by approximately 3 months for ALS patients,¹⁰⁶,¹⁰⁷ therefore the development of a new therapy is important. Mutations in Cu/Zn superoxide dismutase 1 (SOD1), which was first identified in ALS, have been found in more than 20% of familial and 1–4% of sporadic ALS cases.¹⁰⁸–¹⁰⁹ SOD1 binds copper and zinc ions and is one of superoxide dismutases responsible for destroying free superoxide radicals. In the relation between Cu/Zn and MT, G93A SOD1 mice mimicked in ALS reduced life span by crossing MT-1/2 or -3 KO mice.¹¹⁰ Tokuda et al.¹¹¹ reported that endogenous MT-1/2 level and oxidative damage are correlated in the spinal cords of SOD1G93A mice, and that upregulation of MT-1/2 decreased lipid peroxides level in SOD1G93A mice. Hashimoto et al.¹¹² demonstrated that MT-3 prevents neuronal death and extends life span in SOD1G93A mice. From the above, it is clear that MT-1/2 and/or -3 are strongly involved in the pathology of ALS. Furthermore, we considered how MTs participated in ALS pathophysiology. While the immunoreactivities of both MT-1/2 and MT-3 stained dominantly in glial cells and were decreased in the spinal cords with ALS patients,¹¹³ another report showed a high expression of MT-3 in ALS.¹¹⁴ It has been reported that treatment with cytokine leukemia inhibitory factor increases MT-3 expression in glia and boosts expression of superoxide dismutase 3 in neurons.¹¹⁵ Through such cell-specific upregulation, various factors might have the potential to protect multiple cell types against ROS injury. These reports indicate that MTs may have therapeutic potential against motor neuron diseases; however, there is much room for further research.

### 4. CONCLUSION

We have reviewed recent reports regarding the biological functions of MTs in the CNS. Taken together, MTs regulate Zn²⁺ and Cu²⁺ and inhibit ROS production to suppress neu-
rodegeneration, and stabilize neovascularization in the CNS (Fig. 2). These findings suggest that MT-1 and/or -2 can be therapeutic targets for exudative AMD and DR with ocular neovascularization, and MTS may be useful as a treatment to prevent secondary cell death after ischemic stroke. Furthermore, MT-3 might be valuable to diagnose or treat intractable neurological diseases including glaucoma, schizophrenia, depression, SMA, and ALS. Regulation of expression and function of MTS requires further investigation.

Conflict of Interest  The authors declare no conflict of interest.

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