PERSPECTIVE

Focusing on the protective effects of metallothionein-I/II in cerebral ischemia

Ischemic stroke is a leading cause of death and has a remarkable social and economical impact, which rises with the increasing age of the industrial population. Unfortunately, treatment strategies for cerebral ischemia still remain very limited. Acute reperfusion therapies with either systemic thrombolysis using rt-PA (recombinant tissue plasminogen activator) or interventional recanalization procedures were shown to be highly effective. In addition, there is also a long-existing concept to modulate stroke-associated pathophysiological events such as exitotoxicity, peri-infarct depolarizations, apoptosis and inflammation (Dirnagl et al., 1999). Various such neuroprotective treatment regimes were also shown to be highly successful in rodent stroke models and were shown to reduce the ischemic injury and improve the neurological outcome. In contrast to that, almost all of these successful neuroprotective experimental stroke strategies failed in clinical studies. Current attempts to explain the frustrating failure to translate the promising experimental data to the human patients were based on limited group numbers in experimental studies, publication bias, or hypothesis-driven research approaches. Thus, there is an urgent need to extend pre-clinical studies before further human studies are initiated.

One very promising candidate for a future neuroprotective stroke therapy is the metallothionein (MT) family of proteins. These small proteins are ubiquitously expressed in various tissues and species, are highly expressed in the brain, and are highly inducible during tissue stress and ischemia. Accordingly, MT mRNA was identified as the most significant induced transcript in the early phase of ischemic stroke (Trendelenburg et al., 2002). By the use of transgenic mice strains or cell lines, neuroprotective and neuroregenerative effects of MT have been shown in manifold *in vitro* and *in vivo* studies (*e.g.*, models of ischemic stroke or neurodegeneration).

MTs are 6–7 kDa small cytoplasmatic proteins with a high content of cysteine residues and the ability to bind divalent metal ions like zinc, copper and cadmium. Four MT family members are known in mammalians. MT-I/II is ubiquitously expressed, MT-III expression has been mostly found in the brain and choroid plexus, and MT-IV expression is restricted to some stratified squamous epithelia (Pedersen et al., 2009). During physiological circumstances, Zn seems to be the main metal associated with MT *in vivo* (Pedersen et al., 2009). MT contains a domain structure that encloses four or three ions in a tetrahedral formation. The promoter regions of the highly inducible MT-I and -II genes are stimulated by stress hormones (*e.g.*, glucocorticoids and catecholamines), proinflammantory cytokines (*e.g.*, IL6, IL1-β, TNF-α) *via* the JAK-STAT-Pathway, reactive oxygen species (ROS) through antioxidant response elements, and metal ions which bind to metal containing transcription factors (Pederson et al., 2009).

The brain-specific MT-III is expressed in astroglia and neurons and was initially identified as a growth inhibitory factor (Vallee et al., 1995). None of the other MT isoforms exhibit growth inhibitory activity, indicating that this functional characteristic is specific to MT-III. MT-III knockout mice had enlarged ischemic brain damage after focal (but not after permanent) cerebral ischemia. Moreover MT-III has strong ROS scavenging properties and PEP-1-MT-III fusion proteins can protect against oxidative stress *in vitro* and *in vivo*. MT-III also protects against toxic stress, and protects against amyloid-β. Accordingly, a prominent role in the aging brain and for neurodegenerative diseases like Alzheimer's disease was postulated (Howells et al., 2010).

MT-I and MT-II are the best studied MT isoforms and were shown to have strong neuroprotectivity abilities. Similar functions were ascribed to MT-I and MT-II, which differ by only a single amino acid. Both are mainly expressed in the central nervous system (CNS) in astroglia, but also found in microglia, macrophages and endothelial cells (Pedersen et al., 2009). With its cysteine residues, MT-I/II can protect against ROS and it is speculated that MT regulates gene transcription *via* interaction with zinc-finger domain containing transcription factors (Santos et al., 2011). MT-I/II is induced by tissue-stress and damage, inflammation and toxic amounts of metal ions. Interestingly, MT-I/II was recently shown to be secreted in the extracellular space (Lynes et al., 2006), where it is proposed to act immunmodulatory and neuroprotective (**Figure 1**). The protective effects are thought to be mediated either *via* binding to membrane receptors from the low-density-lipoprotein (LDL)-family, especially by LDL receptor-1 and -2 (megalin receptor) or by a direct metal- or ROS-scavenging effect (West et al., 2011). MT-I/II was shown to induce growth and trophic hormone expression and promote angiogenesis, neurogenesis, axonal sprouting and expression of anti-inflammatory cytokines. MT-I/II also displays neuroprotective abilities *in vivo* (Santos et al., 2012), *e.g.*, in murine models of Parkinson's disease or multiple sclerosis.

MT-I/II also modulates the immune response: whereas MT-I/II was shown to stimulate lymphocyte proliferation (Lynes et al., 2006), it has been shown to suppress a T-dependent humoral response. Some of these effects of MT are potentially based on effects on the Zn metabolism, in the whole body, but also in specific cells. The discovery of the interplay between MT and glutathione disulfide provides an explanation for the release of Zn from MT (Pederson et al., 2009). A further important mechanism of MT-I/II actions is the ROS-reducing potential of MT. ROS are activators of the apoptotic cascade and MT-I/II has ROS-scavenging properties, which depend on the associated metals. Antiapoptotic effects of MT-I/II may correlate with the folding of p53 which regulates DNA-binding, and is dependent on zinc concentration (West et al., 2011).

There are various arguments for a significant neuroprotective role of MT-I/II in ischemic brain injury since many years (Santos et al., 2012). This view is further strengthened by the finding that MT mRNA was found to be the most significant induced gene in the early phase of reperfusion in a murine experimental stroke model analyzed by the use of a whole-transcriptome screening assay (Trendelenburg et al., 2002). MT-I/II was found to be induced mainly in reactive glia and phagocytic cells at the infarct border zone. Endogenous MT-I/II protects against ischemic brain injury, which was shown by the use of transgenic mice that over-express MT-I and have reduced infarct volumes and MT-I/II knockout mice that have larger infarct volumes after experimental stroke compared to wild-type mice. It is speculated that the prominent expression of MT in activated astrocytes in postischemic tissue protects against neuronal death and inflammation.

Whereas MT-I/II was long considered as a pure intracellular protein, there is increasing data of an extracelluar role of MT: reactive astrocytes were able to secrete MT-I/II into the extracellular compartement, where it could act together with other neuroprotective astroglial growth factors and could promote axonal regeneration (West et al., 2011). In agreement with that, MT could also be detected in blood and in the cerebrospinal fluid in a rat model of brain injury (West et al., 2011).

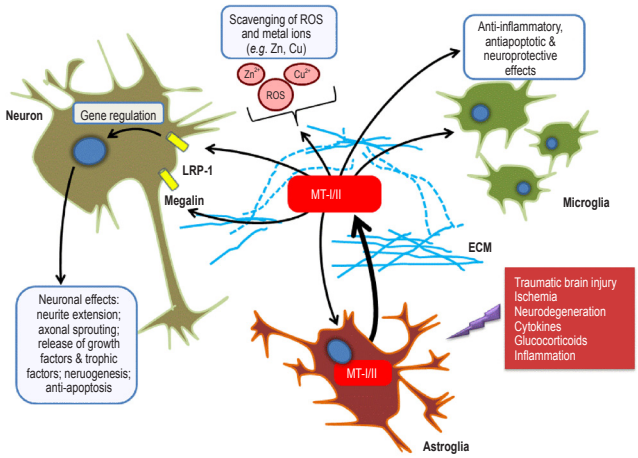

Figure 1 Neuroprotective effects of metallothionein-I & -II (MT- I/II) in the ischemic brain tissue.
ECM: Extracellular matrix; LRP-1: low density lipoprotein receptor-related protein 1; LRP-2: megalin-receptor; ROS: reactive oxygen species.

Following a suspected extracellular role of MT, protective abilities of exogenous applied MT were investigated in different models of neurological diseases. Despite recent speculation that megalin-receptors may help MT to enter the brain there is no proof yet that MT-I/II can cross the intact brain-blood barrier (West et al., 2011). Furthermore, plasma MT-I/II levels drop rapidly after injection due to the rapid loss through the urine (Lewis et al., 2012). Nevertheless, neuroprotective effects of extracellular MT-I/II were shown in different diseases (Santos et al., 2012): exogenous MT-II application decreased the neurological deficit and the mortality in a rat model of multiple sclerosis (MS), and increased neuroregeneration was shown after cerebral ischemia. Intraperitoneal treatment of MT-I/II in a model of focal cryolesion brain injury reduced oxidative stress and neuronal apoptosis. It is speculated that systemically applied MT-II could reach the brain in models of experimental autoimmune encephalomyelitis through the damage of the brain-blood barrier. Very recently intraperitoneal injection (i.p.) of MT-II in a rat model of transient cerebral ischemia was shown to protect against ischemic injury *in vivo*.

Endogenous MT is thought to be actively secreted by astroglia and picked up by neurons through the LRP-2 (megalin) and the LRP-1 receptor (West et al., 2011). Megalin-mediated internalization of MT-II was shown *in vitro*, and megalin is mainly expressed in ependyma and neuronal cells. MT-mediated activation of megalin receptor triggers intracellular activation of transcription factors, which involves the phosphoinositide 3-kinase pathway and the cAMP response element binding protein (CREP).

Due to the existing controversies in the MT field, we wonder if intraperitoneally applied MT-II could protect against cerebral ischemia in a standard model of focal experimental stroke, and if potential effects are counteracted by systemic thrombolysis as a 'gold standard' to treat acute stroke patients (Eidizadeh et al., 2015). Interestingly, reduced infarct volumes and improved neurological outcome after i.p. injection of MT-II were only observed after mild transient cerebral ischemia in mice, whereas i.v. application in a more stringent model of experimental stroke failed to reveal a significant benefit of MT-II application *in vivo* with regard to the resulting infarct volumes. At least, MT-II proved not to cause relevant toxic effects when applied together with rt-PA in murine transient cerebal ischemia *in vivo*, which is a prerequisite before clinical stroke studies can be initiated. Interestingly, mice treated with MT-II and rt-PA had an improved survival ratio after 48 hours of reperfusion after MCAO when compared to rtPA-only treated control mice. Exogenous MT-II also protected primary neuronal cell against oxygen-glucose deprivation *in vitro*. Gene expression analysis revealed decreased expression of pro-inflammatory genes after MT-II treatment *in vivo*; however it remains unresolved if these data should be interpreted as causal relationships or as only secondary bystander effects. In addition to these limitations (*e.g.*, model-specific effects), it must be noted that effect sizes in exogenously MT-treated animals did not reach observed benefits of endogenously (and intracellularly) expressed MT, as revealed by the use of MT-I/II knockout or MT-overexpressing transgenic mice.

In conclusion, there are very strong arguments for a prominent neuroprotective role of MT-I/II in ischemic injury. Uncertainties with regard to pharmacokinetics and the exact place of action still remain and further long-term studies involving different species are needed, before clinical trials with metallothionein could be initiated. However, unresolved exact ways of molecular action should not hinder further evaluation of this exiting small, highly expressed, highly inducible, pro-survival and anti-inflammatory protein, which exists in a whole plethora of organisms, including plants, fungi, bacteria, and mammalians.

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***orcid:** 0000-0001-9277-6241 (George Trendelenburg)*
***doi:** 10.4103/1673-5374.182689* ***http**://www.nrronline.org/*
***How to cite this article:** Eidizadeh A, Trendelenburg G (2016) Focusing on the protective effects of metallothionein-I/II in cerebral ischemia. Neural Regen Res 11(5):721-722.*

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