

**The role of extracellular metallothioneins in the
cellular response to neuronal injury**

By

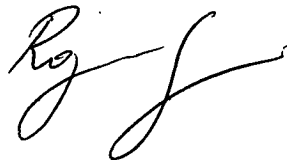
Roger Steven Chung, B.Sc. Hons

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Doctor of Philosophy**

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STATEMENTS

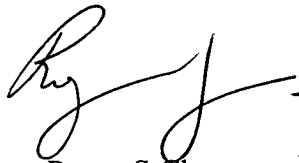
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23/9/03

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SUMMARY

Metallothioneins (MTs) are unusual, cysteine rich proteins, which can sequester heavy metals (including zinc and cadmium), and also have free radical scavenging properties, which allow them to protect cells from cytotoxicity induced by reactive oxygen species. In the last 10 years, interest in the potential roles of these proteins has shifted from metal homeostasis and free radical scavenging to the neurological functions which they may possess. In this regard, it is the brain specific, MT-III isoform, which has been of most interest. This protein exhibits neuronal growth inhibitory properties upon cultured cortical neurons, and also has been proposed to be involved in the development of Alzheimer's disease. The aim of this thesis is to further investigate the proposed neuroactive properties of MTs, at both a functional and biological level, to determine their possible role within the brain.

This study firstly investigated the relationship between structure and biological function of MT-III by investigating the neuronal growth inhibitory activity of a variant MT-III protein previously identified in this laboratory, namely sheep MT-III. This protein was produced recombinantly, and by comparison with recombinant human MT-III, reductions in its metal binding and neuronal growth inhibitory activity upon cultured cortical rat neurons were found. These results indicate the importance of protein structure to MT-III's inhibitory activity, and may also partly explain the susceptibility of sheep to heavy metal induced toxicity.

MT-III has also been proposed to inhibit neurite outgrowth, although all available studies in the literature have alternatively focused upon its ability to inhibit neuronal survival. Using recombinant human MT-III, this study found that MT-III does indeed inhibit initial neurite formation and growth when applied to cultured cortical rat neurons. Furthermore, following axonal transection in culture, MT-III inhibited reactive (or regenerative) neurite sprouting. These results support the hypothesis in the literature that reduced levels of MT-III in the brain allow the aberrant neurite sprouting observed in Alzheimer's disease.

Rather surprisingly, it was found that another MT isoform, human MT-IIA, promoted neurite elongation, reactive sprouting, and growth following injury in the same culture models. At the same time, several reports in the literature demonstrated that MT-I and -II knockout mice had significantly reduced cortical wound healing capacity and that exogenous application of MT-II promoted cortical wound healing. To elucidate whether the neuroactive properties observed in culture within this study were involved in this response, human MT-IIA was applied following focal cortical brain injury in the adult rat. MT-IIA promoted marked neural recovery following injury, suggesting that MT-I and MT-II might act in an extracellular capacity to promote cortical wound healing, as well as their better investigated intracellular roles.

Based upon the demonstration, both within the literature and this thesis, that MTs can modulate neural recovery following injury, it was hypothesized that these properties might relate to their physiological function within the brain. In this regard, this hypothesis would explain the observation within the literature that MT-I and -II are up-regulated within astrocytes in response to various forms of brain injury and neurodegenerative disorders. Using neuron/astrocyte co-cultures, this study found a similar pattern of MT-I and -II up-regulation following scratch wound injury. However, injury to pure astrocyte cultures did not result in changes in MT-I and -II expression, suggesting that MT-I and -II respond specifically to neuronal injury. Based upon these results, this thesis proposes a potential model/hypothesis for MT action within the CNS, where they are up-regulated in astrocytes in response to neuronal injury, released, and subsequently promote/inhibit neuronal recovery.

In summary, this thesis presents data suggesting an important role for extracellular MTs in the cellular response to neuronal injury. Furthermore, the neuroactive properties of MTs discovered in this work reveal the possibility of metallothionein based therapeutics in the context of brain injury.

ACKNOWLEDGEMENTS

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This thesis is dedicated to Alfie. It has always been a great regret that you didn't get to witness this moment. But you have always been with me, and you always will be.

PUBLICATIONS

The following publications have arisen from this thesis to date:

Chung RS, Holloway AF, Eckhardt BL, Harris JA, Vickers JC, Chuah MI and West AK. (2002) Sheep have an unusual variant of the brain-specific metallothionein, MT-III. *Biochem J* 365: 323-328.

Chung RS, Vickers JC, Chuah MI, Eckhardt BL and West AK. (2002) Metallothionein-III inhibits initial neurite formation in developing neurons as well as post-injury, regenerative neurite sprouting. *Exp Neurol* 178: 1-12. (a figure from this publication was also used as the cover image of this issue)

Chung RS, Vickers JC, Chuah MI and West AK (2003) Metallothionein-IIA promotes initial neurite elongation and post-injury reactive neurite growth, and facilitates healing following focal cortical brain injury. *J Neurosci* 23: 3336-3342.

Chung RS, Adlard PA, Dittmann J, Chuah MI, Vickers JC, and West AK (2003) Neuron-glia communication: metallothionein expression is specifically up-regulated by astrocytes in response to neuronal injury. *Journal of Neurochemistry* (in press)

Chung RS and West AK (2003) A role for extracellular metallothioneins in CNS injury and repair. *Neuroscience* (invited commentary; in press)

The following published abstracts have arisen from this thesis to date:

Eckhardt BL, **Chung RS**, Harris JA, Vickers JC, Chuah MI and West AK (2000) Production of neuro-active metallothionein-III isoforms and assay of their action in a rat model of cortical damage. Poster presentation at ComBio 2000 (combined Australian and NZ societies for Biochemistry and Molecular Biology), Wellington NZ.

Chung RS, Dittmann J, Adlard PA, Vickers JC, Chuah MI and West AK (2002) Neuron-glia communication: metallothionein expression is rapidly increased by astrocytes in response to neuronal injury. Poster presentation for the National and International Neurotrauma Societies in Tampa, Florida USA.

Chung RS, Dittmann J, Vickers JC, Chuah MI and West AK (2002) Extracellular, neuroactive properties of metallothioneins. Poster presentation at the Society for Neuroscience Annual Meeting in Orlando, Florida USA.

Chung RS, Dittmann J, Vickers JC, Chuah MI and West AK (2002) An extracellular role for metallothionein in recovery from brain injury. Poster presentation at the 3rd Asia Pacific Symposium on Neural Regeneration, Perth Australia.

Chung RS, Dittmann J, Vickers JC, Chuah MI and West AK (2002) The role of Metallothionein in the cellular response to neuronal injury. Poster presentation at the 3rd Asia Pacific Symposium on Neural Regeneration, Perth Australia.

West AK, Dittmann J, Vickers JC, Chuah MI and **Chung RS** (2003) An extracellular role for metallothionein in recovery from brain injury. Oral presentation at the Annual Meeting of the Australian Neuroscience Society, Adelaide Australia.

Chung RS, Dittmann J, Vickers JC, Chuah MI and West AK (2003) The role of extracellular metallothionein in cortical wound healing. Oral presentation at the Annual Meeting of the Australian Neuroscience Society, Adelaide Australia.

Some of the work presented in this thesis has formed the basis of an Australian Provisional Patent Application:

Chung RS, Chuah MI, Vickers JC and West AK (2002) Metallothionein based neuronal therapeutics and therapeutic methods. Australian Provisional Patent # PS2958 (July 2002)

ABBREVIATIONS

AAS	atomic absorption spectrophotometry
AD	Alzheimer's disease
BSA	bovine serum albumin
CNS	central nervous system
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene diamine tetra-acetic acid
FCS	fetal calf serum
GFAP	glial fibrillary acidic protein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IPTG	isopropyl- β -D-thiogalactopyranoside
LDS	lithium dodecyl sulphate
MES	2-(N-morpholino) ethane sulfonic acid
MT	metallothionein
MW	molecular weight
PBS	phosphate buffered saline
PI	post-injury
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane

MT NOMENCLATURE

Despite the recommendations made by the Committee on the Nomenclature of Metallothionein appointed at the General Discussion Session of the Second International Meeting on Metallothionein and Other Low Molecular Weight Metal-binding Proteins (see Fowler *et al*, 1987), MT nomenclature has not been standardised.

This thesis discusses primarily MT proteins from human, although a variety of other mammalian MTs may be briefly mentioned. The distinction between these different mammalian isoforms is important to note, and has been made throughout the text.

The MT family of proteins consists of 4 primary isoforms, referred to as MT-I, MT-II, MT-III and MT-IV. However, each isometallothionein family consists of a number of specific subforms. Human MT subforms are designated following the guidelines for human gene nomenclature established by Karin *et al* (1984) and West *et al* (1990), i.e. human MT-IIA.

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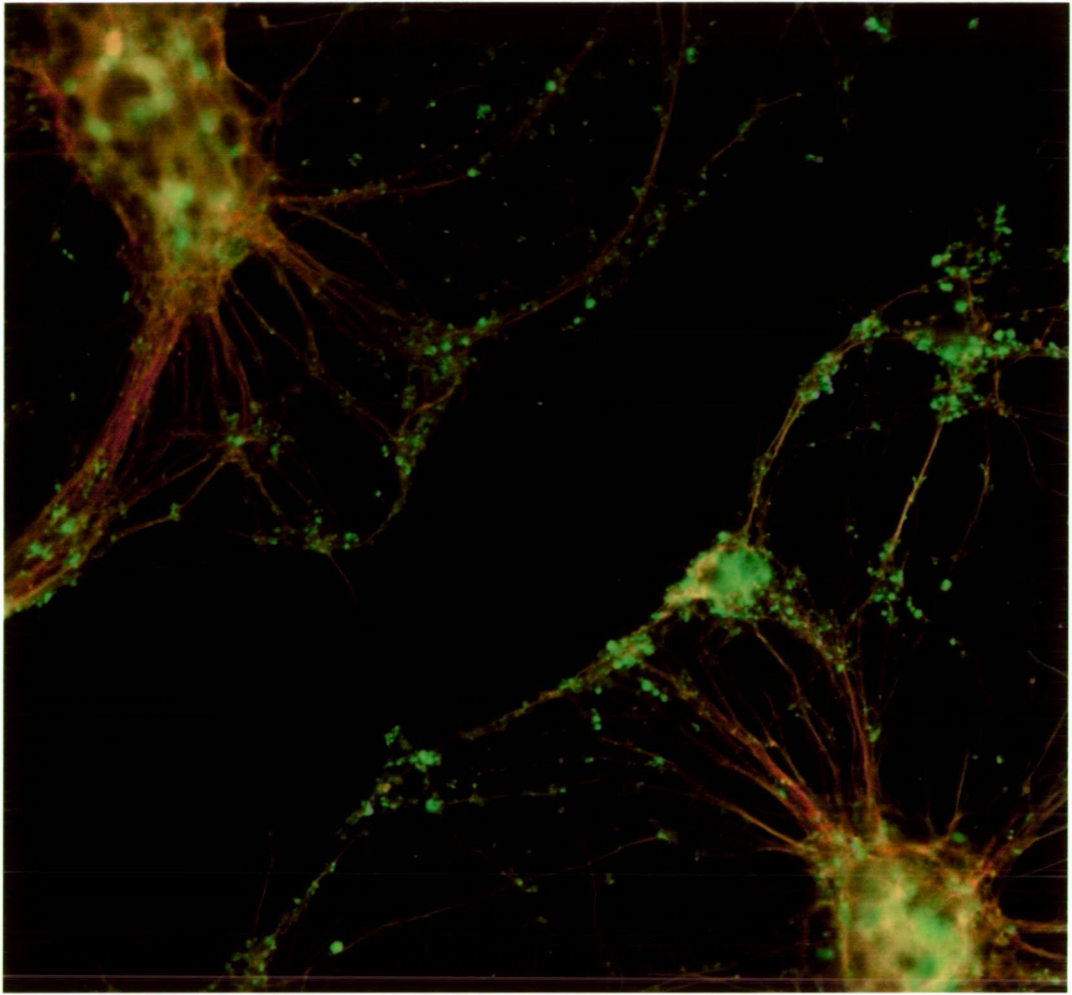
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Embryonic rat cortical neurons were cultured at high cell density, promoting the formation of neuronal clusters. By 21 days *in vitro*, fasciculated axonal bundles formed between clusters, which were transected by microscalpel. By 12 hours post-injury, several reactive axonal sprouts were observed by fluorescent immunocytochemistry entering the injury site (green – β III-tubulin; red – tau). However, treatment with recombinant human MT-III significantly inhibited reactive sprouting. This figure appeared on the cover of *Experimental Neurology* (Issue 178, 2002).

Chapter 1 – Introduction

This introduction will provide a brief overview of the development of the mammalian brain, followed by a summary of the response of the mature CNS to injury. Continuing this theme, an overview of our current knowledge of the metallothionein family of proteins will be presented, based on the recent demonstration that these proteins are intimately involved in the cellular response to brain injury as well as in the pathogenesis of a number of neurodegenerative disorders.

1.1 Development of the neuronal architecture of the brain

The development of the mammalian brain involves the complex interplay of a number of different processes. These include initial neurite formation and outgrowth (a neurite being any process extending from the cell body, including both axons and dendrites), neurite guidance and growth, and finally synapse formation. Following is a brief overview of these processes.

1.1.1 Initial neurite formation, outgrowth and elongation

The initial processes involved in neurite formation have been well characterized (see Tanaka & Sabry, 1995; Tessier-Lavigne & Goodman, 1996; Brandt, 1998). The first step is the formation and outgrowth of multiple, morphologically indistinguishable minor neurites, one of which becomes an axon with a growth cone.

The growth cones are highly motile, hand-like structures which are responsible for the directed growth of the neurite the long distances required for it to reach its correct target. They sample the external environment, and respond to numerous molecular stimuli to guide neurite growth. Guidance cues can be positive (attractive) or negative (repulsive), and the correct reading and integration of these cues by the growth cone is essential for the precise wiring of the brain (as reviewed by Mueller, 1999).

Underlying the outgrowth of neurites from the cell body are changes in the dynamic cytoskeleton of the neuron. The major components of the neuronal

cytoskeleton are filamentous actin (F-actin), which is the primary component of the peripheral domain of the growth cone, and microtubules, of which the neurite shaft, and the central domain of the growth cone are composed (Figure 1.1).

Neurite growth relies upon dynamic microtubules and actin. Initially the filopodia advance by the assembly of new actin filaments at the leading edge of the growth cone, followed by the flow of microtubules from the central core into the newly extended protrusion, leaving behind a new stretch of axon (Figure 1.2).

1.1.2 Synapse formation

When axons reach their appropriate targets they form synapses, structures which permit signaling between neurons. Synapse formation involves three key events: the formation of selective connections between the developing axon and its target, the differentiation of the growth cone into a nerve terminal, and the elaboration of the postsynaptic terminal in the target cell. This process is driven by bi-directional, intercellular interactions that allow the recognition of the appropriate postsynaptic cell followed by the coordinated differentiation of pre- and post-synaptic elements of the synapse. These steps are briefly described in Figure 1.3, using the example of the well characterized neuromuscular junction, the synapse formed between motor neurons and skeletal muscle fibres.

1.2 The neuronal response to injury

Within mature central nervous system neurons, injury to the cell body will often lead to neuronal death. However, damage to the axon results in the neuron exhibiting a characteristic response to injury. Initially this involves two processes, Wallerian degeneration and chromatolysis.

1.2.1 Wallerian degeneration and chromatolysis

Transection of the axon (axotomy) results in a division of the axon into a proximal segment (which remains attached to the cell body) and a distal segment. The distal segment physically degenerates over a period of time, together with the myelin sheath encasing the axon. Axonal debris is removed by phagocytic

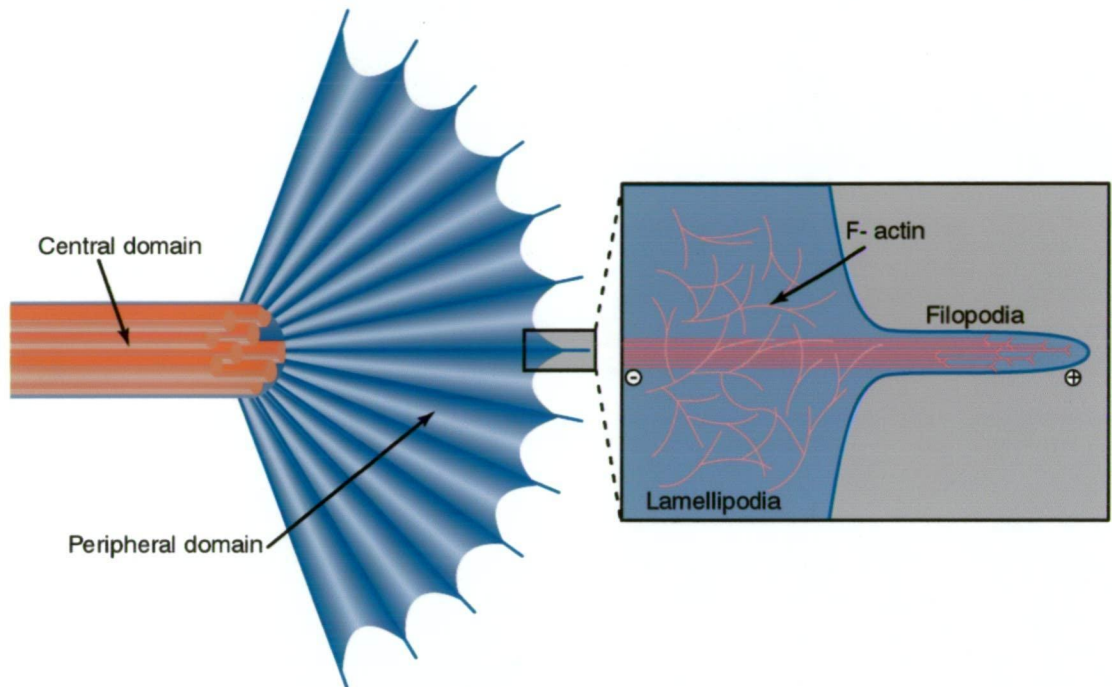


Figure 1.1: The axonal shaft is composed primarily of microtubules, which also extend into the proximal portion of the growth cone. Actin is highly concentrated within the filopodia and leading edge of the growth cone. Within filopodia, actin fibrils are orientated with their growing tips pointed distally. Within the lamellae, actin fibrils are randomly orientated and form a dense meshwork. Modified from Lin *et al* (1994).

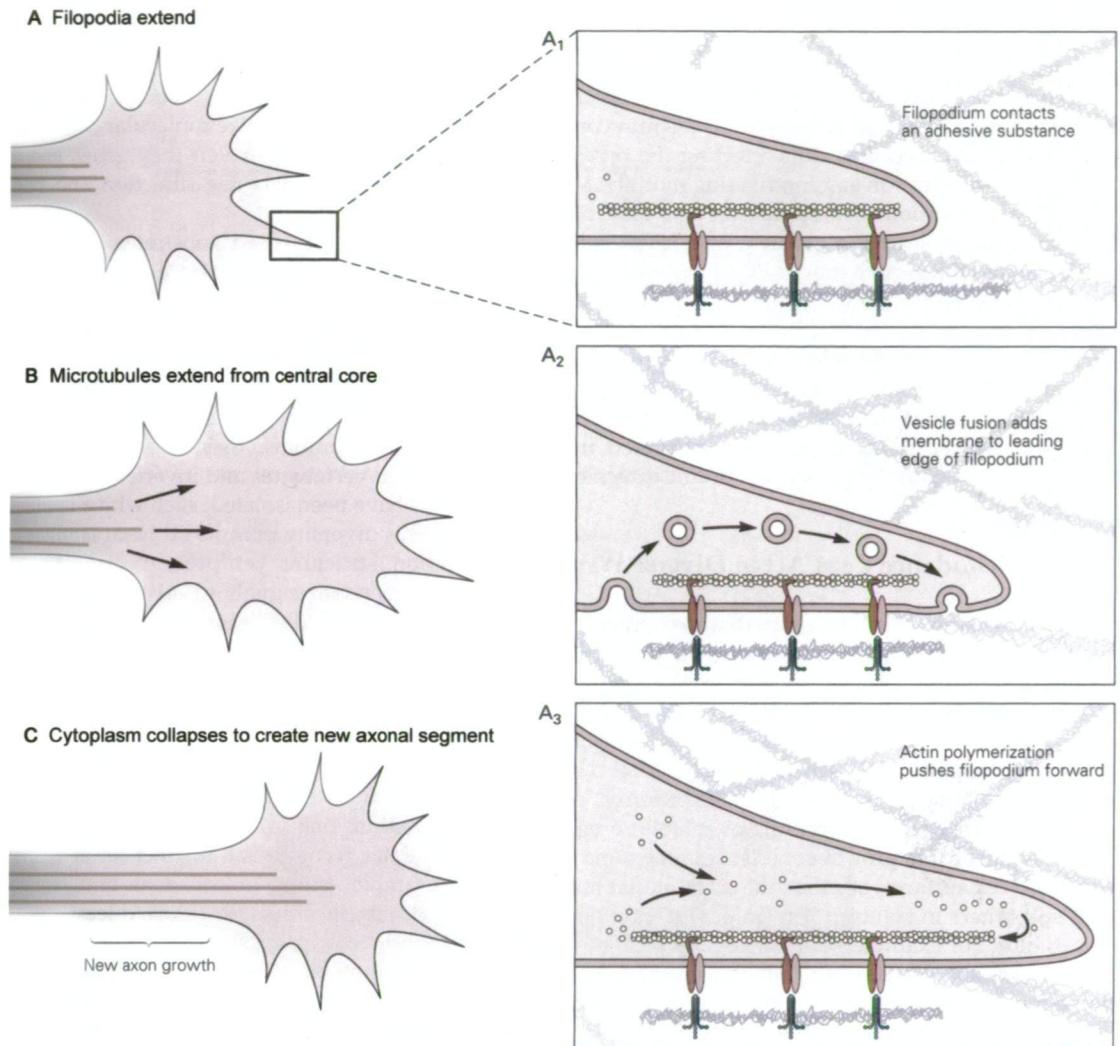


Figure 1.2: Neurite growth is driven by dynamic actin and microtubules. The filopodia respond to adhesive cues by contracting, which pulls the growth cone forward (A). Actin filaments assemble at the leading edge of the filopodia, while disassembling at the trailing edge. The force generated by this flow of actin pushes the filopodia forward (A₁). Membrane is added to the leading edge of the filopodia by exocytosis (A₂). Actin polymerization pushes the filopodia forward. Actin polymers are linked to adhesion molecules on the plasma membrane to provide traction (A₃). These combined actions result in an actin-poor space that is filled by advancing microtubules (B). The microtubules form a new bundle, and the cytoplasm collapses around them to form a new length of axonal shaft. Modified from Heidemann (1996).

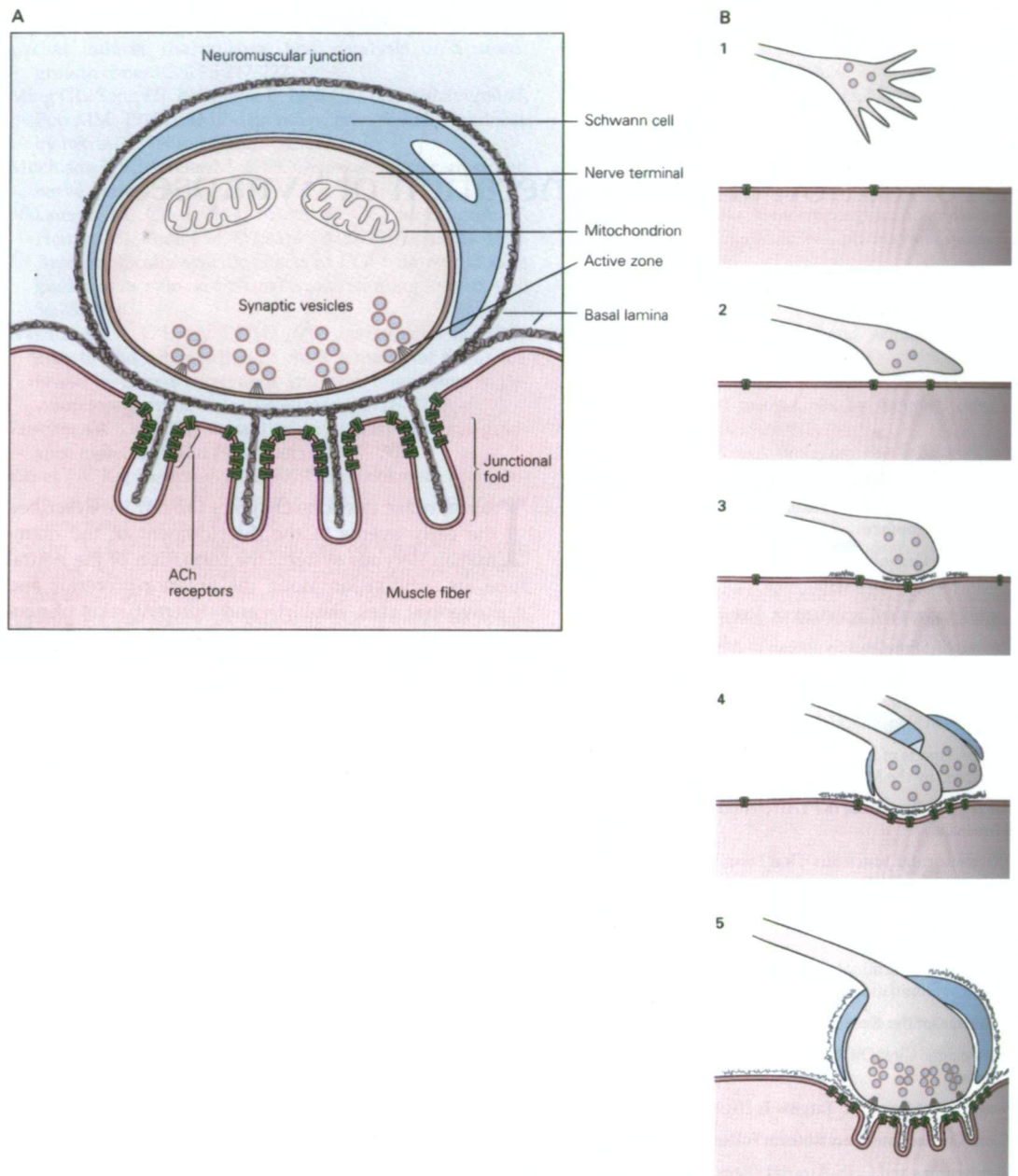


Figure 1.3: At the mature neuromuscular junction (A), the synaptic cleft separates pre- and post- synaptic membranes. Synaptic vesicles are clustered at the pre-synaptic release site, while transmitter receptors are clustered at the post-synaptic membrane. The entire nerve terminal is ensheathed by glial processes. The neuromuscular junction forms in a number of discrete steps (B), which begins with the growth cone approaching the muscle fibre (1), forming a functional but morphologically unspecialized connection (2). The terminal accumulates synaptic vesicles, and a basal lamina forms in the synaptic cleft (3). Multiple axons converge on a single site (4), but all are eliminated except one that matures to form a functional synapse (5). Based on Hall & Sanes (1993).

microglia/macrophages, and this entire process is termed Wallerian degeneration (Figure 1.4).

In many cases, the proximal segment of the axon and the cell body itself die by apoptosis, due to the lack of target derived trophic factors. When this doesn't occur, the cell body undergoes a series of changes termed chromatolysis. The cell body swells, the nucleus moves to an eccentric position, and the rough endoplasmic reticulum fragments (Figure 1.5). Metabolically the cell also changes, by increasing protein and RNA synthesis, as well as the pattern of genes expressed. This process is often viewed as an attempt by the neuron to regenerate, by regrowing the injured axon and re-forming its connections (Deller and Frotscher, 1997; Christman *et al.*, 1997; Pastor *et al.*, 2000). Axonal growth from the proximal stump is termed reactive sprouting, or reactive axonal sprouting (Figure 1.5).

1.2.2 Reactive axonal sprouting

It was Ramón _ Cajal (1928) who first proposed that mature injured CNS neurons are not capable of regeneration. However, more recently, it has been demonstrated that these neurons are indeed able to produce reactive sprouts and regenerate if provided with suitable conditions. Such experiments include those performed by Aguayo and colleagues (David & Aguayo, 1981; Duncan *et al.*, 1981) who implanted peripheral nerve tissue grafts into lesioned spinal cords, and identified a number of reactive axonal processes entering the graft. In more recent times, inhibitory molecules such as myelin associated glycoproteins and Nogo have been identified which inhibit axonal regeneration following injury (see reviews by Fournier *et al.*, 2002; McKerracher & Winton, 2002). These experiments suggest that reactive axonal sprouting is an intrinsic property of neurons, and experiments in culture support this hypothesis. In this regard, it has been demonstrated that injured axons of mature neurons are capable of undergoing a defined series of reactive changes, ultimately culminating in a sprouting response, which occurs independently of the presence or effects of glial cell populations (Dickson *et al.*, 2000; Chuckowree & Vickers, 2003).

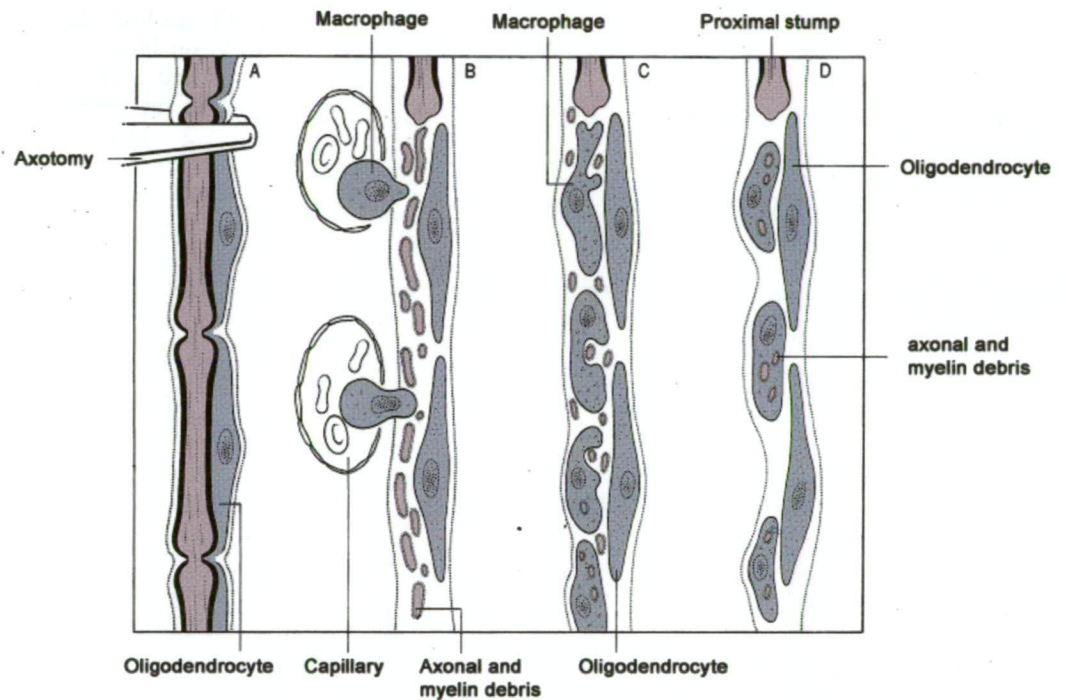


Figure 1.4: Following injury to the axon (A), the axon undergoes Wallerian degeneration. Macrophages migrate into the injury site from the periphery (B), and the distal segment of the damaged axon relative to the original injury site degenerates over time. Degraded axonal and myelin fragments are removed by phagocytic macrophage (C, D). Modified from Jacobsen (1991).

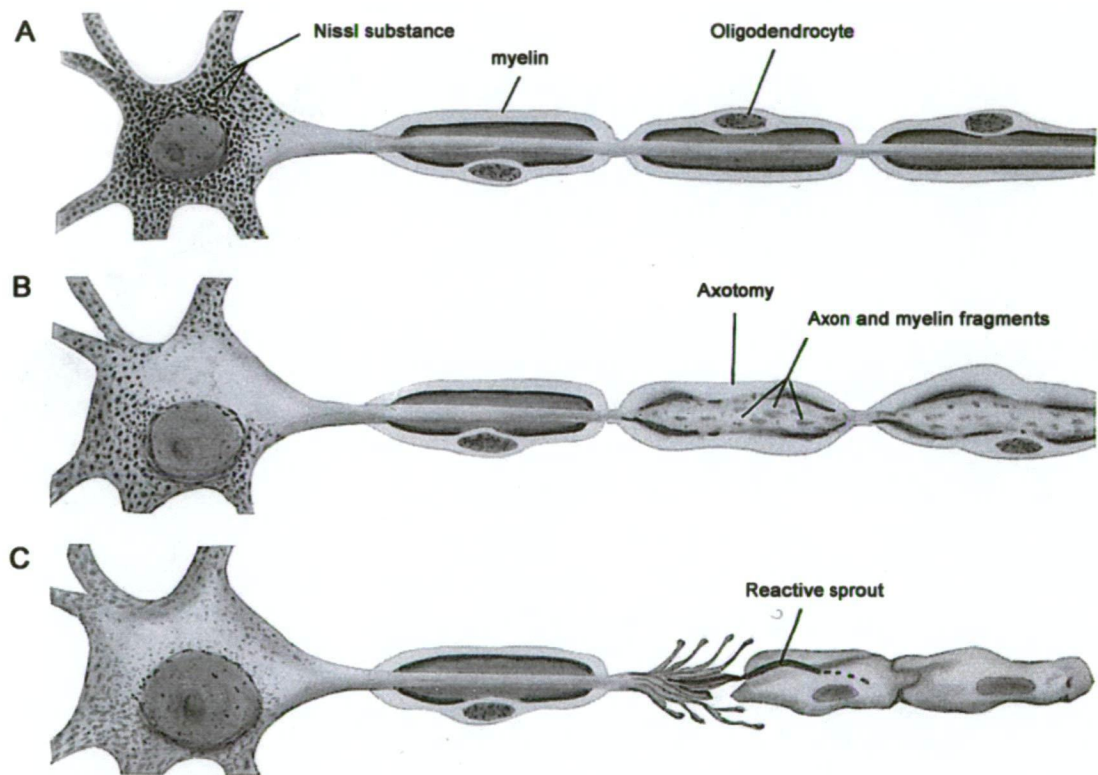


Figure 1.5: Following axotomy (B), the neuronal cell body undergoes a series of changes collectively known as chromatolysis. The cell body swells, the nucleus moves to an eccentric position, and the rough endoplasmic reticulum becomes fragmented (B). Metabolic changes follow, including overall increases in RNA and protein synthesis of a number of regeneration associated genes (such as GAP-43), resulting in a reactive sprouting response (C). Modified from Burt (1993).

The mechanisms underlying reactive axonal sprouting and growth following injury to mature CNS neurons are currently unknown. In this context, it is also unclear whether reactive axonal growth recapitulates the same processes and mechanisms involved in axonal development, although several experiments suggest this. For instance, changes in the expression of a number of cytoskeletal elements (of which GAP-43 is the best characterized) reflects those observed during the sequence of developmental events which occur during neurite formation and growth (Hoffman and Cleveland, 1988; Lee and Cleveland, 1996). Furthermore, following injury to mature axons in embryonic rat cortical neuron cultures, growth cone like structures have been observed at the distal tips of reactive axonal sprouts (Chuckowree & Vickers, 2003). A number of neurotrophic factors, such as the neurotrophin family of proteins, which promote developing axonal growth, have also been demonstrated to promote reactive axonal growth following CNS injury (see review by Blesch & Tuszynski, 2002).

1.3 The cellular response to brain injury

Injury to the brain results in the initiation of a number of repair processes. These include not only direct neuronal responses (as discussed previously), but also changes in the cellular environment of the brain. In this regard, the role of glial cells such as microglia/macrophages and astrocytes are crucial to the brain's response to injury. Their response is characterized by the formation of a dense web of interlacing glial processes that form within the lesioned area.

1.3.1 Microglia/macrophage activation in response to brain injury

As discussed previously, the neuronal response to injury involves degradation of axonal components and the myelin sheath. The resultant axonal debris is removed by phagocytic microglia that are resident within the CNS, and macrophages which migrate into the area of degeneration from outside of the nervous system. These cells respond vigorously to traumatic injury with marked hypertrophy, proliferation, migration and transition to a different morphological phenotype, dubbed "reactive". Reactive microglia/macrophages also exhibit strong secretory properties within the

injury environment, producing a variety of molecules which alter the injury environment, including cytokines, degradative enzymes (such as proteases) and small molecules such as leukotrienes and superoxide (for review, see Banati *et al*, 1993; Streit *et al*, 1999).

1.3.2 Astrocytic response to brain injury

Astrocytes also acquire distinct reactive changes, evidenced both morphologically and functionally (as reviewed by Ridet *et al*, 1997). This process is known as astrogliosis and the astrocytes are considered “reactive”. This process is crucial to the brain’s response to neuronal injury, as reports utilising both chemical treatments (Bush *et al*, 1999) and transgenic null-mice (such as IL-6 null mice; Penkowa *et al*, 2000), which result in reduced astrogliosis, exhibit dramatically reduced recovery following brain injury. Reactive astrocytes express a number of factors that modulate the external injury environment, including cytokines and growth factors. Recently, it has been demonstrated that astrocytes significantly up-regulate metallothioneins in response to injury, and that these proteins are major neuroprotective proteins within the brain (Campagne *et al*, 1999; Penkowa *et al*, 1999a; Trendelenburg *et al*, 2002). Following is a brief discussion of the metallothioneins.

1.4 The metallothioneins

1.4.1 General discussion

The mammalian metallothionein (MT) family of proteins consists of cysteine rich (25-30%), low molecular weight (6-7 kDa), heavy metal binding proteins (Hamer, 1986; Kagi and Schaffer, 1988; Vasak and Hasler, 2000). There are four isoforms; -I and -II (found in most tissues including the brain), -III (brain specific) and -IV (found in squamous epithelial tissue) (Palmiter *et al*, 1992; Quaife *et al*, 1994). Throughout this thesis, MT-I and -II will be considered as a single isoform denoted MT-I/-II, in light of the fact that they exhibit very high structural homology (for instance, >98% in the case of human MT-I and MT-II), and their genes are co-

ordinately expressed and respond in parallel to all MT inducers (Yagle & Palmiter, 1985).

Much of our current understanding of the functional roles of MTs is based upon the comparative analysis of their chemical and structural features. Most intriguing is the fact that the structure of all MTs identified so far, including both mammalian and non-mammalian (such as crustaceous and bacterial), indicate a common monomeric protein structure composed of two globular domains, each composed of a metal-thiolate cluster (Figure 1.6). Furthermore, most non-mammalian MTs generally contain the same number of metal-chelating Cys-Cys and Cys-X-Cys motifs (where X stands for any amino acid other than cysteine) as their mammalian counterparts. This indicates a tight evolutionary constraint of the key structural features of MTs, and suggests the conservation of physiological function. In this regard, their heavy metal binding properties allow them to sequester heavy metals and protect against metal toxicity (in particular cadmium), and it has been suggested that they are able to regulate the availability of metal ions, particularly Zn (II) and Cu (I), to various enzymes and transcription factors (Kelly *et al*, 1996; Aschner, 1997). They also have free radical scavenging properties, which allow them to protect cells from cytotoxicity induced by reactive oxygen species (Sato and Bremner, 1993).

The discovery of MT-III, a brain specific MT isoform, and the report of its down-regulation in Alzheimer's disease (AD), shed light upon the role MTs may play within the brain (Uchida *et al*, 1991). Furthermore, investigation of this isoform indicated that different MT isoforms apparently have different roles within the brain. Following is a brief discussion of the neurologically important MTs (MT-I, -II and -III), and their potential roles within the CNS following injury and in the pathogenesis of a number of neurodegenerative diseases.

1.4.2 Structural differences between MT-I/-II and MT-III

As discussed previously, all mammalian MTs are monomeric proteins composed of two globular domains, each encompassing a metal-thiolate cluster. Furthermore, MT-I/-II and MT-III contain the same number of cysteine residues,

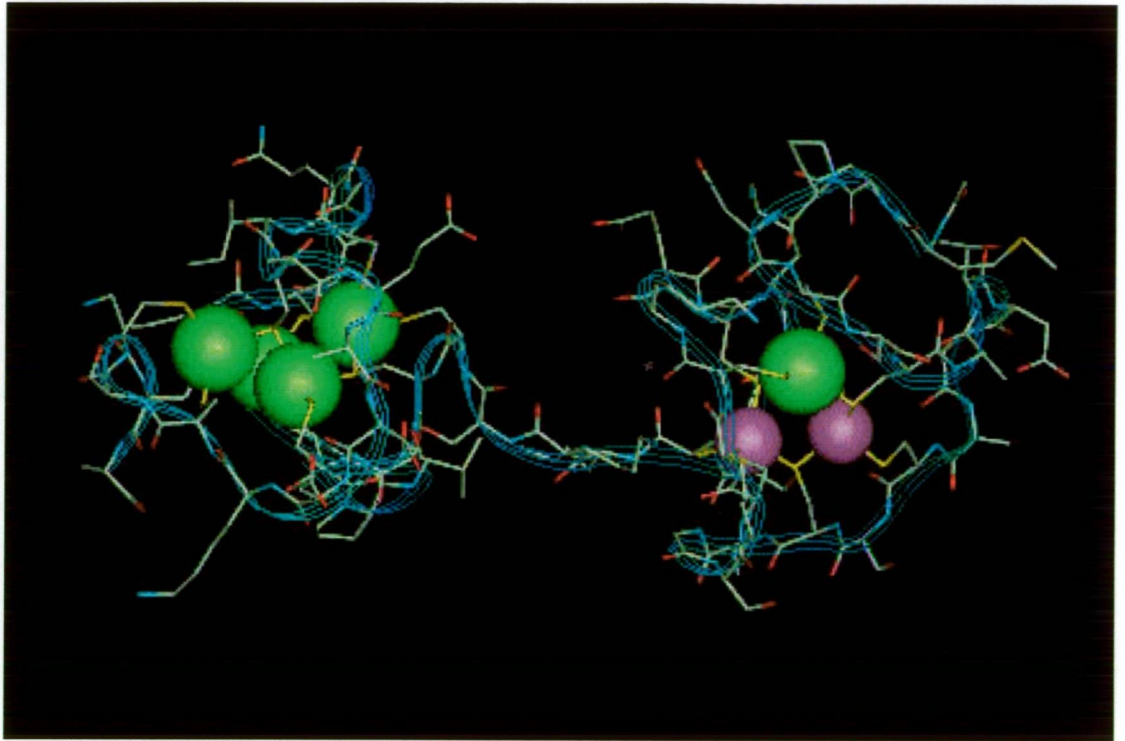


Figure 1.6: Mammalian metallothioneins are composed of two domains that bind 7 divalent transition metals such as cadmium (green spheres) and zinc (purple spheres). Under natural conditions, it is bound primarily to zinc, and some monovalent copper. Figure courtesy of Dr John Beattie.

found as Cys-Cys and Cys-X-Cys motifs (where X stands for any amino acid other than cysteine). However, MT-III has two regions which distinguish it from all MT-I/-II isoforms. These are a C₆PCP motif within the β -domain, and an acidic hexapeptide within the α -domain (Figure 1.7). Interestingly, recent NMR studies have identified dynamic processes within the metal-thiolate clusters of MT-III, which have not been observed in MT-I/-II (Faller *et al*, 1999). Hence, changes in the primary structure of MT-III, when compared with MT-I/-II, result in markedly increased structural flexibility, and may result in variations in the biological activity of MT-III in comparison to MT-I/-II. As an indication of this, the acidic insertion within the α -domain is accommodated in a loop that exhibits structural dynamics, and as such is proposed as a possible interaction site with other proteins (Oz *et al*, 2001).

1.4.3 Expression of MTs within the brain

Within the brain, MT-I/-II comprise approximately two thirds of total brain MT (Erickson *et al*, 1994). The primary cellular source of MT-I/-II within the mammalian brain is astrocytes (Nakajima *et al*, 1991; Nishimura *et al*, 1992), with low levels found in some neuronal populations, and particularly the neurons of the CA3 field of the hippocampus (Masters *et al*, 1994). Glial localized MT-I/-II is found within a limited number of CNS regions, including cortex, hippocampus, brainstem and spinal cord (Choudhuri *et al*, 1995).

In contrast to MT-I/-II, the cellular localisation of MT-III within the mammalian brain is currently unclear. Studies indicate the presence of MT-III at both the transcript and protein level in astrocytes only (Uchida *et al*, 1991; Nakajima & Suzuki, 1995), neurons only (Choudhuri *et al*, 1995; Masters *et al*, 1994) or in both astrocytes and neurons (Yamada *et al*, 1996; Yuguchi *et al*, 1997; Zheng *et al*, 1995). Despite these conflicting results concerning the cellular localisation of MT-III, the general consensus at present is that like MT-I/-II, MT-III is expressed primarily in astrocytes, with low levels (but significantly more than MT-I/-II) in specific subsets of neurons (Uchida *et al*, 2002). In regards to regional distribution, MT-III is expressed within many of the same CNS regions as MT-I/-II, including cortex,

Human MT-IIA	1	mdp ⁿ -c ^s caa	11	g ^d sctcag ^s c	21	k ^c ke ^c ckctsc	31	kk ^s ccsccp ^v
Human MT-III	1	mdpet ^c p ^c p ^s	11	g ^g sctcad ^s c	21	k ^c eg ^c ckctsc	31	kk ^s ccsccp ^a
Human MT-IIA	41	g ^c ak ^c caqg ^c i	51	c ^k g-----a	61	sd ^k csc ^c ca		
Human MT-III	41	e ^c ek ^c akd ^c v	51	c ^k g ^g ea ^a ae ^a e	61	ae ^k csc ^c c ^q		

Figure 1.7: Comparison of the amino acid structure of human MT-IIA (the major human MT-I/-II representative) and human MT-III (the only human MT-III representative). The two major structural differences between MT-I/-II and MT-III isoforms, the C₆PCP motif and acidic hexapeptide insertion, are indicated by boxes. Conserved amino acids between the two proteins are indicated by grey shading. Dashes in the MT-IIA sequence are for alignment purposes.

brainstem and spinal cord (Choudhuri *et al*, 1995). However, MT-III is most abundant within the granule cells of the dentate gyrus in the hippocampus and particularly those neurons that store zinc in their synaptic vesicles (Choudhuri *et al*, 1993; Masters *et al*, 1994).

1.4.4 MTs and brain injury

Expression of MT-I/-II is up-regulated in response to a number of experimental models of brain injury (as reviewed by Hidalgo *et al*, 2001). This would suggest that MT-I/-II have an important role in the brain's response to injury. Indeed, Penkowa and colleagues (1999a) recently demonstrated that MT-I/-II transgenic knockout mice exhibit markedly reduced wound healing ability following cortical cryolesion. This was followed by a similar report indicating that transgenic MT-I/-II over-expressing mice recover much more rapidly than their wild-type littermates following focal cortical ischaemia (Campagne *et al*, 1999).

While there is significant evidence in the literature to suggest that MT-I/-II may promote wound healing within the CNS by protecting tissue from oxidative damage or extracellular zinc (for review, see Hidalgo *et al*, 2001), Penkowa and colleagues have recently demonstrated that MT-I/-II knockout mice exhibit dramatic changes in inflammatory responses following brain injury (Penkowa *et al*, 1999b). Intriguingly, they have further demonstrated that intraperitoneal application of MT-I/-II resulted in alterations in inflammatory mechanisms including cytokine activation and reactive gliosis, leading to significantly improved recovery following cortical brain injury (Penkowa *et al*, 2002). This suggests that MT-I/-II may not only act within the cell to protect against oxidative damage, but also may act outside the cell to promote recovery from brain injury. However the extracellular mechanisms involved are currently unclear. The direct extracellular properties of MTs are discussed in section 1.4.5.

Intriguingly, in most cases where MT-I/-II expression is up-regulated, MT-III expression is often initially down-regulated, before returning to its original levels some time after the original insult (as reviewed by Hidalgo *et al*, 2001). While the reasons underlying the down-regulation of MT-III are not known, this could be due

to the different biological properties which these proteins possess, in contrast to their shared ability to scavenge free radicals. Furthermore, differences in expression profiles following injury may also be due to the different cellular distributions of MT-I/-II and MT-III within the brain (Aschner *et al*, 1997).

1.4.5 Direct effects of extracellular MTs upon neurons and astrocytes

Upon their initial discovery of MT-III, Uchida and colleagues (1991) demonstrated that exogenously applied MT-III exhibits neuronal growth inhibitory activity upon cultured cortical neurons, while other MT isoforms exhibit no such inhibitory activity. To date, a number of reports have confirmed the inhibitory activity of MT-III, although in all cases, a crude brain homogenate was required for the bioactivity of MT-III (for example, see Erickson *et al*, 1994; Sewell *et al*, 1995). In all of these assays, MT-I/-II exhibited no inhibitory effect (Erickson *et al*, 1994; Sewell *et al*, 1995).

Intriguingly, in the absence of brain homogenate, extracellular MT-III promotes neuronal survival (Sewell *et al*, 1995). Furthermore, extracellular MT-III promotes astrocytic migration following scratch wound injury in culture, although MT-I/-II has no such effect (Carrasco *et al*, 1999).

Recently, it has been demonstrated that extracellular MT-I/-II is able to modulate inflammatory responses to injury. In this regard, MT-I/-II applied intraperitoneally following cortical brain injury results in a significant reduction in proinflammatory cytokine expression and also decreases reactive gliosis (Penkowa *et al*, 2002).

These results indicate that MT-I/-II and MT-III exhibit very different extracellular properties, despite their high sequence and structural homology. However, it is important to note that MTs lack signal peptides or other such extracellular trafficking signals, and as such are considered solely intracellular proteins (Palmiter *et al*, 1992). While there are limited reports that cultured cells actively release MTs under various conditions (Trayhurn *et al*, 2000; Uchida *et al*, 2002), the mechanism by which this may occur is unknown. Thus, at present, the

commonly held view is that the extracellular properties of MTs are artificial properties of these proteins, rather than true physiological functions (Palmiter, 1998).

1.4.6 Metallothioneins in neurodegenerative disorders

Neurodegeneration is a primary pathological hallmark of a number of neurological diseases. In accordance with this, changes in MT expression have been noted in a number of these diseases. Following is a brief discussion of some of these:

1.4.6.1 Multiple sclerosis

Multiple sclerosis is a chronic, inflammatory, demyelinating disease of the CNS. Pathologically it is characterised by cellular infiltration (mostly lymphocytes and macrophages), and varying degrees of demyelination and axonal loss (Lassmann *et al*, 1998). As an inflammatory condition, oxidative stress is an obvious candidate for involvement in the early stages of MS. Indeed, there is much evidence indicating the role of oxidative stress in the development of lesions, one of the hallmarks of MS (Lin *et al*, 1993; De Groot *et al*, 1997; Vladimirova *et al*, 1998). Experimental autoimmune encephalomyelitis (EAE) is a mouse model of MS, with close resemblance to MS both clinically and morphologically (Martin *et al*, 1992).

Juan Hidalgo's lab have extensively investigated the possible roles of MTs in EAE, with extremely exciting results. Their initial studies focussed upon the expression profiles of the MTs in EAE, and they found a marked difference between MT-I/-II and MT-III. Mice with EAE showed significant induction of MT-I/-II in the spinal cord white matter, and in the brain, though to a lower extent. These proteins were prominent in areas of cellular infiltration, with reactive astrocytes and activated monocytes/macrophages being the cellular sources of MT-I/-II. Contrastingly, MT-III expression was unaltered in mice with EAE (Espejo *et al*, 2001). The relationship between MTs and EAE was further elucidated by Hidalgo and colleagues, investigating the pro-inflammatory cytokine control of MT expression. Interferon- γ knockout mice suffer a more severe form of EAE, and MT-I/-II induction is significantly higher in these mice (Espejo *et al*, 2001). To confirm their previous findings, MT-I/-II knockout mice were induced into developing EAE. These mice

had a much greater susceptibility to EAE development, and exhibited a significantly greater inflammatory response, evidenced by increased macrophage and T-lymphocytes infiltration in the CNS, while reactive astrogliosis was significantly decreased. In addition, the expression of the pro-inflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha elicited by EAE was further increased in the MT-I/-II knockout mice, and oxidative stress and apoptosis were also significantly increased in MT-I/-II knockout mice compared to wild type controls (Penkowa *et al*, 2001). Hence, there is significant evidence to suggest a strong relationship between the inflammatory response invoked by EAE and metallothionein expression.

1.4.6.2 Alzheimer's disease

Alzheimer's disease (AD) is a debilitating, age-onset disease, characterised clinically by the presence of two pathologic structures found throughout the cortex; amyloid plaques and neurofibrillary tangles (Drachman & Lippa, 1992). The principal component of amyloid plaques is the β -amyloid peptide which is derived from the β -amyloid precursor protein (Glenner & Wong, 1984; Masters *et al*, 1985). A subset of these β -amyloid plaques, that are spatially associated with dystrophic neurites, are referred to as neuritic or senile plaques. Neurofibrillary tangles consist of abnormal filamentous structures (such as paired helical and straight filaments) that are derived from the transformation of normal cytoskeletal proteins. Their composition is unclear due to their high degree of insolubility, but it is generally agreed that the microtubule associated protein, tau, or an abnormal form of this protein, is an integral component of neurofibrillary pathology (see review by Vickers *et al*, 2000). The consequence of these structures is extensive neuronal loss, which is thought to be closely related to the degree and progression of dementia, the central symptom of AD (Vickers, 1997; Vickers *et al*, 2000).

1.4.6.2.1 The discovery of MT-III

Based upon their previous work, Uchida and colleagues (1989) hypothesized that the formation of neurofibrillary tangles, one of the pathological hallmarks of

Alzheimer's Disease (AD), was due to an increase in neurotrophic activity in the AD brain. Furthermore, they postulated that this increase in neurotrophic activity was actually due to the absence of inhibitory factor(s) present within normal brain. This idea contradicted the prevailing view at the time, which was that AD was associated with a loss of neurotrophic factors in the brain (Appel, 1981).

In search of these inhibitory factors, Uchida *et al* (1991) purified and characterised a 68 amino acid protein, which they named growth inhibitory factor (GIF). They found GIF to be greatly reduced in the AD brain compared to control brain. GIF was also found to inhibit survival of cortical neurons *in vitro*.

Rather surprisingly, it was discovered that GIF strongly resembled the MT family of proteins. The most striking feature of this similarity to MTs was the presence of 20 cysteine residues, all of which are conserved between GIF and mammalian MTs. These cysteine residues were arranged in either Cys-X-Cys or Cys-Cys formations (Uchida *et al*, 1991). The protein sequence of GIF was differentiated from other metallothioneins by two amino acid insertions; a single Thr in the N-terminal region, and an acidic hexapeptide in the C-terminal region (Uchida *et al*, 1991; Palmiter *et al*, 1992). GIF also contained a C₆PCP motif, which was not identified in any other metallothionein isoform (Sewell *et al*, 1995).

Further evidence indicating GIF as a member of the metallothionein family was obtained from atomic absorption spectrophotometry. Human GIF was found to bind three zinc and four copper atoms per polypeptide chain, indicating that like the MTs, GIF has a high metal binding activity (Uchida *et al*, 1991). Molecular analysis found the GIF gene to be of similar size, share intron/exon boundaries, and cluster on the same chromosome as the other metallothionein isoforms (Palmiter *et al*, 1992).

Because of the evidence supporting GIF as a member of the metallothionein family, it has been re-named MT-III (Palmiter *et al*, 1992).

1.4.6.2.2 The role of MT-III in Alzheimer's Disease

Since the initial discovery of MT-III, there have been several conflicting reports in regards to the changes in MT-III levels within the AD brain, at both mRNA (Tsuji *et al*, 1992; Erickson *et al*, 1994) and protein level (Uchida *et al*, 1991;

Erickson *et al*, 1994; Carrasco *et al*, 1999). These apparently opposing reports may be related to issues such as populational differences between the AD brains analysed. For instance, Uchida *et al* (1991) and Tsuji *et al* (1992) looked at samples from a Japanese population (which can be considered a homogenous population), whereas Erickson *et al* (1994) and Carrasco *et al* (1999) have looked at North American populations, which can be considered a mixed population. A recent, comprehensive study at both the protein and RNA level involving a large sample size found evidence to indicate a down-regulation of MT-III in AD brains (Yu *et al*, 2001), confirming the original observations by Uchida *et al* (1991). Hence, while MT-III levels in the AD brain are unclear, this may reflect the complex nature of the disease.

The second pathological hallmark of AD, senile plaques, is caused by neurotoxic aggregations of β -amyloid (see review by Selkoe, 1994). Irie & Keung (2001) recently reported that MT-III could protect cultured neurons from neurotoxic β -amyloid by preventing β -amyloid aggregation, suggesting that MT-III may prevent senile plaque formation in AD.

Again highlighting the significant differences between the metallothionein isoforms are the reports that MT-I/-II expression is actually increased in the early stages of AD (Adlard *et al*, 1998; Zambenedetti *et al*, 1998). The role that MT-I/-II may play in the etiology of AD is unclear, but the general consensus is that these isoforms are most likely involved in the response to oxidative stress or the mis-handling of heavy metals, two possible factors in the development of AD (see Hensley *et al*, 1994; Cuajungco & Faget, 2003 respectively)

1.4.6.3 Epileptic seizures

Both MT-I/-II and MT-III knock out mice, while appearing phenotypically normal during both development and adult life, are extremely susceptible to kainic acid induced seizures (Penkowa *et al*, 1999a and Erickson *et al*, 1997 respectively). Conversely, transgenic mice over-expressing MT-I/-II or MT-III are more resistant to kainic acid induced seizures (Penkowa *et al*, 1999a and Erickson *et al*, 1997 respectively). These observations strongly suggest that MTs are involved in seizure etiology. Considering the fact that MTs appear to have zinc modulatory properties,

this suggests that MTs may be involved in zinc regulation during neural stimulation. In this sense, MTs may be important in maintaining intracellular zinc concentrations, through recycling or by acting as a zinc reserve. Thus an absence of MT (as observed in MT knockout mice) may lead to a rapid decline in intracellular zinc during prolonged stimulation (as observed during seizure activity), resulting in a reduced capacity to prevent further seizure activity (Erickson *et al*, 1997).

Alternatively, it may be that during prolonged neuronal activity, potentially toxic influxes of zinc are chelated by intracellular MT. This would suggest that there is either a pool of apo-MT (MT not bound to any metal ions) or that MT redistributes incoming zinc to locations within the neuron where it is less toxic (Erickson *et al*, 1997). Furthermore, intense neuronal excitation also leads to the generation of reactive oxygen species (Coyle & Puttfracken, 1993), so MT may also act to protect against seizure activity by free radical scavenging.

1.4.6.4 Familial amyotrophic lateral sclerosis

Familial amyotrophic lateral sclerosis (FALS) is a progressive disorder leading to the death of motor neurons and ultimately death. It has been reported that MT-I/-II are up-regulated in this disease, both in humans and in experimental animal models of this disease (Blaauwgeers *et al*, 1996; Gong & Elliot, 2000 respectively). One such group of experimental FALS models are those involving mutations in the Cu/Zn superoxide dismutase (SOD1) gene. Experiments using both mutant SOD1 or knockout SOD1 mice have demonstrated that the disease is caused by a toxic gain of function and not by a normal loss of SOD1 activity. While the precise mechanisms underlying the toxicity of mutant SOD1 are unclear, abnormalities in zinc binding to SOD1 have been implicated in disease pathogenesis (Elliot, 2001). Indeed, recent work has found that zinc depleted SOD1 is highly toxic when introduced to cultured motor neurons (Estevez *et al*, 1999).

Based upon the zinc binding properties of MTs, it has been hypothesized that they could be involved in the progression of FALS through regulating the availability of zinc to SOD1. To explore this, the G93A SOD1 mutant mouse was crossed with both MT-I/-II and MT-III knockout mice (Puttaparthi *et al*, 2002). Both of these

mice strains exhibited significant reductions in neuronal survival compared to G93A SOD1 mutant mice. Additionally, motor dysfunction was markedly accelerated in the MT deficient SOD1 mutant mice in a differential manner, in regards to onset (MT-I/-II) and progression (MT-III).

There are a number of ways in which MTs might protect against mutant SOD1-induced toxicity. For instance, *in vitro* experiments have demonstrated that MTs are able to act as a zinc chaperone, ferrying zinc ions to apo-SOD1 (Suzuki & Kuroda, 1995). In this regard, in the absence of MTs an inadequate supply of zinc might be available to apo-SOD1, particularly in the context that mutant SOD1 has a decreased affinity for zinc than wild-type SOD1 (Crow *et al*, 1997). It is also possible that MT may act as a zinc buffer, binding zinc released by mutant SOD1 (Crow *et al*, 1997; Elliot, 2001). Alternatively, MT may act in a protective manner by scavenging free radicals produced from malfunctioning mitochondria, a prominent hallmark of mutant SOD1 induced disease (Dal Canto & Gurney, 1994; Wong *et al*, 1995).

1.5 Concluding remarks

Within the past 10 years, interest in the potential roles of metallothioneins has shifted from metal homeostasis and free radical scavenging to their involvement in the cellular response to brain injury as well as in the pathogenesis of a number of neurodegenerative disorders. In this regard, it is the neuronal growth inhibitory properties of MT-III and the immunosuppressive properties of MT-I/-II that have been of most interest. Perhaps the most important point to note in regard to these findings is that MT is considered an intracellular protein. The results discussed above however, are observations of the effect of MT applied extracellularly either to cultured neurons (in the case of experiments investigating the neuronal growth inhibitory activity of MT-III) or intraperitoneally following cortical brain injury (in the case of MT-I/-II).

While MT literature contains numerous reports based upon the intracellular properties of MTs within the brain, there is little known about the possible extracellular, neuroactive roles that these proteins may play. The aim of this thesis is

further elucidation of the extracellular, neuroactive properties of brain MTs (MT-I/-II and MT-III; MT-IV was not investigated as it is not expressed within the CNS), using tissue culture models and an *in vivo* animal model of neuronal injury. Specifically, this will involve:

- 1) Characterising the neuroactive properties of an unusual MT-III isoform identified previously in this laboratory, sheep MT-III.
- 2) Quantitatively measuring the neurite growth inhibitory properties of recombinant human MT-III in an embryonic cortical neuron assay system (using recombinant human MT-IIA for comparison)
- 3) Determining whether the extracellular, neuroactive properties of MTs are also displayed in an *in vivo* injury situation, using a model of focal cortical brain injury in the adult rat.
- 4) Investigating potential cell to cell signaling mechanisms that regulate the expression of MT in response to neuronal injury.

The implications of this work will be considered in evaluating the role of MTs in the cellular response to neuronal injury, and also in a therapeutic context.

Chapter 2: Materials and Methods

2.1 Media and buffers

LB broth

10g NaCl

10g Tryptone

5g yeast extract

dH₂O to 1 L, pH 7.0

4x NuPAGE LDS SDS-PAGE sample buffer

4g glycerol

0.682g Tris Base

0.666g Tris HCl

0.800g LDS

0.006g EDTA

0.75ml of 1% solution Serva Blue G250

0.25ml of 1% solution Phenol Red

dH₂O to 10ml, pH 8.5

20x NuPAGE MES SDS-PAGE running buffer

97.6g MES (2-(*N*-morpholino) ethane sulfonic acid)

60.6g Tris Base

10g SDS

3g EDTA

dH₂O to 500ml, pH 7.3

SDS-PAGE Coomassie blue staining solution

500mg Coomassie blue R-250

500ml methanol

100ml glacial acetic acid

dH₂O to 1L

SDS-PAGE destaining solution

400ml methanol

100ml glacial acetic acid

dH₂O water to 1L

Bradford protein assay reagent

100mg Coomassie Brilliant Blue G-250

50mls 95% ethanol

100mls 85% orthophosphoric acid

dH₂O to 1L

filtered before use

Recombinant protein resuspension buffer

20mM Tris-HCl, pH 7.6

1mM DTT

0.02% NaN₃

dH₂O to 1L

G-75 elution buffer

20mM Tris-HCl, pH 7.6

1mM DTT

0.02% NaN₃

dH₂O to 1L

DEAE elution buffer

20mM Tris-HCl, pH 7.6

1mM DTT

appropriate NaCl concentration

0.02% NaN₃

dH₂O to 1L

20x NuPAGE western blotting transfer buffer

10.2g Bicine

13.08g Bis-Tris

0.75g EDTA

dH₂O to 125ml, pH 7.2

PBS-Tween buffer (Western Blotting)

10 PBS tablets (Sigma)

1ml Tween 20

dH₂O to 1L

5% Blocking Solution (Western Blotting)

5g Diploma Skim Milk Powder

PBS-Tween buffer to 100ml

2.2 Production of recombinant MT proteins

2.2.1 Overview of bacterial expression of recombinant MTs

PET-3d (Novagen) expression vector constructs containing inserts coding for sheep MT-III, as well as human MT-IIA and human MT-III have been previously constructed in this laboratory (sheep and human MT-III: Chung, 1999, BSc Hons thesis; human MT-IIA: Eckhardt, 2000, BSc Hons thesis). Their structure has been examined by DNA sequencing, confirming the presence of the correct insert and that this insert is in the correct reading frame. Note that previous work had resulted only in the production of these recombinant MT expression vectors.

The MT expression vectors were transformed into BL21(Plys) host cells, an *E.coli* strain which contains a chromosomal copy of the T7 RNA polymerase gene. These hosts are lysogens of bacteriophage DE3, a lambda derivative that carries a DNA fragment containing the lacI gene, the lacUV5 promoter, and the gene for T7 RNA polymerase (Studier and Moffat, 1986). Once a λ DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the lacUV5 promoter, which is inducible by isopropyl- β -D-thiogalactopyranoside

(IPTG). Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid. The bacterial expression protocol is described briefly in Figure 2.1.

2.2.2 Induction of bacterial expression

A 5ml LB broth solution (with 100µg/ml ampicillin) was inoculated with the appropriate expression vector (transformed into BL21 host expression cells), and incubated overnight at 37°C with shaking. In the morning, the cells from this 5ml starter broth were used to inoculate 400mls of fresh LB broth in a 1L conical flask (to ensure good aeration). The flask was incubated with shaking at 37°C until OD₆₀₀ reached 0.6. Bacterial expression was then induced by the addition of IPTG to a final concentration of 0.4mM, and incubation continued for 3 hours. At 30 minutes post IPTG induction, 1mM of ZnSO₄ was added to form Zn-MT complexes from the freshly produced recombinant apo-MTs. This provides the basis of detection of the recombinant MTs during latter stages of purification.

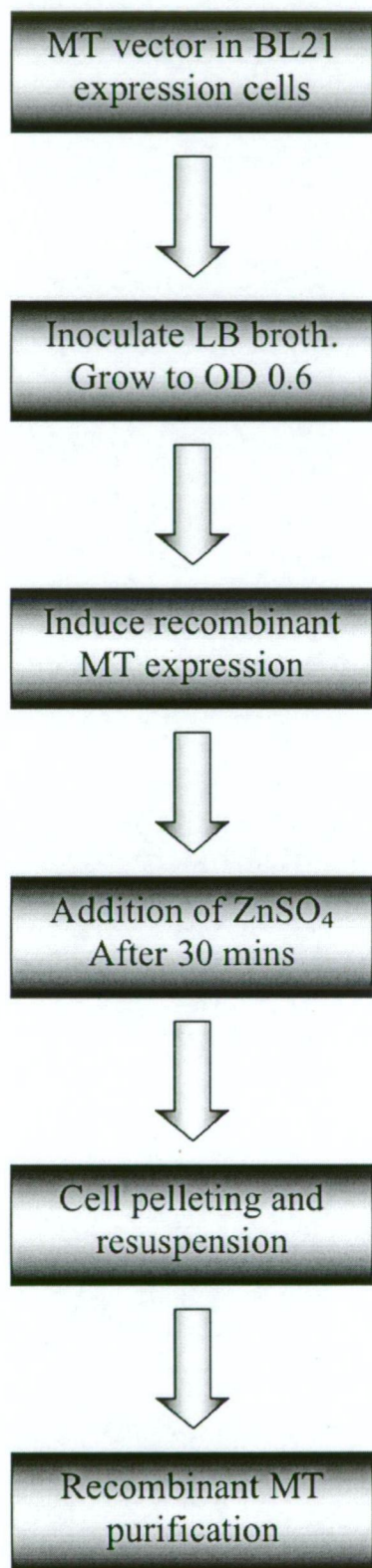
After 3 hours, the cells were placed on ice for 5 minutes. They were then harvested by centrifugation at 5000g for 20 minutes at 4°C. The pelleted cells were then resuspended in 10mls of recombinant protein resuspension buffer, and freeze-thawed twice at -20°C. Cellular debris was pelleted by centrifugation at 2000g for 2 minutes, and the supernatant (containing recombinant Zn-MTs) collected for further purification.

2.2.3 Anion exchange chromatography

Supernatants were applied to a Whatmans DEAE cellulose column (0.75 x 7cm, Pharmacia) equilibrated with elution buffer (20mM Tris-HCl, pH 7.6, 1mM DTT, 0.02% NaN₃). Fractions were eluted using a linear NaCl gradient, at a flow rate of 0.4ml/min, and their zinc content determined by atomic absorption spectrophotometry (AAS, Varian Techtron 1000). Zn-containing fractions were pooled and concentrated by ultrafiltration (MW cut-off 5 kD, Millipore).

To determine the NaCl gradient, fractions were eluted in the absence of MT sample, and the conductivity of each fraction assessed using a conductivity meter

Recombinant MT expression overview



Recombinant MT purification overview

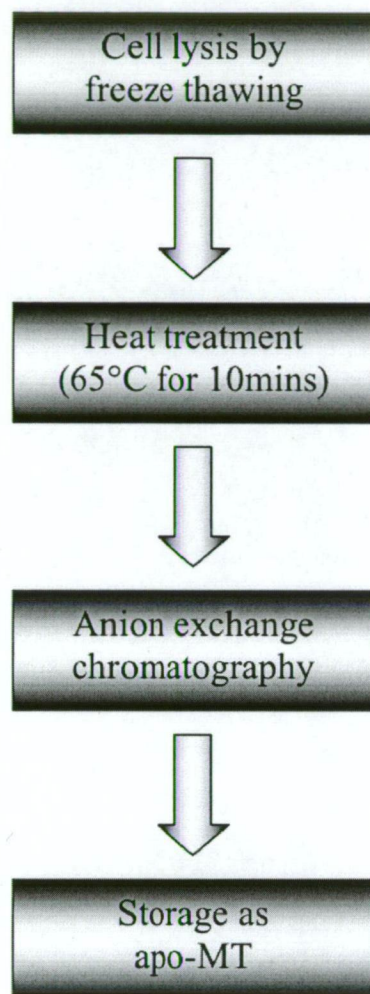


Figure 2.1 A brief overview of the recombinant MT production and purification process.

(ATHOM). These were compared to standards (known NaCl concentrations in DEAE elution buffer) to calculate the NaCl gradient.

2.2.4 Storage of recombinant MTs

Purified recombinant MTs were stored as apo-MTs (metal free MTs) in acidic conditions, as suggested by Denis Winge (personal communication). Briefly, recombinant MTs were acidified with dilute HCl to pH 2.0, and bound Zn(II) ions removed by applying the sample to an ultrafiltration column. The apo-metallothionein was stored in aliquots at -80°C until required. When required, the apo-metallothionein was reconstituted with Zn(II) by the addition of 7.5mol equivalents (for human MT-III and MT-IIA), and 5.5mol equivalents (for sheep MT-III) of Zn(II), followed by neutralization of the sample to pH 8.0 with 20mM Tris-HCl. The sample was desalted by ultrafiltration.

2.2.5 SDS-PAGE of recombinant MTs

Samples were prepared for SDS-PAGE by adding 16.5 μl of sample to 7.5 μl of 4x NuPage LDS sample buffer, vortexing, and addition of 6 μl of NuPage 10x Reducing Agent (0.5M DTT). The samples were heated for 10 minutes at 70°C and electrophoresed on a Novex, ready made 10% NuPage Bis-Tris gel (Invitrogen) in 1x MES buffer, under reducing conditions. Immediately after electrophoresis, the gel was removed and incubated in Coomassie blue staining solution for 2 hours, followed by de-staining.

2.2.6 Metal binding analysis of recombinant MTs

The Bradford protein assay, a sensitive (μg quantities) protein-dye assay (Bradford, 1976) was used to quantify recombinant protein concentrations. Briefly, this involved diluting the MT sample with Bradford reagent, and subsequent protein binding assessed by spectrophotometry at $A_{595\text{nm}}$. Quantification was performed using a standard curve with bovine serum albumin (BSA). Subsequently, the zinc content of known recombinant MT protein amounts was measured by AAS. From this, the molar equivalent of metal ions bound to recombinant MT was determined.

2.2.7 Western blotting analysis of recombinant human MT-IIA

Western blotting was performed using a modified method of Whitacre (1996). Briefly, the proteins were electro-transferred from the SDS-PAGE gel to a 0.45µm nitrocellulose membrane (Advantec MFC). After blocking with 5% skim milk in Phosphate Buffered Saline-Tween 20 buffer (PBS-T), the filter was washed three times in PBS-T, followed by incubation overnight with primary antibody (anti-MT-I/-II, DAKO) at a dilution of 1:500 in PBS-T. The following morning, the membrane was washed in PBS-T, and then incubated with secondary antibody (anti-mouse Ig HRP linked antibody, Amersham) at a dilution of 1:1000 in PBS-T. Following three washes in PBS-T, detection was performed using the Lumi-light Western Blotting Substrate Kit (Boehringer Mannheim), and the membrane placed in a x-ray cassette and exposed to Hyperfilm ECL for varying exposure times. The specificity of the MT -I/-II antibody has been tested previously (Skabo et al, 1997), and shows no cross-reaction with any other MT isoforms.

2.3 Primary cell culture, and immunocytochemical analysis

2.3.1 Low density neuron cultures

All procedures involving animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Cortical tissue was removed from embryonic day 18 (sperm positive day = E1) Hooded Wistar rat embryos and incubated in sterile 10mM HEPES buffer (37°C). This was followed by trypsin digestion (0.25%, Sigma), followed by addition of trypsin inhibitor (40 BTEE units/mg protein; Sigma). Three gentle washes of the cell pellet were made using fresh HEPES buffer. The cell suspension was then triturated carefully using a 1ml pipette, and filtered through gauze of pore size 60µm. Cell counts were performed by trypan blue exclusion. This involved suspending a 10µl aliquot of cell suspension with 10µl of trypan blue, and viable cells (those that exclude dye and are hence white) were counted. Cells were then plated onto glass coverslips (132mm²) pre-coated overnight with poly-L-lysine (0.01%, Sigma), at a

cell density of 5×10^4 cells/well, with 150 $\mu\text{g/ml}$ rat brain extract added. Cultures were maintained at 37°C in humidified air containing 5% CO_2 for 3 days after MT addition. A specific culture medium developed for selective neuronal growth was used (Brewer, 1995; 1997), consisting of NeurobasalTM medium (GIBCO), supplemented with 0.1% (f/c) B-27 supplement (GIBCO), 0.2% N2 supplement (GIBCO) 0.1mM (f/c) L-glutamine (Sigma), and 200U/ml gentamicin (GIBCO).

2.3.2 Neuron cluster cultures

2.3.2.1 Culturing techniques

Neurons were obtained as described above, except that the neurons were plated directly from the unfiltered cell suspension onto glass coverslips (254mm^2 , pre-coated overnight with 0.01% poly-L-lysine) at a cell density of 4.5×10^5 cells/well, and incubated overnight at 37°C , 5% CO_2 . The increased plating density promotes neuronal cluster formation, with thick axonal bundles forming between the clusters (Dickson *et al*, 2000). The next day, the culture medium was replaced with fresh medium, and the neurons maintained for 21 days (initial plating is day 0), with the medium replaced with fresh medium every 4 days. The medium used was as described previously for single cell neuron cultures.

2.3.2.2 Transection of neurite bundles

Transection of axonal bundles was essentially as described previously (Dickson *et al*, 2000). Briefly, at 20 days after initial plating, coverslips were moved to individual petri dishes (approximately 2900mm^2) with 150 $\mu\text{g/ml}$ rat brain extract added. The following day, neurite bundles were transected on an inverted microscope (Leitz Fluovert) using a fine goniotomy knife (Kaisers).

2.3.3 Neuron/astrocyte co-cultures

Cortical tissue was removed from embryonic day 19 (sperm positive day = E1) Hooded Wistar rat embryos and incubated in sterile 10mM HEPES buffer (37°C). This was followed by trypsin digestion (0.25%, Sigma), followed by addition of trypsin inhibitor (40 BTEE units/mg protein; Sigma). Three gentle washes

of the cell pellet were made using fresh HEPES buffer. The cell suspension was then triturated carefully using a 1ml pipette. The cells were counted by trypan blue exclusion, and plated onto glass coverslips (132mm²) pre-coated overnight with poly-L-lysine (0.01%), at a cell density of 1×10^6 cells/well. Cultures were maintained at 37°C in humidified air containing 5% CO₂. The culture medium consisted of NeurobasalTM medium (GIBCO), supplemented with 0.1% (f/c) B-27 supplement (GIBCO), 0.1mM (f/c) L-glutamine (Sigma), and 200U/ml gentamicin (DBL). Media was changed every 3 days for the first week, and then every 7 days for the next two weeks. Only half of the media in the well was replaced at each media change. This allowed astrocytes present within the culture to enrich the medium with growth promoting factors (such as neurotrophins) promoting their survival and proliferation. In some cases, scratch wound injuries were performed. Scratch wounds were performed using a fine goniotomy knife (Kaisers).

2.3.4 Fluorescent immunocytochemical analysis of primary cultures

At the appropriate time, cells were fixed with 4% paraformaldehyde, for 20 minutes at room temperature. Coverslips were then incubated with a variety of primary antibodies (see Table 2.1) diluted in 0.1% PBS, 0.03% tritonX-100 (immunofluorescence diluent).

Coverslips were then incubated with two secondary antibodies (goat anti-mouse IgG conjugated to Alexafluor 488 and goat anti-rabbit IgG conjugated to Alexafluor 594, 1:1,000 dilution; Molecular Probes), applied in 0.1% PBS. Following 3 washes in PBS and drying, the coverslips were mounted onto slides using Permafluor mounting medium (Immunotech). In some cases nuclear yellow staining was performed. Following immunocytochemistry, a 0.1% solution of nuclear yellow was applied to the coverslip, and incubated at room temperature for 5 minutes, and the coverslip subsequently washed 3 times with PBS.

In some experiments, triple immunofluorescence was performed using three primary antibodies: anti-MT-I/-II, anti- β III tubulin (monoclonal mouse antibodies) and anti-GFAP (polyclonal rabbit). Coverslips were incubated with anti-MT-I/-II (diluted 1:200 in diluent) for 1 hour at room temperature, with shaking. Following

Antibody name	Type	Immunoreactivity	Dilution	Source	Reference
anti-MAP2	M	Microtubule-associated protein 2	1:1,000	Chemicon	Binder <i>et al</i> , 1984
anti- β III tubulin	M	Neuron specific β -tubulin subunit of the tubulin monomer	1:10,000	Promega	Lee <i>et al</i> , 1990
anti-MT-I/-II	M	Metallothionein-I and -II isoforms only	1:500	DAKO	Skabo <i>et al</i> , 1997
anti-tau	R	Microtubule-associated tau protein, axonal	1:5,000	DAKO	Goedart <i>et al</i> , 1988
anti-NFM	R	Neurofilament, medium subunit	1:1,000	Serotec	Karlsson <i>et al</i> , 1987
anti-GFAP	R	Glial fibrillary-associated protein	1:500	DAKO	Viale <i>et al</i> , 1991

Table 2.1: A list of the various primary antibodies used for immunohistochemical staining. Antibodies were either monoclonal mouse (M) or polyclonal rabbit (R).

three washes in PBS, coverslips were incubated with goat anti-mouse IgG conjugated to Alexafluor 488 (Molecular Probes) at 1:1,000 (in PBS) for 1 hour at room temperature with shaking. This was repeated, and coverslips subsequently washed three times in PBS. This, and all later steps were performed in the dark to preserve fluorescence. Anti- β III tubulin (1:5,000) and anti-GFAP (1:2,000) were applied to coverslips in PBS for 1 hour at room temperature, with shaking. Following three PBS washes, goat anti-rabbit IgG conjugated to Alexafluor 594 (1:2,000; Molecular Probes) and sheep anti-mouse IgG conjugated to AMCA (1:500; Vector) were applied to coverslips in PBS for 1 hour. Following three PBS washes, coverslips were dried and mounted onto slides using Permafluor mounting medium (Immunotech).

In some experiments, cell viability was determined by propidium iodide uptake. Briefly, 50 μ g/ml of propidium iodide was added to cultures for 30 minutes, followed by fixation as described previously. Those cells which take up propidium iodide (and which are hence unviable) were visualized on fluorescent microscope at 594nm (Texas Red filter).

2.4 Rat focal cortical brain injuries, and immunohistochemical analysis

2.4.1 Rat focal cortical brain injuries

Rat focal cortical injuries were made as reported previously (King *et al*, 1997). Briefly, 250g male rats were anaesthetised with 0.1ml/100g of Nembutal (sodium pentobarbitol), the head shaved, and the rat then immobilised in a Stoelting stereotaxic frame. An incision down the midline of the skull was made, and a hole carefully drilled through the skull above the Par 1 region of the somatosensory cortex using a Dremel surgery drill. A Hamilton syringe with a 25 gauge bevelled needle was aligned with the hole, and then lowered 1.5mm into the brain. After 10 minutes, the syringe was slowly removed and the hole within the skull filled with gelfoam (such that the gelfoam sits above the injured cortex), pre-treated with either 20 μ l of phosphate buffered saline (PBS) or 20 μ l of MT-IIA (5 μ g/ml concentration). Antiseptic powder was applied, and then the skin pulled back over the skull and held together by 2 staples.

2.4.2 Vibratome sectioning

At the appropriate time, rats were re-anaesthetised and transcardially perfused with 100ml PBS followed by 100ml 4% paraformaldehyde at a flow rate of 8ml/min. Brains were removed and post-fixed overnight in 4% paraformaldehyde at 4°C. They were then embedded in 5% agar (in 0.1% PBS), and sectioned by vibratome at a thickness of 50µm (Leica VT1000E). Sections were then used in fluorescent immunohistochemistry as described below or stored in PBS (containing 0.01% sodium azide) at 4°C.

2.4.3 Fluorescent immunohistochemistry of brain sections

Brain sections were incubated with a combination of two primary antibodies; mouse SMI-312/rabbit anti-ferritin (1:2,000 Sternberger Monoclonals; 1:10,000 ICN), or SMI-312/rabbit anti-GFAP (1:500 Sternberger Monoclonals; 1:2,000 DAKO). Anti-ferritin was used as a marker of activated microglial cells (King *et al*, 2001). SMI-312 labels the phosphorylated form of neurofilaments (NF), and is considered an axonal marker (Ulfig *et al*, 1998). The antibodies were diluted in 0.1% PBS, 0.03% tritonX-100. Sections were then incubated with two secondary antibodies (goat anti-rabbit IgG conjugated to alexafluor 488, 1:250 dilution, Molecular Probes; and horse anti-mouse rat adsorbed IgG, 1:250 dilution, Vector) applied in 0.1% PBS. Brain sections were mounted using Permafluor mounting medium (Immunotech). Specimens were viewed on the BX-60 (Olympus) fluorescence microscope.

2.5 Miscellaneous techniques

2.5.1 G-75 gel filtration chromatography

To calibrate the column, a 10ml sample of protein standards (10mg each of Blue Dextran, cytochrome c, and potassium ferrocyanate) was evenly applied to a Sephadex G-75 column (31.5 x 2.75 cm, Pharmacia) equilibrated with G-75 elution buffer (20mM Tris-HCl, pH 7.6, 1mM DTT, 0.02% NaN₃). The sample was allowed to run down until only a very small amount covered the top of the Sephadex beads.

Elution buffer was then evenly applied to the top of the column and a gravity feed attached. The sample was eluted with elution buffer at a flow rate of 1.2ml per minute, and collected in 3ml fractions. Protein content of these standards was determined using ultraviolet (UV) spectrophotometry (BIOCHROM Ultraspec II) at 230nm.

Samples were applied to the column, and eluted under the same conditions as above. The protein content of each fraction was assessed by the method of Bradford (1976).

Chapter 3: An unusual mammalian MT-III variant, sheep MT-III

3.1 Introduction

The mammalian MT family consists of 4 isoforms, within which a number of sub-isoforms exist. It is striking, however, that none of these exhibit alterations in the number or position of the consistently conserved cysteine residues. This suggests a tight evolutionary constraint, with these conserved cysteine residues being essential to the physiological role of the MTs. However, characterization of cDNAs of sheep MT-III in our laboratory by Adele Holloway (Holloway, 1996, PhD thesis) suggested that the sheep MT-III gene might encode a variant protein (Figure 3.1). Using standard metallothionein protein isolation techniques (including heat treatment, solvent precipitation, gel filtration and anion exchange chromatography) the sheep MT-III protein has been isolated and purified by the author, as part of his BSc Honours studies, confirming that sheep do indeed produce a naturally occurring MT-III variant (Chung, 1999, BSc Hons thesis). Indeed, partial amino acid sequencing confirmed the aberrant nature of the sheep MT-III protein.

Of particular interest within the sheep MT-III protein is the absence of 3 cysteine residues, 2 by deletion and one by substitution (with reference to other characterised MT-III proteins). Previous studies have found that experimental alterations in the cysteine residues of MTs significantly affects the conformation of the protein, its metal binding properties (Cismowski & Huang, 1991), and consequent physiological functions such as metal detoxification (Cismowski & Huang, 1991; Chernaik & Huang, 1991). As such, the absence of 3 cysteine residues in sheep MT-III is likely to alter the conformation of the protein, and it would seem feasible then, that sheep MT-III would have altered metal binding properties (Figure 3.2), and as a consequence altered physiological properties. Intriguingly, sheep have been found to exhibit a high sensitivity within the brain to dietary fluctuations in heavy metals, and in particular copper (Alleyne *et al*, 1998; Doherty *et al*, 1969; Howell *et al*, 1964). It is known that expression and structure of sheep MT-I/-II isoforms are similar to their other mammalian counterparts (Peterson *et al*, 1988), so this marked sensitivity may be related to the variant sheep MT-III protein.

Sheep MT-III	1	11	21	30
	MDPEACPCPT	GGSCTCSDSC	KCEGCTCASS	
		*	*	*
Human MT-III	1	11	21	30
	MDPETCPCPS	GGSCTCADSC	KCEGCKCTSC	
Mouse MT-III	1	11	21	30
	MDPETCPCPT	GGSCTCSDKC	KCKGCKCTNC	

Sheep MT-III	31	41	51	61	68
	KK - - -	SCCPA	ECEKCAKDCV	CKGGEGAEAE	EKKCGCCQ
				*	*
Human MT-III	31	41	51	61	68
	KKSCCSCCPA	ECEKCAKDCV	CKGGEGAEAE	AEKCSCCQ	
Mouse MT-III	31	41	51	61	68
	KKSCCSCCPA	GCEKCAKDCV	CKGEEGAKAE	AEKCSCCQ	

Figure 3.1: The predicted protein sequence (from mRNA) of sheep MT-III was compared to known human and mouse MT-III protein sequences. There is a 3 amino acid deletion (SCC) in sheep MT-III, normally conserved at positions 33-35 of MT-III proteins, as well as a C_S change at position 30. Amino acids found in the sheep MT-III protein but not in human or mouse MT-III, are indicated by asterisks. Data from Holloway, 1996, PhD thesis.

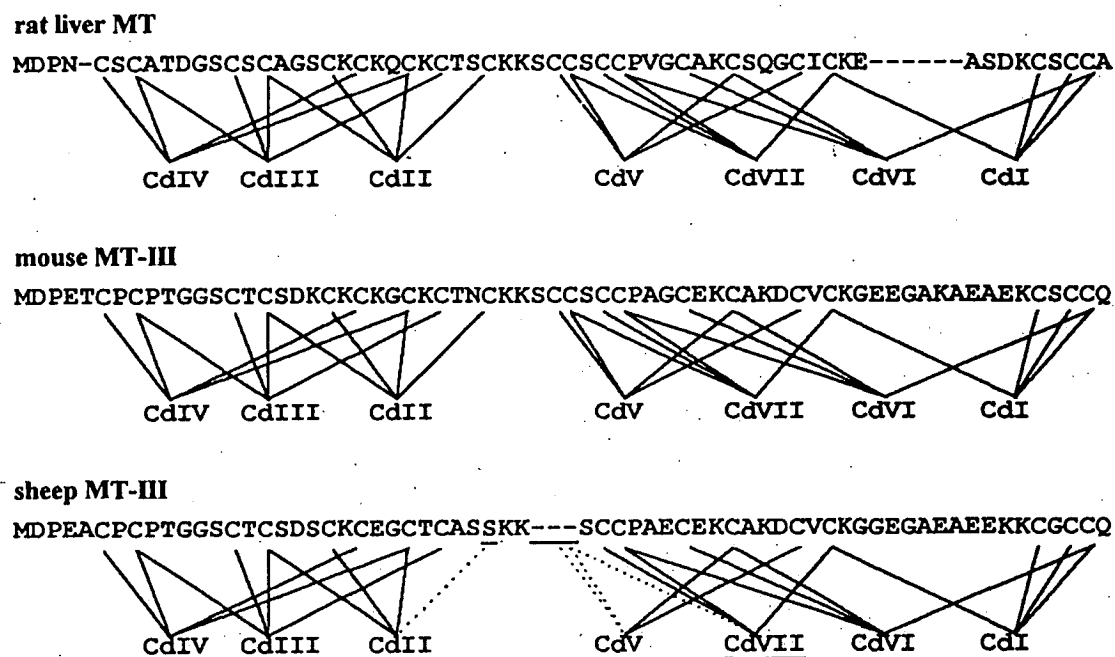


Figure 3.2: Cadmium-thiolate linkages in rat liver $^{113}\text{Cd}^7$ -thionein have been determined by NMR spectroscopy (adapted from Kagi and Schaffer, 1988) and using this structure as a model, a proposed structure for the cadmium-thiolate linkages of mouse MT-III is shown. This structure is based upon reports indicating that MT-III exhibits a similar metal-protein conformation to the MTI/II class of isoforms (as reviewed by Romero-Isart & Vasak, 2002). In comparison, the altered cysteine complement of sheep MT-III is likely to disrupt metal-thiolate linkages affecting binding to at least three metal atoms (dotted lines). Adapted from Holloway, 1996, PhD thesis.

While the physiological role of MT-III is unknown, MT-III has been demonstrated to have neuronal growth inhibitory properties in culture models (for example see Uchida *et al*, 1991). Later studies defined the C₍₆₎PCP region of the protein as responsible for MT-III's inhibitory properties (Sewell *et al*, 1995). All mammalian MT-III isoforms isolated to date possess this C₍₆₎PCP motif, and subsequent inhibitory properties. Interestingly, the variant sheep MT-III protein also contains this motif (Figure 3.1), suggesting that it also shares the inhibitory properties of other MT-III isoforms.

The aim of this chapter is two-fold. Firstly, because sheep MT-III is the only known MT variant expressed at the protein level to exhibit changes in its cysteine distribution, the physical consequences of these changes will be compared to other, well characterized MTs, namely human MT-IIA and human MT-III. These experiments may elucidate potential mechanisms underlying the marked sensitivity of sheep to heavy metal induced neurotoxicity. The sheep MT-III variant will also be assessed functionally, by comparing its biological activity (namely neuronal growth inhibition) to human MT-IIA and MT-III. This may indicate the importance of this novel extracellular property of MT-III, and also provide further insight into the structural features of the MT-III molecule responsible for this activity.

3.2 Experimental Procedures

3.2.1 Overview of recombinant MT production

Metallothionein is well suited to recombinant bacterial production, because it is a small, single-chain peptide with no known post-translational modifications, or internal disulphide linkages. pET-3d expression vector constructs (Novagen) containing inserts coding for sheep MT-III, as well as human MT-IIA and human MT-III have been previously constructed in this laboratory (sheep and human MT-III: Chung, 1999, BSc Hons thesis; human MT-IIA: Eckhardt, 2000, BSc Hons thesis) The important features of this vector are ampicillin resistance, and inducibility of target gene expression by addition of the galactose analogue isopropyl- β -D-thiogalactopyranoside (IPTG). The addition of IPTG induces the production of T7 RNA polymerase within the host bacterium, which in turn transcribes the MT insert

in the plasmid. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression. As such, the desired protein product can comprise more than 50% of the total cell protein after a few hours of induction (Studier & Moffat, 1986). Mammalian MT sequences were derived from mRNA by RT-PCR, at the same time incorporating *Nco*I and *Bam*HI restrictions sites at appropriate locations using mismatched primers. The structure of all constructs has been examined by DNA sequencing, confirming the presence of the correct insert, and that this insert is in the correct reading frame (Figure 3.3).

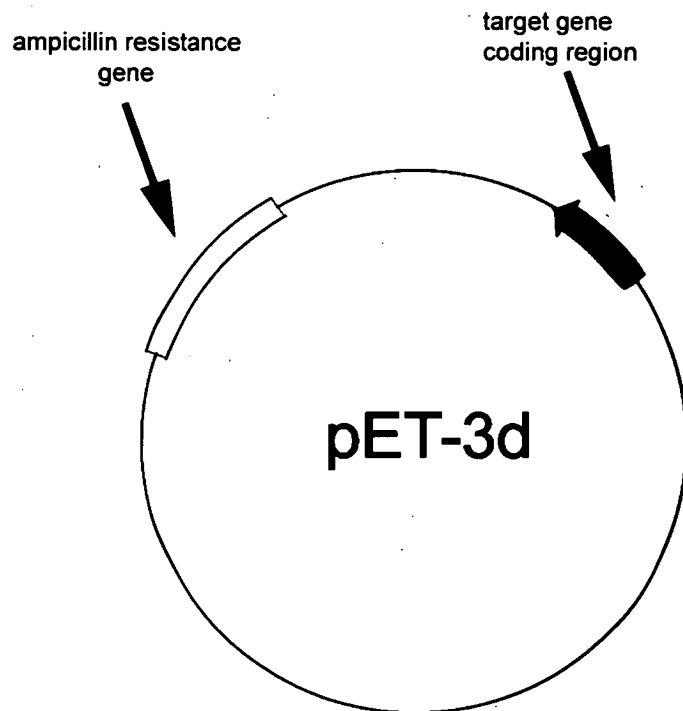
All of the recombinant proteins were expressed in *E.coli* in the presence of zinc, to produce Zn-MT conjugates, which are less readily oxidized than apo-MT (Hong *et al*, 2001). Furthermore, zinc forms the basis of recombinant MT detection during purification by chromatography. Each recombinant MT isoform was produced in a separate run, to avoid possible contamination between batches. Proteins were isolated from bacterial pellets by a combination of heat treatment, anion-exchange chromatography and ultrafiltration as described in Figure 2.1 (Chapter 2).

3.2.2 Functional analysis of recombinant sheep MT-III

The metal binding properties of recombinant MT proteins were determined by firstly quantifying recombinant protein concentrations using the Bradford protein assay (Bradford, 1976). Subsequently, the zinc content of known recombinant MT protein amounts was measured by atomic absorption spectrophotometry (AAS). From this, the molar equivalent of metal ions bound to recombinant MT was determined.

3.2.3 Neuronal growth inhibitory activity of recombinant sheep MT-III

The neuronal growth inhibitory activity of sheep MT-III was investigated using the model first described by Uchida *et al* (1991). Recombinant MTs were applied to low density (5×10^4 cells/well) embryonic cortical neuron cultures immediately after initial plating, together with 150µg/ml brain extract. Control cultures received brain extract only. For analysis, 10 digital images were captured



pET-3d cloning/expression region

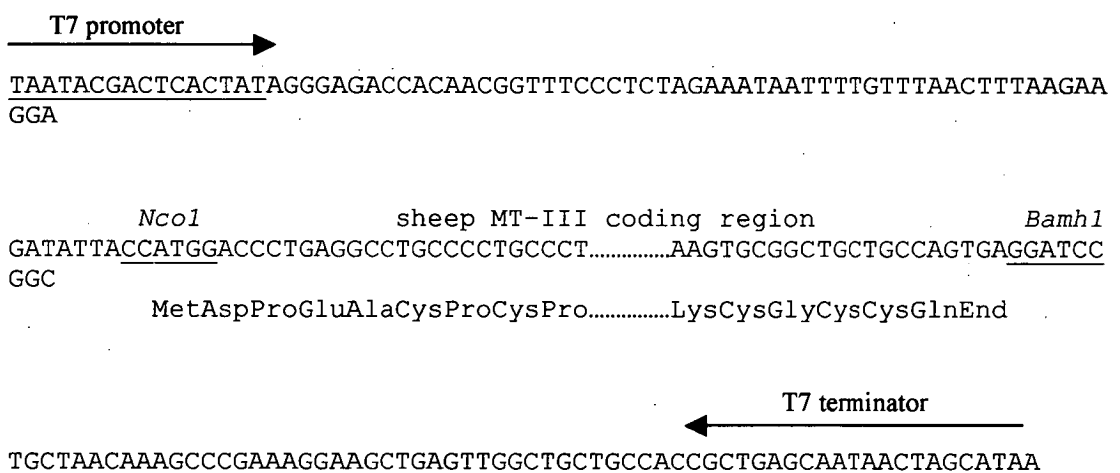


Figure 3.3: cDNA encoding the sheep MT-III protein (indicated in red) was inserted into the pET-3d expression vector, using the *Nco*I and *Bam*HI restriction enzyme sites. Expression of MT-III protein is controlled by the T7 promoter (arrow). The expression vector is transferred to a host *E.coli* strain with a chromosomal copy of the T7 RNA polymerase gene, under the control of the lacUV5 promoter, which can be induced by IPTG. Hence, addition of IPTG induces T7 RNA polymerase production, which subsequently transcribes the sheep MT-III DNA.

from each coverslip at 3 days post MT treatment, at a magnification of 40x (Olympus BL-51 microscope, Olympus DP-50 digital camera). The number of neurons counted per coverslip ranged from 50 to 120 cells. Experiments were replicated in triplicate. Growth inhibitory activity was determined in a similar manner to that of Uchida *et al* (1991) as a percentage by the following formula: percentage inhibition = $100 - (100 \times \frac{\text{the number of neurons per field of view in the presence of added recombinant MT}}{\text{the number of neurons in control cultures}})$. Student t-test analysis was performed using SigmaStat (Jandel Scientific Software Corporation; San Rafael, CA).

3.3 Results

3.3.1 Bacterial expression of recombinant MTs

At the commencement of this work, pET-3D expression vectors for sheep MT-III and human MT-IIA and MT-III had been constructed (Chung, 1999, BSc Hons thesis; Eckhardt, 2000, BSc Hons thesis), but protocols for bacterial expression, MT isolation and purification, and characterization had not been developed in this laboratory. Therefore, the initial aim of this chapter was to complete this work, so as to provide recombinant MT protein for further experimentation. Briefly, bacterial cultures were grown to an optical density of 0.6, at which time MT protein expression was induced by addition of IPTG for a period of 3 hours in the presence of 1mM zinc sulphate. Recombinant protein expression was confirmed by SDS-PAGE, using samples at the start and end of the expression period (Figures 3.4A and 3.4B). Before induction of MT protein expression, it was clear that there was no protein band at the 7kD size range (the approximate size of MTs as assessed by SDS-PAGE is 6-7kD). At 3 hours post induction, a dense protein band was clearly visible at 7kD. Furthermore, there was little difference in the intensity of higher molecular weight protein bands between time points, indicating that the bacterial cells are not dividing and are almost exclusively producing recombinant protein.

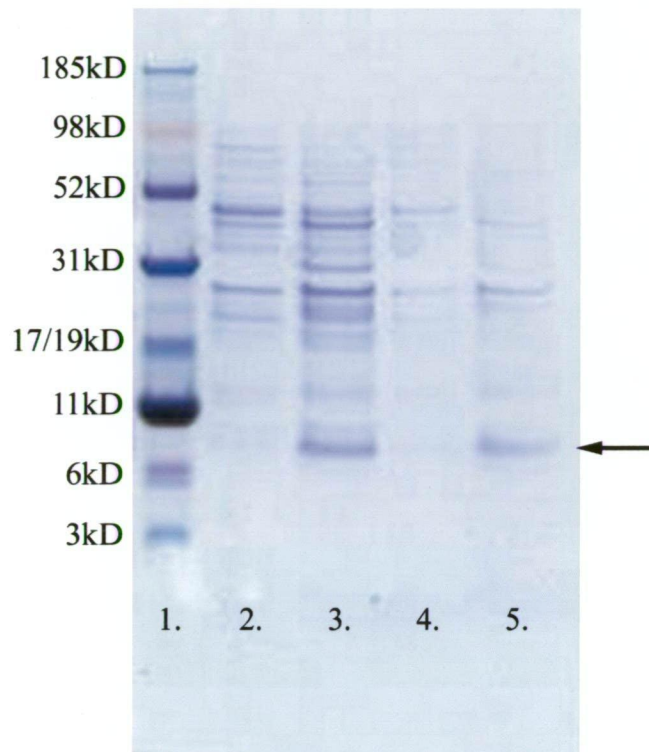


Figure 3.4A: SDS-PAGE analysis of recombinant MT-III protein expression. Samples were taken both prior to IPTG induction of protein expression and after 3 hours of expression. Lanes contained the following:

- Lane 1 – molecular weight markers
- Lane 2 - human MT-III, bacterial lysate prior to IPTG induction
- Lane 3 - human MT-III, bacterial lysate 3 hours post induction
- Lane 4 - sheep MT-III, bacterial lysate prior to IPTG induction
- Lane 5 - sheep MT-III, bacterial lysate 3 hours post induction

At 3 hours post induction, a protein band corresponding to recombinant MT-III was clearly visible at 7kD (arrow).

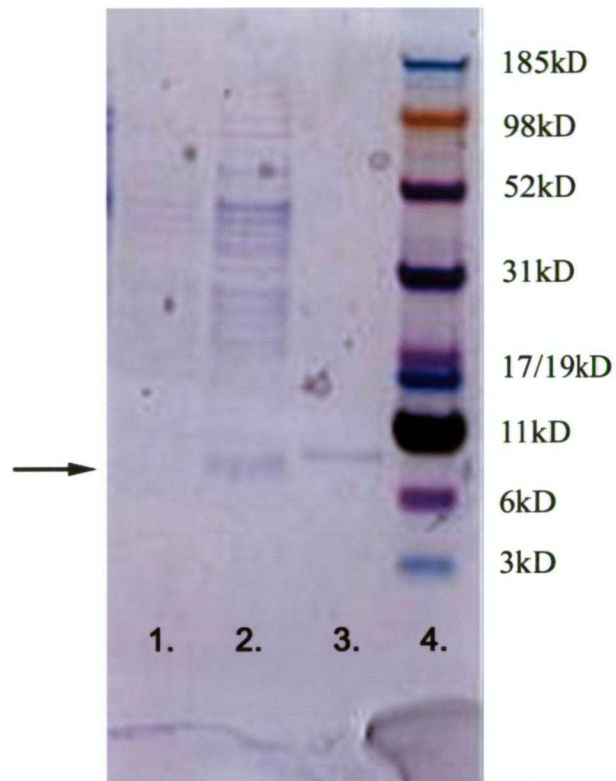


Figure 3.4B: SDS-PAGE analysis of recombinant human MT-IIA protein expression. Samples were taken both prior to IPTG induction of protein expression and after 3 hours of expression. Lanes contained the following:

- Lane 1 – human MT-IIA, bacterial lysate prior to IPTG induction
- Lane 2 – human MT-IIA, bacterial lysate 3 hours post induction
- Lane 3 – native sheep MT-I/-II (used as a molecular weight marker for MT, and also as a positive control for western blotting)
- Lane 4 – molecular weight markers

At 3 hours post induction, a protein band corresponding to recombinant MT-IIA was clearly visible at 7kD (arrow), which was also similar to the electrophoretic position of native sheep MT-I/-II.

3.3.2 Isolation and purification of recombinant MTs

Following expression, the bacterial cells were pelleted and carefully lysed by freeze thawing. Following lysis, bacterial cell debris was pelleted, and the supernatant (containing recombinant MT) was heat treated at 65°C for 10 minutes. Heat treatment resulted in the precipitation of a number of proteins, which were removed by centrifugation.

Further purification of the recombinant MTs was achieved by anion-exchange chromatography using DEAE cellulose at pH 7.6 (see Chapter 2.2.3). A NaCl gradient was used to elute recombinant proteins from this column. These proteins were found to elute at various salt concentrations, ranging from 100mM (human MT-IIA-Figure 3.5A) to 200mM (human MT-III-Figure 3.5B) to 250mM (sheep MT-III-Figure 3.5C). These points of elution are similar to those observed previously for native MTs. For instance, MT-II isoforms have been eluted at salt concentrations ranging from 80-120mM (Nordberg *et al*, 1972; Hidalgo *et al*, 1989), whilst various mammalian MT-III isoforms have been eluted at concentrations near 200mM (including 200mM for human MT-III, Uchida *et al*, 1991; 230mM for porcine MT-III, Chen *et al*, 1996). Native sheep MT-III has been eluted at 250mM NaCl concentration (Chung, 1999, BSc Hons thesis).

Collected fractions were concentrated by ultrafiltration, and analysed by SDS-PAGE (Figure 3.6). All recombinant MT proteins electrophoresed as a single band at the expected size (approximately 7kDa), indicating the absence of any contaminating bacterial proteins, or oxidation of the recombinant MTs during the purification process.

Identification of the purified recombinant proteins as MTs was based upon their electrophoretic position in SDS-PAGE analysis, their zinc binding ability and their elution profiles when purified by anion-exchange chromatography. Western blotting analysis has also been performed for recombinant human MT-IIA as further confirmation of the protein expression process (results not shown). This was not possible for recombinant MT-III due to the lack of antibodies which bind to MT-III.

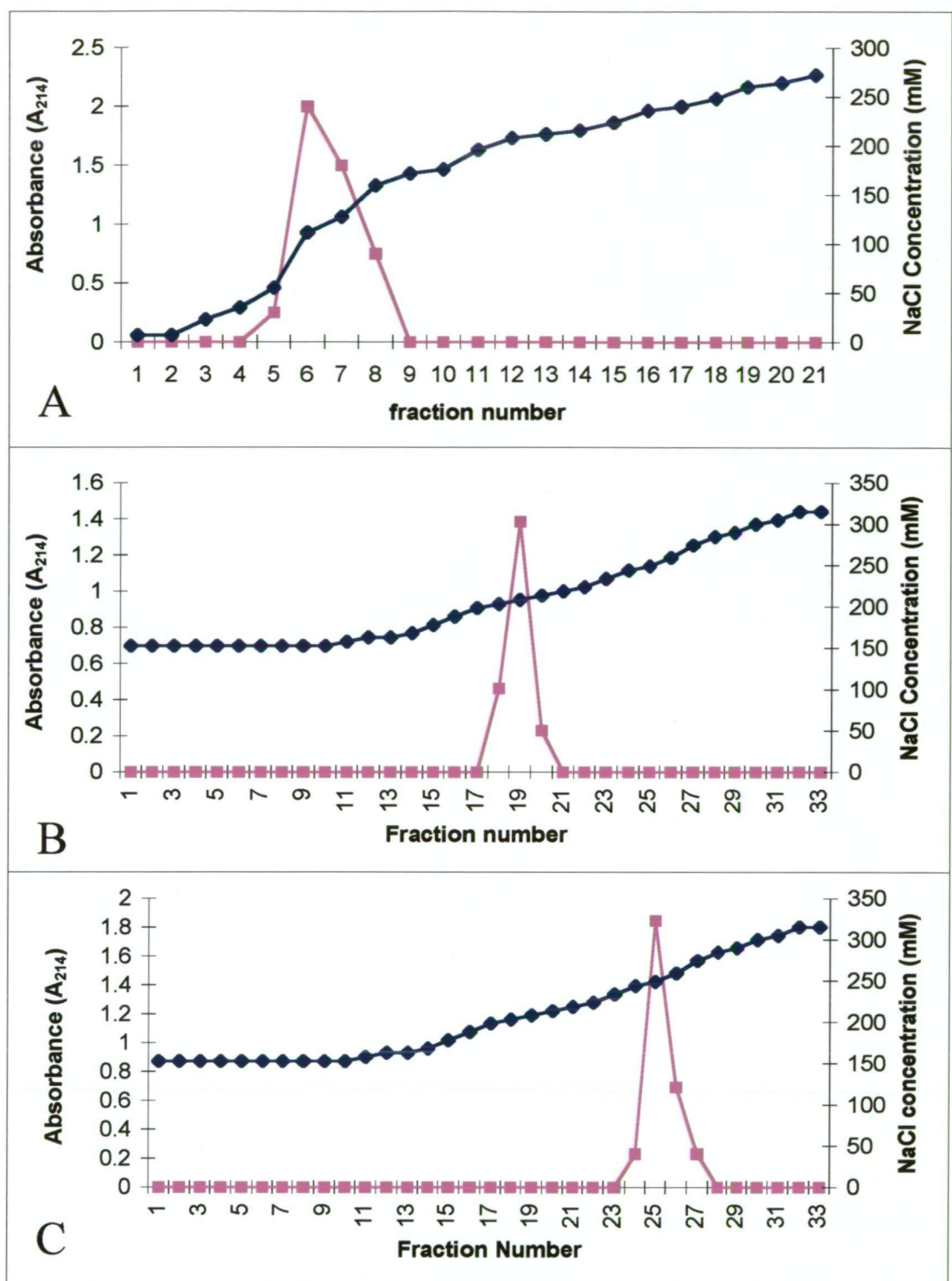


Figure 3.5: Recombinant MT proteins were purified by anion-exchange chromatography. MTs were eluted using a salt gradient (\blacktriangle), and detected by the zinc content of individual fractions using AAS at a wavelength of 214nm (\blacksquare). Human MT-IIA was eluted from the column at 100mM salt concentration (A). Human MT-III (B) eluted from the column at 200mM salt concentration and sheep MT-III (C) at a concentration of 250mM.

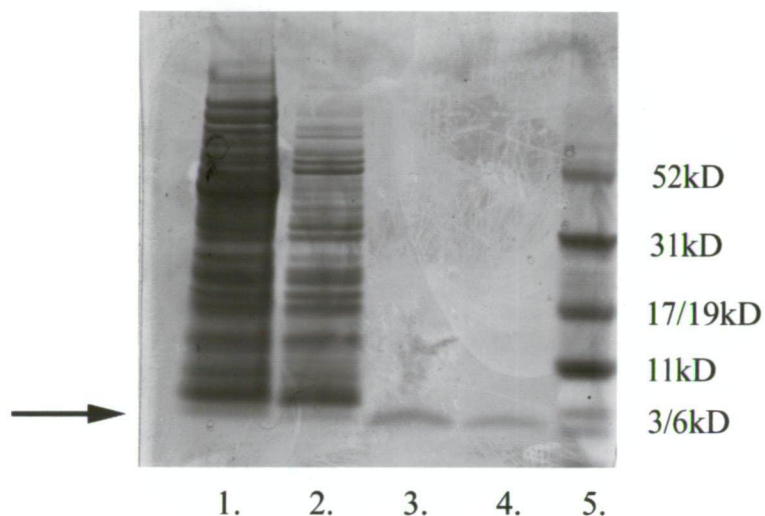


Figure 3.6: SDS-PAGE analysis of recombinant MT-III purification by anion exchange. Following anion-exchange chromatography of crude bacterial extracts, the peaks exhibiting high zinc content were pooled and concentrated by ultrafiltration. These samples were then analysed by SDS-PAGE. Lanes contained the following:

- Lane 1 – human MT-III, crude bacterial extract
- Lane 2 – sheep MT-III, crude bacterial extract
- Lane 3 – anion-exchange purified human MT-III
- Lane 4 – anion-exchange purified sheep MT-III
- Lane 5 – molecular weight markers

Purified recombinant MT-III electrophoresed as a single protein band at approximately 6-7kD (as indicated by arrow).

3.3.3 Metal binding analysis of recombinant MT proteins

The stoichiometry of metal-protein binding of recombinant sheep MT-III was compared to the known properties of human MT-IIA and human MT-III by atomic absorption spectrophotometry. Recombinant sheep MT-III bound 5 zinc ions per molecule, while both recombinant human MT-III and human MT-IIA were found to bind 7 zinc ions per molecule.

3.3.4 Neuronal growth inhibitory activity of recombinant sheep MT-III

The neuronal growth inhibitory activity of recombinant sheep MT-III protein (compared to vehicle treated cultures and determined as % inhibitory activity) was found to be 14% and 37% at 1 μ g and 5 μ g/ml respectively. This was compared to the activity of other MT isoforms, namely human MT-IIA and human MT-III (Figure 3.7). Human MT-IIA, was found to have almost no inhibitory effect at any of the concentrations tested. This is in accordance with other MT-I/-II isoforms (recombinant rat MT-I/II, Erickson et al, 1994; recombinant mouse MT-I, Sewell et al, 1995). The inhibitory activity of sheep MT-III was found to be significantly smaller than human MT-III ($p < 0.01$; student's t-test), which exhibited 22% and 53% inhibition at 1 μ g and 5 μ g/ml respectively, similar to that reported previously (Erickson et al, 1994).

3.4 Discussion

3.4.1 Functional consequences of the altered protein structure of sheep MT-III

Most of the proposed functions of MT are dependent upon the unusual structure of this protein. In particular, it is the presence of 20 conserved cysteine residues which are essential for MT's metal binding and redox characteristics. No significant variation in the number or relative position of these cysteines has been observed in previously isolated mammalian MTs (Kagi, 1993). Furthermore, MT-III's neuronal inhibitory properties have been ascribed to the presence of a cysteine motif, C₍₆₎PCP, present in the neural-predominant MT-III isoform, but not in other MTs (Erickson *et al*, 1994).

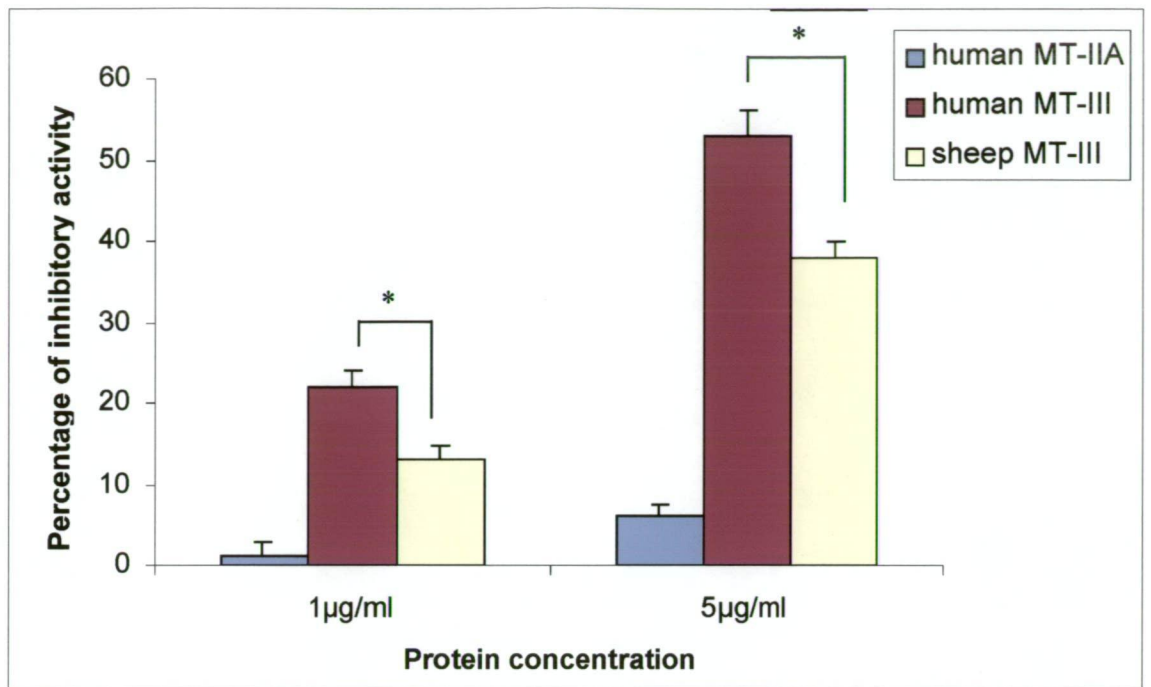


Figure 3.7: Rat cortical neurons were cultured with either 1 or 5 µg/ml of human MT-IIA, MT-III, or sheep MT-III, all in the presence of rat brain extract (150 µg/ml). The percentage of growth inhibitory activity was calculated by the following formula: percentage inhibition = $100 - (100 \times \frac{\text{the number of neurons per field of view in the presence of added recombinant MT}}{\text{the number of neurons in control cultures}})$. * - $p < 0.01$; paired t-test. Error bars represent standard error values.

Sheep MT-III has unique structural features compared to the previously characterised MT-III proteins and therefore may be useful in determining the physiological role of MT-III isoforms and their relationship to other MTs. The most striking feature of the sheep MT-III protein in comparison to any mammalian MT is a deletion of 3 amino acids (SCC) at positions 33-35. This is a deletion in the **S₍₃₃₎CCSCC** (deleted amino acids are highlighted in bold) sequence that occurs directly after the 2 lysine residues which form the bridge between the α - and β -domains of the MT protein. This sequence forms the start of the β -domain of the protein, and is conserved among other mammalian MT protein sequences documented thus far (Bogumil *et al*, 1996). In earlier work (Holloway 1996, PhD thesis; Chung 1999, BSc Hons thesis), it was demonstrated that these changes were maintained in cDNAs from 12 individual sheep, representing two separate breeds (Merino and Pol Dorset). The number of cDNAs sequenced, in combination with Southern blotting, strongly suggested that only one expressed MT-III gene exists in sheep. In this chapter, it was demonstrated that the absence of these 3 cysteine residues has directly altered the metal binding properties of the sheep MT-III protein since the sheep isoform binds 5 zinc atoms per metallothionein molecule, whereas under the same conditions, human MT-III, and human MT-IIA bind seven. This is supported by Cismowski & Huang (1991), who found Chinese hamster MT mutants C₍₁₃₎Y, C₍₅₀₎Y and C₍₁₃₎₍₅₀₎Y (in which cysteine 13, cysteine 50 or both cysteine 13 and 50 are mutated to tyrosine residues) had metal-binding domains which tended to be folded less tightly and all three mutants bound 6 rather than 7 mole equivalents of cadmium. Furthermore, while some mutants retained their ability to protect against cadmium toxicity, others had reduced detoxification function (Cismowski & Huang, 1991; Chernaik & Huang, 1991), and this would suggest that by analogy sheep MT-III also has altered properties *in vivo*.

3.4.2 Sheep MT-III exhibits neuronal growth inhibitory activity

The neuronal growth inhibitory assay used in this chapter is based upon the assay used previously by Uchida *et al* (1991) and Erickson *et al* (1994). The basis of this assay is that the embryonic neurons are plated at a low cell density (5×10^4

cells/well) in serum-free culture media, which results in less than optimal culture conditions. To enhance survival, adult rat brain extract is added, in essence “rescuing” the neurons and it is against the action of this extract that the action of MT-III can be observed and quantified. Addition of recombinant human MT-III, in the presence of the brain extract, results in a significant decrease in neuron survival, hence exhibiting a neuronal growth inhibitory effect. Using this assay, it was found that recombinant sheep MT-III also exhibits inhibitory activity (13 and 37% inhibition; 1 and 5 µg/ml respectively), supporting the hypothesis first proposed by Sewell *et al* (1995) that the C₍₆₎PCP motif, retained in sheep MT-III as well as the other known mammalian MT-III isoforms, confers this property. However, its growth inhibitory properties are not as potent as recombinant human MT-III (22 and 53% inhibition; 1 and 5 µg/ml respectively; $p < 0.01$; student’s t-test, suggesting that perhaps other aspects of the physical structure of the MT-III molecule are intimately involved in its ability to inhibit neuronal growth. As an indication of this, Romero-Isart *et al* (2002) have engineered the C₍₆₎PCP motif found in MT-III into mouse MT-I. While addition of the C₍₆₎PCP motif alone into mouse MT-I did not confer inhibitory activity, introduction of the conserved T₍₅₎ residue found immediately prior to the C₍₆₎PCP motif in native MT-IIIs, to create the sequence T₍₅₎CPCP, resulted in bioactivity. They concluded from their results that the structure/cluster dynamics of MT-III are critical for its neuroactivity. Interestingly, in sheep MT-III the T₍₅₎ residue is substituted with an A₍₅₎. Because threonine and alanine share similar physical characteristics, it is unlikely that this substitution would substantially alter the structural characteristics of sheep MT-III. However, the fact that sheep MT-III retains the C₍₆₎PCP motif, yet exhibits reduced inhibitory activity, lends support to the suggestion of Romero-Isart and colleagues (2002) that the nature of the amino acid at position 5, and its effect upon the structure/cluster dynamics of the molecule, affects its growth inhibitory properties. However, it is also possible that other features specific to sheep MT-III, including other amino acid differences or even its altered metal binding properties, might be important in reducing its growth inhibitory ability.

3.4.3 Physiological significance of the evolutionary conservation of the C₆PCP motif

While sheep MT-III bears only 81% and 76% protein sequence homology with human and mouse MT-III respectively, it does retain the C₆PCP motif common to all mammalian MT-III isoforms (but not found in any other MT isoforms). Furthermore, sheep MT-III also possesses the neuronal growth inhibitory properties conferred by this motif, although to a lesser extent. The fact that this motif is retained in all MT-III members characterized so far, including this unusual variant, is highly suggestive that this motif, and associated inhibitory activity, are an integral part of the physiological role of extracellular MT-III. While MT-III is considered a solely intracellular protein (Palmiter *et al*, 1992), Uchida *et al* (2002) have recently demonstrated that nanogram quantities of MT-III are actively released in culture by astrocytes, the primary cellular source of MT-III in the brain. Based on calculations that concentrations of MT-III in both rat and human adult brain range from 4-20µg/g of brain (Erickson *et al*, 1994; Uchida *et al*, 2002), it is possible to envisage that extracellular levels of MT-III in the brain could feasibly reach the levels used in the work reported here (1-5 µg/ml). Therefore, the hypothesis first raised by Uchida *et al* (1991) in the context of Alzheimer's disease that MT-III has an actual extracellular, trophic role, presumably through modulating the aberrant reactive sprouting involved in neurofibrillary tangle formation, must remain a possibility. The following chapter continues this theme by precisely defining, for the first time, the action of human MT-III on neuronal growth and recovery from defined physical lesions.

3.4.4 Susceptibility of sheep to heavy metal induced neurotoxicity

It has been well documented that sheep are particularly prone to both deficiency (swayback disease; Alleyne *et al*, 1998; Howell *et al*, 1964) and overloading with dietary copper (chronic copper toxicity; Doherty *et al*, 1969). Both conditions, while initially affecting the liver and kidney, ultimately lead to dramatic neurological deficiencies, ranging from numerous lesions in the white matter (swayback; Tan & Ulrich, 1983) to severe swelling and vacuolation of the white matter and swelling of astrocytes (chronic copper toxicity; Doherty *et al*, 1969;

Morgan, 1973). The underlying biochemical mechanisms which lead to the neurological progress of these diseases is unknown, but it has been suggested that they may be caused by the miss-handling of copper in the brain. In light of the work reported here, it can be hypothesized that this might be related to the variant sheep MT-III protein, which has reduced metal binding capacity compared to other mammalian MT-IIIs. It must be noted that MT-III knockout mice have been found to exhibit no apparent phenotypic changes up to two years of age (Erickson *et al*, 1997). However, sheep are much longer lived than rats, and unlike laboratory animals, sheep in agricultural situations are frequently exposed to environmental fluctuations in heavy metals such as copper. Thus, it may be that sheep provide a model for revealing important physiological information on the role of MT-III.

Chapter 4: Investigation of the “neurite inhibitory” properties of human MT-III

4.1 Introduction

In the previous chapter, the neuronal growth inhibitory activity of human and sheep MT-III isoforms was evaluated using an assay first described by Uchida and colleagues (1991), which has become the standard means of measuring the inhibitory activity of MT-III (for instance, Erickson *et al*, 1994; Sewell *et al*, 1995). The basis of this assay is a low cell plating density of rat E17-18 cortical neurons, which results in culture conditions which are not optimal for cell survival. Addition of either an adult human brain or Alzheimer’s disease brain extract to the neurons promotes survival over the three days of the assay, in essence “rescuing” the neurons. Subsequent addition of MT-III (either recombinant human or rat MT-III bound to 7 zinc atoms per molecule) results in a significant decrease in neuron survival. As discussed by Uchida & Tomonaga (1989), this assay measures cell number either directly, or relative to accumulation of MAP-2, and hence is therefore an indicator of neuronal survival, rather than growth inhibition. However, Uchida *et al* (1991) further suggest that MT-III exhibits neurite growth inhibitory properties in culture, that is, that MT-III specifically inhibits the outgrowth of new neurites from newly plated embryonic neurons. However, this specific property has never, at the time of conducting the work described in this chapter, been reported within the literature by Uchida’s group, or others. This is an important issue because the hypothesis that a deficiency of MT-III is involved in the abnormal sprouting thought to underlie the formation of the neurofibrillary tangles which characterize Alzheimer’s disease, is based upon the putative neurite growth inhibitory properties of MT-III (Uchida *et al*, 1991). In this regard, MT-III is often referred to as a neurite inhibitor, although as stated there is no evidence within the contemporary literature to support this contention.

The aim of this chapter therefore is to investigate whether MT-III does indeed have neurite growth inhibitory properties, as opposed to its well documented effect on neuronal survival. This was performed in two different tissue culture models. In the first model, the effect of MT-III upon the early developmental processes of initial neurite formation and growth was investigated in single cell, embryonic cortical

neuron cultures similar to that established by Uchida *et al* (1989). In the second model, neurons were plated at a higher density, promoting the formation of neuronal clusters. By 21 days *in vitro*, these clusters form fasciculated axonal bundles between them, which can be transected (Dickson *et al*, 2000), and the effect of MT-III upon the subsequent regenerative response can be examined. Because aberrant reactive sprouting is a possible process in the development of the neurofibrillary tangles seen in Alzheimer's disease, these experiments may elaborate the role that MT-III has in this disease. The final aim of this chapter was elucidation of the role that brain extract has in mediating MT-III's activity, in light of previous reports that it is a necessary co-factor for observing MT-III's biological activity (Erickson *et al*, 1994).

4.2 Experimental Procedures

4.2.1 Brief description of the low density neuron culture

To maintain consistency, the culture conditions used within this chapter were based upon the neuron assay first described by Uchida *et al* (1991). The basis of this assay is a low cell plating density of rat E17-18 cortical neurons, which results in culture conditions which are not optimal for cell survival. Addition of either an adult human brain or Alzheimer's disease brain extract to embryonic rat cortical the neurons promotes survival over the three days of the assay, in essence "rescuing" the neurons. Addition of either recombinant human or rat MT-III (bound to 7 zinc atoms per molecule) results in a significant decrease in neuron survival. As an internal control for the experiments conducted in this chapter, recombinant human MT-IIA, bound to 7 zinc atoms, was used. Adult rat brain extract was used instead of human or AD brain extract due to availability issues. This was not expected to significantly alter the culture conditions however, as Uchida and colleagues have demonstrated that within their culture system, adult rat brain extract exhibits similar effects to human brain extract.

4.2.2 Low density neuron culture calculations and analysis

For quantitative analysis of the effect of MT-III on initial neurite formation, 10 bright field digital images from random areas of the coverslip were captured, for each timepoint, at a magnification of 400x (Olympus BL-51). Groups of three coverslips were used for each data point. Neuron viability was determined by trypan blue exclusion staining, and only viable cells were counted for all analyses. The total number of cells counted per coverslip ranged from 300-500 cells.

The percentage of surviving neurons was determined by dividing the average number of neurons per field in the presence of MT-III (or vehicle treated) by the average number of neurons per field upon initial plating. Propidium iodide uptake was also performed to assess neuronal survival.

Inhibition of neurite formation was investigated in two ways; the percentage of neurite-bearing neurons, and the number of neurites per neuron. A neurite (which includes both axons and dendrites) was defined as any process of at least 20 μ m extending from the cell body. The percentage of neurite-bearing neurons (or more accurately the percentage of neurons with one or more neurites) was calculated by dividing the average number of neurite-bearing neurons per field by the average total number of neurons per field. Of the neurite-bearing neurons, the number of neurites per cell was calculated by dividing the average number of neurites per field by the average number of neurite-bearing neurons per field. ANOVA, student's t-test and standard error analysis of results was made using SigmaStat (Jandel Scientific Software Corporation; San Rafael, CA).

Neurite length measurements were manually performed using NIH Image analysis software, with at least 300 neurites being measured per treatment group. To determine the rate of neurite elongation between timepoints, the average length of neurites at the earlier timepoint was subtracted from the average length of neurites at the later timepoint.

4.2.3 Neuron cluster culture calculations and analysis

Cytoskeletal changes following axonal transection were identified by double labelling immunocytochemistry, using both anti-tau and anti- β III-tubulin primary

antibodies. For analysis of tau/ β III-tubulin immunolabeled coverslips at 12 hours post transection, 5 digital images of different injury sites of the coverslip were captured (Olympus BX-60). Each group, unless otherwise stated, consisted of at least 3 coverslips. All neurite measurements were performed using NIH Image analysis software. T-test and standard error analysis of results was made using SigmaStat.

4.3 Results

4.3.1 *MT-III inhibits neuronal survival at high concentrations*

The objective of this chapter was to investigate whether human MT-III has a real neurite inhibitory ability, as opposed to its ability to inhibit neuronal survival which has been shown previously (Uchida *et al*, 1991; Erickson *et al*, 1994; Sewell *et al*, 1995). Therefore, to ensure that any neurite inhibitory effects of MT-III were not attributable to non-specific neurotoxicity, an experiment was conducted to establish the range in which no reduction in neuronal survival was observed. To determine such doses, a range of MT concentrations was applied to one day old neuron cultures in the presence of brain extract. The brain extract consisted of a homogenate of adult rat brain, which was applied to neuronal cultures at a concentration of 150 μ g/ml total protein. The percentage of surviving neurons was determined after a further three days in culture (Figure 4.1). At lower doses (0.01, 0.1 and 1 μ g/ml), MT-III had no significant effect on neuron survival, although the percentage of surviving neurons at all of these concentrations was lower than that of the vehicle (PBS) treated group. At the highest dose (5 μ g/ml), MT-III had a significant inhibitory effect upon neuron survival, with survival reduced to 63% compared to 88% neuron survival in vehicle treated cultures ($p < 0.01$, student's t-test). These results were confirmed by propidium iodide uptake (Figure 4.1), which labels only dying cells. For subsequent studies investigating the neurite inhibitory properties of MT-III, levels which had no significant effect upon neuronal survival (0.1 and 1.0 μ g/ml; less than 10% reduction in neuron survival compared to vehicle treated cells) were used. It must be noted that in these experiments human MT-III exhibited reduced inhibitory activity compared to

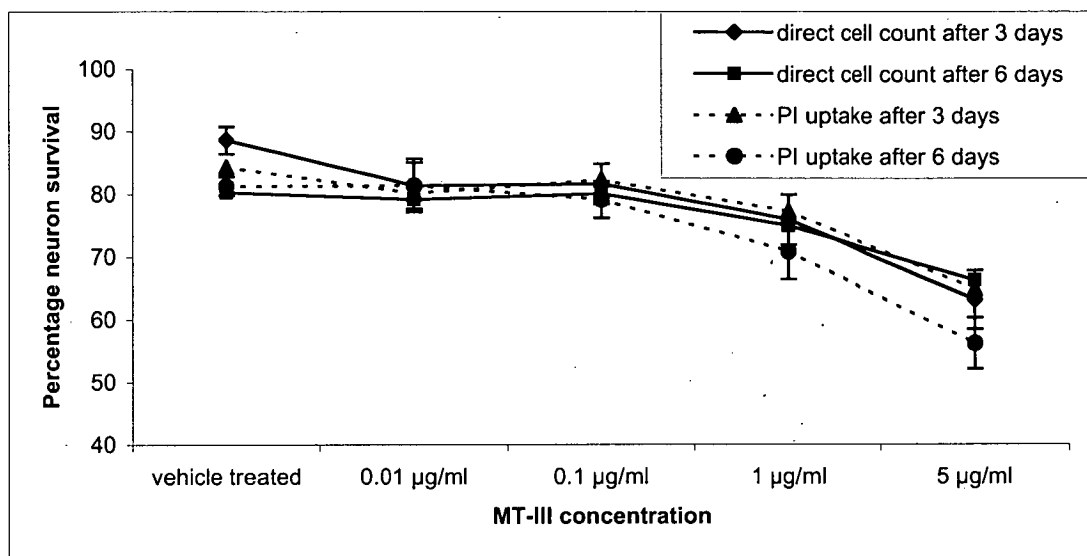


Figure 4.1: Human MT-III has a dose dependant, inhibitory effect upon neuronal survival in the presence of adult rat brain extract (equivalent to 150µg/ml protein). Neuronal survival was determined by direct cell counting and propidium iodide (PI) uptake. MT-III only significantly inhibited neuronal survival at 5µg/ml ($p < 0.01$, student's t-test). Error bars represent standard error values.

those observed in Chapter 3 (3.3.4). However differences in the culture conditions could account for this. In Chapter 3, human MT-III was applied to neurons immediately upon plating, while in this chapter, human MT-III was applied one day post-plating.

As a control, human MT-IIA did not affect neuronal survival at up to 5µg/ml dosages (results not shown), which is in accordance with other reported experiments using MT-I/-II isoforms (recombinant rat MT-I/II, Erickson *et al*, 1994; recombinant mouse MT-I, Sewell *et al*, 1995; Chapter 3, 3.3.4).

4.3.2 MT-III inhibits neurite formation of developing cortical neurons

To investigate the effect of MT-III on neurite development in culture, the process of initial neurite formation in the presence of MT-III was evaluated. Neurons were plated immediately in the presence of MT-III and adult rat brain extract, and maintained in culture for 3 days. MT-III clearly inhibited neurite formation of developing cortical neurons (Figures 4.2A, B), in a dose dependant manner (up to 45% inhibition compared to vehicle treated by day 3, 1µg/ml MT-III; Figure 4.2C). While these results demonstrate that there was a significant decrease in the number of neurite bearing neurons, MT-III mediated inhibition of neurite formation was not complete. Investigation of neurons with at least one neurite indicated that MT-III inhibited multiple neurite formation, with more than half of the neurons being unipolar, compared to the bipolar morphology of vehicle treated neurons (neurites per neuron values of 1.4 and 1.98 by day 3, 1µg/ml MT-III and control cultures respectively; Figure 4.2D). Under similar conditions, MT-IIA did not affect initial neurite formation, expressed as either the percentage of neurite bearing neurons, or the number of neurites per neuron (Figure 4.2E, F), suggesting that the observed effect of MT-III was not due to addition of recombinant MT per se, or its associated zinc ligands.

To further investigate the effect of MT-III (1µg/ml) on initial neurite formation, the previous experiment was repeated, but neurite formation was identified by immunocytochemistry, using a combination of anti-tau and anti-MAP-2 primary antibodies. A number of neurites were noted, which were both tau and

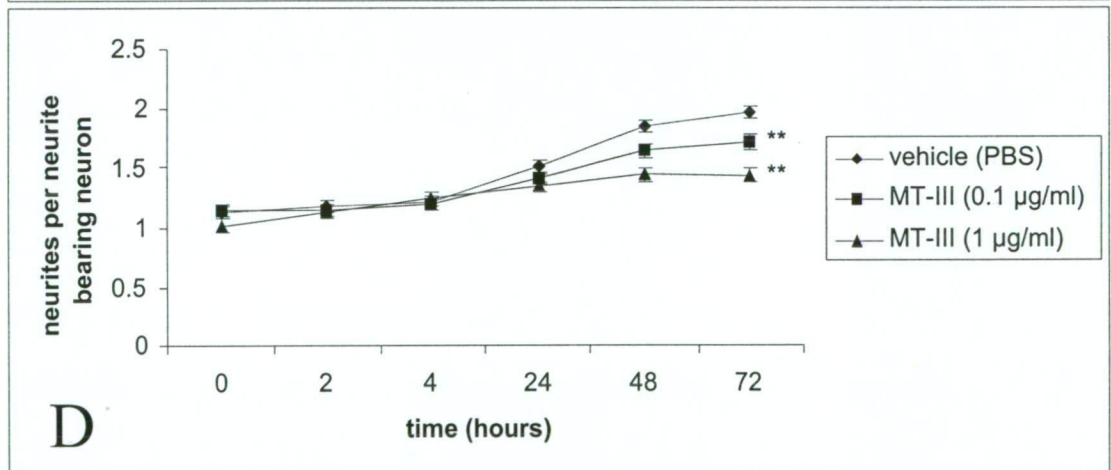
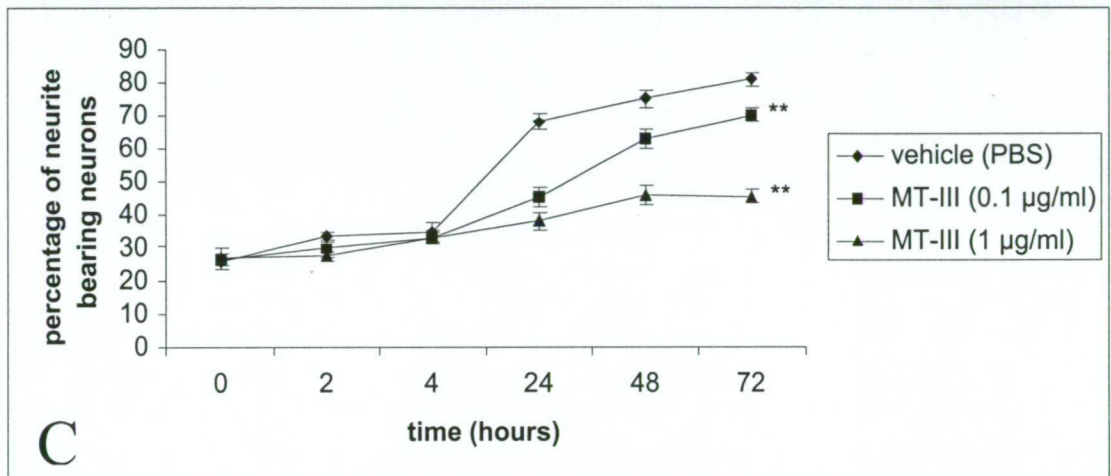
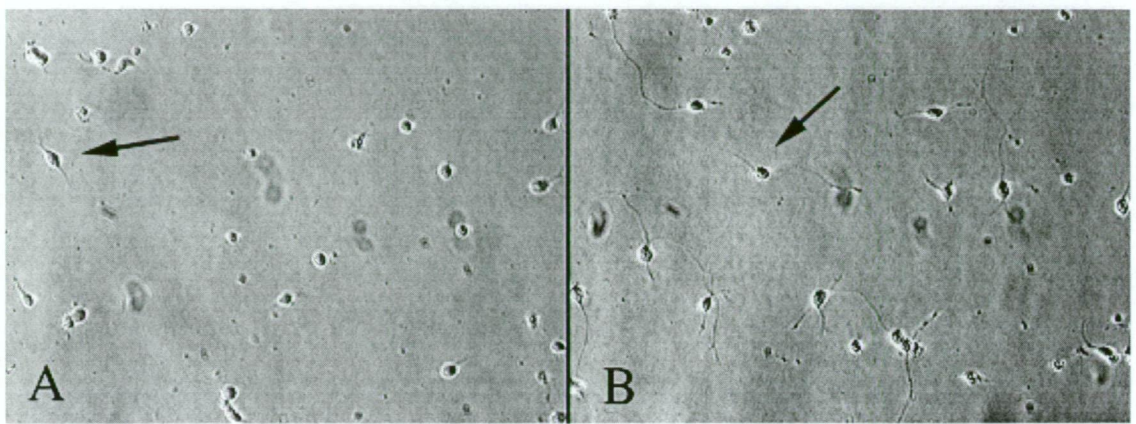


Figure 4.2: The effect of human MT-III on neurite formation in the presence of adult rat brain extract (150µg/ml) after 3 days (A – MT-III, B – vehicle; arrows indicate neurite bearing neurons) was determined by the percentage of neurite bearing neurons (C), and the number of neurites per neuron (D). MT-III significantly inhibited neurite outgrowth in both instances, at both concentrations tested ($p < 0.01$, ANOVA). Error bars represent standard error values.

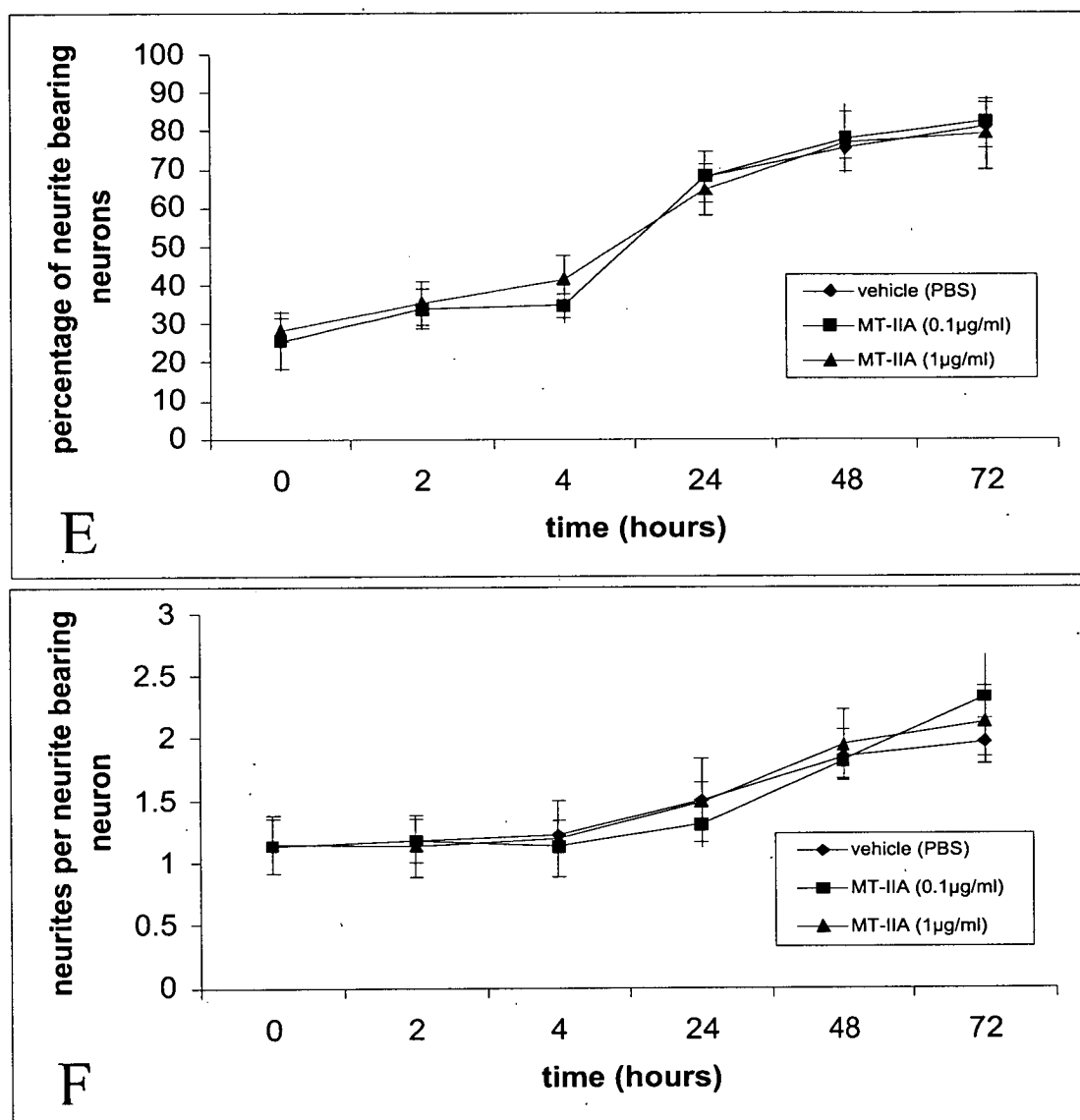


Figure 4.2: Human MT-IIA had no effect on initial neurite outgrowth over 3 days, as assessed by both the percentage of neurite bearing neurons (E) or number of neurites per neuron (F). [$p > 0.01$, ANOVA] For all graphs, error bars represent standard error values.

MAP-2 immunoreactive (Figure 4.3A), which has been reported previously (Kosik & Finch, 1987). It was apparent that the bipolar morphology of vehicle (brain extract only) treated neurons discussed previously is due to two tau immunoreactive processes, suggesting that these processes are precursors to axons. Analysis revealed that only 14% of all neurites were solely MAP-2 immunoreactive dendrites in vehicle treated neurons, but this was reduced to less than 3% following MT-III treatment (Figure 4.3B). This indicates that MT-III not only inhibits axonal formation (as noted by the unipolar morphology of neurons described previously), but also dendrite formation.

4.3.3 MT-III delays neurite elongation of cortical neurons

As noted above (Figures 4.2C, D), MT-III did not completely inhibit neurite formation. To investigate those neurites which were able to develop in the presence of MT-III, their rate of neurite elongation was measured. In the vehicle (PBS) treated group, there was an initial retraction by the neurites (between 0 and 2 hours), most likely in response to the medium change made in conjunction with MT-III addition (Figure 4.4A). From 2 hours onwards the rate of neurite elongation gradually increased, although there was a significant slowing of neurite elongation between 24 and 48 hours, compared to 0-24 hours and 48-72 hours ($p < 0.01$, student's t-test).

In the presence of MT-III (both 0.1 and 1 $\mu\text{g/ml}$) however, the initial retraction by the neurites, presumably in response to the medium change, was prolonged for an extra 2 hours (from 2 to 4 hours). Thus it would appear that MT-III delayed initial neurite elongation (between 2 and 4 hours). However, from 4 hours onwards there was no difference in the relative pattern of neurite elongation between vehicle (PBS) treated and MT-III neurites, although MT-III clearly reduced the absolute rate of neurite growth (Figure 4.4A). In the presence of MT-IIA (both 0.1 and 1 $\mu\text{g/ml}$), the rate of neurite elongation was significantly increased compared to control cultures (Figure 4.4B).

The average total length of vehicle treated neurites at 3 days in vitro was $139 \pm 3 \mu\text{m}$. MT-III significantly reduced average neurite length to $109 \pm 4 \mu\text{m}$ (0.1 $\mu\text{g/ml}$) and $97 \pm 5 \mu\text{m}$ (1 $\mu\text{g/ml}$) (student's t-test, $p < 0.01$; \pm values represent

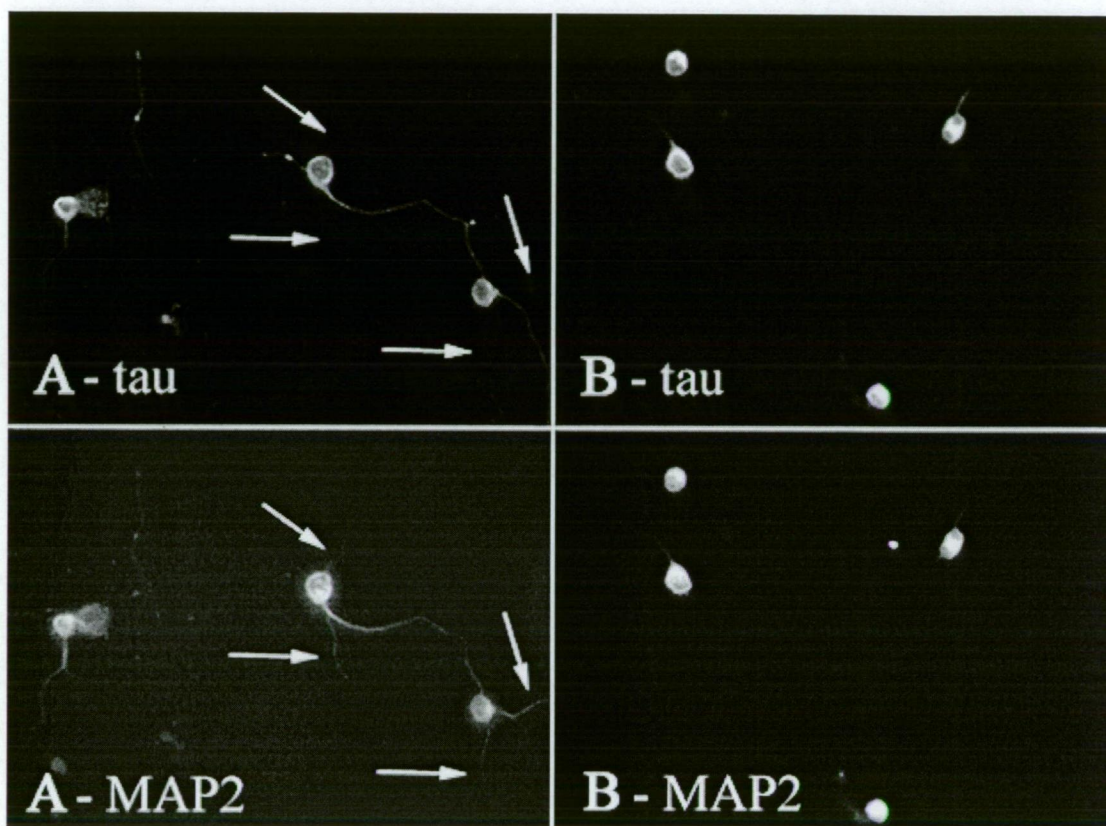


Figure 4.3: Immunocytochemistry of control (brain extract only) neuronal cultures to distinguish between axonal (tau) and dendritic (MAP-2) formation indicated a high degree of localisation of tau and MAP-2 in axons (A), as well as several MAP-2 immunoreactive dendrites (indicated by arrows). Following human MT-III treatment, there were significantly fewer, shorter axons, and no MAP-2 positive, tau negative dendrites (B).

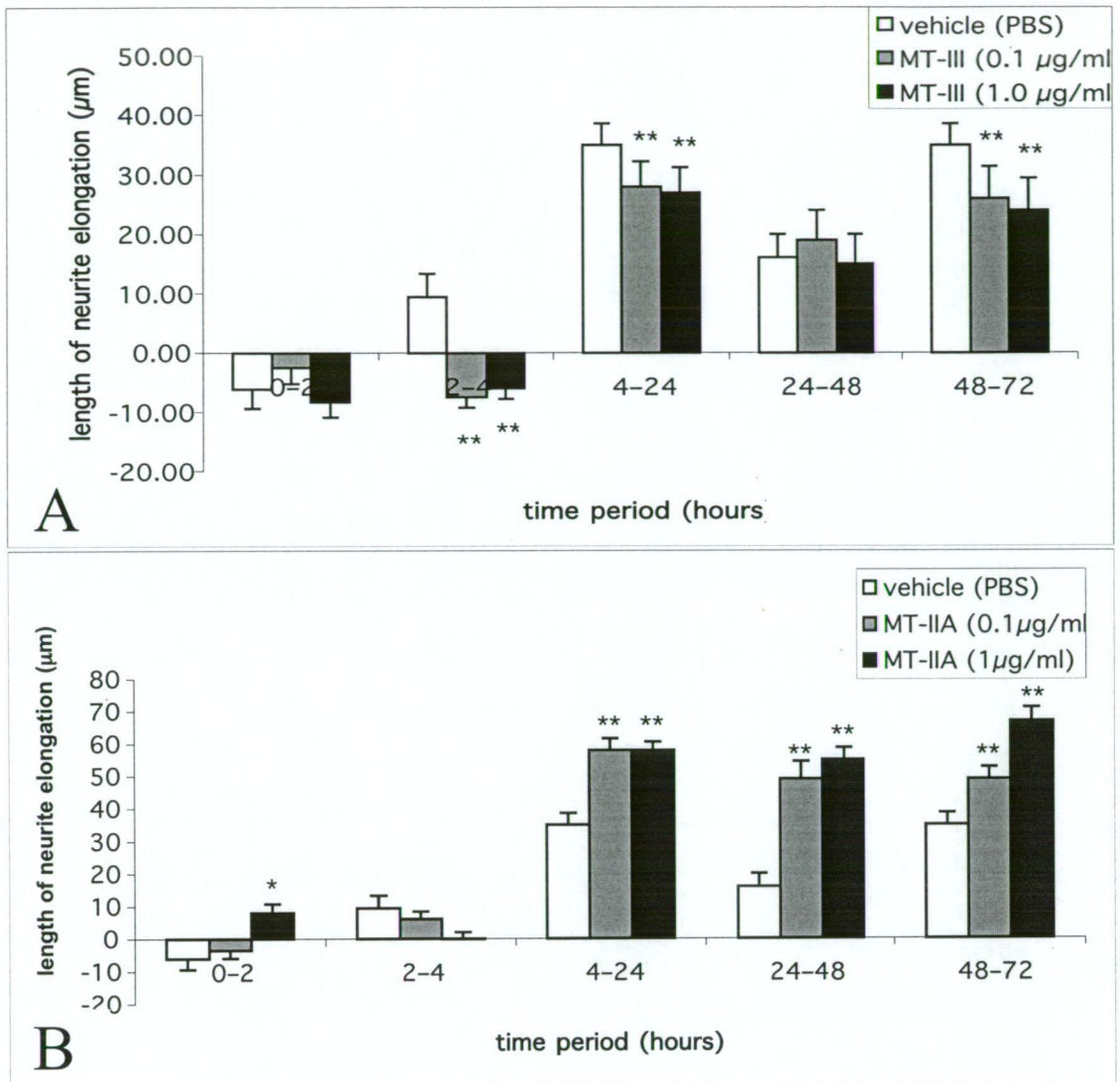


Figure 4.4: Human MT-III prolongs the process of neurite retraction from 0-2 and 2-4 hours post plating (A), after which the rate of neurite elongation proceeds at a reduced rate. In contrast, human MT-IIA significantly promoted the rate of neurite elongation (B). * - $p < 0.05$; ** - $p < 0.01$ (ANOVA). Error bars represent standard error values.

standard error values). However, calculation of the average neurite length of MT-III treated neurons is misleading, as measurements were not normally distributed (ie: in a bell-shape distribution), and many MT-III treated neurites were much shorter than the actual average neurite length (Figure 4.5A). Hence, it is important to note that while MT-III significantly reduced the growth of the majority of neurites, a small number of neurites appeared to be unaffected by MT-III and actually grew to lengths comparable to those observed in vehicle treated cultures.

Contrastingly, the average total length of MT-IIA treated neurites at 3 days in vitro was $204 \pm 10\mu\text{m}$ and $234 \pm 10\mu\text{m}$ (0.1 and $1\mu\text{g/ml}$ MT-IIA respectively), which was significantly longer than vehicle treated neurites at the same time point ($p < 0.01$, student's t-test). Furthermore, the distribution of MT-IIA treated neurites was also not normal, with many MT-IIA treated neurites much longer than the average neurite length (Figure 4.5B).

4.3.4 MT-III is not inhibitory in the absence of brain extract

Previously, Uchida *et al* (1991) and Erickson *et al* (1994) have shown that MT-III inhibits neuronal survival in the presence of brain extract from normal humans, and brain extract from Alzheimer's disease sufferers. Furthermore, it was also demonstrated that, in the absence of brain extract, MT-III actually promoted neuronal survival in the embryonic cortical neuron culture assay (Erickson *et al*, 1994). This suggests that MT-III is interacting with a constituent of the brain extract to inhibit neuron survival. To investigate whether MT-III can inhibit neurite outgrowth in the absence of brain extract, neurons were plated into medium containing either $1\mu\text{g/ml}$ MT-III or PBS, all in the absence of brain extract. Analysis of the percentage of neurite bearing neurons (Figure 4.6A) and neurites per neuron (Figure 4.6B) after 3 days revealed that MT-III had no significant inhibitory effect upon initial neurite formation in the absence of brain extract.

4.3.5 Elucidation of the specific interactions between MT-III and brain extract

To begin to elucidate the factors within brain extract with which MT-III interacts, whole brain extract was fractionated by gel filtration using a Sephadex G-

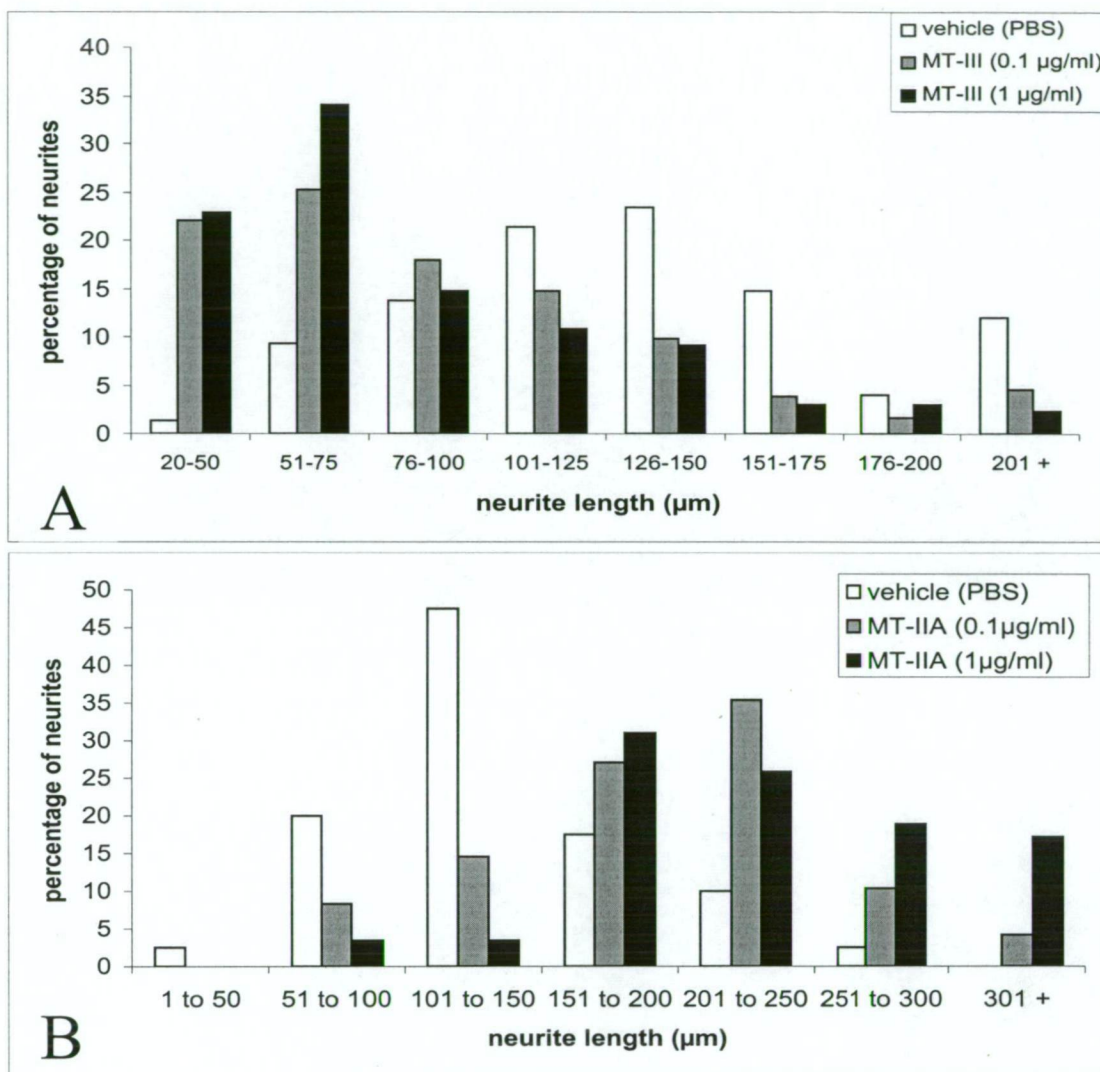


Figure 4.5: The distribution of neurite lengths at 3 days post MT-III treatment (A) indicates that while MT-III significantly inhibited neurite growth, a small percentage of neurites were unaffected, and grew to lengths comparable to vehicle (brain extract only) treated neurites. The distribution of neurite lengths following MT-IIA treatment indicated that a number of neurites grew to lengths greater than that of vehicle treated neurites (B).

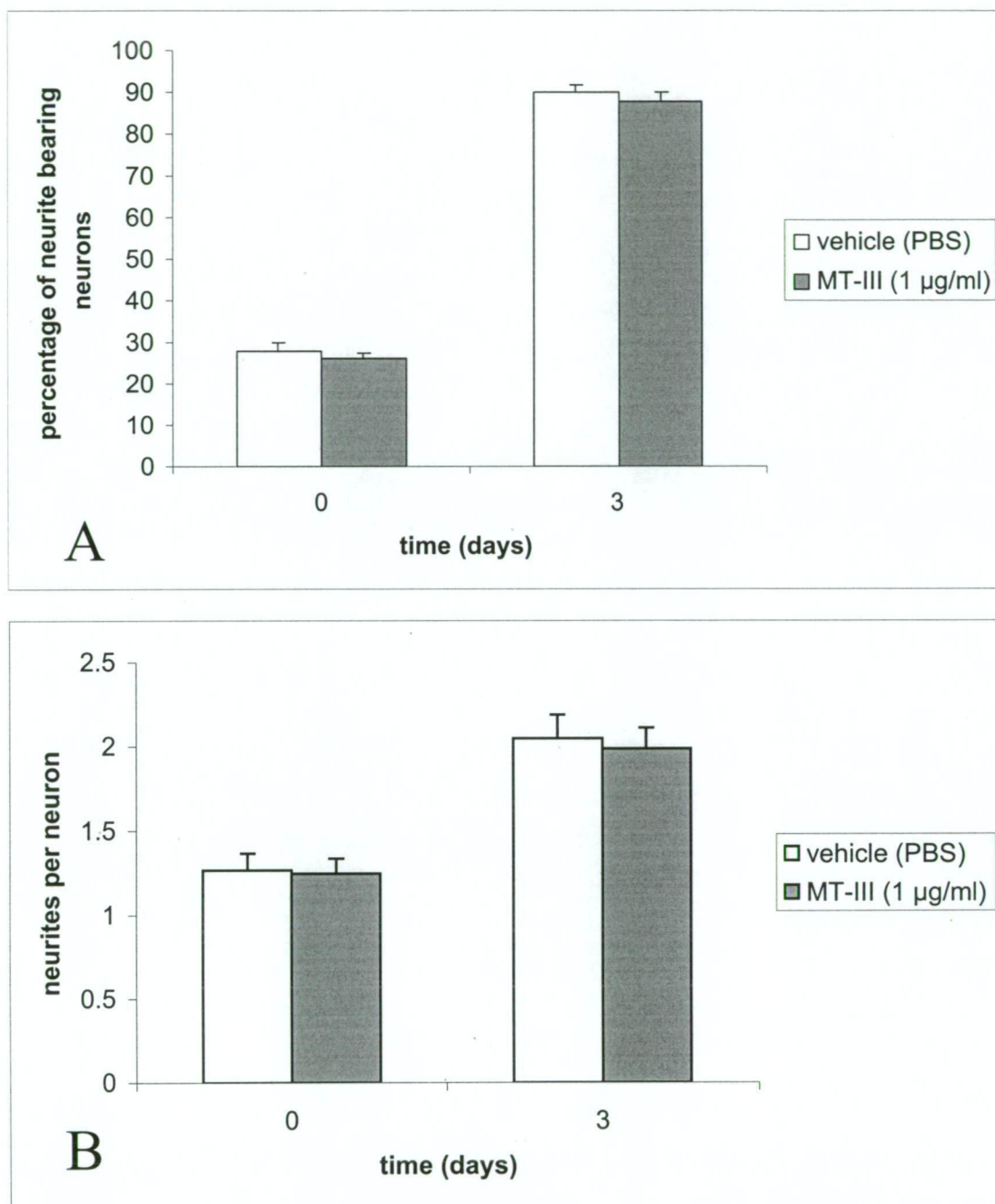


Figure 4.6: MT-III does not exhibit neurite inhibitory properties in the absence of brain extract, either expressed as the percentage of neurite bearing neurons (A), or as the number of neurites per neuron (B). Error bars represent standard error values.

75 column. An aliquot from each fraction was added to neuronal cultures at a protein concentration proportional to the contribution of that fraction to the original 150 ug/ml protein supplied by the unfractionated brain extract, together with 1µg/ml MT-III, and neurite inhibition assessed relative to cultures that had received unfractionated brain extract and no MT-III. Two peaks were observed (Figure 4.7), indicating that MT-III is possibly interacting directly with more than one component of the brain extract. Based on calibration of the gel filtration column with a series of samples of known molecular weight, it was estimated that these two peaks correspond to approximately 150kD and 15-20kD.

4.3.6 MT-III's inhibitory activity can be reversed

Three days after MT-III addition (1µg/ml) the culture medium was replaced with fresh medium (lacking MT-III), and brain extract, and the neurons incubated for a further 3 days. While neurite formation was inhibited in the presence of MT-III during the first 3 days (comparable to the results observed in Figures 4.2C, D), neurons were able to extend neurites following the medium change (as observed by the percentage of neurite bearing neurons and number of neurites per neuron; Figures 4.8A and 4.8B respectively). Indeed, by 3 days post MT removal, the percentage of neurite bearing neurons was not significantly different to vehicle treated neurons (Figure 4.8A). This demonstrates that the effect of MT-III is reversible and suggests that MT-III is acting by a specific mechanism, rather than by a mechanism which involves reduction of neuron viability.

4.3.7 MT-III inhibits reactive neurite sprouting following axonal transection

The previous experiments utilised a culture model which is well suited to quantitating the effect of MT-III on neurite development in relatively immature neurons. Recently, a more complex neuron culture model has been developed in this laboratory, which allows the investigation of the changes involved in reactive neurite sprouting which follows axonal transection to established clusters of mature (21 days *in vitro*) neurons in culture (Dickson *et al*, 2000). By analogy to the response seen in neurons *in vivo* following traumatic injury, the process of reactive neurite sprouting is

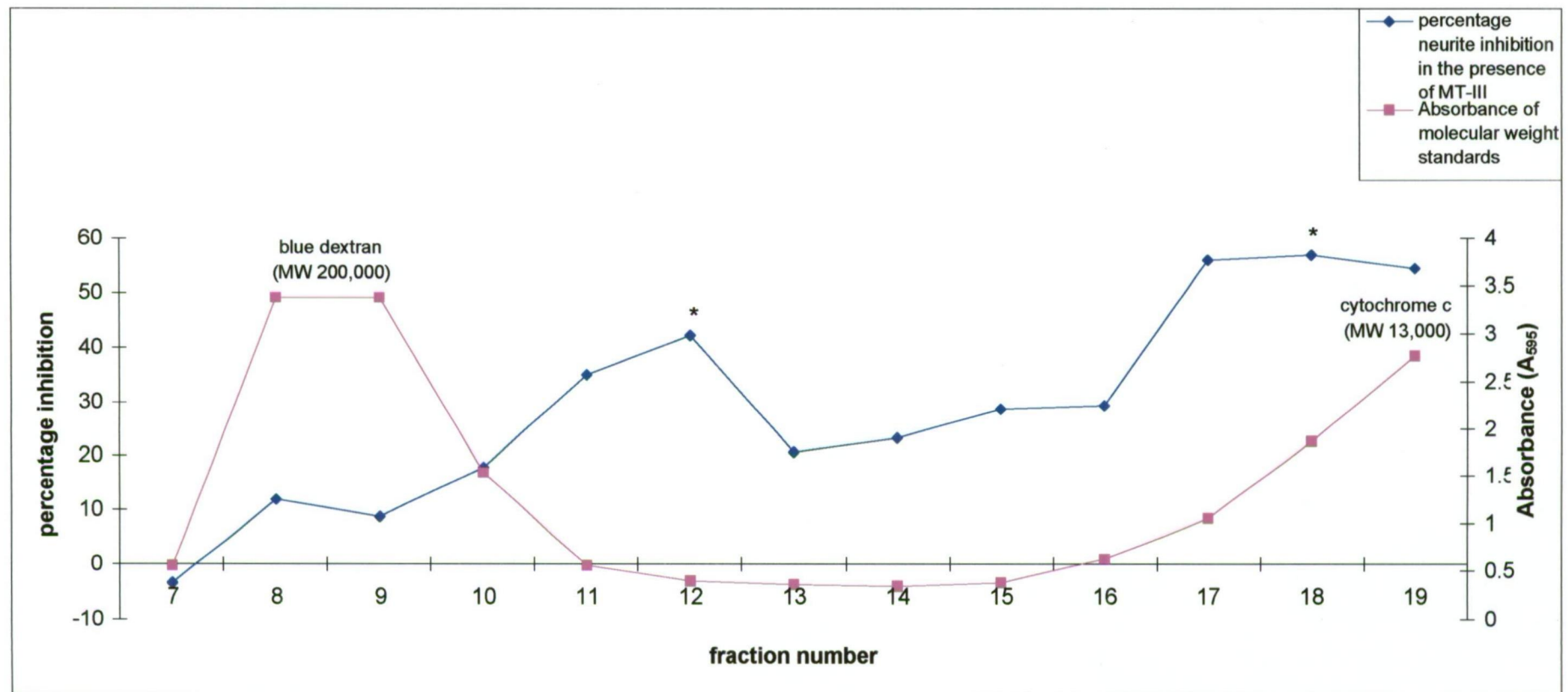


Figure 4.7: Brain extract was fractionated by gel filtration chromatography using a Sephadex G-75 column, and each fraction applied individually to low density neuron cultures, together with 1 μ g/ml MT-III. When MT-III induced inhibition of neurite outgrowth was determined (\blacklozenge), two peaks of inhibitory activity were observed (indicated by asterisks). Several proteins of known MW were eluted from the column to identify the approximate size range of proteins within each brain extract fraction (\blacksquare).

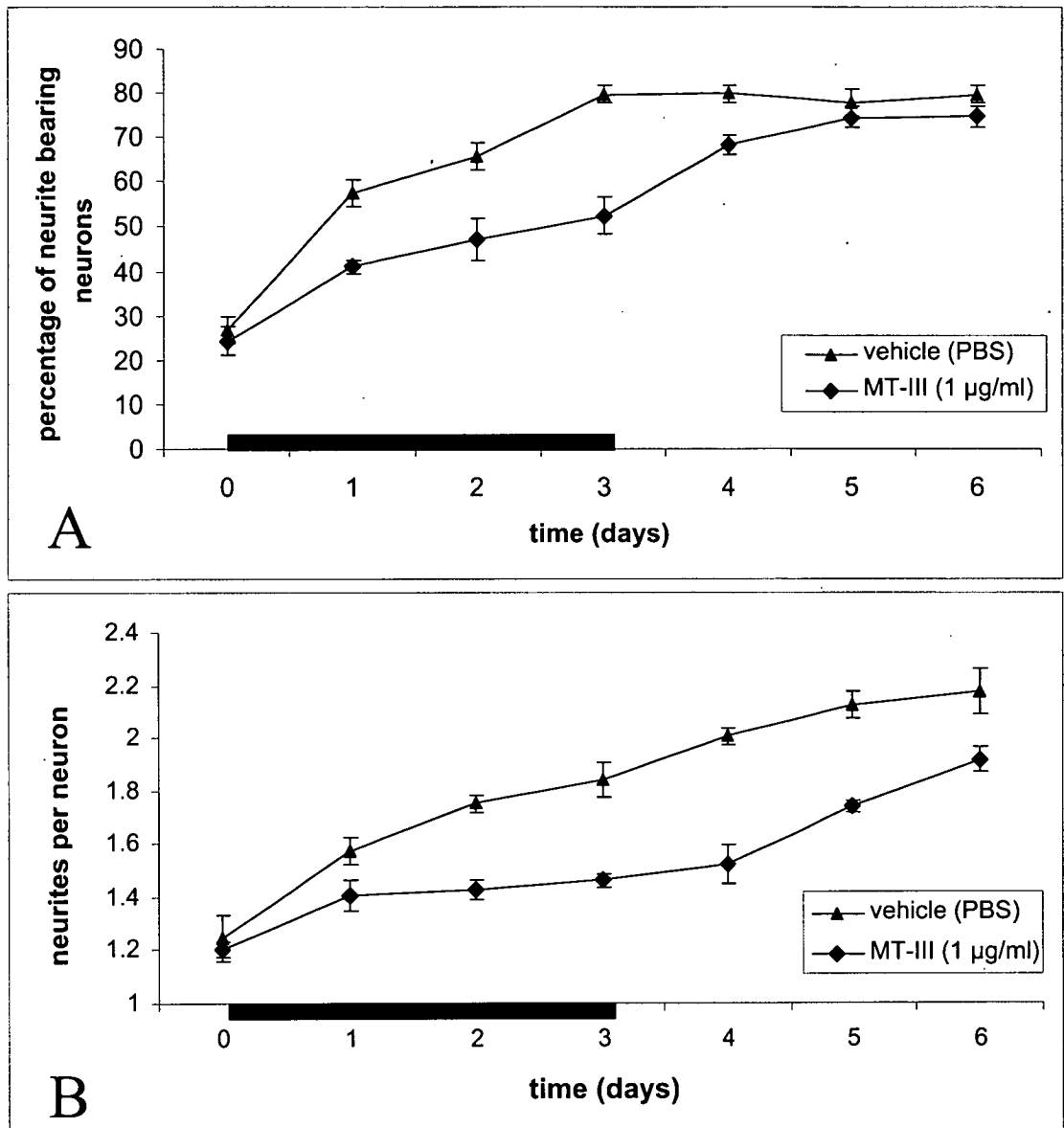


Figure 4.8: Inhibition of neurite formation by MT-III was reversed by replacing MT-III containing medium with fresh medium which lacked MT-III. This was observed in both the percentage of neurite bearing neurons (A), and the number of neurites per neuron (B). The solid black bar represents the time in presence of MT-III. Error bars represent standard error values.

interpreted as part of the subsequent regenerative process. To investigate the effect that MT-III has upon this neuritic response in culture, MT-III together with 150µg/ml of brain extract was applied to cortical neuron cluster cultures immediately after axonal transections.

By 12 hours post transection in cultures that had received brain extract only, there was a marked retraction of the transected stumps from the lesion site (indicated by the broken line). Furthermore, there were a number of tau immunoreactive neurites identified extending from these transected stumps (Figures 4.9A, B). In the presence of MT-III (1µg/ml) however, there was a reduction of over 50% in the number of tau immunoreactive sprouts (15.8 ± 1.3 sprouts/100µm linear distance along the cut compared to 6.9 ± 0.9 sprouts/100µm; $p < 0.01$, student's t-test; Figure 4.10A). Neurite length measurements indicated that the MT-III treated tau immunoreactive processes (mean neurite length = $15.9\mu\text{m} \pm 1.4\mu\text{m}$) were significantly shorter than vehicle (PBS) treated processes (mean neurite length = $41.5\mu\text{m} \pm 1.7\mu\text{m}$) at this time point ($p < 0.01$, student's t-test; Figure 4.10B). In comparison, in the presence of MT-IIA (1µg/ml), a number of reactive processes were identified extending from the transected stumps (Figures 4.9C, D). Quantitative analysis indicated that there were more reactive sprouts compared to the vehicle (20.8 ± 0.8 vs 15.8 ± 1.2 ; $p = 0.07$, student's t-test; Figure 4.10A), and that these processes were significantly longer (mean neurite length = $70.1\mu\text{m} \pm 3.4\mu\text{m}$ vs $41.5\mu\text{m} \pm 1.7\mu\text{m}$; $p < 0.01$, student's t-test; Figure 4.10B). By 18 hours post transactions, MT-IIA treatment had promoted the growth of reactive sprouts across the entire transection tract, which was not observed following vehicle or MT-III treatment (Figure 4.11).

4.4 Discussion

4.4.1 MT-III is a specific inhibitor of initial neurite formation in culture

Human MT-III significantly inhibited initial neurite formation, as assessed by both the percentage of neurite bearing neurons, and the number of neurites per neuron. This was observed with concentrations of MT-III that did not significantly reduce neuronal survival (less than 10% reduction in neuronal survival compared to vehicle treated neurons). Propidium iodide studies confirmed that these levels of

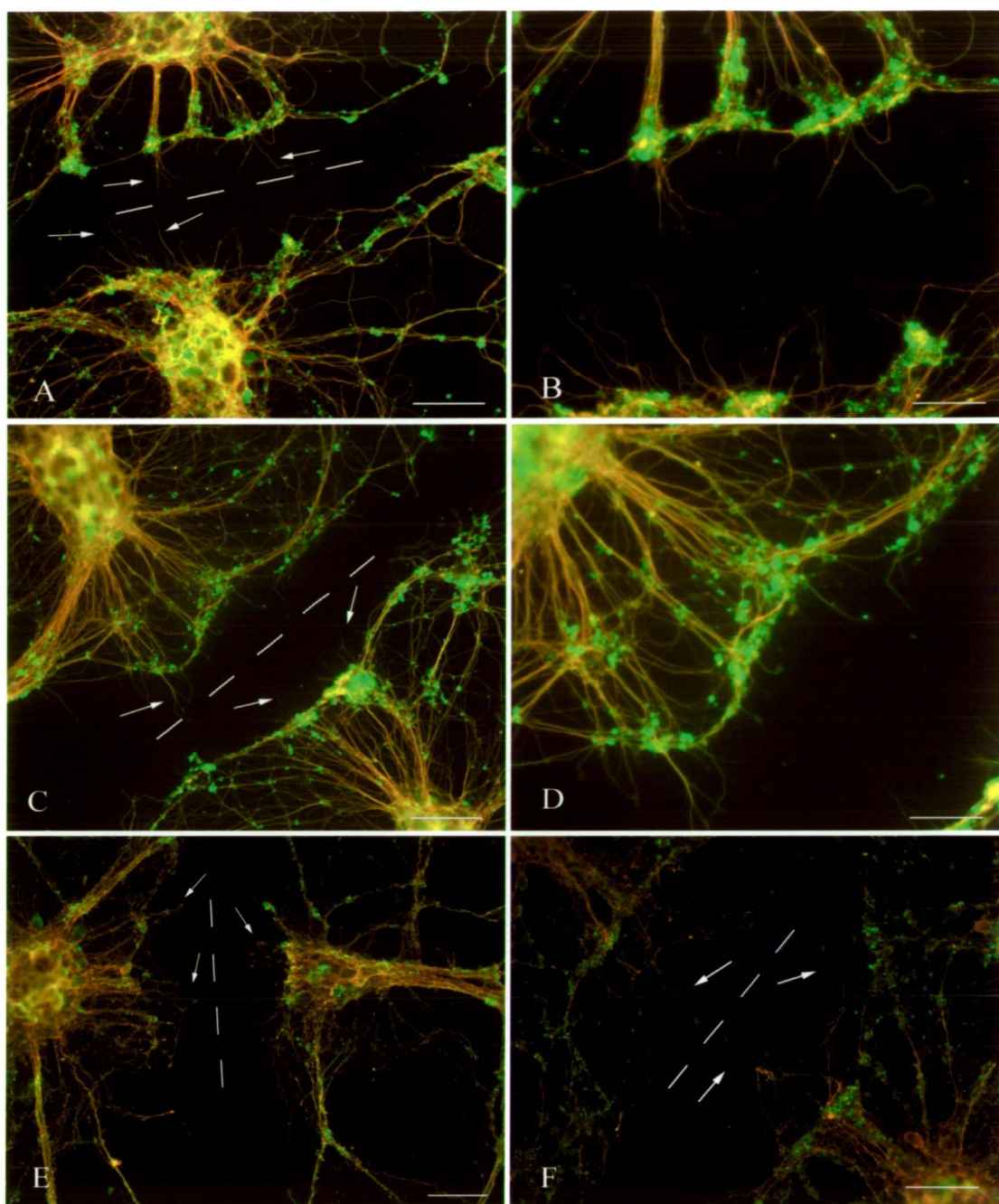


Figure 4.9: Fluorescent double immunocytochemical labeling of cytoskeletal changes, both tau (red) and β III-tubulin (green), 12 hours after axonal transection (A, B). Treatment with MT-III (1 μ g/ml; C, D) reduced regenerative neurite sprouting compared to vehicle treated. The transection site is indicated by the broken line, and there is a large area of retraction away from this line. Sprouting neurites are indicated by arrows. MT-IIA treatment increased both the number and length of reactive sprouts following injury (E – vehicle, F – MT-IIA, 1 μ g/ml). Scale bars represent 60 μ m (A, C, E, F) and 30 μ m (B, D).

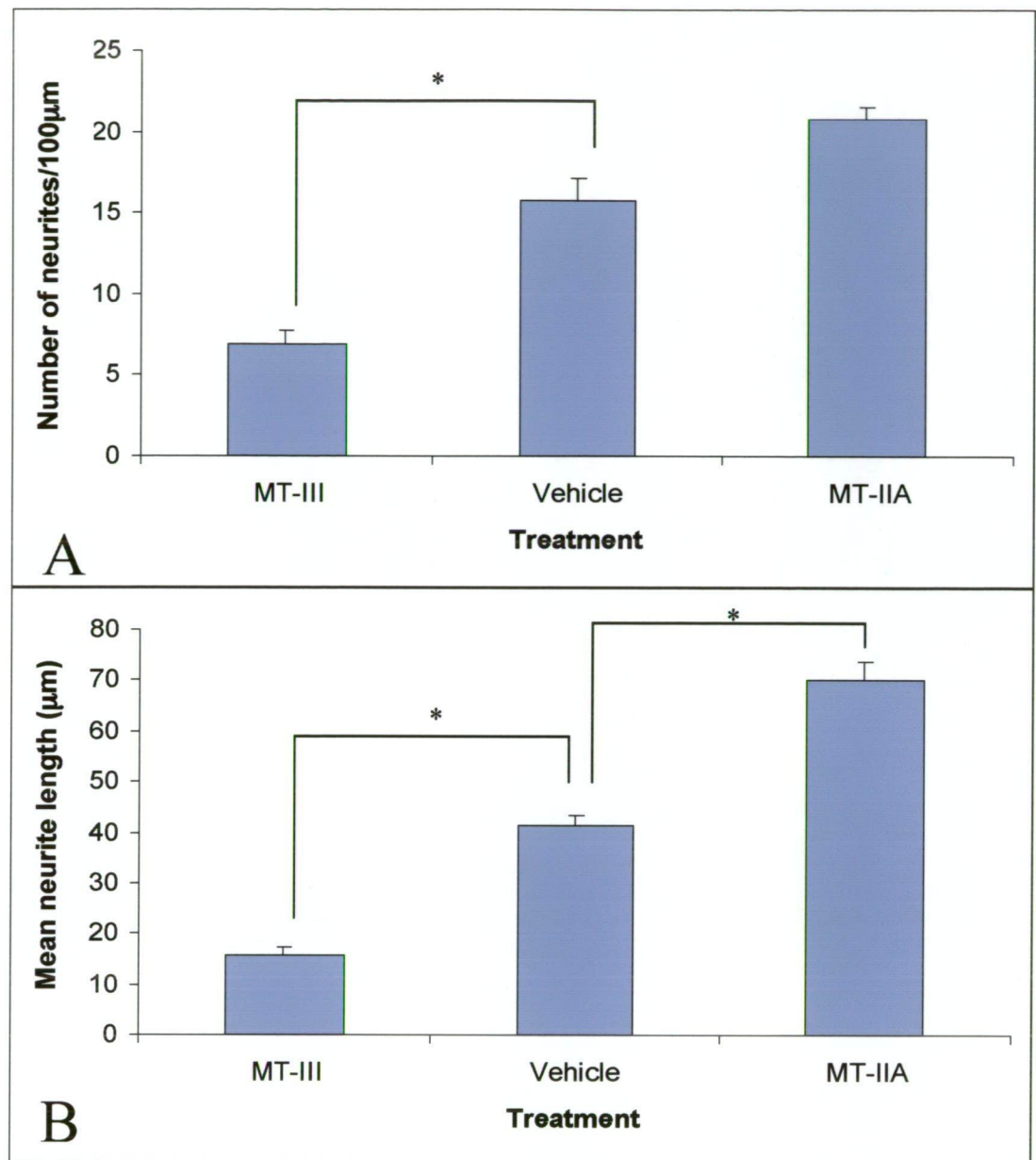


Figure 4.10: Treatment with human MT-III significantly inhibited both the number (A) and length of reactive sprouts (B) at 12 hours post- axonal transection in culture. However, human MT-IIA significantly increased the mean neurite length of reactive sprouts (B). * - $p < 0.01$, (paired t-test). Error bars represent standard error values.

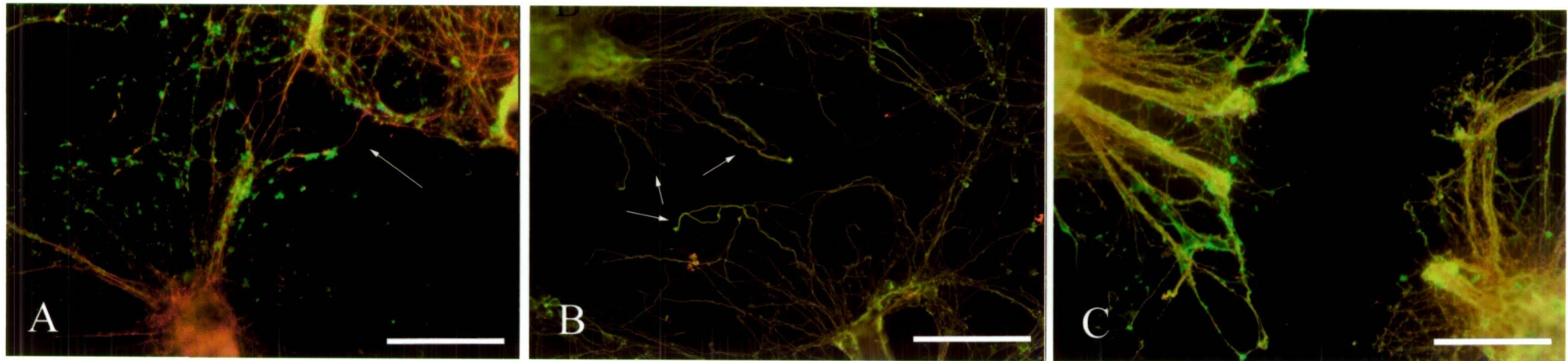


Figure 4.11: Fluorescent double immunocytochemical labeling of cytoskeletal changes, both tau (red) and β III-tubulin (green), 18 hours after axonal transection. MT-IIA promoted reactive axonal growth across the entire transection site (A), which was not observed following treatment with either vehicle (B) or MT-III (C). scale bars: A = 100 μ m; B, C = 50 μ m

MT-III were not significantly toxic at up to 6 days post MT-III treatment. This is in accordance with a report, which was published following the completion of the work discussed in this chapter, that also indicates that MT-III inhibits initial neurite extension of cortical neurons in culture (Uchida *et al*, 2002). Furthermore, the inhibitory activity of MT-III was reversible, suggesting that MT-III is a specific inhibitor of initial neurite formation and growth. To account for the possibility that zinc (released from MT-III) or an artifact from the recombinant protein purification process was responsible for the observed results, experiments were also conducted with another recombinant human MT isoform, MT-IIA (also bound to 7 zinc atoms per molecule). MT-IIA did not alter the initial formation of neurites, but surprisingly significantly accelerated the rate of neurite elongation. Furthermore, MT-IIA also significantly promoted recovery following axonal transection. These unexpected properties of MT-IIA will be discussed further in Chapter 5. These results suggest that MT-III is a true neurite inhibitor, separate from its ability to inhibit neuronal survival.

4.4.2 The role of brain extract in MT-III mediated neurite inhibition

The absence of any inhibitory activity of MT-III when brain extract was not applied to cultures indicates that adult rat brain extract contains a necessary co-factor for the neurite inhibitory activity of MT-III, or that it is inhibiting a neurite growth promoting molecule present within brain extract. Further experiments suggested the possibility of MT-III interacting with more than one component of brain extract, as evidenced by MT-III induced reduction in neurite outgrowth in the presence of two different fractions obtained from gel filtration of whole brain extract. Based upon calibration of the column with samples of known molecular weight, the brain extract fractions correspond to sizes of approximately 150kD and 15-20kD. The 15-20kD size range is of particular interest, as this is the size range of many neurotrophic factors such as neurotrophins (Bibel & Barde, 2000). Indeed, a possible mechanism for protein-protein interaction between MT-III and another protein has been recently described by Oz *et al* (2001). They report that the acidic insertion to the alpha-domain of MT-III (relative to all other MT isoforms) is accommodated within a loop,

which exhibits dynamics identifying it as a possible interaction site with other proteins (See Chapter 1.4.2). As further evidence of the potential interactions between MT-III and other molecules, several reports suggest that MT can bind ATP under physiological conditions (Maret *et al*, 2002; Jiang *et al*, 1998). Interestingly, it has been demonstrated that inosine, the breakdown product of ATP, is capable of promoting significant neurite growth in culture and *in vivo* (Benowitz *et al*, 1999, 2002). Because inosine is the major break-down product of ATP, it is possible that any ATP released from lysed cells during the production of brain extract will accumulate as inosine. In this regard, it is feasible to envisage that MT-III may bind to inosine, in essence titrating it out of the brain extract, to exhibit its neurite inhibitory activity. This specific hypothesis can be readily tested in further work.

To further identify the potential binding partners of MT-III within brain extract, classical pulldown assays could be performed, by biotinylating MT-III and binding the conjugate to streptavidin bound Dynabeads. Bound substrates would be analysed by SDS-PAGE and mass spectrometry.

4.4.3 MT-III in neurological disorders

An exciting potential role for MT-III was suggested by its apparent deficiency in the Alzheimer's disease brain, and the idea that this deficiency was a causative factor in the development of neurofibrillary tangles (Uchida *et al*, 1991). However, reports of MT-III levels in the AD brain are conflicting, with findings of both deficiency (Uchida *et al*, 1991; Tsuji *et al*, 1992; Yu *et al*, 2001) and no deficiency (Erickson *et al*, 1994; Carrasco *et al*, 1999). While this issue is clouded, there is separate biochemical evidence for a link between MT-III and AD. For instance, oxidative stress has been implicated in the neuropathy of AD (Hensley *et al*, 1994), and thus a deficiency in MT-III, a known free radical scavenger *in vitro*, may be involved in the development of AD. It has also recently been reported that MT-III can inhibit the formation of neurotoxic β amyloid aggregations *in vitro* (Irie & Keung, 2001), and protect against free radical damage induced by the β -amyloid peptide 25-35 (Ren *et al*, 2001). The observation here that MT-III inhibits tau immunoreactive neurite sprouting (hypophosphorylated tau is the major constituent

of NFTs) indicates that a deficiency in MT-III in the brain could indeed be a possible mechanism involved in NFT formation.

MT-III may also have a role to play in other neurological disorders. For instance, the fact that MT-III null mice are highly susceptible to kainic acid seizures (Erickson *et al*, 1997) suggests that MT-III is involved in the pathology of epileptic seizures. Indeed, MT-III over-expressing mice exhibit much less kainic acid induced brain damage than control mice (Campagne *et al*, 1999). It is possible that the zinc binding properties of MT-III would allow it to regulate electrically stimulated synaptic zinc release, preventing possible seizure occurrence. However, Cole *et al* (2000) using ZnT3/MT-III double knockout mice (which lack vesicular zinc) have concluded that MT-III is unlikely to protect against kainic acid induced seizures by modulating intracellular zinc release. Interestingly, McKinney *et al* (1997) have reported abnormal neurite sprouting following axonal injury to CA3 hippocampal neurons in brain slice culture (one of the most MT-III rich regions in the brain). Furthermore, these injured pyramidal neurons were hyperexcitable, and synaptic responses often displayed unusual prolonged polysynaptic components. Hence, it may be that MT-III protects against seizures by preventing abnormal neurite sprouting following axonal injury.

Chapter 5: Human MT-IIA promotes reactive neurite sprouting and cortical wound healing

5.1 Introduction

During the investigation of the neurite growth inhibitory properties of human MT-III described in the previous chapter, it was surprisingly found that human MT-IIA exhibited different neuroactive properties when applied to cultured cortical neurons. That is, MT-IIA significantly promoted neurite elongation (but not initial neurite formation), and also promoted reactive axonal sprouting and growth following injury *in vitro*. At about the same time that this work was performed, several reports indicated a correlation between the presence of MT-I/-II and the ability of animals to recover from a variety of brain lesions. These were based primarily upon studies utilising transgenic knockout MT-I/-II mice, which exhibit dramatically reduced ability to recover from cortical cryolesion injury (Penkowa et al, 1999a) and focal cortical ischaemia (Campagne et al, 1999). Furthermore, these animals exhibited significant impairment of glial cell responses to injury, which resulted in changes at the molecular level including reductions in cytokine expression (such as IL-1, IL-6 and TNF- α ; Penkowa et al, 1999b). Neuronal apoptosis was also significantly increased in the MT-I/-II knockout mice. Based on these results, Penkowa et al (1999b) proposed the hypothesis that the protective effect of MT-I/-II involves glial cell activation and reduction of post injury inflammation. Surprisingly, some neuroprotective activity was also observed when Zn-MT-II was intraperitoneally given to MT-I/-II knockout mice (Penkowa et al, 2002), indicating that MT may be acting by a previously unsuspected, extracellular mechanism.

Based upon the results of Chapter 4 which suggest an ability of MT-IIA to promote reactive neurite sprouting and regeneration following injury in culture, it was hypothesised that the neuroprotective properties of MT-I/-II observed previously might be due to a direct action of MT-IIA on neurons, in addition to the proposed action of MTI/II on glial cells and the CNS cytokine profile (Penkowa et al, 1999b). This possibility has not been identified by Penkowa and colleagues since the main focus of their work has been on glial cells and cells of the CNS immune system, and to date they have not reported an extensive investigation of the neuronal response to

injury of exogenous metallothionein. Hence, the aim of this chapter was to investigate the neuronal response to rat cortical brain injury following recombinant human MT-IIA treatment. The injury model used was a focal cortical needle stick injury model, which has been well characterised in this laboratory (King *et al*, 1997; 2001). In particular, temporal profiles for changes in glial cell activation and migration, and reactive neurite sprouting have been characterised for this model. These changes are observed immunohistochemically using antibodies against ferritin (activated microglia), GFAP (astrocytes) and SMI-312 (a cocktail of antibodies directed against phosphorylated neurofilaments, which are found only in axons).

5.2 Experimental Procedures

5.2.1 Cortical brain needle stick injury analysis

Focal cortical brain injuries were performed on 250g male adult rats, using a brain injury model developed previously in this laboratory (King *et al*, 1997; 2001), and described in detail in Chapter 2 (2.4.1). MT-IIA was applied to the lesion site (in gelfoam placed directly above the lesion; 100ng total MT-IIA in 20µl PBS) following cortical needle stick injury. For analysis of tissue sections, digital images were captured using the Olympus BX-60 digital camera (Olympus BL-51 microscope). A total of 16 rats were treated with MT-IIA (in 4 different experiments), and maintained for 4 days post injury. The same number of control animals (treated with PBS) were also used. A further 12 rats received MT-IIA (in 4 different experiments) for 7 days post injury studies, and a similar number of animals received vehicle (PBS). All measurements were performed using NIH Image. T-test analysis was performed using SigmaStat (Jandel Scientific Software Corporation).

The volume of tissue exhibiting ferritin immunoreactivity was used as a measure of inflammatory response. The volume of the inflammatory response to injury was determined from the area of ferritin immunoreactivity in the epicentral tissue section, and the next two consecutive sections in either direction. The percentage of tissue degradation was determined by subtracting the area of empty space from the total area of ferritin immunoreactivity within a brain section. For both of these analyses, 9 animals (over 3 different experiments) were used.

5.2.2 Protein isolation from gel foam, SDS-PAGE and western blotting

Absorbant gel foam, which had been used to seal the burr hole in the skull during the initial surgery, was recovered at a variety of time points post-injury (3 animals per group). Soluble proteins were removed from the gel foam by the addition of 100 μ l of PBS, followed by vortexing. Crude gel foam extracts were examined by Western blotting analysis, as discussed in Chapter 2.

5.3 Results

5.3.1 MT-IIA promotes wound healing and reactive axonal growth following cortical brain injury

To investigate the effect of human MT-IIA upon wound healing and reactive cytoskeletal changes following brain injury in the rat, MT-IIA was applied to the lesion site (as discussed in 5.2.1) following cortical needle stick injury. At 4 days post injury in vehicle (PBS) treated rats, the area of the injury site was clearly demarcated by ferritin immunoreactivity (Figure 5.1A). The volume of inflammatory response (defined by ferritin immunoreactivity) was not significantly affected by MT-IIA application ($p = 0.096$, student's t-test), although the volume in all MT-IIA animals (average volume = $2035 \pm 54\text{mm}^3$) was smaller than vehicle treated rats (average volume = $2772 \pm 78\text{mm}^3$). From the pial surface down, MT-IIA treatment resulted in the formation of a cellular bridge enclosing the injury site, resulting in a teardrop-like injury site (Figure 5.1B). The percentage of tissue degradation within the area of inflammation was significantly greater within vehicle treated animals ($54\% \pm 4$ vs $12\% \pm 11$; $p < 0.01$, t-test).

MT-IIA promoted the growth of a number of SMI-312 immunoreactive axonal processes into the injury site, at all cortical levels injured, as well as at the pial surface (Figures 5.1C, D). This was in marked contrast to vehicle treated rats, in which very few SMI-312 immunoreactive processes were visualized entering the lesion site (Figures 5.1E, F). As reported previously, these SMI-312 immunoreactive reactive axonal sprouts exhibited a higher degree of labeling for neurofilaments than adjacent, uninjured processes (King *et al*, 2001).

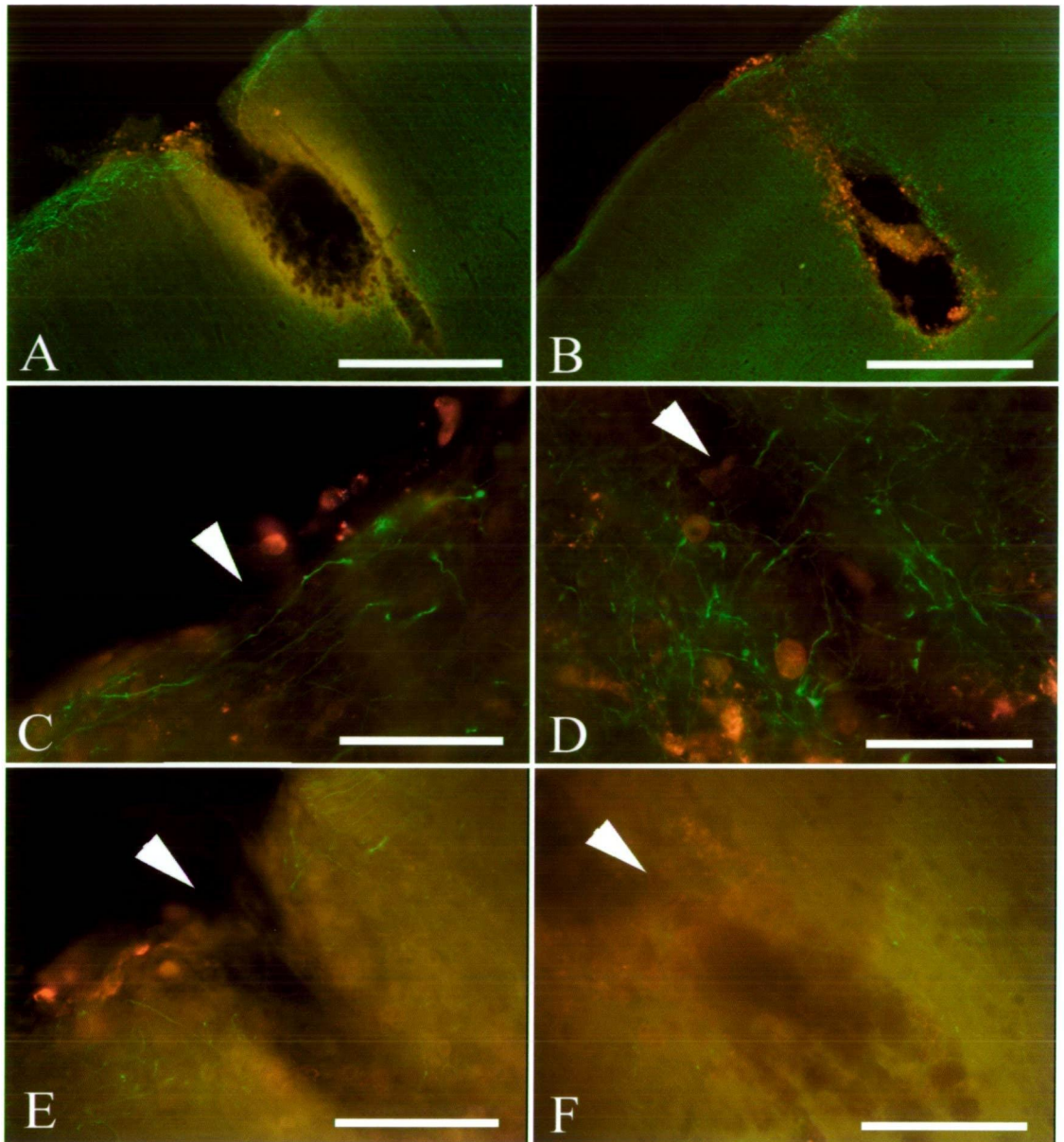


Figure 5.1: Immunohistochemistry for SMI-312 (green; axonal marker) and ferritin (red; microglial marker) at 4 days post injury. Needle stick injury resulted in a large injury tract, and microglial migration into and surrounding the injury site (A). MT-IIA treatment promoted the formation of a tissue bridge enclosing the lesion site from the pial surface down, forming a teardrop like invagination (B). MT-IIA promoted axonal sprouting into the lesion site at both the pial layer (C) and deeper cortical layers (D). In contrast, very few axonal sprouts were visualised in control rats, at the pial level (E) or deeper cortical layers (F). Arrowheads indicate the injury tract. scale bars: A, B = 100 μ m; C-E = 25 μ m

By 7 days PI in vehicle treated rats, the injury tract was significantly smaller than at 4 days PI, although a cavity was often evident (Figure 5.2A). Reactive sprouting was observed in all vehicle treated animals at this timepoint (Figure 5.2A). Reactive astroglial migration resulted in a thick line along the borders of the injury tract, and reactive astrocyte density gradually diminished away from the injury site (Figure 5.2B). In MT-IIA treated rats, the entire injury tract was enclosed. Microglial inflammation was decreased, and was found as a thin line of ferritin immunoreactive cells demarcating the injury site (Figure 5.2C). Astrocytes also formed a thin line marking the injury tract. The density of reactive astrocytes decreased further from the injury site (Figure 5.2D). Numerous SMI-312 immunoreactive processes were observed within the injury tract, although these processes often did not exhibit a higher degree of labeling than neighbouring, uninjured processes (Figure 5.2E). At the pial surface however, long reactive axonal processes were observed entering the injury site, which exhibited increased labeling compared to adjacent, uninjured processes (Figure 5.2F).

5.3.2 Western blotting analysis of endogenous MT-I/-II following cortical brain injury

The previous experiments examined the effect of exogenous human MT-IIA on recovery from cortical lesions; however, it is possible that the treatment with recombinant MT-IIA is acting over and above that mediated by endogenous MT-I/-II. As a first step to investigate this, studies by Paul Adlard within this laboratory have demonstrated that MT-I/-II is indeed induced in the vicinity of this type of cortical injury. While MT-I/-II immunoreactivity was not evident in normal un-injured neocortex, MT-I/-II labelling was significantly up regulated by 7 days PI, decreasing by 14 days PI (Adlard, 2000, PhD thesis). MT-I/-II immunoreactivity was confined to astrocytes, and MT-I/-II positive astrocytes were often observed aligned along the injury tract (Figure 5.3). Intriguingly, MT-I/-II immunoreactivity was maximal at the same time that reactive sprouting is observed within this animal injury model (King et al, 1997; 2001). This suggests that in analogy to the ability of MT-IIA to promote reactive axonal sprouting, endogenous MT-I/-II might be acting to directly

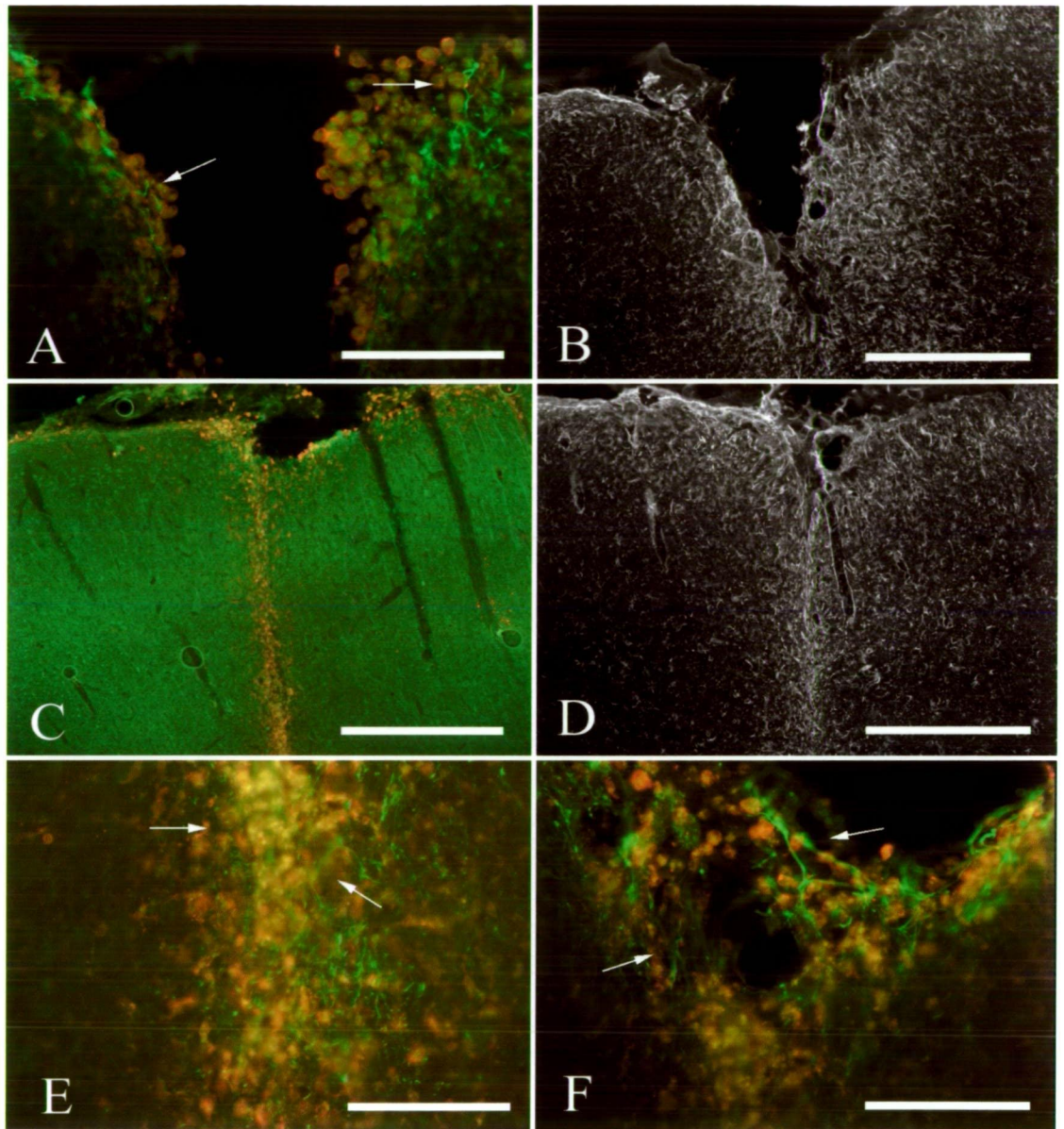


Figure 5.2: Immunohistochemical staining against SMI-312 (green), ferritin (red) and GFAP (black and white images; astrocytic marker) of brain sections at 7 days post injury. In vehicle treated rats, the injury tract was smaller compared to 4 days post injury, although it had not completely closed over. A degree of reactive sprouting was evident (arrows) in all animals at this timepoint (A). Reactive processes exhibited greater SMI-312 reactivity than background neuritic processes. Reactive astrocytes also aligned along the borders of the injury tract (B). In MT-IIA treated rats, the entire injury tract had closed over, and was demarcated only by a fine line of ferritin immunoreactivity (C). Reactive astrocytes also enclosed the injury tract, and were found at lower density in adjacent uninjured tissue (D). In MT-IIA treated animals, numerous reactive axonal processes were observed (arrows) within the injury tract, at both deeper cortical levels (E) and the pial level (F). scale bars: A = 50µm; B-D = 100µm; E, F = 25µm

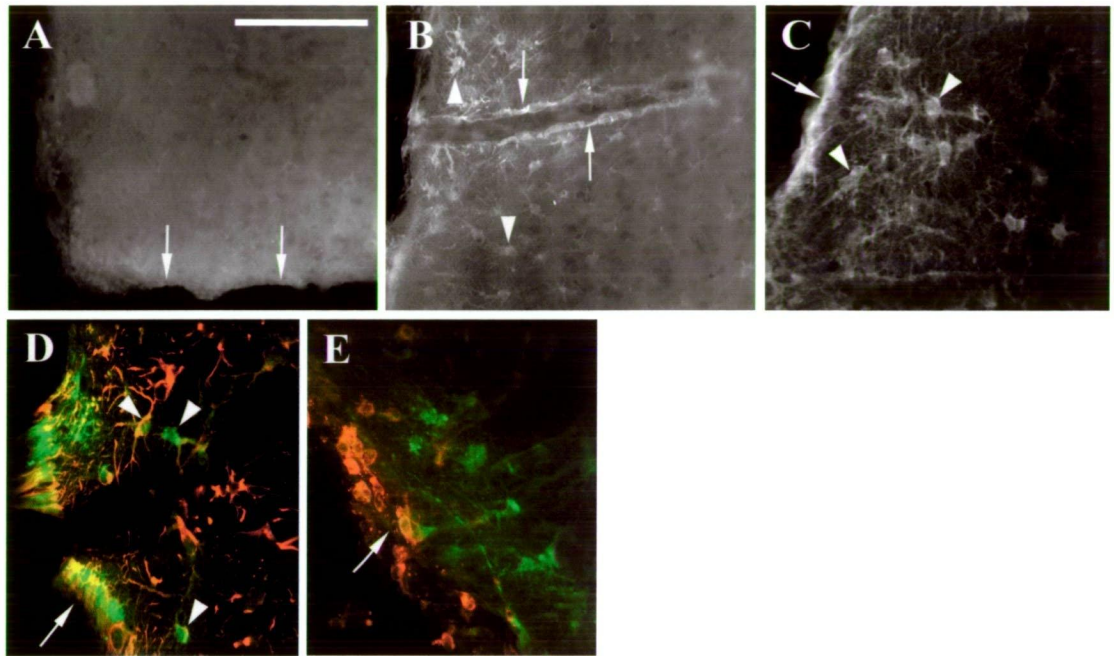


Figure 5.3: Immunohistochemical analysis following focal cortical brain injuries. At one day post-injury (PI), MT-I/-II labelling is absent (A). Arrows indicate the location of the needle tract. At four (B) and seven (C) days PI, MT-I/-II immunoreactivity is localised to glia-like cells (arrowheads), cells and processes surrounding blood vessels (arrows in B), and processes along the pial surface (arrow in C). At 7 days PI (confocal microscopy, D) cells labelled for MT-I/-II (green; arrowheads) have a cell body and/or processes labelled for GFAP (red). The pial surface is indicated (arrow). In contrast, at the same time, ferritin immunoreactive microglia (E; red) are not co-labelled for MT-I/-II (green). The border of the needle tract is indicated (arrow). Modified from Adlard, 2000, PhD thesis.

promote neuronal recovery. To investigate whether endogenous MT-I/-II is present extracellularly following cortical brain injury, the gel foam overlying the lesion (in vehicle treated animals) was recovered following injuries, and the crude gel foam extract analysed by western blotting. MT-I/-II immunoreactivity was observed at both 7 and 14 days PI (Figure 5.4). Re-probing of blots for ferritin and GFAP did not exhibit immunoreactivity, suggesting that there were not significant numbers of either astrocytes or microglia present in the gel foam.

5.4 Discussion

5.4.1 MT-IIA promotes wound healing and reactive axonal growth following cortical brain injury

Exogenous application of human MT-IIA following cortical brain injury in rats significantly promoted wound healing. This was observed by the rapid closing over of the injury tract by 7 days PI, as well as the presence of numerous reactive processes bridging the injury tract. In previous studies using this model, which did not include MT administration, it has been noted that it requires up to 21 days PI for the injury tract to become almost indistinguishable from surrounding, un-injured cortical tissue (King *et al*, 2001). Hence, it appears that MT-IIA application has accelerated the wound healing process. To quantitatively assess the effect of MT-IIA upon reactive neurite sprouting, brain tissue from the injury tract could be collected and western blotting performed for SMI-312 immunoreactivity. Measurement of band intensity between vehicle and MT-IIA treated animals would provide quantitative evidence that MT-IIA promotes reactive sprouting into the injury tract.

Based upon these results and those discussed in Chapter 4, it is possible that the previously reported ability of MT-I/-II to promote cortical wound healing (Penkowa *et al*, 1999a; Campagne *et al*, 1999) could be attributed to two distinct mechanisms. Firstly, work by other groups suggests that MT-I/-II is essential for a number of post-injury recovery processes, such as astrocytic activation and migration, as well as decreasing inflammatory responses (such as microglial activation and pro-inflammatory cytokine expression). Indeed it was noted in the

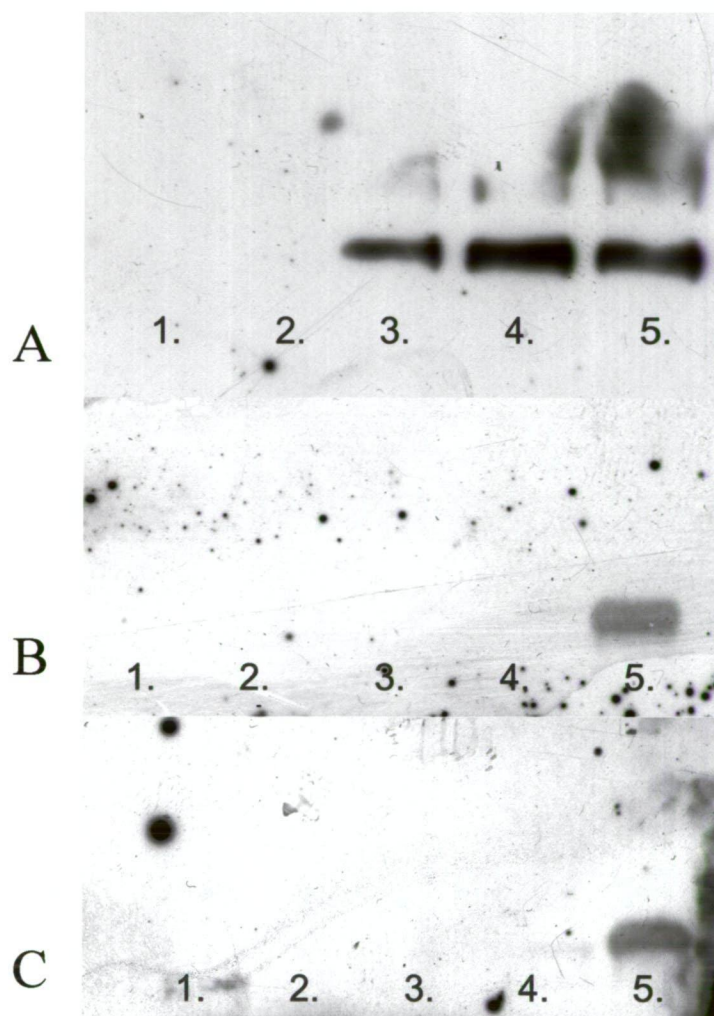


Figure 5.4: Western blotting analysis of protein extracted from gel foam recovered from cortical brain injury sites, using antibodies against MT-I/-II (A), ferritin (a marker of activated microglia; B) and GFAP (a marker of astrocytes; C). Lanes contained the following:

- Lane 1 – gel foam extract, 1 day post injury (PI)
- Lane 2 – gel foam extract, 4 days PI
- Lane 3 – gel foam extract, 7 days PI
- Lane 4 – gel foam extract, 14 days PI
- Lane 5 – crude brain homogenate of an area of tissue encompassing the injury site at 7 days PI. This tissue acts as a positive control, as all three antigens are expected to be present within this sample.

work presented here that there was clearly a reduced inflammatory microglial response at 4 days PI following MT-IIA treatment. And secondly, based upon both the results within this chapter and that of the previous chapter, MT-I/-II may also promote cortical wound healing by acting directly upon neurons to promote neural recovery. Both of these mechanisms appear to be generic to the MT-I/-II isoforms, as similar results have been observed with a commercial MT-I/-II source (results not shown; rabbit mixed Zn-MT-I/-II, Sigma).

To define the relative contribution of the direct effect of MT-IIA upon neurons as opposed to their proposed ability to suppress immune response in the injured brain environment, a series of experiments could be conducted in animals with a compromised immune system. One such experiment would be to assess the ability of MT-IIA to enhance recovery from lesions in cyclosporine A treated rats. The use of a rat model is advantageous since it will allow reference to our previous work. While cyclosporine A has been demonstrated to have a small effect upon neurite extension and sprouting (Sugawara *et al*, 1999), based upon the significant effect of MT-IIA administration to lesioned rat cortex, it would be expected that MT-IIA treatment would result in a quantifiable difference over and above that due to cyclosporine A alone. MT-IIA could also be tested in nude mice, which lack a competent immune system. While MT-IIA has not been administered to mouse models in this laboratory to date, based upon an extensive literature including MT knockout- and over expressing- transgenic mice, it seems feasible that similar results to those observed in rats would be expected.

It has been demonstrated within the literature that the pro-inflammatory cytokine interleukin-6 (IL-6) mediates MT-I/-II induction by inflammation, through its responsive transcription factor Stat3 (Hernandez *et al*, 2000). Intriguingly, it has been recently reported that IL-6 may also have an important role in plasticity and regeneration, which is separate from its role in the inflammatory response associated with brain injury (Parish *et al*, 2002). Briefly, Parish and colleagues (2002) demonstrated the lack of reactive sprouting following injury to the substantia nigra pars compacta in IL-6 knockout mice. Additional reports, both in culture (Schafer *et al*, 1999) and *in vivo* (Shuto *et al*, 2001), support the notion that IL-6 is able to

directly promote neurite regeneration. This suggests that both IL-6 and MT-I/-II have dual functional roles within the brain following injury, whereby they act initially in an inflammatory role, followed later by a role in plasticity and regeneration. In this regard, these proteins probably act in a differential temporal profile to promote neuronal recovery, as IL-6 (together with a number of other inflammatory cytokines) is rapidly expressed in response to cortical brain injury (Yan *et al*, 1992), and decreases coincident with the increased expression of MT-I/-II (Penkowa *et al*, 1999b have demonstrated that MT-I/-II can suppress IL-6 expression) which occurs several days after the original injury (Adlard, 2000, PhD thesis).

A limitation of the experimental injury model used within this chapter is that functional recovery cannot be assessed. In this regard, alternative experimental models may be more effective in evaluating the ability of MT-I/-II to promote neuronal recovery following injury. One possible model is experimental spinal cord injury. In parallel to cortical brain injury, MT-I/-II are significantly up-regulated following injury to the adult rat spinal cord (Carmel *et al*, 2001), indicating the potential involvement of these proteins in the cellular response to such injury. Furthermore, a number of tests have been devised to assess the functional recovery of animals following a variety of different models of spinal cord injury (as reviewed by Schwab & Bartholdi, 1996). Perhaps a future challenge will be to investigate whether MT-IIA is able to promote neural recovery in complex injury paradigms such as spinal cord injury.

5.4.2 Release of endogenous MT-I/-II in response to neuronal injury

Work in our laboratory by Adlard (2000, PhD thesis) has shown that the cortical injury model used here is characterised by a dramatic upregulation of MT about 4 days after the lesion. Interestingly, it was found here that endogenous MT-I/-II was detectable within gel foam retrieved following rat brain injuries in vehicle treated animals, in the absence of astrocytes and microglia, suggesting that this MT-I/-II is extracellular. It is possible that these two observations are related, that is, the extracellular MT recovered from the gel foam is actually released by the reactive

astrocytes surrounding the lesion. One alternative suggestion is that the detected MT-I/-II is released from dying astrocytes rather than through an active release mechanism. Preliminary work performed concurrently to this thesis by Justin Dittmann indicated that MT-I/-II may be actively released from cultured astrocytes following induction by zinc (Dittmann, 2002, BSc honours thesis). These results support those in the literature reporting the detection of MT-I/-II in the extracellular environment *in vivo* (Bremner *et al.*, 1987; Garvey, 1984; Hidalgo *et al.*, 1988), and in culture (Trayhurn *et al.*, 2000). Based on these results, it is therefore hypothesized that the extracellular neuroactive properties of MTs discussed within this thesis occur physiologically within the brain. If so, this implies that the exogenous human MT-IIA is able to promote reactive sprouting over the top of endogenous MT-I/-II. One possibility is that human MT-IIA is more effective than the endogenous rat MT-I/-II isoforms at promoting neuronal recovery. However, based on protein sequence alignment, there is very little difference in the structure of these proteins (Figure 5.5). An alternate possibility is that exogenous MT-IIA stimulates astrocytes surrounding the injury tract to up-regulate endogenous MT-I/-II at greater levels, or in a different temporal timeframe in comparison to untreated animals. Another possibility is that exogenous MT-IIA accelerates repair processes when it is applied acutely after injury, in contrast to endogenous MT-I/-II which is not observable until 4 days after the original injury (Adlard, 2000, PhD thesis).

5.4.3 The possible physiological role of endogenous MT-I/-II following brain injury and in neurodegenerative diseases

It is well documented that MT-I/-II expression is up-regulated not only in response to a number of different models of experimental brain injury to the rat (including focal injury, freeze lesion and ischaemia; see review by Hidalgo *et al.*, 2001), but also a number of neurodegenerative disorders including Alzheimer's disease, EAE (an animal model of multiple sclerosis) and familial amyotrophic lateral sclerosis. The general consensus is that this is in response to oxidative stress or to elevated cytokine levels, a downstream by-product of cellular injury. However, it is also possible to hypothesise that the up-regulation of endogenous MT-I/-II is

	1	11	21	
human MT-IIA	mdpncsca	ag	dsctcagsck	ckeckctsck
		* *	* ** *	
rat MT-II	mdpncscatd	gscscagsck	ckqckctsck	
rat MT-I	mdpncscstg	gsctcssscg	cknckctsck	
	31	41	51	61
human MT-IIA	kscscsccpvg	cakcaqgcic	kgasdkcscc	a
		* * *	* *	
rat MT-II	kscscsccpvg	cakcsqgcic	keasdkcscc	a
rat MT-I	kscscsccpvg	cskcaqgcvc	kgasdkctcc	a

Figure 5.5: The protein sequences of human MT-IIA, rat MT-I and rat MT-II were aligned to identify structural differences. Amino acids which vary between human MT-IIA and either rat MT-I or rat MT-II are indicated by asterisks, while amino acids which are different to both rat isoforms are highlighted in boxes.

directly induced by neuronal injury as a post-injury regenerative response, which has evolved to specifically protect neurons, and to enhance regenerative sprouting, and which involves an extracellular component of action as suggested by the work in this chapter. As an indication of this, MT-I/-II expression peaks at 7 days PI within the focal cortical injury model used within this chapter, which coincides with the appearance of reactive axonal sprouts entering the injury tract (Adlard, 2000, PhD thesis). To further explore this hypothesis, neuronal/astrocyte co-cultures were established, to attempt to generate a model in which neuronal-astrocyte interactions, possibly involving metallothionein induction and release, can be observed in the presence or absence of injury. These experiments will be presented in the next chapter.

Chapter 6: Investigation of astrocytic expression of MT-I/-II in a neuron/astrocyte co-culture model

6.1 Introduction

In the previous chapter, it was demonstrated that endogenous MT-I/-II is present in the extracellular environment following cortical brain injury. An exciting possibility is that, analogous to the ability of human MT-IIA to promote reactive sprouting in culture, endogenous MT-I/-II is able to directly promote neuronal recovery following injury in vivo. Interestingly, there is considerable literature indicating that MT-I/-II are up-regulated in response to various other experimental models of brain injury, including cryolesion, chemical induced injury and experimental autoimmune encephalomyelitis (for review, see Hidalgo et al., 2001). Furthermore, numerous reports indicate an up-regulation of MT-I/-II within astrocytes in a number of neurodegenerative disorders, ranging from early stages of Alzheimer's disease (Adlard et al, 1998), to amyotrophic lateral sclerosis (Gong & Elliott, 2000). Taken together, this evidence is highly suggestive that MT-I/-II are involved in the cellular response to neuronal injury.

Based on the work of Hidalgo and colleagues, proinflammatory cytokines such as IL-6 and TNF- α are important inducers of MT-I/-II expression following brain injury. They have demonstrated that in both IL-6 and TNF- α knockout mice, there is a dramatic deficit in MT-I/-II response to brain injury (Carrasco et al, 1998; Penkowa et al, 2000). This is in accordance with tissue culture experiments that report the induction of MT-I/-II by a number of inflammatory cytokines (Coyle et al, 1995).

An intriguing observation in our laboratory is that the up-regulation of MT-I/-II by astrocytes observed either following cortical brain injury (Adlard, 2000, PhD thesis) or in the pre-clinical Alzheimer's brain (Adlard et al, 1998) is not uniformly distributed. That is, while all MT-I/-II immunoreactive cells and processes co-localise for GFAP positive astrocytes, not all GFAP immunoreactive astrocytes are MT-I/-II positive. This suggests that the induction of MT-I/-II expression in response to brain injury might not be solely attributable to cytokines. Based on this, the primary aim of this chapter was to try and elucidate mechanisms other than

inflammation related molecules such as cytokines, which could contribute to the changes in MT-I/-II expression observed following brain injury, using tissue culture techniques.

6.2 Experimental Procedures

6.2.1 Brief description of neuron/astrocyte co-cultures

As discussed in Chapter 2.3.3, neuron/astrocyte co-cultures were obtained from cortical tissue from embryonic day 19 (sperm positive day = E1) rat embryos, using a method modified from that reported by Schlag et al (1998). Two types of co-culture were maintained, either neuron enriched (more than 50% neurons) or astrocyte enriched (more than 50% astrocytes). Neuron enriched co-cultures were obtained by performing a full medium change every three days, while astrocyte enriched co-cultures were obtained by performing a half medium change once a week.

6.2.2 Analysis of MT-I/-II immunoreactivity

For analysis, 25 digital images were taken from 3 coverslips per group. In the case of coverslips with scratch wounds, images were captured aligning the injury tract. All images within an individual experiment were captured at the same exposure time at 400x magnification. Furthermore, all coverslips within an individual experiment underwent immunocytochemistry at the same time. Experiments, unless otherwise stated, were performed in triplicate (that is separate cultures derived from fetuses from three different pregnant rats). Changes in MT-I/-II expression were qualitatively determined by mean intensity values, using a method based on that of Levadoux and colleagues (2000). Briefly, images were converted to black and white, and the mean intensity value of each image measured (Adobe Photoshop). Standard error and student's t-test analysis were performed using SigmaStat (Jandel Scientific Software Corporation, San Rafael, CA). Using this method, it was possible to directly compare different treatment groups within an individual experiment using quantitative tools. However, it was not possible to

normalise intensity values between experiments performed in two or more different cultures (derived from separate fetuses). This is because the basal levels of MT-I/-II varied between different cultures, and immunocytochemistry and further analysis was not performed at the same time. In these cases, where results from different cultures were compared to each other, the overall changes in MT-I/-II expression (trendline) were qualitatively compared.

To validate the use of mean MT-I/-II intensity values as a means of observing changes in MT-I/-II expression at the immunocytochemical level, changes in MT-I/-II expression were observed following treatment of astrocyte cultures with zinc, a well known inducer of MT-I/-II. Real-time PCR and western blotting results were similar to changes in mean MT-I/-II intensity values (Dittmann, 2002, BSc honours thesis), indicating that this is a suitable method for qualitatively observing changes in MT-I/-II expression.

6.3 Results

6.3.1 Changes in MT-I/-II expression following physical injury to astrocytes*

* The work in 6.3.1 was performed by Justin Dittman (Dittman, 2002, BSc honours thesis), with supervision from the author.

The simplest explanation for the up-regulation of MT following cortical injury is that astrocytes respond directly to physical trauma by inducing MT expression. To investigate this possibility, primary neonatal astrocyte cultures (21 days in vitro) were mechanically injured, by performing scratch wound injuries using a microscalpel. Based on mean intensity values of MT-I/-II immunoreactivity, there was no change in MT-I/-II expression in response to physical injury, at up to 24 hours post-injury (PI). This was observed directly adjacent to the injury tract, or at up to 200µm from the scratch wound site (Figure 6.1).

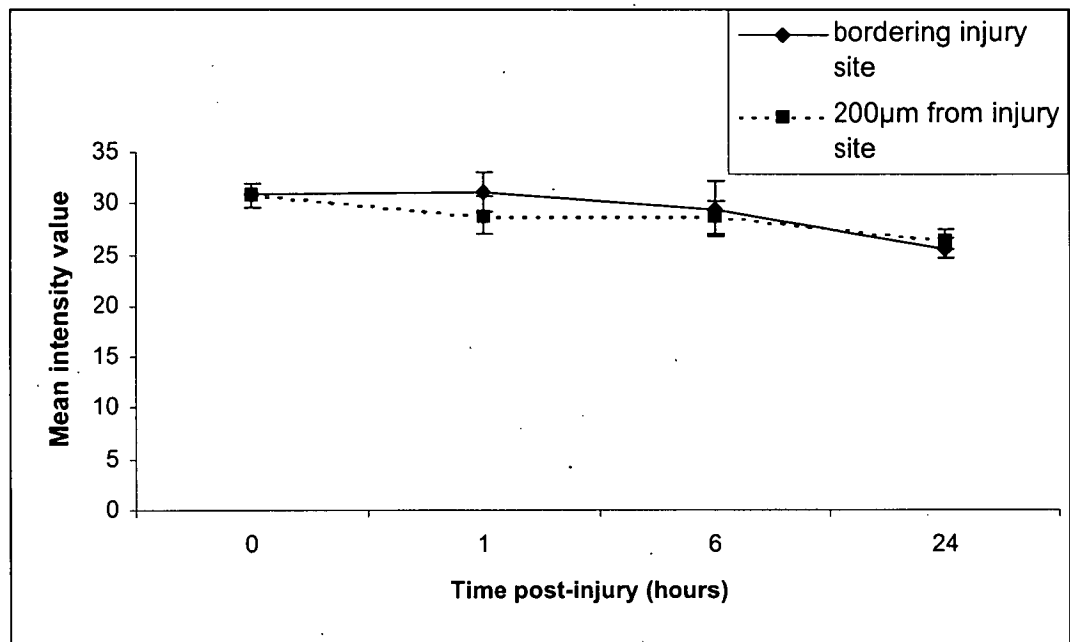


Figure 6.1: Scratch wound injury in primary astrocyte cultures did not result in up-regulation of MT-I/-II at up to 24 hours post injury, either immediately bordering the injury site, or up to 200µm away. Taken from Dittmann, 2002, BSc hon's thesis.

6.3.2 MT-I/-II immunoreactivity following scratch wound injury in neuron/astrocyte co-cultures

To investigate a culture model that more closely resembles the environment of the brain, rat neuron/astrocyte co-cultures (21 days *in vitro*) were used. Although derived from E18 rats, these cells are considered representative of those in the older brain, since they have matured in culture. Co-cultures were prepared as either neuron-enriched (more than 50% neurons) or astrocyte-enriched (more than 50% astrocytes). Mechanical (scratch wound) injury was performed using a fine microscalpel, and changes in MT-I/-II expression evaluated by fluorescent immunocytochemistry. In un-injured co-cultures, MT-I/-II was detected at a basal level in some astrocytes (Figure 6.2A). MT-I/-II immunoreactivity was often nuclear, and not all astrocytes exhibited MT-I/-II immunoreactivity. Indeed, an MT positive astrocyte was often found beside an MT-I/-II negative astrocyte (Figure 6.2A).

At 1 hour post injury (PI), a number of MT-I/-II immunoreactive astrocytes were observed aligning along the scratch wound boundary (Figure 6.2B). MT-I/-II labelling was found not only within the nucleus, but also throughout the cell body, as well as astrocytic processes. Only astrocytes in close proximity to the scratch wound exhibited elevated MT-I/-II labelling.

By 6 hours PI, a number of broad, flat, fibrous astrocytic processes were observed entering the scratch wound area, which were both GFAP and MT-I/-II immunoreactive (Figure 6.2C). The distribution of astrocytes with elevated MT-I/-II levels beside the scratch wound was similar to that observed at 1 hour PI, although there appeared to be more of these cells at 6 hours PI. Many of the astrocytes within 100µm of the scratch wound exhibited increased MT-I/-II immunoreactivity (Figure 6.2C).

While levels of MT-I/-II expression were not different in either neuron- or astrocyte- enriched co-cultures at up to 6 hours PI, differences in the response between the two culture models appeared by 24 hours PI. In neuron enriched co-cultures (50% or more neurons), MT-I/-II immunoreactivity had significantly diminished by 24 hours PI, with few astrocytes exhibiting very low levels of MT-I/-II

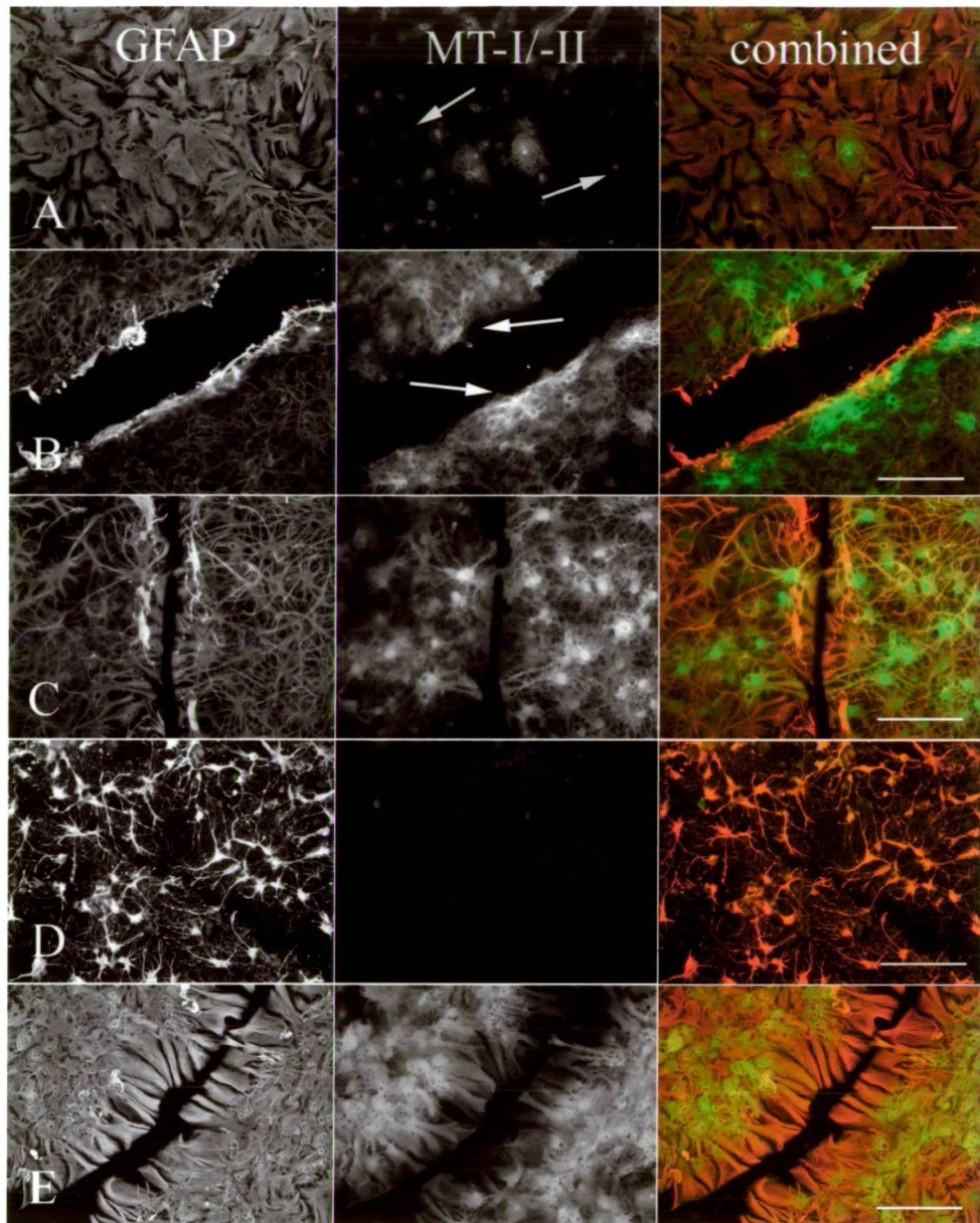


Figure 6.2: MT-I/-II (green) and GFAP (red) double-immunocytochemical analysis following scratch wound injuries in neuron/astrocyte co-cultures. In un-injured cultures (A), MT-I/-II immunoreactivity was primarily nuclear. While all MT-I/-II immunoreactive cells were GFAP positive astrocytes, not all astrocytes were MT-I/-II positive (arrows). At 1 hour PI (B), MT-I/-II was significantly increased in some astrocytes aligned along the injury border (arrows), and was found throughout the cell body and associated processes. At 6 hours PI (C) a number of astrocytes exhibited MT-I/-II immunoreactivity throughout the cell body and processes. While some MT-I/-II immunoreactive astrocytes were still aligned along the injury border, the majority of MT-I/-II positive astrocytes were dispersed up to 100 μ m from the injury site. By 24 hours PI in neuron enriched co-cultures (D), MT-I/-II immunoreactivity had decreased to below basal, un-injured levels. All astrocytes exhibited increased GFAP immunoreactivity, which often appeared thin and fragmented. At 24 hours PI in astrocyte enriched co-cultures (E), MT-I/-II immunoreactivity had significantly increased, and was most prominent in the cell body, but also in some processes. Scale bars = 100 μ m

labelling (Figure 6.2D). A number of astrocytic processes were observed entering, and in some cases crossing the injury site. The density of the astrocyte layer was reduced compared to previous time points. This was highlighted by a significant decrease in astrocytic process meshwork, which was replaced with thin, fragmented GFAP labelling (Figure 6.2D). Further, astrocytes appeared thin, spindly and filamentous. All astrocytes across the coverslip displayed increased GFAP immunoreactivity, throughout the cell body and processes. Astrocyte layers often detached from the coverslip, and were found floating in the medium. Furthermore, neurons exhibited punctate β III-tubulin staining, indicating significant neuronal degeneration (results not shown).

Contrastingly, in astrocyte enriched co-cultures (comprised of less than 50% neurons), MT-I/-II immunoreactivity had significantly increased by 24 hours PI, and was found in almost all astrocytes across the entire coverslip (Figure 6.2E). MT-I/-II staining was found throughout the cell body and fine processes, but was noticeably much lower and diffuse within the large, flat processes extending into the injury site.

Measurements of the mean intensity of MT-I/-II immunoreactivity (Figure 6.3) in neuron-rich cultures (more than 50% neurons) indicated a statistically significant, increasing trend from un-injured (average mean intensity value = 37.6 ± 0.6) to 1 hour PI (52.0 ± 2 ; $p < 0.01$, student's t-test) to 6 hours PI (70.2 ± 2.1 ; $p < 0.01$, student's t-test). At 24 hours PI, MT-I/-II immunoreactivity had significantly decreased below the basal levels observed in un-injured cultures (22.5 ± 0.6 ; $p < 0.01$, student's t-test). In cultures with less than 50% neurons, MT-I/-II immunoreactivity increased with time (Figure 6.3), from un-injured (24.1 ± 1.0), to 24 hours PI (96.6 ± 2.4 ; $p < 0.01$, student's t-test).

6.3.3 Changes in un-injured cultures following addition of media from injured cultures*

Based on the observation that only astrocytes co-cultured with neurons up-regulate MT-I/-II expression in response to cellular injury, it was hypothesised that induction was neuron dependent. To investigate this hypothesis, a series of media

* Astrocyte cultures were prepared by Justin Dittmann.

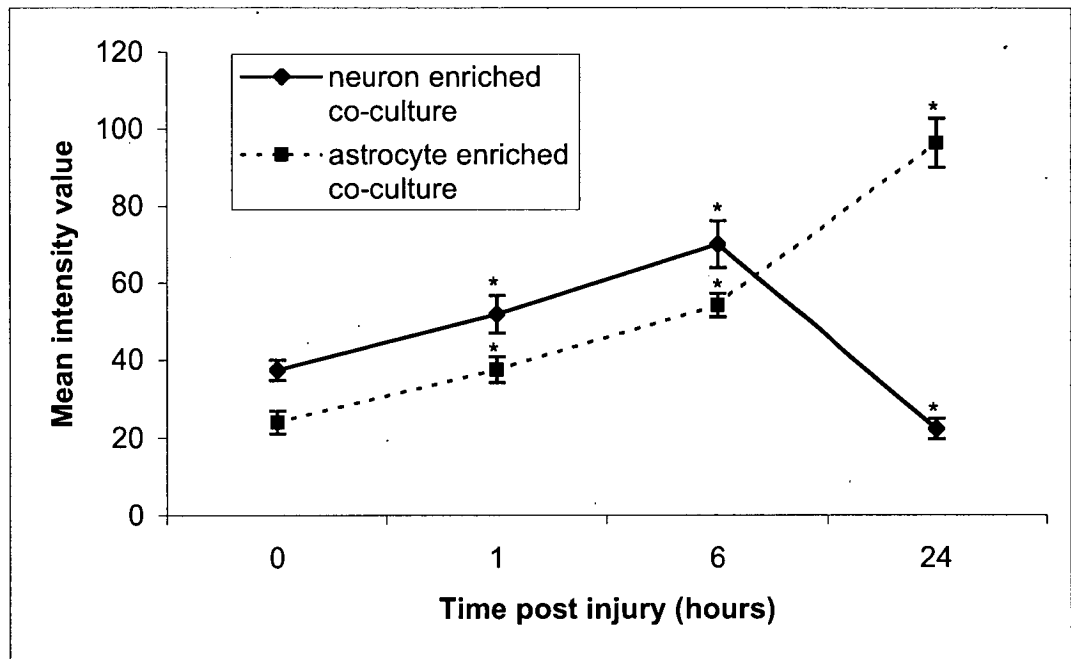


Figure 6.3: The mean intensity of MT-I/-II immunoreactivity surrounding the scratch wound site was measured at various time points post injury (PI). Un-injured co-cultures exhibited a low basal expression of MT-I/-II. In neuron enriched co-cultures, scratch wound injury resulted in a significant increase in MT-I/-II intensity, increasing from 1 hour to 6 hours PI. By 24 hours PI, MT-I/-II levels had decreased to below basal, un-injured levels. In astrocyte enriched co-cultures, MT-I/-II intensity increased with time. * - $p < 0.001$ compared to un-injured, student's t-test. Similar intensity value patterns were observed in replicate experiments. Intensity values from the two different cultures (neuron-rich and astrocyte-rich co-cultures) were not quantitatively comparable to each other because they were not processed for immunocytochemical analysis at the same time (see 6.2.1 for detailed explanation).

exchange experiments were conducted. Briefly, culture medium from injured astrocyte, neuron and astrocyte/neuron co-cultures, all at 24 hours PI, was applied to un-injured co-cultures. Based upon changes in MT-I/-II immunoreactivity (Table 6.1), only the culture medium from injured neuron cultures caused induction of MT-I/-II expression. In further, preliminary experiments, culture medium was collected from injured astrocyte, neuron and neuron/astrocyte co-cultures (24 hours PI), and applied to un-injured astrocyte cultures. In all cases, there was no observable change in MT immunoreactivity after 24 hours (results not shown).

6.3.4 Response of un-injured cultures to glutamate

The requirement of a neuronal component for up regulation in MT-I/-II expression in astrocytes following injury suggests communication between injured neurons and surrounding astrocytes. One of the major communication systems between neurons and astrocytes is based upon glutamate signalling (for review, see Vesce *et al.*, 1999). To investigate whether glutamate could be the potential mechanism responsible for the previous observations, un-injured neuron/astrocyte co-cultures were treated with 1mM glutamate. This resulted in rapid MT-I/-II induction within astrocytes. Un-treated co-cultures exhibited no MT staining, or staining confined to the nucleus (Figure 6.4A). Within an hour of glutamate treatment, a number of astrocytes exhibited strong MT-I/-II staining, although not all astrocytes exhibited MT-I/-II immunoreactivity. Indeed, MT-I/-II immunoreactive astrocytes were often found next to non-immunoreactive astrocytes (Figure 6.4B). By 6 and 24 hours post treatment, almost all astrocytes were strongly MT-I/-II immunoreactive, throughout the cytoplasm and associated processes (Figures 6.4C, D). Preliminary experiments performed by Justin Dittmann found that MT-I/-II expression in primary astrocyte cultures was unaltered by addition of 1mM glutamate (Dittmann, 2002, BSc honours thesis).

Injured culture media source	Average MT Immunoreactivity value
no media change	1
injured astrocytes	0.96 \pm 0.08
injured neurons	2.13 \pm 0.07*
injured co-cultures	0.99 \pm 0.13

Table 6.1: Culture media from injured cultures (astrocytes, neurons and neuron/astrocyte co-cultures) were applied to un-injured co-cultures. The average MT immunoreactivity (as assessed by mean intensity values measured from digital images – see 6.2.1) was determined, and normalised to cultures from the same batch that had received no media change. Note that in this experiment, all immunocytochemical and subsequent processing was completed in the same run, so that intensity data could be compared between cultures. Only culture media from injured neuron cultures resulted in an increase in MT immunoreactivity. * - $p < 0.01$; student's t-test. Note: these experiments have been replicated in separate cultures derived from two pregnant rats.

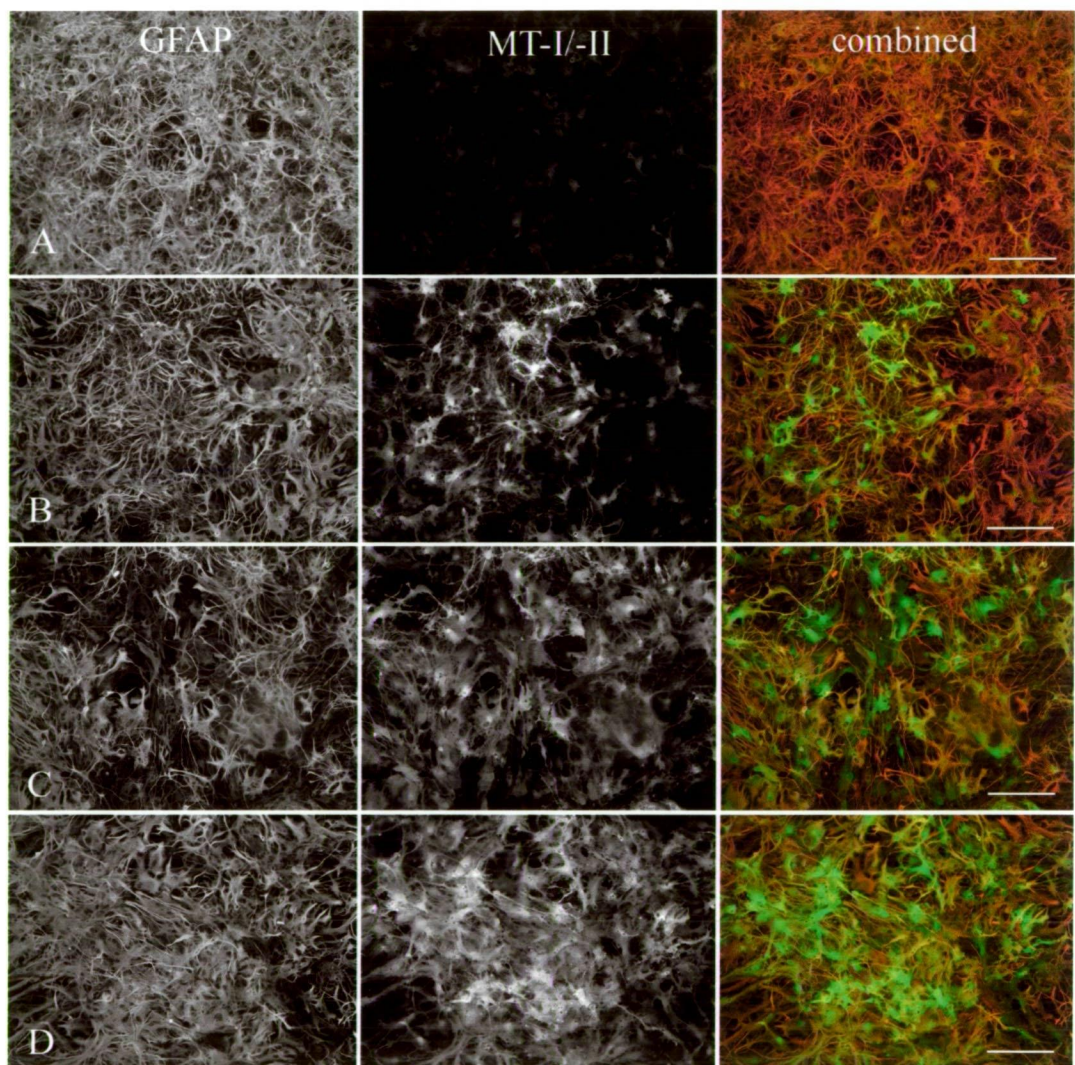


Figure 6.4: MT-I/-II (green) and GFAP (red) double-immunocytochemical analysis following 1mM glutamate treatment in neuron/astrocyte co-cultures. In un-treated cultures (A), MT-I/-II immunoreactivity was primarily nuclear. While all MT-I/-II immunoreactive cells were GFAP positive astrocytes, not all astrocytes were MT-I/-II immunoreactive. At 1 hour post-treatment (PT), a number of astrocytes exhibited strong MT staining (B), but not all astrocytes were MT immunoreactive. By 6 hours PT, almost all astrocytes were strongly MT immunoreactive (C), and this was similarly observed at 24 hours PT (D). Scale bars = 100 μ m

6.4 Discussion

6.4.1 MT-I/-II expression is upregulated in response to neuronal injury

This chapter details the use of tissue culture models to investigate the possible mechanisms responsible for up-regulation of MT-I/-II expression following neuronal injury. Following scratch wound injury in these co-cultures, MT-I/-II was found in astrocytes aligned along the injury site. At later time points, almost all astrocytes within the culture were MT-I/-II immunoreactive. Induced MT-I/-II was found both within the cell body and processes, while in un-injured controls, MT-I/-II staining was either absent or nuclear in localisation. These observations were analogous to those observed previously by Adlard (2000, PhD thesis) following focal cortical brain injury in live animals. Intriguingly, work performed concurrently to this thesis and presented in section 6.3.1 demonstrated that scratch wound injury in pure astrocyte cultures resulted in no change in MT-I/-II expression (Dittmann, 2002, BSc honours thesis). This suggests that MT induction was specifically elicited by neuronal injury, and that MT-I/-II plays an important role in the cellular response to neuronal injury.

It was noted that there was a temporal difference in up-regulation in MT-I/-II expression in response to injury between the *in vitro* (within hours) and rat brain (within several days) injury models. This suggests that following cortical brain injury, a number of factors might act together in a complex manner to regulate MT expression in response to injury.

6.4.2 The role of glutamate in MT-I/-II upregulation in response to neuronal injury

Culture medium exchange experiments provided further evidence supporting the hypothesis that MT-I/-II is specifically up-regulated in response to neuronal injury. Un-injured co-cultures did not respond to culture media from injured astrocytes, but MT-I/-II expression was increased when medium from injured neurons was applied, suggesting that an extracellular factor released by neurons is responsible for MT-I/-II induction. Surprisingly, addition of culture media from injured co-cultures did not induce MT-I/-II expression. This suggests the possibility

that the astrocytes within the co-culture have removed the factor released by injured neurons. In a set of preliminary experiments, un-injured primary astrocyte cultures did not respond to any of the injured culture media, including that from injured neurons (results not shown). This suggests that astrocytes cultured in the absence of neurons are unable to respond to the trophic stimulus responsible for MT-I/-II induction.

A possible explanation for the medium exchange experiments discussed above is that neurons rapidly communicate with neighbouring astrocytes via an extracellular signalling factor. It is well known that rapid cellular communication occurs between neurons and astrocytes based upon reciprocal glutamatergic signalling (for review, see Vesce *et al*, 1999). Indeed, the involvement of glutamate possibly explains the results observed in the media exchange experiments. Glutamate is released by neurons in response to injury, which would explain why medium from injured neurons causes MT-I/-II induction. The fact that media from injured co-cultures did not alter MT-I/-II expression could be due to astrocytic uptake of extracellular glutamate. Furthermore, the observation that astrocytes cultured in the absence of neurons do not respond to the stimulus (either media from injured neurons or 1mM glutamate) could be because in the absence of neurons they exhibit very low levels of glutamate receptor expression (Schlag *et al*, 1998). As further confirmation of this hypothesis, experiments indicated that un-injured co-cultures rapidly respond to extracellular glutamate and up-regulate MT-I/-II in a similar temporal pattern to that observed following scratch wound injury. The fact that in neuron enriched co-cultures, scratch wound injury resulted in significant cellular death by 24 hours PI, suggests that the signalling mechanism is toxic at high concentrations, a known property of high extracellular levels of glutamate. To further elucidate the hypothesised role of glutamate in this system, culture medium could be analysed for glutamate content by HPLC.

It has also been previously demonstrated that both MT-I/-II and MT-III transgenic knockout mice are highly susceptible to kainic acid induced seizures (Penkowa *et al*, 1999a; Erickson *et al*, 1997 respectively), while over expressing mice are protected from such treatment. Furthermore, Montoliu and colleagues

(2000) have recently demonstrated that exogenous MT-III is able to protect neurons from glutamate neurotoxicity (1mM). Based on the high structural similarity between MT-I/-II and MT-III, it seems possible that MT-I/-II exhibits similar protective properties. Combined with the numerous observations that MT-I/-II is significantly up-regulated following neuronal injury, this is suggestive that MT-I/-II is important in the brain's response to glutamate neurotoxicity caused by either neuronal injury or prolonged synaptic activity.

Whilst the work here presents preliminary evidence suggesting glutamate as a putative mediator of the interaction between neurons and astrocytes, it should be noted that there are a large number of other possible candidates. Possibilities include other neurotransmitters, uncharacterised agents, neuronal components such as cytoskeletal elements. It should, however, be possible to use the model system established in this work to characterise the agent using standard biochemical and cell biological techniques.

It is important to note that while advantageous in some respects, an important limitation of the culture models discussed in this chapter is the absence of immune cells. This means that the role of other agents capable of inducing MT expression, such as the proinflammatory cytokines IL-6 and TNF- α that are produced by immune cells, cannot be assessed within these experiments. In this regard, it is possible that a number of factors, including glutamate, zinc and cytokines, regulate MT expression in response to brain injury. However, differences both spatially and temporally in these factors might allow for quite precise, controlled regulation of MT expression. This would be in contrast to the prevailing thought that MTs are regulated as a generalised response to a variety of cellular stresses. Furthermore, it is possible that these factors regulate MT expression by different mechanisms (for instance, zinc acts through MT's metal responsive element while IL-6 induces MT expression through its own Stat3 transcription factor), suggesting that some inducers may be able to over-ride other signals.

Chapter 7: Summary and concluding remarks

7.1 Brief summary

The aim of this thesis was to investigate possible extracellular actions of both major isoforms of MT found in the brain, MT-III and MT-I/-II. This research originated from the report more than 10 years ago by Uchida et al (1991), who identified MT-III as an inhibitor of neuronal survival. They also proposed that MT-III inhibited neurite sprouting, although no detailed quantitative assessment of this property was initially presented. This thesis describes a detailed quantitative analysis of this proposed property of MT-III, and indicates that human MT-III inhibits initial neurite formation and outgrowth in cultured cortical neurons. Rather surprisingly, while human MT-IIA did not affect initial neurite formation, it was found to significantly promote neurite elongation. Based on these findings, the importance of these properties under physiological conditions and within several models of neuronal injury were examined. Both MT-III and MT-IIA exhibited these neuroactive properties in an in vitro model of neuronal injury and regeneration (cluster neuron cultures). However, it was found that exogenously applied MT-III and MT-IIA elicit a variety of responses following axonal transection in culture. For instance, MT-III inhibited reactive neurite sprouting and growth, while MT-IIA increased reactive sprouting and growth. These results are relevant from an experimental point of view, but they also indicate the possibility that we have revealed an actual, and previously unsuspected, component of the physiological role for these proteins. Furthermore, they may lead to further work to explore the possibility of therapeutic action, especially for MT-IIA.

7.2 MTs exhibit isoform specific neuroactive properties

An intriguing feature of the MT family of proteins is the existence of multiple isoforms in mammals. It has been hypothesised that the various MT isoforms provide functional diversity, but some MT literature does not support this. For instance, all MT isoforms exhibit heavy metal binding activity and the ability to scavenge free radicals (as reviewed by Aschner *et al*, 1997). However, the work reported in this thesis indicates that human MT-IIA (the major representative at the

protein level of the human MT-I and MT-II isoforms) and MT-III exhibit remarkably opposing extracellular, neuroactive properties. Human MT-IIA and MT-III share a 70% sequence homology, and also exhibit similar metal binding properties. Indeed, the 20 conserved cysteine residues, which characterise the mammalian MTs, are conserved within these isoforms (with the notable exception of sheep MT-III, discussed in this thesis). Interestingly, Sewell *et al* (1995) found that by changing the C₍₆₎PCP motif of MT-III to the C₍₆₎SCT motif found in MT-I/-II isoforms, MT-III's ability to inhibit neuronal survival was abolished. Likewise, engineering of the T₍₅₎CPCP motif found in MT-III into the MT-I sequence resulted in neurotoxic activity (Romero-Isart *et al*, 2002). This indicates that relatively small changes in protein structure result in quite marked biological differences within these proteins. The opposing neuroactive properties of these remarkably similar MT isoforms, combined with their specific spatial and temporal expression patterns following injury, suggests that the neuroactive properties of MTs are specialised, true physiological functions of these proteins. As a further indication of this, the C₍₆₎PCP motif, which confers MT-III its inhibitory effect upon neuronal survival, is conserved throughout all mammalian MT-IIIs identified to date, including the unusual sheep MT-III described in Chapter 3.

7.3 Elucidation of a potential physiological role of MTs within the brain

It is following neuronal injury that the neuroactive properties of MTs appear most important. Indeed, the work presented in this thesis, together with substantial MT literature, suggests a novel physiological role for MTs within the brain following injury. Clearly, neuronal injury results in significantly increased expression of MT within astrocytes (based on work presented in this thesis, this could be due to a number of different factors such as cytokines, zinc and glutamate), both within the rat brain and in culture. While it has not been well explored, there is evidence to suggest that under certain physiological conditions, astrocytes release MT into the extracellular environment. This released MT could then exhibit its extracellular functions including suppression of inflammatory response, protection from apoptosis

or direct neuroactive effects (such as neuroprotection and enhancing neuronal regeneration)(see Figure 7.1).

While it is possible to postulate that within the brain, MTs play an important role in the cellular response to neuronal injury, investigation of the extracellular release of MT is crucial to elucidation of this idea. While there is evidence to suggest that cultured astrocytes release MT into medium (Uchida *et al*, 2002), the mechanism involved is not known. For example, MTs do not possess known signal sequences or other motifs associated with release into the extracellular environment (Palmiter *et al*, 1992). MT-I/-II was observed in gel foam following cortical brain injury, in the apparent absence of astrocytes and microglia, indicating that this MT-I/-II is a component of the extracellular fluid (Chapter 5). MT-I/-II was detected at both 7 and 14 days post-injury, in parallel to the astrocytic up-regulation of MT-I/-II surrounding the injury tract. However, this detection method is rather crude. Microdialysis techniques have been applied to monitor the changes in the extracellular brain environment, including changes in extracellular glutamate, lactate and neurotrophic factors (Di *et al*, 1999; Mendelowitsch *et al*, 2001; Humpel *et al*, 1995 respectively). Such techniques could be readily adapted to accurately measure the changes in MT levels within extracellular brain fluid in response to brain injury. It must be noted that the prevailing dogma is that MTs are intracellular proteins, which have functional roles inside the cell (Palmiter *et al*, 1992). However, the work described in this thesis indicates that MTs also have extracellular functions. Further investigation of the release and activity of extracellular MT in physiological conditions may reveal the elusive function of these proteins.

7.4 MTs – a potential family of neurotrophic factors within the brain

It is over half a century since the discovery of the first peptide growth factor, nerve growth factor (NGF). Indeed, NGF has become the model neurotrophic factor (a factor which can regulate neuronal death, survival, growth and differentiation; as discussed by Barde, 1989). While this classical definition still holds true, it has been extended to reflect that neurotrophic factors may exhibit multiple effects, as well as having distinct functions at different times during development and following

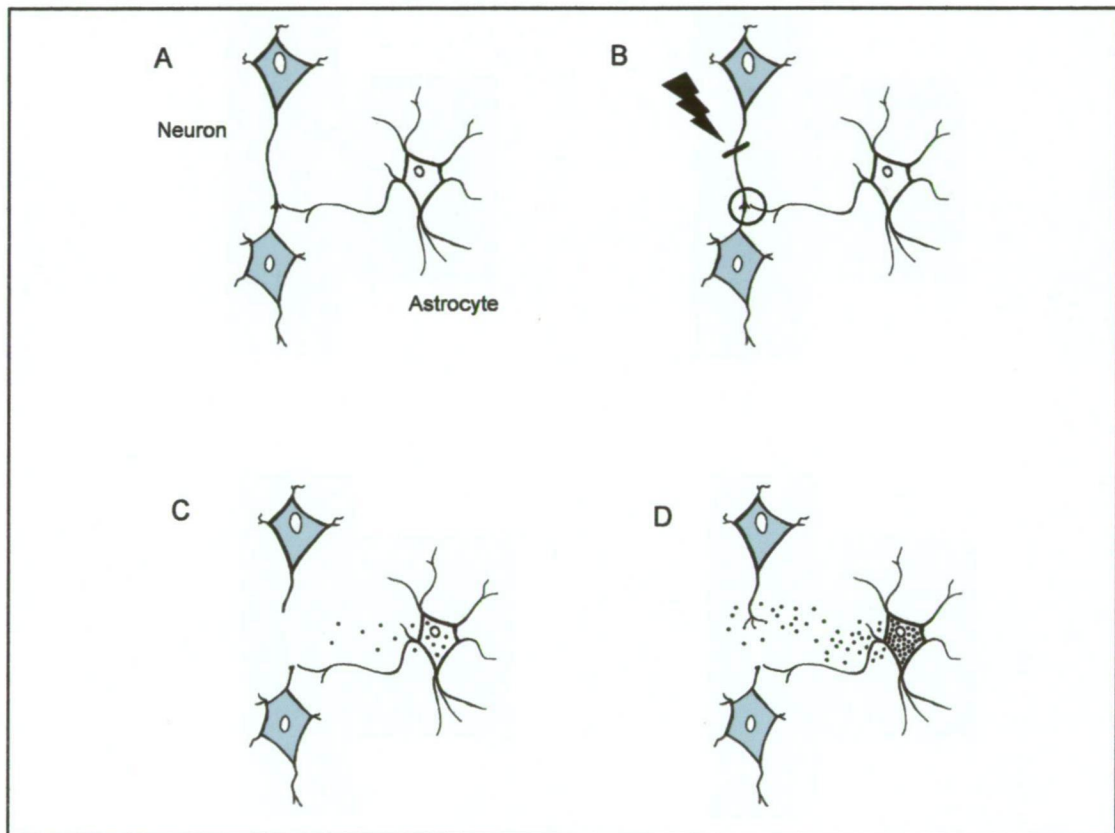


Figure 7.1: A diagrammatic model describing a possible physiological role of extracellular MT within the brain. Astrocytes are closely associated with neurons within the CNS (A). Chemical or physical injury to the axon (B) is detected by astrocytes, perhaps by a diffusible factor or by direct cell-to-cell contact, for example in the vicinity of the synapse (circle). This induces MT production concurrent with axonal degeneration (C), leading to increased extracellular levels of MT, thus promoting axonal sprouting (D). Thanks to Dr Adrian West for designing the above figure.

neuronal injury (Unsicker *et al*, 1992). According to this definition, MTs could be considered as a family of neurotrophic factors. Indeed, MTs exhibit neuronal death, survival and growth regulating actions, as demonstrated both in culture and within the brain. The different neuroactive properties of various MT isoforms is not uncommon within families of neurotrophic factors. For instance, the epidermal growth factor family, the glial derived neurotrophic factor family and the related neuregulin family of neurotrophic factors contain a number of isoforms which exhibit differing functional properties within the nervous system, such as enhancing survival and inhibiting apoptosis of post-mitotic neurons, and promoting proliferation, migration and differentiation of neuronal precursors (for review, see Xian & Zhou, 1999).

7.5 Therapeutic potential of MTs

Within this thesis, the ability of MT-I/-II (namely human MT-IIA) to modulate CNS injury has been examined following cortical needle stick injury to the rat brain. The rat cortical brain injury model used in this study results in a sequence of cytoskeletal changes which are similar to the reactive changes observed in both human and experimental brain injury studies (Maxwell *et al*, 1997). Hence, human MT-IIA may be a valuable therapeutic agent in treating human brain injuries. Furthermore, research by Juan Hidalgo and associates indicates that MT treatment can also alleviate the symptoms of EAE in rats, an animal model of multiple sclerosis (Penkowa & Hidalgo, 2000; 2001). This suggests that MT-IIA could potentially be used for the treatment of a broad range of CNS injuries or neurodegenerative diseases (see Table 7.1). Examination of the efficacy of MT-IIA treatment in other animals models of both CNS injury (such as spinal cord injury) and neurodegenerative diseases (such as β -amyloid transgenic mice) will provide significant insight into the therapeutic potential of MTs.

In contrast to situations following neuronal injury that result in a lack of neural regeneration, there are also neurodegenerative conditions that result in excessive and aberrant neurite sprouting. These include AD (as evidenced by neurofibrillary tangle formation) and seizure related disorders such as epilepsy and

Disease	Indication	Role of MTI/II (IIA)
Alzheimer's disease	Promote neuronal survival and regeneration. Buffer metals implicated in development of pathological hallmarks.	Our lab has demonstrated that MTI/II is upregulated in early stages of the disease (Adlard <i>et al</i> , 1998)
Parkinson's disease	Promote neuronal survival and regeneration. Buffer metals implicated in toxicity.	Evidence of abnormal metal homeostasis in the brain, as well as neurodegeneration.
Motor neuron disease	Promote nerve cell survival. Promote neuronal regeneration. Buffer metals implicated in toxicity. Reduce oxidative stress implicated in neuronal degeneration.	Evidence of abnormal metal homeostasis in the brain and spinal cord as well as neurodegeneration.
Head injury	Promote nerve cell survival. Promote neuronal regeneration.	It has been shown that MT I/II is upregulated at zone of injury (Adlard, 2000). Recombinant protein promotes brain healing and axonal regeneration (Chapter 5)
Spinal cord trauma	Promote nerve cell survival. Promote neuronal regeneration.	Evidence of delayed neurodegeneration and spinal cavitation following injury. Chapter 5 indicates that the recombinant protein is capable of promoting neural healing and regeneration.
Glaucoma	Promote nerve cell survival. Promote neuronal regeneration.	Axonal damage followed by neurodegeneration underlies the disease. MTI/II may potentially promote survival of nerve cells and/or appropriate regeneration.

Table 7.1: Neurodegenerative diseases which could potentially be treated with human MT-IIA. Note that this is not an exhaustive list, but is designed to indicate the range of conditions which could potentially be treated with MT-IIA.

schizophrenia. It is possible to envisage that the neurite inhibitory properties of MT-III may be utilised as a therapeutic agent to treat these conditions. Furthermore, the opposing effects of MT-III and MT-IIA indicate that treatment could involve a combination of these isoforms. For example, MT-III could be used to initially prevent reactive neurite sprouting following injury, allowing time for the modulation of the injury environment, at which point MT-IIA could be applied to promote regenerative neurite growth.

MTs possess a number of generic properties which promote their possible use as a therapeutic agent. Unlike chemical based treatments for neuronal injury, MT is a naturally occurring protein found in the human brain. Further, MT (as a zinc-thionein) is known to be non-toxic in the doses used within this thesis (Suzuki *et al*, 1979). MT following intraperitoneal application has also been shown to rapidly enter the CNS in certain experimental situations (Penkowa & Hidalgo, 2001), indicating a possible therapeutic route for MT.

The therapeutic potential of MTs, while extremely exciting, requires further investigation. For instance, it will be important to determine the relative contribution of MT's direct action upon injured neurons *in vivo* with its previously established ability to suppress CNS inflammatory response following injury. This could be elucidated by examining MT action in immunosuppressed rats (by the use of cyclosporin A), or in nude mice (mice lacking a functional immune system). Furthermore, there are critical functional regions of the MT molecule, which are responsible for its neuroactive activity. Definition of these regions (such as the C₍₆₎PCP motif of MT-III responsible for neurotoxicity) will allow the design of active peptide analogues of MT with enhanced neuroactivity, which may be suitable for therapeutic use.

Because MT over-expressing mice exhibit increased tolerance to cortical brain injury (Campagne *et al*, 2000; Giralt *et al*, 2002) and chemically induced seizures (Erickson *et al*, 1997; Giralt *et al*, 2002), it is possible to envisage the use of MTs as a preventative treatment. Perhaps in the future, MT supplementation may protect people from CNS damage caused by a variety of insults (such as oxidative stress) or neurodegenerative disorders (such as AD). As an indication of this,

Hidalgo and co-workers have used intra-peritoneal injections of Zn-MT-II (Sigma) to ameliorate the neurological damage which occurs during EAE, a rat model of multiple sclerosis (Penkowa & Hidalgo, 2000, 2001).

Alternatively, the work described within this thesis presents the hypothesis that MTs have an important intrinsic role in promoting cortical wound healing. This suggests that less invasive enhancement of this protective mechanism could be considered as a therapeutic treatment. While the literature discusses a number of inducers of MT expression, such agents (including zinc, cytokines such as IL-1 and IL-6) have multiple effects within the brain. In this regard, the identification of a specific inducer of MT expression could provide an avenue to enhance the endogenous protective role of MT within the brain.

7.6 Conclusion

This thesis presents evidence indicating that MT-I/-II and MT-III possess remarkably different properties when applied to several culture and *in vivo* models of neuronal damage. Furthermore, the changes in their expression following brain injury and in some neurological disorders, in combination with these neurotrophic properties, suggest that these proteins have an important physiological role within the cellular response to neuronal injury. Based upon their neurotrophic properties, MTs also have therapeutic potential for the possible treatment of a broad range of CNS injuries and neurodegenerative diseases.

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