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Metallothionein in the central nervous system: roles in protection, regeneration and cognition

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Abstract

Metallothionein (MT) is an enigmatic protein, and its physiological role remains a matter of intense study and debate fifty years after its discovery. This is particularly true of its function in the central nervous system (CNS), where the challenge remains to link its known biochemical properties of metal binding and free radical scavenging to the intricate workings of brain. In this compilation of four reports, first delivered at the 11th International Neurotoxicology Association (INA-11) meeting, June 2007, the authors present the work of their laboratories, each of which gives an important insight into the actions of MT in the brain. What emerges is that MT has the potential to contribute to a variety of processes, including neuroprotection, regeneration, and even cognitive functions. In this article, the properties and CNS expression of MT are briefly reviewed before Dr Juan Hidalgo describes his pioneering work using transgenic models of MT expression to demonstrate how this protein plays a major role in the defence of the CNS against neurodegenerative disorders and other CNS injuries. His group's work leads to two further questions, what are the mechanisms at the cellular level by which MT acts, and does this protein influence higher order issues of architecture and cognition. These topics are addressed in the second and third sections of this review by Dr Adrian West, and Drs Edward Levin and Donnie Eddins, respectively. Finally, Dr Michael Aschner examines the ability of MT to protect against a specific toxicant, methylmercury, in the CNS.

Introduction

Fifty years ago, an unusual cadmium binding protein was isolated from horse kidney (Margoshes and Vallee, 1957). Due to its high content of metals and cysteine residues, this protein was named metallothionein (MT) (Kagi and Vallee, 1961; Kagi and Vallee, 1960). In these 50 years, an overwhelming flow of information about these proteins has been published. It is clear that they constitute a superfamily of proteins, widely distributed in the animal

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kingdom as well as other phylogenetic groups (Andrews, 2000; Bremner, 1987a; Coyle et al., 2002; Ghoshal and Jacob, 2001; Hamer, 1986; Vasak and Hasler, 2000). In humans, the MT genes are tightly clustered in the q13 region of chromosome 16 (Karin et al., 1984a; Palmiter et al., 1992; Quaife et al., 1994; West et al., 1990), consisting of 7 functional MT-I genes (*MT-1A*, *-B*, *-E*, *-F*, *-G*, *-H* and *-X*) and a single gene encoding each of the other MT isoforms, namely MT-II (the *MT-2A* gene), MT-III and MT-IV. Mice have a much simpler MT gene structure, with only one functional gene for each isoform MT-I to MT-IV, all located on chromosome 8 (Cox and Palmiter, 1983; Palmiter et al., 1992; Quaife et al., 1994; Searle et al., 1984). The genes *Mt1* and *Mt2* are expressed coordinately in most tissues including the central nervous system (CNS) (Campagne et al., 2000; Searle et al., 1984; Yagle and Palmiter, 1985) *et al.*, 2000), while *Mt3* and *Mt4* show a much more restricted tissue expression (mainly CNS and stratified squamous epithelia, respectively). The evolutionary retention of MT genes argues that they contribute important physiological properties; nonetheless these remain elusive to some extent.

All CNS MTs are composed of a single polypeptide chain of 61–68 amino acids, 20 of which are highly conserved cysteine residues, and there are no disulfides, aromatic amino acids or histidine. A major feature of their amino acid sequences is the occurrence of Cys-Xaa-Cys, Cys-Xaa-Yaa-Cys and Cys-Cys motifs, where Xaa and Yaa stands for an amino acid residue other than Cys (Kagi and Schaffer, 1988). These proteins usually bind 7 divalent metal ions (eg Zn(II)) and up to 12 monovalent copper ions, partitioned into two metal-thiolate clusters (Bogumil et al., 1998; Faller et al., 1999; Vašák M, 1994). Each cluster is located in a separate protein domain designated α (residues 32–61) and β (residues 1–31). When compared with the classical 61–62 amino acid sequences of MT-I and -II (hereafter referred to generically as MT-I/II, unless otherwise indicated) the sequence of MT-III (68 amino acids) shows two inserts: a single Thr in the N-terminal region and an acidic hexapeptide in the C-terminal region (Uchida et al., 1991a).

It is generally accepted that the expression of MT-I/II proteins is highly inducible in response to a range of stimuli, including metals, hormones, cytokines, oxidative agents, inflammation and stress (Bremner, 1987b; Sato and Bremner, 1993). *Mt-1* and *Mt-2* genes are co-ordinately regulated in mice by metals and glucocorticoids (Searle et al., 1984) (Yagle and Palmiter, 1985). Metal induced synthesis is mediated through the action of short cis-acting DNA sequences known as metal responsive elements (MREs), which are present in the promotor region of all mammalian MT genes (Karin et al., 1984b); (Radtke et al., 1993), and is mediated mainly by metal response element-binding transcription factor (MTF-1), a zinc sensitive trans-acting factor (Andrews, 2000; Radtke et al., 1993; Westin and Schaffner, 1988). Similarly, glucocorticoid responsive elements (GREs) are responsible for expression of some MT genes in response to glucocorticoids (Karin et al., 1984b) (Kelly *et al.*, 1997). MT gene expression is also regulated by antioxidant response elements (AREs), although some MREs also respond to oxidants, again MTF-1 being involved (Samson and Gedamu, 1998). Increased levels of MT-I/II in response to inflammatory factors is very likely to be influenced by cytokines such as interleukin (IL-6) through signal transducer and activator of transcription (STAT) factors (Lee *et al.*, 1999). Nevertheless, it is feasible that multiple signals participate in stress, inflammation and oxidative stress induction of the MT genes (Figure 1). The regulation of MT-III and MT-IV gene expression is poorly known.

It has been demonstrated that MT-I/II occur throughout the brain and spinal cord, and that the main cell expressing these MT isoforms is the astrocyte, especially the reactive astrocyte (Holloway *et al.*, 1997). Nevertheless, MT-I/II expression is also found in ependymal cells, epithelial cells of choroid plexus, meningeal cells of the pia mater and endothelial cells of blood vessels. Neurons appear to express MT-I/II to a much lower extent than astrocytes, while in the normal brain, oligodendrocytes and microglia are essentially devoid of MT-I/II, but the

latter cells do upregulate MT-I/II expression in response to injury (Hidalgo *et al.*, 2001). MT-III was discovered as a putative factor important in Alzheimer disease (Uchida *et al.*, 1991b). In contrast to MT-I/II, there is still significant uncertainty regarding the cellular source of MT-III in the CNS, and astrocytes as well as neurons have been suggested to be the main cellular sources (Kobayashi *et al.*, 1993); (Masters *et al.*, 1994b; Uchida, 1994b);(Hidalgo *et al.*, 2001).

It is clear from the literature that MT-I/II and MT-III not only show a distinct pattern of expression in the CNS, but also respond differently to a number of insults. MT-I/II are typically upregulated in response to tissue injury, even to subtle insults. For instance, brain MT-I/II are known to be upregulated by psychogenic stress (Hidalgo *et al.*, 1990) or by the administration of bacterial endotoxin (Palmiter *et al.*, 1992; Searle *et al.*, 1984), glutamate analogues (Acarin *et al.*, 1999b; Dalton *et al.*, 1995), cryogenic injury (Penkowa *et al.*, 1999a; Penkowa *et al.*, 1999c), or by stroke/ischemia (Campagne *et al.*, 1999; Campagne *et al.*, 2000; Neal *et al.*, 1996; Tang *et al.*, 2002; Trendelenburg *et al.*, 2002);. It is thus clear that MT-I/II may play an important role in the overall response of the brain to damage, a response that is thought to be orchestrated by a number of cytokines in a complex manner (Allan, 2001). Results obtained with microarrays and transgenic mice with null mutations have demonstrated that IL-6 (Poulsen *et al.*, 2005) and TNF- α (Quintana *et al.*, 2007) play major roles in the response of the cortex to injury. These studies also demonstrated that both cytokines regulate MT-I/II expression, which is in line with results obtained with transgenic mice overexpressing either IL-6 (Carrasco *et al.*, 1999; Hernandez *et al.*, 1997) or TNF- α (Carrasco *et al.*, 2000a) and with the presence of STAT response elements in the promoters (Lee *et al.*, 1999). In contrast to MT-I/II, MT-III expression shows up- or downregulation depending on the model, time, etc. in animal models of brain injury. MT-III expression has been shown to be increased by stab wounds (Anezaki *et al.*, 1995; Hozumi *et al.*, 1995; Hozumi *et al.*, 1996) and kainic acid administration (Anezaki *et al.*, 1995), but decreased by cortical ablation of the somatosensory cortex (Yuguchi *et al.*, 1995a), facial nerve transection (Yuguchi *et al.*, 1995b), and middle cerebral artery occlusion (Inuzuka *et al.*, 1996). A biphasic response of MT-III to CNS injury, with initial downregulation followed by upregulation, was observed in response to N-methyl-D-aspartate (NMDA) (Acarin *et al.*, 1999a) or to a cryolesion (Penkowa and Hidalgo, 2000b; Penkowa *et al.*, 1999c).

Tissue injury elicits an inflammatory response and oxidative stress. There is increasing experimental evidence that oxidative stress contributes significantly to the neuropathology of several adult neurodegenerative disorders as well as to stroke, trauma, seizures and neuronal degeneration caused, among other reasons, by persistent activation of glutamate-gated ion channels (Coyle and Puttfarcken, 1993). As could be expected, brain MT-I/II levels have been consistently reported to be increased in Alzheimer's disease (Duguid *et al.*, 1989; Uchida, 1994a) (Adlard *et al.*, 1998; Chuah and Getchell, 1999; Zambenedetti *et al.*, 1998), Pick's disease (Duguid *et al.*, 1989) short-course Creutzfeld-Jakob disease (Kawashima *et al.*, 2000), amyotrophic lateral sclerosis (Sillevis Smitt *et al.*, 1992) (Sillevis Smitt *et al.*, 1994) (Blaauwgeers *et al.*, 1996), multiple sclerosis (Lock *et al.*, 2002; Penkowa *et al.*, 2003b), and aging (Suzuki *et al.*, 1994).

Taken together, the above studies strongly suggest a significant role of MT-I/II and MT-III during neurodegenerative diseases and in response to brain injury, but demonstration of their involvement in specific processes in the CNS has been problematical, due in large part to the broader lack of a definitive understanding of the physiological role for MT in any tissue. In the sections to follow, four research groups present their investigations into the involvement of MT in physiological and protective CNS processes. It is interesting, and consistent with the long history of MT investigation, that convincing arguments can be made for a multi-faceted role of MT, spanning normal physiological processes and also a major reactive role in the face of injurious events such as toxicity or lesion.

Use of transgenic models to explore the role of MT in CNS disorders (J.H.)

The generation of genetically modified mice which overexpress (Dalton et al., 1996) or do not produce MT-I and MT-II (Masters et al., 1994a; Michalska and Choo, 1993) has been a key strategy for understanding the roles of these proteins in the CNS. Mice overexpressing MT-I were partially protected against mild focal cerebral ischemia and reperfusion, since the volume of affected tissue was smaller and the motor performance (3 weeks after the lesion) better (Campagne et al., 1999). Conversely, mice with null mutations in *Mt-1* and *Mt-2* (hereafter MT-I/II null mice) developed approximately threefold larger infarcts than wild-type mice and a significantly worse neurological outcome (Trendelenburg et al., 2002). These results highlight an important role of MT-I/II to cope with ischemic damage of the brain. Other studies have clearly demonstrated a similar essential role to cope with damage elicited by kainic acid-induced seizures (Carrasco et al., 2000b), the gliotoxin 6-aminonicotinamide (Penkowa et al., 2002; Penkowa et al., 1999b), 6-hydroxydopamine (Asanuma et al., 2002), mutated Cu,Zn-superoxide dismutase (Nagano et al., 2001; Puttaparthi et al., 2002a, 2002), multiple sclerosis models (Penkowa et al., 2001a; Penkowa et al., 2003a), traumatic brain injury (Giralt et al., 2002b; Penkowa et al., 1999b; Penkowa and Hidalgo, 2000a), and transgenic IL-6-induced neuropathology (Giralt et al., 2002a; Molinero et al., 2003; Penkowa et al., 2003a). Overall the results obtained in these studies are compatible with a role of MT-I/II decreasing oxidative stress, inflammation and apoptosis in the CNS, which is in accordance with results in other tissues (Kang YJ, 1997; Kondo et al., 1995; Lazo et al., 1995; Youn et al., 2002). It is worth noting again that besides being potent antioxidant proteins, MT-I/II are induced by oxidative stress (Andrews, 2000; Aschner, 1996a; Sato and Bremner, 1993). Alzheimer disease is the most prevalent neurodegenerative disease, and as stated above MT-I/II levels are consistently elevated; recently MT induction in response to amyloid plaques/production has been demonstrated in three different mouse models of AD (Carrasco *et al.*, 2006), and thus are suitable models for analyzing the putative neuroprotective role of MTs.

The exact mechanisms through which MT-I/II elicit these neuroprotective effects are ill defined, and may include not only the putative roles of the intracellular protein but also those of the extracellular one: we have shown that exogenously applied MT-II protein mimics MT-I transgenic overexpression, causing a significant clinical improvement in the animal model of multiple sclerosis experimental autoimmune encephalomyelitis (Penkowa and Hidalgo, 2000a; Penkowa and Hidalgo, 2003), and protecting against traumatic brain injury (Chung et al., 2003a; Giralt et al., 2002a), which opens exciting perspectives about the use of the MT family as therapeutic agents. A microarray study comparing MT-I/II null mice with wildtype mice clearly shows the complexity of the challenge of the understanding fully the roles of these proteins (Penkowa et al., 2006).

MT-III was discovered unexpectedly while pursuing putative mechanisms underlying the neuropathology of Alzheimer's disease (Uchida et al., 1991b). It was originally suggested that MT-III is down-regulated during AD, but unfortunately this is not a consistent finding (Amoureux et al., 1997; Carrasco et al., 1999; Erickson et al., 1994; Tsuji et al., 1992; Uchida, 1994b; Uchida et al., 1991b; Yu et al., 2001). Also, in line with the unclear nature of its response following injury, MT-III expression increases or decreases in other human diseases such as Down syndrome (Arai et al., 1997), Creutzfeldt-Jakob disease (Kawashima et al., 2000), pontosubicular necrosis (Isumi et al., 2000), Parkinson disease, meningitis, or amyotrophic lateral sclerosis (Uchida, 1994b). As with MT-I/II, the generation of genetically modified mice (Erickson et al., 1997; Erickson et al., 1995) could help significantly to understand the potential biological roles of MT-III. In normal conditions, MT-III null mice do not show an appreciable phenotype (Erickson et al., 1997), but if challenged with the seizures elicited with the glutamate analogue kainic acid, they showed enhanced sensitivity, convulsing longer and having greater mortality than littermate controls, and showing increased neuronal death in the CA3 pyramidal

cell layer of hippocampus; transgenic mice overexpressing MT-III showed the opposite trends (Erickson et al., 1997). Consistent with these results, MT-III was found to prevent glutamate neurotoxicity in primary cultures of cerebellar neurons (Montoliu et al., 2000). The increased neuronal death in the CA3 following kainic acid treatment of MT-III null mice has been confirmed recently, but these authors have also shown a *decreased* neuronal death in the CA1 area (Lee et al., 2003), indicating a protective or detrimental role of MT-III depending on the brain area. In the transgenic G93A SOD1 mice (a mouse model of ALS), crossed with MT-III null mice (Puttappathi et al., 2002b), the deficiency of MT-III potentiated motor neuron impairment as revealed by stride length and grip strength, likely because of increased motor neuron death. In line with this study, a recent report shows that an adenoviral vector encoding rat MT-III prevents the degeneration of injured motoneurons (Sakamoto et al., 2003). In the cryolesion model of cortical injury, MT-III deficiency did not affect the inflammatory response, oxidative stress and apoptotic death (Carrasco et al., 2003), in sharp contrast to what is observed in MT-I/II deficient mice (see above). However, MT-III deficiency did increase the expression of some neurotrophins and other factors that may significantly affect neuronal survival and/or growth, such as GAP43 (Benowitz and Routtenberg, 1997; Bibbel M, 2000), which would support an inhibitory role of MT-III in line with some *in vitro* bioassays (Chung et al., 2002a; Chung et al., 2002; Chung et al., 2003b; Chung and West, 2004b; Erickson et al., 1994; Sewell et al., 1995; Uchida et al., 1991b). A recent report analyzing the response of MT-III null mice in a peripheral nerve model supports a role of MT-III as an inhibitory factor of neuronal sprouting (Ceballos et al., 2003), since axonal regeneration was faster as substantiated electrophysiologically and histologically. Thus, it might be envisaged that MT-III could serve different functions in the CNS, promoting neuronal survival (perhaps only in specific neuronal populations such as motor neurons or hippocampal CA3 neurons) or death (hippocampal CA1 neurons) while inhibiting neuronal sprouting. The mechanisms underlying these effects remain to be established despite much effort with *in vitro* studies since again significant discrepancies are observed in the literature (i.e. (Uchida et al., 2002) vs. (Chen et al., 2002; Shi Y, 2003) for instance).

Thus, a considerable body of work has demonstrated that transgenic models in which MT expression is modified develop important phenotypes after injury, excitotoxicity or after the onset of neurological disorders, with a strong theme that MT-I/II have regenerative and protective properties. The interesting properties of MT-III following challenge are less clear. These animals will continue to be a valuable resource for investigating the role of MT specifically, and the pathological basis of neurological damage in general.

Interactions between neurons, astrocytes and metallothionein after CNS injury (A.K.W.)

An unexpected outcome of investigation of MT in the CNS is that it is shedding light on how glial cells in the CNS interact to help or hinder the recovery of neurons from physical and chemical injury. Following neuronal injury, astrocytes respond in a variety of ways that change over time and according to their distance from the lesion site (Myer et al., 2006). A common theme however is that the astrocytic response is complex and contains both inhibitory and stimulatory components towards regenerative neuronal growth after injury, each of which contributes to the overall outcome following injury. Understanding this response is essential to the development of therapeutic strategies for any CNS injury or degenerative condition and is likely to contain multiple points at which a therapeutic strategy might act. As an example of how astrocytes can influence neuronal regeneration, Penkowa and Hidalgo (Giralt et al., 2002a; Penkowa et al., 1999a), as discussed above, showed that mice lacking proteis produced predominantly by astrocytes in the CNS, MT-I/II exhibited significantly worse outcomes following a range of CNS injuries. Likewise, MT-I/II deficient animals had severely worse outcomes following stroke, the onset of EAE (a model for MS) and motor neuron disease

(Campagne et al., 1999; Penkowa and Hidalgo, 2001; Puttapparthi et al., 2002b). Hence, perturbation of a purely astrocytic protein has major consequences in the injured CNS, including an increase in apoptotic neurons and deficient neuronal regenerative growth. Indeed, genetically modified animals have been produced that exhibit the full range of possible astrocytic MT-I/II expression, ranging from null to overexpressing strains, and there is an extremely robust correlation between MT-I/II expression and the ability of the animal to recover from CNS insult or degenerative disease (Hidalgo et al., 2001; Penkowa et al., 1999b; Penkowa et al., 2001b; Xie et al., 2004). No definitive mechanism has been demonstrated for these observations, including the key question of how these astrocytic proteins promote the recovery of injured neurons. MT-I/II are not significantly expressed in neurons in either the normal or injured state (Chung et al., 2004a; Dittmann et al., 2005; Holloway et al., 1997; West et al., 2004).

There are a number of ways that MT-I/II might potentially enhance the ability of astrocytes to promote neuronal regeneration, for example by zinc binding, with roles in metal homeostasis, or free radical scavenging (Hidalgo et al., 2001; West et al., 2004) although their function in any tissue remains to be definitively described. Since they demonstrably accumulate in the astrocytic cytoplasm after neuronal injury (Chung et al., 2004b), MTs likely act within the astrocyte itself, and they may, for example, be part of the mechanism by which astrocytes handle toxic intermediates such as reactive oxygen species. Astrocytes produce a number of proteins which are induced following injury and which are known to be strongly protective and as well as MT-I/II (Chung et al., 2004b; Holloway et al., 1997) include proteins such as Cu,Zn, superoxide dismutase (Turner et al., 2005), HSP70 (Robinson et al., 2005), prion protein (Lima et al., 2007) and S100 β (Businaro et al., 2006). In this model, MT-I/II and other protective proteins act primarily to allow astrocytes to reduce the noxious environment surrounding a lesion thereby increasing the likelihood of neuronal survival and regeneration (Chung and West, 2004a; Chung et al., 2007). This may be a partial explanation for the phenomenon of reactive astrocytes in which these cells respond to neuronal stress with a program of morphological and gene expression changes.

However, we have shown that the role of MT-I/II is more complex than simply acting within astrocytes and we have demonstrated, for example, that MT-I/II strongly increases post-injury regenerative sprouting when added directly to injured neurons in culture (Chung et al., 2003b). In these experiments there are no glial or immune system cells present, showing that MT-I/II can act directly on injured neurons, outside of the context of the astrocyte cytoplasm. These experiments have been extended by work done elsewhere and we now know “exogenous” MT-I/II strongly promote regenerative neurite growth of cortical (Chung et al., 2003b), cerebellar (Ambjorn et al., 2007; Kohler et al., 2003) and retinal ganglionic cells (Fitzgerald et al., 2007) suggesting that there is a robust and generic neuronal response to extracellular MT-I/II. The physiological significance of these findings therefore hinges on whether MT-I/II are actually released by astrocytes, something which would initially appear to be unlikely since they do not have a secretory leader sequence. However, we have now unequivocally demonstrated that i) cultured astrocytes can release significant amounts of MT-I/II into medium after stimulation by zinc and Il-1, in an active, non-lytic manner and ii) substantial amounts of MT-I/II can be observed in the extracellular milieu of lesions by western blotting and immunohistochemistry (Chung *et al.*, under review). These observations are consistent with the hypothesis that a major component of MT-I/II action in the injured CNS follows secretion by astrocytes and thus direct action on injured neurons to enhance regenerative growth.

How does MT-I/II act on neurons to promote regenerative sprouting and growth cone turning? We have shown that MT-I/II tethered to the substrate does not promote neurite extension *in vitro* (Fung, Chung and West, unpublished data). When MT-I/II was directly bound to glass

coverslips, neuronal extension was not observed, yet this could be overcome by adding soluble MT-I/II to the system. One interpretation of this experiment is that MT-I/II must be internalised by neurons to promote neurite extension. To investigate this, we have labelled MT-I/II (Alexofluor₅₉₄) and successfully observed uptake into cultured cortical neurons and retinal ganglionic cells both *in vitro* and *in vivo*. We have also shown that other labelled proteins, including albumin, are not similarly taken up.

We have further shown that a significant proportion of MT-I/II is transported into neurons by the cell surface protein megalin, a scavenger receptor which has been implicated in the uptake of cadmium-MT in the kidney (Klassen et al., 2004; Wolff et al., 2006). Our evidence is that i) neurons in culture and *in vivo* express megalin receptor, ii) the competitive inhibitor of megalin, RAP significantly blocks MT-I/II uptake in cultured cortical neurons and iii) anti-megalín antibodies block MT uptake (Chung and West, unpublished data). Furthermore, anti-megalín antibodies block the ability of MT-I/II to promote neurite outgrowth in mixed retinal cultures (Fitzgerald et al., 2007). The concept that MT-I/II promotes regenerative neurite growth in part by a receptor-mediated mechanism is novel.

Therefore, we now have strong evidence that MT-I/II is produced in astrocytes following neuronal damage, that it can be released by astrocytes *in vitro* and *in vivo*, and that it can be internalised by neurons to produce regenerative growth following injury. Taken together, our recent findings have two key implications. Firstly, the ability of MT-I/II to act directly on neurons to promote regenerative sprouting has major therapeutic potential. Indeed, we have demonstrated that exogenous MT promotes exuberant neuronal extension when applied to cortical lesions (Chung et al., 2003a) and to the cell bodies of lesioned retinal ganglionic cells (Chung *et al.*, under review). The second major implication is that the properties of MT-I/II offer insight into the unanswered question of how astrocytes are able to support neuronal regeneration after injury: we propose they are able to produce and release proteins normally considered to be cytoplasmic which then act on neurons to promote axonal recovery, in this case by a mechanism dependent in part on neuronal uptake. We have proposed a theoretical model on the basis of our experimental findings (Chung and West, 2004a; Chung et al., 2007) which may have general applicability to protective astrocytic proteins other than MT-I/II. In particular, it is now becoming clear that proteins such as Cu,Zn superoxide dismutase (Turner et al., 2005), HSP70 (Robinson et al., 2005), prion protein (Lima et al., 2007) and S100 β (Businaro et al., 2006) can be found outside the cell, and that these might confer protective effects on injured neurons.

Both of these implications are important. Current therapeutic agents to assist in CNS injury and disease are few and are generally unsatisfactory, whilst our understanding of the interaction between glia and injured neurons is a poorly understood field which limits our attempts to develop treatments for injury and neurodegenerative illness. In ongoing investigations, we are examining the potential of MT-I/II as a therapeutic agent in CNS disorders and have obtained promising outcomes in animal models of cortical injury (Chung et al., 2003a) and optic nerve lesion..

A number of important questions remain unanswered at this time. For example, little is known about how the interaction between megalin and MT-I/II promotes regenerative growth, and which intracellular pathways mediate this effect. It remains possible that the action of MT-I/II depends on its zinc binding property and that the role of MT-I/II uptake by injured neurons is to ferry zinc to a physiologically important location within the cell. Likewise, the free radical scavenging properties of MT-I/II might also be similarly targeted to a sensitive intracellular site after neuronal uptake. Alternatively, a megalin MT-I/II complex might activate signalling pathways within the cell that promote regenerative neuronal growth (Fig 3). The conundrum

that the related isoform, MT-III appears to inhibit neuronal growth under some conditions is also interesting; it has not yet been established whether this isoform interacts with megalin.

In summary, the observation that extracellular MT-I/II have a previously unsuspected capacity to influence neuronal regeneration after injury has implications for our understanding of the interaction of astrocyte and neurons, and also suggests that MT-based molecules might have therapeutic potential in a range of injury and neurodegenerative disorders. It also raises the question of whether MT-I/II might have roles outside regenerative growth of neurons, for example, in development of the CNS or in cognitive processes.

Cognitive Effects of Metallothionein 1 and 2 Knockout in Mice (E.D.L. and D.E.)

MTs are known to act in key ways in the physiology of transition metals, but their roles in neurobehavioral function have not been well characterized. There are reasons to believe that alterations in MT function could alter neural function in ways that would impair behavioral function. For example, the hippocampus, which plays a central role in cognitive functions such as memory depends on correct physiological actions of zinc, which is heavily concentrated in the hippocampal mossy fiber system (Daumas et al., 2004). MT-I/II protect against the adverse effects of both zinc under and overload (Kelly et al., 1996). In the striatum, alterations of copper functions can lead to cognitive and behavioral impairments seen in Wilson's disease (Portala et al., 2001). Other transition metals such as mercury have no identified normal physiological role, but are environmental contaminants, which have known neurotoxic actions, which result in behavioral abnormalities including cognitive impairment (Goulet et al., 2003; Peixoto et al., 2007; Yoshida et al., 2005). Abnormalities in MT function could alter neurobehavioral function through dysregulation of all of these transition metals.

To begin determining the potential roles MTs may play in neurobehavioral function we have tested the effects of knocking out *Mt1* and *Mt2* genes (MT-I/II null mice) on cognitive function. The impact of MT-1/II knockout on choice accuracy and locomotor behavior in the 8-arm radial-arm maze was determined during acquisition and maintenance of response, with drug treatment and after toxicant challenge.

In our initial set of studies (Figure 1) we found that MT-I/II null mice (Jackson Labs) showed a significant ($p < 0.001$) impairment in choice accuracy on the 8-arm radial maze test of spatial learning and memory (Levin et al., 2006). This was seen both in younger adult and older adult mice (Levin et al., 2006). Interestingly, as shown in Figure 2, the impairment of the MT-I/II null mice vs. their wildtype controls (129 strain) was significantly ($p < 0.05$) ameliorated by acute nicotine treatment (Levin et al., 2006). This indicated that the MT-I/II knockout-induced impairment was not due to learning *per se* because the effect of nicotine was immediate and did not depend on further training in the maze. More likely it was due to attentional or memory aspects of the task which could be immediately changed from session to session as the nicotine treatments changed.

Developmental heavy metal exposure has long been known to impair neurobehavioral function. Physiologic mechanisms controlling metal distribution and metabolism such as MTs can greatly influence the actions of toxic as well as essential metals. However, only now is their role in neurobehavioral functioning being determined. In a recent study we have shown that MT-I/II null mice show an enhanced effect of neonatal mercury (HgCl_2) exposure (Eddins, Levin et al. under review). In that study, a significant impairment in choice accuracy on the radial-arm maze was seen in MT-I/II null mice treated with a history of neonatal mercury exposure during the initial phase of training. During this initial phase neither mercury alone nor the MT-1/2 null mutation alone caused an impairment. To further characterize the role of

metallothionein in learning and memory, mice with deletions of genes for MT-1/II were trained on a win-shift task in an 8-arm radial maze. MT-1/II null mice had similar choice accuracy levels as wildtype controls in the early stage of training, but had poorer accuracy during the later stages as the controls continued to improve. Acute nicotine treatment, which can improve working memory, eliminated the impairment suggesting that the MT-1/II null mice had learned the task across sessions, but that they were deficient in the attentional or memory requirements for accurate performance within each session. Then we replicated this effect and demonstrated that MT-1/II null mice were more vulnerable to the persisting adverse cognitive effects of developmental mercury exposure. Mercury, as mercuric chloride, was injected (0, 2 and 5 mg/kg) on days 7, 14 and 21 after birth. During the early stage of training the MT-1/II null mice exposed to 5 mg/kg mercuric chloride had significantly lower accuracy scores than either wildtype mice exposed to the same mercury dose or MT-1/II null mice not exposed to mercury. Frontal cortical dopamine (DA) showed a differential effect of developmental mercury exposure in MT-1/II null and wildtype mice. The 5 mg/kg mercury dose caused a significant increase in frontal DA levels in the wildtype mice. No such effect was seen in the MT-1/II null mice. MT knockout significantly increased frontal cortical levels of serotonin and its metabolite 5-HIAA regardless of mercury condition. The mercury-induced increased frontal cortical DA level seen in wildtype but not MT-1/II null mice may be relevant to the protection against memory impairment since the MT-1/II null mice showed a significant initial learning deficit with this dose of mercury while the wildtype controls did not. MT-1/II null mice were more sensitive to the persisting adverse cognitive effects of developmental mercury exposure. Alterations in frontal cortical DA response may be related to the functional impairment.

Thus, the absence of MT correlates with quantifiable cognitive changes, and furthermore, this work demonstrates that cognitive changes produced by neonatal exposure to mercury are sensitive to the presence or absence of MT. These pivotal observations are exciting because they provide a direct connection between higher order processes such as cognition and our understanding of the molecular properties of MT in metal binding and cell specific expression. In particular, the linkage with mercury toxicity allows reference to the large body of work in this area, and thereby suggests several specific mechanisms by which MT might interact with neurobehavioural pathways.

The Role of Metallothioneins in Methylmercury (MeHg)-Induced Neurotoxicity (M.A.)

Mercury is a global pollutant, which knows no environmental boundaries. Even the most stringent control of man-made sources of mercury pollution will not eliminate human exposure to potentially toxic quantities, given its ubiquitous presence in the environment. Environmental exposure to mercury occurs primarily via the food chain due to accumulation of methylmercury (MeHg) in fish. Human poisoning outbreaks as a result of food-borne MeHg consumption are evidenced by the tragic epidemics of MeHg poisoning in Japan and Iraq. Because methylation of inorganic mercury species to MeHg by microorganisms is known to take place in waterways, resulting in its accumulation in the food chain, any source of environmental mercury represents a potential source for MeHg poisoning. Anthropogenic sources culminating in the acidification of freshwater streams and lakes in North America, and the impoundment of water for large hydroelectric schemes have led to increases in MeHg concentrations in fish, posing increasingly greater risks to human populations. Latest statistics in the US indicate that 46 states have fish consumption advisories covering 40% of the nation's rivers, lakes and streams. In addition, mercury is a common pollutant in hazardous waste sites in the nation (EPA, 2001). It is estimated that 3–4 million children live within one mile of at least one of the 1,300 + active hazardous waste sites in the USA (EPA, 2001).

Astrocytes make up ~50% of human brain volume (Chen and Swanson, 2003) and perform several functions that are essential for normal neuronal activity, including glutamate uptake (80% of synaptic glutamate), glutamate release, K⁺ and H⁺ buffering, and water transport. The “foot” processes of these cells are known to be closely associated with synapses, axonal tracts, nodes of Ranvier and capillaries. Astrocytes also induce the high electrical resistance (tightness) of the blood-brain barrier (BBB) that limits the transport of noxious substances entering the brain, and modulates optimal transport of nutrients and metabolites (reviewed by (Aschner et al., 1999). During neurodevelopment, cues for axon guidance of migrating neurons are regulated by interactions between axons and astrocytes (radial glia). The understanding of molecular signals that regulate neuron-glia interactions has increased greatly with the advent of molecular and cellular biological techniques, as well as genetically modified mice. Studies in which cell ablations are genetically targeted with ectopic gene expression, and gene knockout with single cell specificity have established distinct roles played by astrocytes during development (reviewed by (Aschner et al., 2002)).

We *do not* maintain that astrocytes are the only cell type affected by methylmercury (MeHg). Nevertheless, there is abundant evidence in support of their *pivotal* role in mediating neurotoxicity, establishing astrocytes as a unique and relevant experimental model for the assessment of mechanisms underlying MeHg-induced cytotoxicity.

- a. Chronic exposure to MeHg in primates is associated with preferential accumulation of MeHg in astrocytes (and to some extent in microglia) (Charleston et al., 1996; Charleston et al., 1994).
- b. MeHg inhibits astrocytic glutamate uptake and stimulates its efflux (Allen et al., 2001a; Aschner, 1996b; Aschner et al., 1993; Brookes and Kristt, 1989), increasing *in vivo* glutamate concentrations in the extracellular fluid (ECF) and sensitizing neurons to excitotoxic injury (Juarez et al., 2002). Damage associated with MeHg correlates with brain areas with dense glutamatergic innervation (Miyamoto et al., 2001). The ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) antagonist, dizocilpine (MK801) protects against neuronal damage induced by *in vivo* MeHg (Miyamoto K, 1999; Miyamoto et al., 2001) (Lafon-Cazal et al., 1993).
- c. MeHg selectively inhibits astrocytic (but not neuronal) uptake systems for cystine and cysteine transport (Allen et al., 2001b; Shanker et al., 2001a; Shanker et al., 2001b), compromising glutathione (GSH) synthesis and the CNS redox potential. Compared with astrocytes, neurons have lower levels of GSH as well as of a second putative antioxidant, MT (Maret, 1994; Philbert et al., 1991) making them more susceptible to the effects of increased ROS. Since GSH synthesis is dependent upon precursors derived from astrocytes (Dringen et al., 1999; (Wang and Cynader, 2000), MeHg-induced inhibition of cystine transport and astrocytic GSH production would ultimately lead to decreased neuronal GSH levels and increased glutamate toxicity.
- d. MeHg-induced ROS formation in astrocytes can be attenuated by antioxidants (Shanker et al., 2002), reversing its functional effects on glutamate uptake inhibition (Allen et al., 2001a; Aschner et al., 2007; Yin et al., 2007).
- e. Co-application of non-toxic concentrations of mercury with glutamate results in the appearance of typical neuronal lesions associated with excitotoxic stimulation (Matyja and Albrecht, 1993).
- f. In human and non-human primates chronic *in vivo* exposure to MeHg is associated with swelling of astrocytes (Charleston et al., 1996; Charleston et al., 1994; Oyake et al., 1966; Vahter et al., 1994), a process associated with release of glutamate into the extracellular fluid.

- g. In the absence of glutamate, neurons are unaffected by exposure to mercury, suggesting that neuronal dysfunction is secondary to disturbances in astrocytes (Brookes, 1992).
- h. Additional data invoke astrocytic cPLA₂ as a target for MeHg toxicity, supporting the notion that cPLA₂-stimulated hydrolysis and release of arachidonic acid (AA) play a role in MeHg-induced neurotoxicity (Shanker et al., 2002).

Thiol groups play fundamental structural and functional roles in protein chemistry, being located mainly within the active catalytic sites of many enzymes. Mercury compounds react specifically with active sulfhydryl (-SH) groups forming complexes of defined stoichiometry, -S-Hg-R. The high affinity of MeHg for the anionic form of -SH groups is responsible for the toxicological behavior of this compound (Hughes, 1957) (Bramanti et al., 1999). The affinity of MeHg for the anionic form of -SH groups (log K, where K is the association constant) is extremely high, on the order of 15–23, whereas its affinity constants for oxygen-, chloride-, or nitrogen-containing ligands such as carboxyl or amino groups are about 10 orders of magnitude lower (Carty AJ, 1979). Indeed, wherever a MeHg compound has been identified in biological media, it has been complexed to -SH-containing ligands. Complexes with cysteine and GSH have been identified in blood (Naganuma and Imura, 1979; Rabenstein and Fairhurst, 1975), and complexes with GSH have been identified in brain (Thomas DJ, 1979), liver (Omata et al., 1978), and bile (Refsvik and Norseth, 1975).

An intriguing concept is that MTs not only account for the preferential accumulation of MeHg in astrocytes, but also endow these cells with tolerance to MeHg. Owing to their inducibility, MTs provide astrocytes with a potential mechanism to attenuate, at least temporarily, the toxicity of MeHg. An astrocyte can be envisioned to experience several stages, beginning with non-exposure, a state of grace that evidently no longer exists in nature, and ending with cell death or pervasive cell dysfunction once tolerance mechanisms have been overwhelmed. Between these extremes lies a continuum of metabolic changes consisting of initial injuries that take place at discrete molecular sites, followed by the metabolic amplification of such injuries. The toxic effects of MeHg become more pervasive until the astrocytes exhibit significant biochemical impairments, affecting the function of juxtaposed neurons. Additional MeHg exposure would presumably hasten the process. Because MeHg turnover in the CNS is very slow, the process may continue without additional exposure. During all stages, the cells may develop tolerance for MeHg, brought about by amplification of the MT genes.

While numerous studies support the ability of inorganic mercury to induce MT expression in various *in vivo* and *in vitro* models (reviewed by (Aschner et al., 2006), 2006), there are few studies on the interaction between MeHg and MT and the ability of MTs to attenuate MeHg-induced toxicity. MeHg was shown to be ineffective in inducing MTs, both in cultured neurons and astrocytes (Kramer et al., 1996a; Kramer et al., 1996b), consistent with *in vivo* findings (Yasutake et al., 1998) in which brain MT levels in rats exposed for 10 days to MeHg (40 µmol/kg per day × 5 days, po) remained unchanged even in the presence of neurological symptoms. The effects of dietary MeHg administration on gene expression were examined by (Gonzalez P, 2005) in three organs (liver, skeletal muscle, and brain) of the zebrafish. Adult male fish were fed for 7, 21, and 63 days with one of three different diets: control diet and two diets contaminated by MeHg at 5 and 13.5 µg of Hg/g. Genes (13 in total) known to be involved in antioxidant defenses, metal chelation, active efflux of organic compounds, mitochondrial metabolism, DNA repair, and apoptosis were investigated by quantitative real-time reverse transcription–polymerase chain reaction and normalized according to actin gene expression. Surprisingly, no change in the expression levels of these genes, including MT-II, was observed in brain samples, although this organ accumulated the highest Hg concentration. Thus, all studies conducted refute the inability of MeHg to induce MT. In rats exposed to MeHg by gastric gavage (5 × 10 mg/kg body wt over a 15-day period), or in drinking water (20 mg

MeHgCl for 14 or 42 days), Hg was detected in undamaged Purkinje neurons and Bergmann glial cells; no Hg was detected in granule cells although these cells had a high incidence of pyknotic nuclei (Leyshon-Sorland et al., 1994). (1994). MT was detected primarily in Bergmann glial cells, Purkinje cells, astrocytes, and glial cells of white matter and no MT was detected in granule cells. The resistance of Purkinje cells to MeHg-induced damage was hypothesized to reflect their ability to transform organic mercurials to inorganic mercury, which in turn, induces the synthesis of radical-scavenging MT molecules.

Similar to the “housekeeping” functions ascribed to MTs in other mammalian tissues, brain MTs are implicated in metal metabolism, cellular repair processes, growth and differentiation where they are likely to serve as the source of zinc for newly synthesized apoenzymes, as well as regulator molecules in gene expression. Additional functions include control of intracellular redox potential, and metal detoxification. The adult human brain consumes >20% of the oxygen utilized by the body although it comprises only 2% of the body weight, indicating that ROS are generated at high rates during oxidative metabolism of the brain. Arrayed against oxygen-free radical-induced damage is an extensive system of defenses capable of scavenging and transforming oxygen-free radicals into non-toxic species. These systems include a range of specific antioxidants such as catalase, GSH peroxidases, and non specific antioxidants such as reduced glutathione (GSH), ceruloplasmin, and transferrin. MT itself has also been invoked as a thiol donor and free radical scavenger, thus providing a line of protection against oxidative damage (Lazo et al., 1995). Accordingly, studies in our laboratory have specifically addressed the ability of MT to attenuate MeHg-induced neurotoxicity in astrocytes. To explore the possibility that astrocytic MTs afford a protective role in heavy metal-induced neurotoxicity, we investigated the effects of MeHg on the induction of MTs both at the mRNA and protein level, and the ability of cultures which express high MT levels (by pre-treatment with ZnSO₄ or CdCl₂) to protect against the cytotoxic effects of MeHg (Aschner et al., 1998; Rising et al., 1995; Yao et al., 1999).

In the first series of studies (Rising et al., 1995), the cytotoxic effect of MeHg on cultured astrocyte monolayers was assayed by measuring its effect on the initial rate of uptake of radiolabeled D-aspartic acid, a non-metabolizable analog of L-glutamate. Treatment of astrocytes with 10 μM MeHg had a significant inhibitory effect on the initial rate (30 minutes) of radiolabeled aspartate uptake. This effect could be completely reversed by pre-treatment of astrocytes with CdCl₂ (48–96 hours) at a concentration as low as 1 μM. To correlate the temporal sequence between astrocytic MT protein levels and resistance to MeHg additional studies were carried out to determine MT levels in astrocytes upon CdCl₂ pre-treatment by means of western blot analysis. MT-I/II protein levels increased in astrocytes pre-treated with CdCl₂ in a time-dependent fashion, with maximal inducibility at 72 hours. Thus, associated with the protective effect of CdCl₂ on MeHg-induced inhibition of D-aspartate uptake is a marked increase in astrocytic MT levels.

Additional studies (Aschner et al., 1998) revealed that pretreatment of astrocytes for 24 hours with 100 μM ZnSO₄ are also associated with a significantly higher MT expression level compared to controls (2.9 fold increase). The relative abundance of MT-I mRNA transcripts in ZnSO₄ pretreated cells showed a 5.6 fold increase compared with controls. Exposure of astrocytes to MeHg (10 μM) led to approximately 20% cell shrinkage, followed by marked swelling with approximately 30% increase in cell volume over baseline. Pretreatment of astrocytes for 24 hours with 100 μM ZnSO₄ abolished the stimulatory effect of MeHg on cell volume (p<0.01), such that the Zn pretreated group was indistinguishable from controls. Since exposure of astrocytes to MeHg is associated with the activation of the Na⁺/H⁺ antiporter, studies were also performed to determine whether the Zn-associated attenuation of MeHg-induced swelling could be correlated with reduced influx of Na⁺. MeHg exposure led to a significant increase in the initial uptake of ²²Na⁺, which was completely abolished by 24-hour

pretreatment of the cells with 100 μM ZnSO_4 . Since a common mechanism of regulatory volume decrease whereby astrocytes re-establish basal cellular volume involves activation of conductive K^+ and Cl^- channels, we determined whether the Zn-associated attenuation of MeHg-induced swelling could be correlated with reduced astrocytic efflux of Rb^+ , a marker of K^+ efflux. Swelling induced by MeHg led to a significant increase in the release of Rb^+ . This effect was significantly attenuated by pretreatment with ZnSO_4 .

In additional studies (Yao et al., 1999), we used routine transfection techniques to assess the potential of MT to mitigate MeHg-induced neurotoxicity. The pGfa2-cLac plasmid, which contains a 2.2 kb promoter of the human GFAP gene was placed in front of the *Escherichia coli* LacZ gene, which in turn was followed by a fragment of the mouse protamine-I gene as a polyadenylation signal. Construction of the recombinant plasmids entailed excision of the LacZ gene by digestion with *Bam*HI and its replacement by blunt end ligation with a 0.55 kb *Hind*III–*Xba*I fragment containing the entire MT-I coding region. These recombinant plasmids were identified with enzyme digestions and confirmed by DNA sequence analysis. Transfection with the pGFAP-MT-I plasmid was associated with a significant increase both in MT-I mRNA and MT-I protein levels compared to controls.

To determine the relationship between the cytotoxicity of MeHg and the relative abundance of MT expression, $\text{Na}_2^{51}\text{CrO}_4$ efflux measurements were carried out. The fractional release of ^{51}Cr is a good index of membrane integrity, and under non-toxic conditions, the basal release of the radiolabeled compound is exceedingly small. As expected, MeHg induced a concentration-dependent increase in the fractional release of ^{51}Cr as its concentration in the perfusion buffer increased. The release of ^{51}Cr in the MT-I transfected groups was lower in pGFAP-MT-I transfected astrocytes compared to their controls, indicative of the neuroprotective effect of MTs.

The series of studies presented above shows that chemical treatments (CdCl_2 or ZnSO_4) or transient transfection with the MT-I gene, are associated with increased expression of MT-I mRNA transcription and MT-I protein synthesis. Although the astrocytic increase in MT-I expression is in most cases relatively modest (<10-fold over controls) it is in close agreement to those published by others. For example, Liu et al. (Liu J, 1992) reported a relatively small 4-fold increase in astrocytic MT protein in response to metal exposure compared to a robust 10-fold increase in MT protein in similarly treated hepatocytes. Kramer et al. (Kramer KK, 1995) reported a 3–4-fold increase in MT expression in cultured murine astrocytes upon zinc exposure. (Hidalgo et al., 1994) reported a 2.25-fold increase in astrocytic expression of MTs subsequent to zinc treatment. The same authors suggested, that compared to other cell types, astrocytes might not be capable of mounting a dramatic MT response to metal exposure, because they possess only a limited capacity to overexpress this protein. The reason for this limited ability to overexpress MTs is unknown, but undoubtedly it may reflect the fact that constitutive MT levels are 10 times higher in astrocytes compared to neurons (Hidalgo et al., 1994). Other reports suggested that MT could be increased between 2.5- and 15-fold in a variety of cell types by homologous and heterologous MT gene transfection (Abdel-Mageed and Agrawal, 1997; Morton et al., 1992), but to the best of our knowledge, such studies in primary astrocyte cultures are lacking.

The results of our studies support the hypothesis that intracellular MT levels confer resistance to MeHg toxicity. MT-I can be efficiently expressed both in primary astrocyte cultures by MT-I gene transfection under the control of the astrocyte-specific GFAP promoter or treatments with CdCl_2 or ZnSO_4 , known potent inducers of MTs. As mentioned above, by virtue of their high -SH content, MTs have a very high affinity for MeHg, potentially providing for a concentrative mechanism for MeHg in astrocytes. Furthermore, induction of MTs may endow the astrocytes, at least during early stages of heavy metal accumulation, with tolerance to

MeHg. The MeHg-MT complex is well suited for the detoxification of MeHg. The stability of MTs against proteolysis is greatly increased after binding to MeHg and the degradative release of the metal results in its association with other MTs. This cycle can keep MeHg in a relatively non-toxic form within the astrocytes, protecting both the astrocytes themselves and juxtaposed neurons from the cytotoxic effects of MeHg. Thus, we propose that astrocytes can play a “double” role in MeHg intoxication. Whereas, at high levels and chronic exposure MeHg can short circuit astrocytic function, at low concentrations of MeHg, MTs may serve to “buffer” MeHg itself, or indirectly attenuate its toxicity and protect vulnerable astrocytic -SH groups, such as those on the glutamate transporter, from MeHg. However, once MT induction mechanisms are overwhelmed, pervasive astrocytic dysfunction leads to failure to control the extracellular fluid, and neuronal impairment. *In vitro* MT enrichment confers resistance against the cytotoxicity of MeHg in multiple assays. This interpretation is consistent with the observation that resistance to heavy metal toxicity is closely related to MT expression levels and raises interesting questions regarding the potential involvement of heavy metals in neurodegeneration where MT synthesis may be compromised.

SUMMARY

Using various experimental approaches we have established that [1] in transgenic models of MT expression this protein plays a major role in the defence against neurodegenerative disorders and other injuries, [2] MTs influence tissue architecture and [3] cognition. Finally, [4] MTs protect against mercury neurotoxicity. The review establishes multi-functional and critical roles afforded by MTs describing a diverse range of processes that are mediated by the unique MT protein family. Fifty years after its discovery, a greater appreciation exists for the MT’s metal binding and free radical scavenging properties, yet clearly its role in the intricate workings of brain is much broader and yet to be fully detailed.

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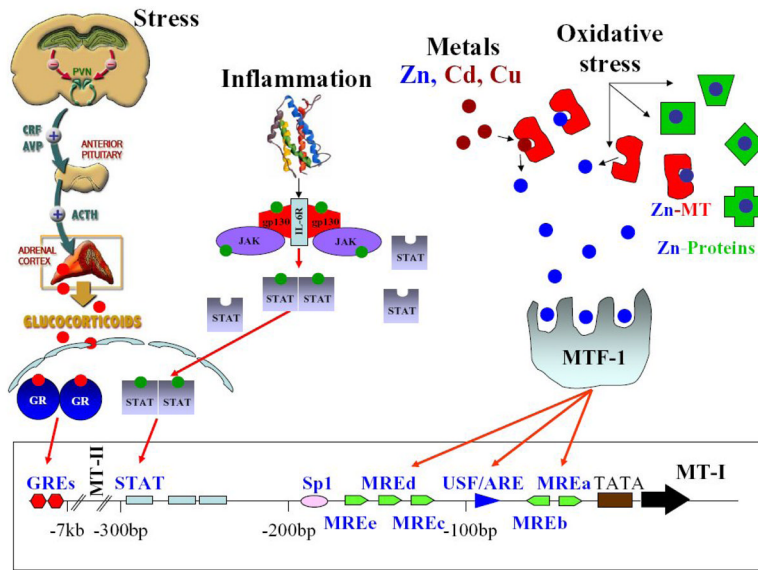


Figure 1.
Metallothionein-I&II regulation

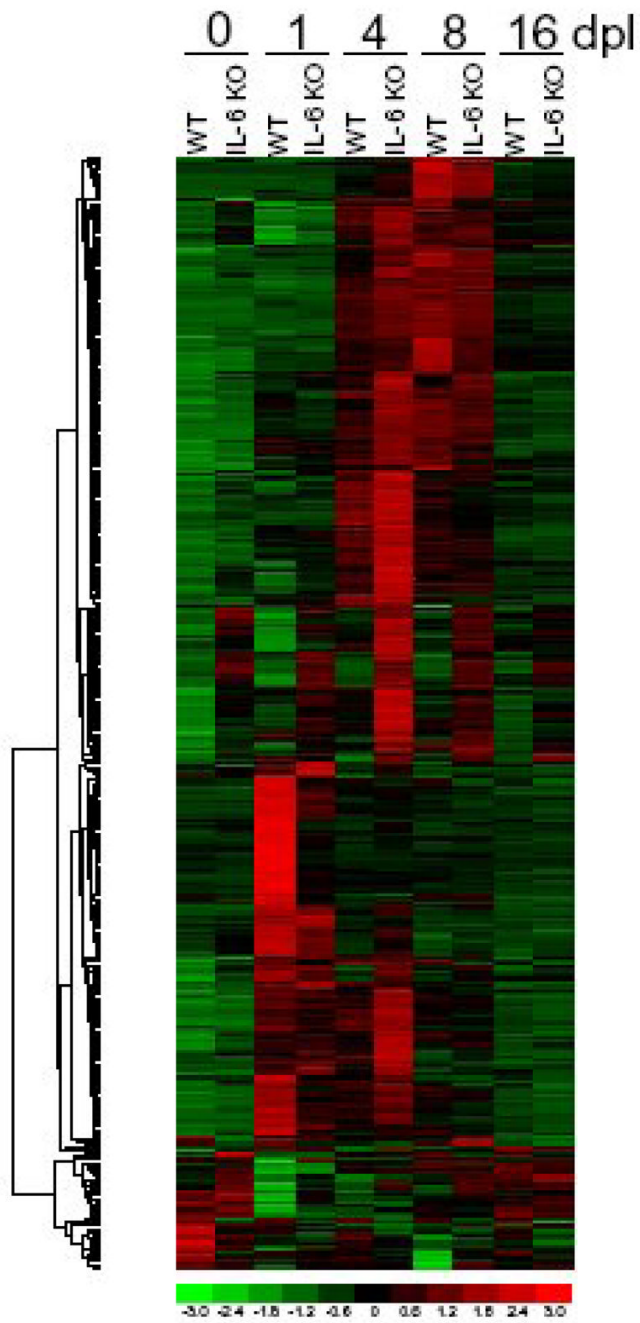


Figure 2. Microarray study with WT and IL-6 KO mice, with 1, 4, 8 and 16 days post lesion as well as unlesioned mice (Poulsen et al., 2005).

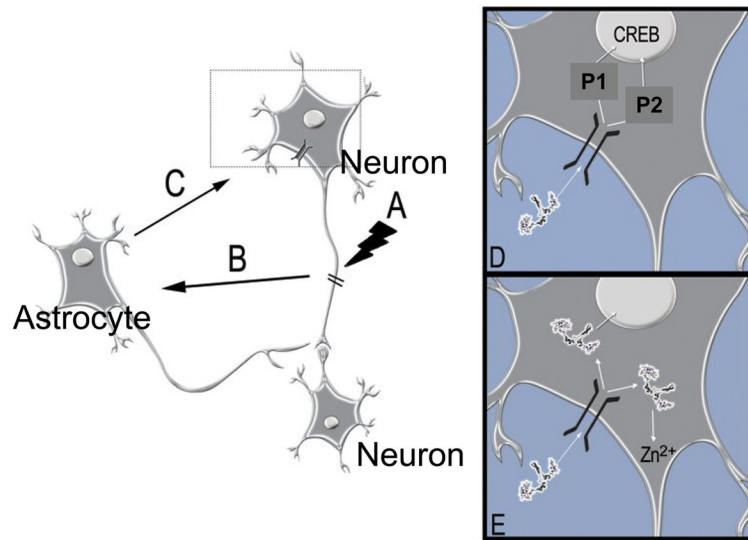


Figure 3.

A model of astrocyte-neuron interaction after injury. Injury to neurons (A) causes signaling (B) to adjacent astrocytes leading them to increase synthesis of intracellular MT-I/II. Following non-lytic release (C), MT-I/II interacts with neurons and promotes regenerative neurite growth via an interaction with the cell surface receptor megalin, either by interaction of the megalin-MT-I/II complex with potential intracellular pathways or (D) by the targeted release of zinc ions into the neuron interior (E).

Radial-Arm Maze Learning in Older Adult Metallothionein Knockout Mice

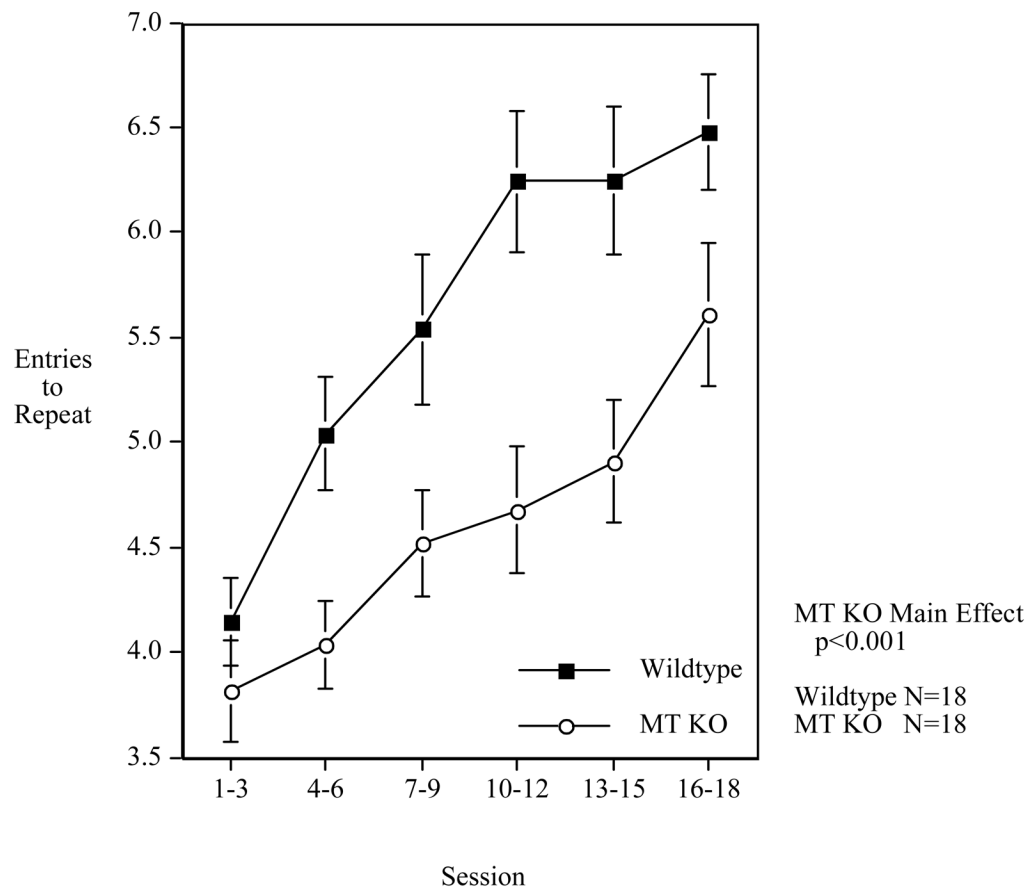


Figure 4. Metallothionein 1 and 2 knockout effects vs. wildtype control (129 strain) performance on choice accuracy during acquisition of choice accuracy (entries to repeat) on the 8-arm radial maze (mean±sem) (Levin et al., 2006)

Radial-Arm Maze Choice Accuracy in Aging Wildtype and MT KO Mice Acute Nicotine

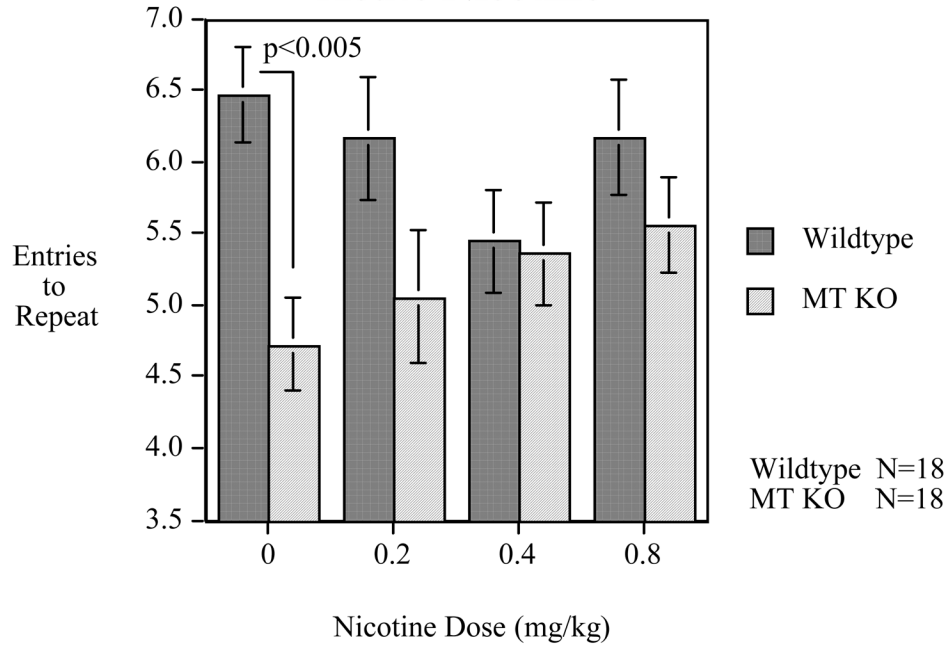


Figure 5. Acute nicotine effects on radial-arm maze choice accuracy deficits (entries to repeat) in Metallothionein 1 and 2 knockout mice vs. wildtype control (129 strain) performance (mean \pm sem).