The Role of Metallothionein I/II in Promoting

Axonal Sprouting in Both Central Nervous System

and Peripheral Nervous System

By

Jacqueline Yee Kei Leung

B.Biotech (Hons)

University of
Tasmania

<u>Declaration of Originality</u>

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Poster presentation

Jacqueline Y.K. Leung, M. Inn. Chuah, Adrian K. West, R. Douglas Fields, Phillip R.Lee, Hiroaki Wake, Roger S. Chung. Expression of metallothionein I/II and its possible role in the peripheral nervous system. Poster presented as part of the Society of Neuroscience (SfN) Annual conference, San Diego, CA, USA. 12-17th November, 2010.

<u>Publications</u>

Adrian K West, Jacqueline Y. K. Leung, Roger S. Chung (2011). Neuroprotection and regenerationby by extracellular metallothionein via lipoprotein receptor-related proteins. J.Chem. Inorg. Biochem. (in press).

Jacqueline Y.K. Leung, William R. Bennett, Rosalind P. Herbert, Adrian K. West, Philip R. Lee, Hiroaki Wake, R. Douglas Fields, Meng Inn Chuah, Roger S. Chung. (2011) Metallothionein Promotes Regenerative Axonal Sprouting of Dorsal Root Ganglion Neurons After Physical Axotomy. CMLS. (in press)

This manuscript contains most of the data presented in Chapter 4 of this thesis.

Statement of Co-Authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1 - Neuroprotection and regenerationby by extracellular metallothionein via lipoprotein receptor-related proteins.

        Adrian K West (45%)

        Jacqueline Y K Leung (5%)

        Roger S Chung (45%)

Paper 2 - Metallothionein Promotes Regenerative Axonal Sprouting of Dorsal Root Ganglion Neurons After Physical Axotomy.

        Jacqueline Y K Leung (40%)

        William R Bennett (5%)

        Rosalind P. Herbert (5%)

        Adrian K West (5%)

        Philip R Lee (10%)

        Hiroaki Wake (5%)

        R Douglas Fields (5%)

        Meng Inn Chuah (5%)

        Roger S Chung (20%)

Details of the authors roles:

Jacqueline Y K Leung, Roger S Chung, M Inn Chuah, Adrian K West and R Douglas Fields contributed to the design of experiments, analysis of data and writing the manuscript.

William R Bennett, Philip R Lee and Hiroaki Wake assisted with analysis and presentation of data.

Christopher W Butler, Rosalind P. Herbert and Emma D. Eaton provided technical support for some of the experimental procedures.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Assoc. Prof. Roger S Chung
Supervisor
Menzies Research Institute
University of Tasmania

Assoc. Prof. M. Inn Chuah
Supervisor
Menzies Research Institute
University of Tasmania

Head of School
Menzies Research Institute
University of Tasmania

Date:_____

<u>Statement of Ethical Conduct</u>

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University

Abstracts

Metallothioneins are a family of low molecular weight, metal-binding proteins with an unusually high cysteine content. The metallothionein I/II isoforms (hereafter referred to as MT) have been demonstrated extensively in a number of studies to promote axonal outgrowth of a variety of central nervous system (CNS) neurons, both *in vivo* and *in vitro*, after traumatic injury. However, the precise mechanisms through which MT interacts with injured neurons to promote their regeneration after injury remains largely unknown. The goal of this thesis is to identify some of these molecular mechanisms in both the injured CNS and peripheral nervous system (PNS).

The first part of this thesis aimed to identify how exogenous MT interacts with glia and neurons in the CNS after traumatic injury using the *in vivo* focal needle-stick injury model. It was found that injection of fluorescently labeled-MT (488-MT) into the injury site led to a decrease in overall injury size compared to vehicle treatment (fluorescent-unincorporated dye, AlexaFluor-488). Histological analysis found that 488-MT was present in both neurons as well as reactive microglia surrounding the injury site. The internalization of 488-MT by cortical neurons suggests that the injected 488-MT might interact directly upon injured neurons to promote regenerative axonal sprouting, which would be in accordance with prior studies demonstrating that neuronal internalization is required for MT to promote initial neurite outgrowth and elongation.

To identify the signaling pathway through which MT exerts its effect upon neurons, the MT were co-injected with receptor-associated protein (RAP), an antagonist for megalin (a recently identified receptor for MT) into the injured cortex using the same injury model. The results demonstrated that the addition of RAP has blocked the effect of MT in

promoting regenerative axonal sprouting after injury, thus confirming that MT acts through LRP receptors-dependent pathways to exert its regenerative effect upon injured cortical neurons.

The uptake of MT by microglia led to a further investigation into the direct effect of MT on the microglial response after injury. *In vivo* data suggested that the injection of 488-MT caused a morphological change in reactive microglia. There was also enhanced physical association between reactive microglia and regeneratively spouting neurons. Using a series of neuron-microglia co-culture and media exchange experiments, it was found that MT could block the inhibitory effect of cytokine-activated microglia had upon neurite outgrowth of cultured cortical neurons. Hence this demonstrates that MT might potentially modulate the microglial response, which indirectly leads to improvement in regenerative axonal sprouting after injury.

The second half of this thesis evaluated the potential role of MT in promoting nerve regeneration in the PNS. The rationale for this study relates to recent reports that another neuroprotective protein, transthyretin, can promote neurite outgrowth of dorsal root ganglion (DRG) neurons via interaction with the megalin receptor (also a putative receptor for MT). To investigate the effect of MT in promoting neurite regeneration in the DRG neurons, MT was applied to DRG neuron cultures, which led to an improvement in axonal sprouting after scratch injury. To investigate in further detail the mechanism of action of this effect, a compartmentized *in vitro* injury model was established, which allowed the precise and restricted application of MT to either the axonal processes or neuronal soma immediately after axonal scratch injury. The length of regenerative axonal sprouts was measured 24 hours after injury, and a 4-fold or 1.4-fold

increase in regeneration was observed when MT was administered to either the soma or axonal compartments respectively (fold change relative to vehicle-treated control). Interestingly, this correlated with the expression of megalin, which was restricted primarily to the neuronal soma, with very little megalin labeling observed in axons. Finally, MT was found to promote DRG neuron regeneration via LRP- and MAPK- (mitogen-activated protein kinase-)-dependent pathway.

Overall, this thesis has provided new insight to the field in understanding some of the precise mechanisms through which MT promotes neural regeneration in both CNS and PNS. It demonstrated that MT may potentially act directly upon microglia to modulate the extracellular environment in response to brain injury, which may then facilitate neuronal sprouting after injury in the CNS. This thesis also demonstrates that MT promotes peripheral nerve regeneration after injury via a megalin and MAPK-dependent mechanism.

Acknowledgement

I would like to thanks my supervisors Assoc. Prof. Roger Chung and Assoc. Prof. Inn Chuah for their help and support throughout the project. Thank you for inspiring me with ideas as well as giving me positive comments all the way through until the writing up stage of this thesis. Thank you Prof. Adrian West for the ideas as well as your ongoing comments as the project developed.

I would also like to express my great appreciation to Prof. Douglas Fields as well as his lab members, Dr. Philip Lee and Dr. Hiroaki Wake for allowing me to use their labs during my visit and taught me all the techniques into setting up the chamber model. Moreover, this visit wouldn't have happened without the generous support from the Adam J Berry Memorial Fund from the Australian Academy of Science, so I would like to thank you once again for giving me this opportunity.

Most importantly, I also would like to thank you my family and friends especially my parents Leo and Ida Leung, for being the best parents and supporting me through all those long-distance phone calls. An important person not to be missed is my fiancé, James Kocharunchitt, thank you for being so patience and supportive. Special thanks should be given to Somsak and Sripet Kocharunchitt for their support as well as being my personal nutritionists.

Last but not least, I would like to acknowledge other lab members from the MT group, Dr. Bill Bennett, Roselind Herbert, Emma Eaton, Kate Lewis and Chris Butler for their help in the lab as well as the occasion cakes and chocolate sessions, which surely are the best stress relieve remedies.

List of Abbreviations

488-MT	Fluorescently Labeled MT
ADP	Adenosine Diphosphate
APTS	3-Aminopropyltriethoxysilane
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
CCI	Controlled Cortical Impact
chABC	Chondroitinase ABC
CNS	Central Nervous System
CSPG	Chondroitin Sulphate Proteoglycan
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal Root Ganglion
DSPG	Dermatan Sulphate Proteoglycan
EAE	Experimental Autoimmune Encephalomyelitis
GFAP	Glial Fibrillary Acid Protein
HBSS	Hanks Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPG	Heparan Sulphate Proteoglycan
IFNγ	Interferron Gamma
IL-1	Interleukin-1
IL-4	Interleukin 4
IL-6	Interleukin-6
IL-10	Interleukin 10
IL-12	Interleukin 12
JAK	Janus Kinase
KSPG	Keratan Sulphate Proteoglycan
LRP	Low-Density Lipoprotein Receptor-Related Protein-1
MAG	Myelin-Associated Glycoprotein
MAPK	MItogen-Activated Protein Kinase
MAI	Myelin-Associated Inhibitor
MCSF	Macrophage Colony Stimulating Factor
MEM	Minimum Essential Medium
MT	Metallothionein
NGF	Nerve Growth Factor
OCT	Optimal Cutting Temperature
OMgp	Oligodendrocyte Myelin Glycoprotein
PBS	Phosphate Buffered Salin
PNS	Peripheral Nervous System
RAP	Receptor Associated Protein
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
STAT	Signal Transducers and Activators of Transcription
TNFα	Tumor Necrosis Factor α

Tables of Contents

Chapter 1 Literature Review

1.1 Neuronal Response After Injury

Injury to the nervous system has a devastating and often long-lasting detrimental impact upon the physical capabilities of people. The nervous system consists of the central nervous system (CNS), which includes the brain and spinal cord; and the peripheral nervous system (PNS), encompassing all nerves and ganglia external to the brain and spinal cord. Damage to the CNS in adults generally recovers poorly, in contrast to that of the injured PNS. Understanding the differences in regenerative capacity between the CNS and PNS may identify new therapeutic strategies for improving recovery of the injured CNS. Hence, this review will focus on the factors that contribute to the different in regenerative outcome of neurons after injury in the CNS and PNS.

Neurons are structurally composed of three primary cellular compartments: the soma or cell body (containing the nucleus), axons (transmit information to other neurons via synapses that connect with other neurons), and dendrites (the receiving terminals which form synapses with the pre-synaptic axons). Notably, the majority of published studies in this field have been focused upon the axonal response to injury and factors that facilitate axonal regrowth and re-connection. Several major areas of focus have been upon improving the regenerative sprouting of injured axons as well as modulating the extracellular environment to be more permissive and supportive for regenerative axonal growth. Therefore, the following review will be mainly focused upon the current

knowledge of the axonal response to injury, as well as the factors that influence the differences in the regenerative capacity after injury between the central and peripheral neurons.

1.2 Axonal Degeneration in Response to Injury

After injury axons generally undergo a degenerative response which can be separated into two categories: events that occur at the proximal nerve stump, (the proximal section of the injured axon that remains connected to the cell body), and the distal nerve stump (comprising the distal part of the injured axon that has became disconnected from the cell body after injury). It should be noted that axonal regeneration has generally been best characterised in peripheral nerves, due to their high accessibility *in vivo* as well as their ability to form long axonal bundles *in vitro*. Therefore the following sections will mainly summarize the current knowledge in the field collected from studies using peripheral nerves, unless otherwise specified.

1.2.1 Degenerative Response to Axotomy at the Proximal Nerve Stump

After complete axotomy (either primary or secondary axotomy), an initial 'die-back' or retraction of the proximal axon occurs. Axonal retraction generally ceases when the retraction reaches the first node of Ranvier, with the cell bodies staying intact after the injury (reviewed by S. Y. Fu and T. Gordon, 1997). Following axonal retraction, swelling occurs within the proximal end of the injured axon adjacent to the lesion site. This swelling is comprised of the accumulation of cellular contents, such as cytoskeletal proteins, smooth endoplasmic reticulum, mitochondria, which is presumably caused by

disruption in the normal transport of these contents along axons (reviewed by J. W. Fawcett and R. J. Keynes, 1990 S. Y. Fu and T. Gordon, 1997). This progresses to the degeneration of the axons, through a process known as Wallerian degeneration, which will be discussed further in the next section (S. Y. Fu and T. Gordon, 1997).

However, traumatic injury in the CNS does not always lead to complete axotomy of axons. Instead, a diffuse form of axonal injury commonly occurs. While axotomy is absent, this diffuse form of injury generally leads to the substantial blockage of axonal transport, resulting in the accumulation of cellular contents and axonal swelling described above (as reviewed by M. Coleman, 2005). It has been reported in studies utilising an *in vitro* stretch-injury model performed upon cultured cortical neurons, that the stretch injury led to a significant change in ion permeability in the injured area of the axon, leading to the influx of ions such as calcium (A. Iwata et al., 2004). The unregulated influx of ions such as calcium triggers a range of intracellular mechanisms that contribute to secondary cellular degeneration. This will be further discussed in the following sections.

1.2.3 Degenerative Response of the Distal Nerve Stump Following Axotomy

The distal nerve stump of axotomised axons undergoes Wallerian degeneration, which is a sequence of events that led to the degeneration of the proximal nerve segment that has became separated from the cell body after injury. (as reviewed in S. Y. Fu and T. Gordon, 1997; B. Beirowski et al., 2005). Transgenic mice that express Yellow Fluorescent Protein (YFP) in subsets of neurons (and their axons) has allowed continual time-lapse

imaging of individual axons after injury in living animals. This study has demonstrated that immediately after either crush or complete transection injury to sciatic nerves, an initial latency period occurs during which time the majority of the axons are observed to remain intact for 36-44 hours after injury (B. Beirowski et al., 2005). This latency period was defined as the time during which injured axons were still able to conduct action potentials, and able to display limited ultrastructural changes as assessed by electron microscopy (B. Beirowski et al., 2005). The use of YFP-expressing transgenic mice has also allowed the monitoring of the spatiotemporal pattern of axonal degeneration that occurs after different types of injury. It has been reported hat both complete nerve transection and crush injury led to a wave-like pattern in the propagation of axonal fragmentation (B. Beirowski et al., 2005). These results suggest that the degenerative process is active and regulated by a precise series of intracellular processes, rather than a set of passive events associated with the discontinued supply of cellular proteins to the distal parts of axons (J. T. Finn et al., 2000; B. Beirowski et al., 2005).

One of the major biochemical drivers of this degenerative process is the disruption of ion homeostasis (including Na^+ and Ca^{2+} ions) in axons after injury (as reviewed in W. Young, 1992). It is thought that injury that is sufficient to cause permeabilisation of the cell membrane of axons leads to an influx in Na^+ ions as well as the passive diffusion of Ca^{2+} ions into the axoplasm (A. Iwata et al., 2004 and reviewed by B. Beirowski et al., 2005). Notably, the post-injury ion homeostasis is a complicated process, which involves a vast diversity of ion channels. Hence, it is difficult to conclude the involvement of particular ions. However, studies using tetrodotoxin, which blocks the activity of voltage-

gated sodium channels have resulted in blocking the increase of intracellular calcium that normally occurs after experimental axotomy in cortical neuron cultures, thus demonstrating that sodium influx might be coupled together with calcium influx (A. Iwata et al., 2004). This influx of calcium then leads to the activation of intracellular calcium-dependent proteases known as calpains, which trigger the induction of axon degeneration after injury (A. Buki et al., 1999). This is supported by studies reporting that application of both calcium channel blockers and calpain antagonists considerably delay or prevent the degenerative response of dorsal root ganglion (DRG) explants after axotomy *in vitro* (E. B. George et al., 1995).

1.3 Regenerative Axonal Sprouting After Injury

Studies have demonstrated that axons have an intrinsic capability to sprout in response to injury, which has largely been considered as an active response mediated by fundamental changes in gene expression profile, i.e. from "transmitting mode" to "growth mode". These changes involve downregulating the expression of neurotransmission related proteins, and upregulating the expression of growth associated proteins (such as the cytoskeletal proteins, actin and tubulin), which bear similarities to the gene expression profile observed during neuron development (as reviewed by S. Y. Fu and T. Gordon, 1997). These changes ultimately led to the formation of axonal sprouts (emanating either from injured processes or from the cell body) from neurons that have survived the initial degenerative process. While more than one sprout might originate from each neuron, similar to what is observed during development, only one sprout that ultimately reaches and re-connects with a target cell would be maintained, while the others would be

withdrawn (as reviewed by S. Y. Fu and T. Gordon, 1997; D. Muir, 2010). Interestingly, the initiation of the axonal sprouting response after injury has been demonstrated to be independent of upregulation of cytoskeletal proteins in the cell body. This is based upon the very quick timeframe in which regenerative sprouting is initiated, as well as the observation that regenerative sprouting can occur from injured axons that have been completely isolated from the cell body. Therefore, initial regenerative sprouting and outgrowth appear to be an innate property of injured axons, and appear to be induced by the axonal transport of tubulin and actin into the proximal stump (M. A. Bisby and W. Tetzlaff, 1992). Further illustrating the significant role of microtubules in axon sprouting, the use of the microtubule stabilizing agent Taxol has been shown to improve neurite sprouting following injury *in vitro* and *in vivo* (V. Sengottuvel et al., 2011). Moreover, in primary cell culture preparations used for *in vitro* studies using both CNS and PNS neurons have shown that after the tissue preparation, these neurons were readily sprout regenerative neurites after being isolated, therefore suggesting that neurons have an intrinsic ability to sprout following injury. This highlights the importance in understanding the factors that contribute to the poor regenerative outcomes observed in the CNS after injury in comparison to the PNS.

Interestingly, a process known as pre-conditioning has been identified whereby a conditioning lesion to the peripheral axon of a DRG neuron (which has both a peripheral and central axon) one week prior injury to the central axon significantly improves nerve regeneration into the lesion site of the spinal cord. The conditioning lesion leads to an increase in the expression of regeneration-associated genes, hence improvement in the

overall regenerative response after the second injury to the central axons (S. Neumann and C. J. Woolf, 1999). Several studies have reported that pre-conditioning injuries modulate the intrinsic regenerative properties of neurons through the activation of the JAK/STAT (Janus kinase / Signal transducers and activators of transcription) pathway (J. Qiu et al., 2005). This further demonstrated that if neurons are able to upregulate the proper cell machinery, they have the potential to regenerate after injury.

1.4 Environmental Factors that Influence Regenerative Sprouting in Central and Peripheral Neurons After Injury

As discussed previously, both central and peripheral neurons have an innate capacity to extend regenerative sprouts after injury. This suggested that the difference between the relative regenerative capacity of CNS and PNS is due to the different extracellular environments present within these systems. To illustrate this, the inhibitory elements present in the CNS will first be discussed, followed by review of the known regeneration-promoting elements present in the PNS.

1.4.1 Factors that Affect Axonal Sprouting in CNS - Reactive Astrogliosis and Glial Scar Formation

The formation of the glial scar is one of the major inhibitory factors that hinder regenerative sprouting after CNS injury. The glial scar represents a cellular and molecular barrier that encapsulates the cavity surrounding the wound area after injury (M. T. Fitch et al., 1999). The CNS glial scar is primarily comprised of reactive astrocytes, which in

response to the injury migrate robustly to surround the injury site (M. T. Fitch et al., 1999; A. R. Filous et al., 2010).

In response to injury, the astrocytes along the injury site first become reactive, which is classified by the upregulation of the expression of intermediate filament glial fibrillary acid protein (GFAP), and undergo hypertrophy and proliferation (as reviewed by R. Biran et al., 1999; A. Araque et al., 2001). Proteoglycans are one of the major inhibitory substrates present in the glial scar (as reviewed by J. Silver and J. H. Miller, 2004). There are four major types of proteoglycans produced by astrocytes: heparan sulphate proteoglycan (HSPG), dermatan sulphate proteoglycan (DSPG), keratan sulphate proteoglycan (KSPG) and chondroitin sulphate proteoglycan (CSPG) (P. C. Johnson-Green et al., 1991). The CSPGs are the most inhibitory to neurite extension of both adult and embryonic neurons (as reviewed in J. Silver and J. H. Miller, 2004). Furthermore, CSPGs also inhibit the effect of growth promoting molecules such as laminin in cerebellar granule neuron cultures (C. L. Dou and J. M. Levine, 1994).

Given that CSPGs expressed by reactive astrocytes are highly inhibitory to regenerative neurite sprouting after injury, it is feasible that modulating the activation of reactive astrocytes might lead to improvement in regenerative axonal sprouting after injury. This has been shown to be successful in studies using the enzyme chondroitinase, which digests the sidechains of CSPGs rendering them inactive (J.M. Massey et al., 2006).

Notably however, recent studies have demonstrated that reactive astrocytes also have important protective roles after CNS injury. Hence, studies that utilize transgenic mice with a GFAP promotor-driven herpes simplex virus-thymidine kinase transgene have revealed the complexity of the reactive astrocyte and glial scar formation in response to CNS injury. This strain of transgenic mice allows the selective ablation of proliferating and GFAP-expressing astrocytes using ganciclovir (antiviral agent) treatment, hence allowing the selective ablation of cells that express thymidine kinase, which in this case are reactive astrocytes (T. G. Bush et al., 1999; J. R. Faulkner et al., 2004; D. J. Myer et al., 2006). In these studies, brain injuries, including stab injury (T. G. Bush et al., 1999) or moderate controlled cortical impact (CCI) injury (D. J. Myer et al., 2006), were performed on both transgenic and non-transgenic wildtype mice treated post-injury with gancyclovir, to determine the effect of ablation of reactive astrocytes in these brain injury models. These studies have found that selective ablation of reactive astrocytes in injured transgenic mice results in an increase infiltration of leukocytes into the lesion area, together with greater numbers of inflammatory microglia/macrophages, (hence increased inflammatory response). Rather surprisingly however, the ablation of reactive astrocytes led to an exacerbation of neuronal loss in the injury site and loss of cortical volume (D. J. Myer et al., 2006;T. G. Bush et al., 1999). This suggests that reactive astrocytes might also have important protective roles in the injured CNS.

The same transgenic mice have also been used to study the role of reactive astrocytes in either stab or crush injury models, representing either mild or moderate spinal cord injuries respectively (J. R. Faulkner et al., 2004). Similar to previous injury models the

targeted ablation of reactive astrocytes in both the stab and crush injured spinal cord led to increased inflammation and increased infiltration of inflammatory cells (especially macrophages) into the injury site. The depletion of astrocytes also led to the exacerbation of neuronal loss and neural degeneration, as well as the failure in repairing the blood brain barrier. Moreover, both of these injuries have diminished motor recovery in the absence of reactive astrocytes (J. R. Faulkner et al., 2004). Overall, these studies have demonstrated that the presence of reactive astrocytes is necessary for protection of neurons, as well as recovery from the injury.

The injury environment is dynamic in which the cellular and molecular response of both astrocytes and neurons undergo rapid changes over time. This could explain the conflicting results shown by studies demonstrating that reactive astrocytes can be both protective and detrimental to neurons. The process of reactive astrogliosis and the formation of a glial scar are a rapid response to CNS injury that contained the injury site, and may subsequently prevent further spreading of damage from the injury. However, at later stages in the post-injury process when recovery and regeneration are important, a permissive cellular and molecular environment is required. Hence, the inhibitory factors should be cleared to allow a more supportive environment for axonal growth. This has been demonstrated in studies using chondroitinase ABC (ChABC), a bacterial enzyme that digests the glycosaminoglycan side chains from the proteoglycan protein and renders the CSPGs molecules inactive. The injection of chondroitinase ABC leads to an improvement in axon regeneration as well as functional recovery after CNS injury (D. Crespo et al., 2007). The premise underlying these experiments is that injecting the

ChABC led to the degradation or digestion of CSPGs in the glial scar, therefore reducing the inhibitory activity of the external environment to regenerative axonal sprouting (J. M. Massey et al., 2006).

1.4.2 Myelin, a Major Inhibitory Component Presence in CNS

Apart from CSPGs, Myelin-associated inhibitors (MAIs) are another class of important proteins that exert inhibitory effect upon regenerative axonal outgrowth in the injured CNS. The MAIs are associated with myelin, which is produced by oligodendrocytes. Myelin is the lipid-rich multilayer membrane that enwraps axons, and creates different axonal segments referred to as the internode (Node of Ranvier). The MAIs consist of several major proteins including Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (as reviewed by E. A. Huebner and S. M. Strittmatter, 2009). Among these MAIs, Nogo-A has been shown to inhibit axon regeneration after lesioning of the CNS in adult rats (as reviewed by E. A. Huebner and S. M. Strittmatter, 2009). Furthermore, antibodies against Nogo-A, IN-1, have been shown to improve the contralateral forepaw function in rats that received an ischemia-induced cortical lesion at the right hemisphere of the cortex an experimental ischemic stroke model (C. Wiessner et al., 2003; A. B. Seymour et al., 2005). Interestingly, most of the MAIs are not found in the PNS, except for MAG (PNS-specific MAG). However, both CNS and PNS myelin have been reported to be inhibitory to neurite growth of both PNS and CNS axons *in vitro* (S. Hirsch and M. Bahr, 1999)). Hence the presence of MAIs alone may not be sufficient to explain the difference in regenerative capacity between injury CNS and PNS *in vivo*. However, several studies have proposed that the

different rate of clearance of myelin debris following injury to the PNS and CNS may contribute towards this difference. The clearance of myelin debris is primarily achieved by inflammatory microglia and macrophages (as reviewed by E. A. Huebner and S. M. Strittmatter, 2009;S. Hirsch and M. Bahr, 1999), which will be further discussed in the next section.

1.5 Inflammatory Cells Response After Injury in Both CNS and PNS

One of the important cellular and molecular components of the injury site in both CNS and PNS is the inflammatory response, which is primarily mediated by microglia and macrophages that rapidly migrate to the site of injury. Both of these cells also participate actively in the phagocytosis of cellular and myelin debris, as well as secreting cytokines and other inflammatory factors in response to injury. It should be noted that during injury both the resident inflammatory cells (i.e. microglia in CNS and macrophages in PNS) as well as those perivascular macrophages that migrate into the injured brain contribute to the inflammatory response. However this review will focus primarily upon the actions of the resident inflammatory cells, as these are the best characterized in post-injury situations.

1.5.1 Microglia Response After CNS Injury

Microglia are the resident immune cells present in the CNS. Similar to astrocytes, in response to injury microglia rapidly undergo a change in morphology, from ramified to ameboid structure, which is commonly known as the "activated" form of microglia (J. A. Amat et al., 1996;F. Gonzalex-Scarano and G. Baltuch, 1999).

In their ramified morphology under basal physiological condition, microglia rapidly extend and retract processes, actively "scanning" the external environment for abnormalities (D. Davalos et al., 2005). In response to injury, microglia undergo a rapid series of changes, becoming "reactive". These changes involve an increase in dynamic movement and hypertrophy of their processes. Reactive microglia extend their processes towards areas of trauma, forming a "barrier" around the injury induced by either laser with two–photon microscopy or needle-stick stab injury in the brains of adult rats (J. A. Amat et al., 1996; D. Davalos et al., 2005). Furthermore, this change in phenotype also occurs over a spatial and temporal gradient, with microglia nearest to the injury site first to develop an ameboid morphology, follw by a shift in morphology in neighbouring microglia radiating outwards away from the injury site over time (J. A. Amat et al., 1996). Subsequent studies have demonstrated that ATP (adenosine triphosphate) released from injured neurons is one of the triggers for these rapid morphology change in microglia (D. Davalos et al., 2005). The "barrier" formed by reactive microglia is believed to act as a cellular and molecular boundary that limits the spread of cell death from the lesion site. It has been shown that reactive microglia inhibit neurite outgrowth as well as causing neurite retraction *in vitro* (G. Münch et al., 2003). Therefore, it is generally considered that the barrier formed by reactive microglia also contributes to the formation of the inhibitory glial scar following CNS injury.

In response to injury, microglia rapidly respond by re-orientating their processes towards and migrating to the injury site. This has been demonstrated in rat hippocampal slice cultures *in vitro* (maintained for 1-7 days), where a slice injury was performed and

resulted in an immediate microglial response, including increased motility (chemotatic activity) towards the injury site (M. A. Petersen and M. E. Dailey, 2004). Studies have identified that the chemotactic behavior of microglia can be stimulated by ATP, an intracellular factor that is rapidly and transiently elevated in the extracellular space following CNS injury. Using transgenic mice bearing a GFP-transgene under a microglia-specific promotor, local release of ATP induced by a microelectrode inserted into the cortex led to a rapid microglial respond similar to that observed after the application of laser ablation (D. Davalos et al., 2005). This chemotactic effect of ATP upon microglia has also been observed *in vitro*, with the application of both ATP and adenosine diphosphate (ADP) inducing chemotaxis in microglia culture (S. Honda et al., 2001). Application of inhibitors (such as pertussin toxin) into microglia cultures have further identified that the chemotactic response observed in microglia is regulated in a P2Y-receptor dependent manner (S. Honda et al., 2001).

Another important function of reactive microglia within the injured brain is phagocytosis. This has been demonstrated in live imaging experiments undertaken on corpus callosum brain slices, revealing that activated microglia rapidly migrate towards the injured surface (within minutes) and phagocytose dead or damaged cells and debris (J. Brockhaus et al., 1996). Furthermore, studies using hippocampal slice culture maintained for 7 days have demonstrated that the phagocytic behaviour of microglia is a regulated process, with microglia selectively phagocytosing cells that undergo apoptosis) (M. A. Petersen and M. E. Dailey, 2004).

1.5.2 Macrophage Response After PNS Injury

Similar to microglia in the CNS, macrophages undergo similar responses to injury in the PNS, including changes in morphology, chemotactic responses towards sites of injury, as well as production of cytokines and other inflammatory factors. There are two major pools of macrophages in the PNS; a small pool of resident macrophages within peripheral nerves, and a larger population of macrophages present in the blood. (M. Muller et al., 2010). Macrophages (both blood-borne or resident) have an important role after PNS injury, when they rapidly respond by upregulating the expression of pro-inflammatory cytokines including IL-1 (interleukin-1), IL-6 (interleukin-6) and TNFα (Y. Murata et al., 2004). Furthermore, reactive macrophages also upregulate the expression of neurotrophic factors such as BDNF (brain-derived neurotrophic factor) and NGF (nerve growth factor) in response to injury (A. Salegio et al., 2011). Interestingly, reactive macrophages can modulate inflammation by producing anti-inflammatory cytokines as well as inducing T-cell apoptosis (reviewed in Y. Murata et al., 2004). Together, these studies demonstrate that macrophages have an important role in modulating the inflammatory response after injury. Macrophages also participate in phagocytosis of myelin and cell debris after injury, especially in response to Wallerian degeneration (W. Brück et al., 1994).

1.5.3 Are Microglia / Macrophages Beneficial or Harmful to CNS / PNS Injury?

Overall, studies have demonstrated that microglia and macrophages have both harmful and protective functions in response to peripheral and central injury. Recent studies have revealed that there are two major functional subtypes of macrophage activation in response to injury. These are referred to as M1, which are the neurotoxic and

proinflammatory macrophages, and M2, which are non-neurotoxic and anti-inflammatory macrophages (K. A. Kigerl et al., 2009). Both microglia and macrophages have been demonstrated to be able to interchange between these two phenotypes in response to different cytokines. Hence, IFN-γ (Interferron-γ) and IL-12 (Interleukin-12) lead to the formation of the M1 phenotype, while, IL-4 (Interleukin-4) and IL-0 (Interleukin-10) are associated with the formation of the M2 phenotype (R. D. Stout et al., 2005). *In vitro*, M2 macrophages have been shown to not only support neuronal growth but also facilitate neuritic outgrowth to overcome the presence of growth inhibitory substrates (K. A. Kigerl et al., 2009). Because of these different functions, it has been proposed that the M1:M2 macrophage ratio is an important contributor to the rate of nerve regeneration after injury (K. A. Kigerl et al., 2009).

1.6 Schwann Cells Make a Major Contribution to the Permissive Environment that Support Axonal Sprouting After Injury in PNS

It is striking that the PNS is substantially more capable of undergoing a successful neural regenerative neural response than the injured CNS. This appears to be primarily mediated by the relative permissiveness of the extracellular environment of the injured PNS to nerve regeneration versus the strongly inhibitory environment of the injured CNS.

One of the seminal examples of the permissiveness of the PNS to neural regeneration were studies using peripheral nerve grafts transplanted into the injured spinal cord. Studies of this type have demonstrated that the different regenerative capacity between CNS and PNS is largely due to the difference in structure and composition of the extracellular matrix (D. Muir, 2010). David, S. and Aguayo, A.J. (1981), were the first to

demonstrate that considerable amounts of regenerative axonal sprouting from the injured spinal cord could be induced by the transplantation of a segment of the sciatic nerve into the completely transected spinal cord to act as a structural "bridge" between the cut ends of the spinal cord. Importantly, regenerating spinal axons growing through peripheral nerve grafts have been reported to form functional connections with target neurons (P. Gauthier et al., 2002). Therefore, these studies demonstrate that if the appropriate extracellular environment was provided, central neurons are able to successfully regenerate after injury (Review by S. Hirsch and M. Bahr, 1999).

One of the major contributing factors to the permissive environment of the PNS to nerve regeneration is thought to be provided by Schwann cells. Schwann cells are the major glial cell within the PNS, and have important roles in both development as well as stressful situations. In some regards, Schwann cells perform the collective functions that astrocytes and oligodendrocytes perform in the CNS. Schwann cells are involved in myelinating peripheral nerves, as well as regulating the extracellular environment within the PNS through the production of neurotrophic factors and chemokines (Kalichman, 1998). Schwann cells can generally be divided into two distinct classes based upon these functions, myelinating and non-myelinating Schwann cells present depending upon the extrinsic signal received from surrounding axons. Non-myelinating Schwann cells associate with small caliber axons, while myelinating Scwann cells ensheath large caliber axons (R. Mirsky and K. R. Jessen, 1999). Schwann cells are also involved in producing the basal lamina, a layer of extracellular matrix that forms the endoneurial tubes that ensheath both Schwann cells and the surrounding peripheral nerve (J. W. Fawcett and R.

J. Keynes, 1990). The basal lamina is mainly composed of laminin, collagen type IV and heparan sulfate proteoglycan. The basal lamina is considered a permissive substrate to axonal growth and guidance (as reviewed in S. Hirsch and M. Bahr, 1999; R. Mirsky and K. R. Jessen, 1999). The importance of basal lamina in post-injury regeneration has been demonstrated following sciatic nerve crush injury, which does not result in loss of the basal lamina. Axonal regeneration is faster following crush injury as opposed to complete sciatic nerve transection. This has been attributed to the intact basal lamina in the crush injury, which acts as a permissive structural and molecular support for regenerating axons. This is in contrast to complete transection of the sciatic nerve where the basal lamina was also damaged, which resulted in regenerative axonal sprouting that appeared to occur in an unguided manner (as reviewed by A. J. Sumner, 1990; D. Tonge et al., 1997).

After injury, Schwann cells have a major modulatory role in Wallerian degeneration, by initially phagocytosing myelin debris which then led to the secretion of chemoattractant molecules by Schwann cells to stimulate macrophages recruitment (U. M. Liu et al., 1995). The recruited macrophages then continued to phagocytose the degenerating axons as well as the surrounding myelin debris (O. A. R. Sulaiman and T. Gordon, 2009). In response to injury, Schwann cells can undergo de-differentiation, reverting to a non-myelinating phenotype, which facilitates their activity in guiding regenerating axons in the endoneurial tubes. The Schwann cells that line the inner side of the basal lamina also provide structural support to guide regeneration of the axons as well as providing trophic

support through production of various neurotrophic factos such as laminin (S. Hirsch and M. Bahr, 1999; O. A. R. Sulaiman and T. Gordon, 2000).

1.7 Permissive VS Nonpermissive Environment for Axonal Sprouting After Injury

The balance between the permissive and nonpermissive extracellular environment has a major role in contributing to the overall regeneration rate in axons after injury in both PNS and CNS. For further comparison of the factors that affect the permissivity of the environment to regeneration between the CNS and PNS refer to Table 1.1 attached below. Treatment strategies for CNS injury have been focused in modulating the permissiveness of the environment, such as the usage of chondroitinase as well as IN-1, which target specific inhibitory components present in the injury site. This thesis focus mainly upon the neuroprotective protein, metallothionein, which has been shown previously to have a neuroprotective effect directly upon central neurons after injury, as well as to act on glial cells to modulate their injury response.

	Central Nervous System (CNS)	Peripheral Nervous System (PNS)
Neurons Intrinsic Growth Rate	Slow upregulation of regenerative-associated gene	Fast upregulation of regenerative-associated gene
	(Bisby and Tetzlaff, 1992)	(Huebner and Strittmatter, 2009)
Glial Response	Astrocytes Production of CSPGs Associate with glial scar formation	Schwann Cells Secrete growth promoting factors e.g. laminin Produce basal lamina
	(Hirsch and Bahr, 1999)	(Hirsch and Bahr, 1999)
Myelin Composition	Oligodendrocytes Contain MAIs (include MAG, NogoA, Omgp)	Schwann Cells Contain PNS specific MAG
	(Silver and Miller, 2004)	(Sulaiman and Gordon, 2009)
Clearance of myelin and cellular debris	Microglia Slower and incomplete clearance	Macrophages Fast clearance
	(George and Griffin, 1994)	(George and Griffin, 1994)

Table 1. Summary of the Factors that Contribute to the Different Regenerative Rates Following Injury in Both CNS and PNS.

This table summarizes the factors that contribute to the different regenerative outcomes observed following injury in CNS and PNS. These differences mostly contribute to the different permissivity of the extracellular environment to nerve regeneration.

1.8 Role of Metallothionein in Neuron Regeneration

Metallothioneins (MTs) are a family of low molecular weight (6-7kDa) metal binding proteins, which consist of two domains (α- and β). The protein structure is characterized by a highly conserved cysteine content (20 cysteine residues across all mammalian isoforms) (reviewed by J. Hidalgo et al., 2001; P. Coyle et al., 2002). There are 4 isoforms of MT in mammalian species, which have different tissue distribution in the body. Briefly, MT-I and -II have high sequence homology and appear to be ubiquitously

expressed throughout the body (e.g. brain, liver, kidney). In contrast, MT-III is primarily expressed in the brain, while MT-IV is mostly expressed in squamous epithelial tissue (for further review refer to (A. T. Miles et al., 2000; J. Hidalgo et al., 2001; P. Coyle et al., 2002). This section will primarily discuss the action of MT-I and-II in promoting repair of injured neurons, which will be the main focus of this study.

Metallothionein I/II (hereafter referred to as MT) has been shown in a number of studies to confer protection to injured neurons both *in vitro* and *in vivo*. It has previously been reported that application of exogenous MT-IIA (the major MT form expressed in the mammalian brain) into the lesion site following focal needle stick injury to the adult rat neocortex led to preservation of tissue damage (characterised by a smaller lesion volume) and an increase in regenerative axonal sprouting compared to vehicle-treated animals (R. S. Chung et al., 2003). Furthermore, the exogenous application of MT also improved the initial neurite outgrowth response in dissociated cortical neuronal culture *in vitro* (R.S. Chung et al., 2003). It has been identified that MT also has a major role in the cellular interaction between astrocytes and neurons after injury, where Chung et al. (2004) have demonstrated that the MT expression was upregulated and secreted by astrocytes in response to neuronal injury both *in vitro* and *in vivo* (R.S. Chung et al., 2004). MT also appeared to have a role in regulating the response of astrocytes after neuronal injury. In which, the addition of MT in culture media of cultured astrocytes has led to an alter activated morphology as well as decrease in CSPGs expression, hence providing a more permissive environment which support the growth of neurites *in vitro* (Y.K. Leung et al., 2009).

The importance of the endogenous MT could be further demonstrated in studies where a cryolesion cortical injury was applied in MT-deficien mice. In this study, cryolesion led to substantially greater tissue damage in MT-deficient mice in comparison to wild type mice (M. Penkowa et al., 1999). Furthermore, a significant delay in astrogliosis and microgliosis was also observed in the cryolesion-injured MT-deficient mice when compared to the wild type mice (M. Penkowa et al., 1999). To further demonstrate the ability of MT to protect neurons against trauma, genetic overexpression of MT-II in transgenic mice leads to substantially improved recovery in motor function after focal cerebral ischemic damage in comparison to wild type mice (M. van Lookeren Campagne et al., 1999). In summary, these studies demonstrate that MT is capable of improving neuronal survival and recovery following various types of experimental traumatic brain injury.

Recently, evidence has emerged identifying a specific molecular pathway through which MT can promote survival and regeneration of injured neurons. In several studies, it has been shown that the megalin receptor (low-density lipoprotein receptor-related protein -1, LRP1) is directly involved in the molecular signaling pathway underlying the neuro-protective effect of MT (M. Fitzgerald et al., 2007; R. S. Chung et al., 2008). Chung et al (2008) reported that the megalin receptor is responsible for uptake of MT into cultured cortical neurons, and that uptake is required for neuritogenic activity. Furthermore, siRNA-mediated knockdown of megalin expression blocked the ability of MT to promote initial neurite outgrowth *in vitro* (R. S. Chung et al., 2008). More recently, an artificial peptide comprised of a small section of the native β-domain of MT-II, named Emtin B,

has been shown to promote neurite outgrowth in primary cerebellar granule neuron cultures in a similar manner to native MT via interaction with the megalin receptor (M. Ambjorn et al., 2008). Overall studies using different neuronal models suggested that megalin is an important component in the signaling pathway through which MT or Emtin B are able to promote neurite outgrowth of CNS neurons.

1.9 Aims of This Project

This project will characterise some of the mechanisms that regulate the ability of exogenous MT to promote axon regeneration after injury. Prior studies have shown that MT directly promotes regenerative axonal sprouting after brain injury. However the precise mechanisms underlying this effect remain unknown. Aim 1 of this project is to investigate whether exogenous MT interacts directly with neurons in the injured brain to promote regenerative sprouting, and whether this involves interaction with low-density lipoprotein receptors such as megalin. As part of the findings, it was surprisingly found that MT might also indirectly promote regenerative axonal sprouting in the injured brain via modulation of the microglial response to traumatic brain injury. Therefore, Aim 2 of this study was to investigate the effect of exogenous MT upon reactive microglia, with particular focus upon whether exogenous MT alters the permissivity of reactive microglia to facilitate nerve regeneration after injury.

Interestingly, a recent study has identified the presence of megalin, the putative MT receptor, in dorsal root ganglion neurons. Therefore, the second part of this study was to investigate whether MT also exerts neuroprotective properties upon injured peripheral neurons. To investigate this, cultures using DRG neurons will be used, where exogenous MT will be applied to injured neurons to investigate what effect exogenous MT might have on DRG neurons after injury.

Aim 1. To identify the molecular mechanism through which exogenous MT promotes regenerative sprouting in cortical neurons after injury.

Aim 2. To investigate how MT might influence or potentially modulate the microglial response after CNS injury.

Aim 3. To investigate whether MT promote peripheral nerve regeneration, and whether this involves signaling through low-density lipoprotein receptors

Chapter 2 Defining the Molecular Mechanisms Underlying the Ability of MT to Promote Regeneration of Injured Cortical Neurons

2.1 Introduction

The precise mechanism/s through which exogenous MT promotes improvement in regenerative axonal sprouting when injected directly into the injured CNS remains unclear. Some insight has been gained from studies utilising tissue culture models, which have clearly demonstrated that exogenous administration of MT acts directly upon neurons to modulate neurite growth. Studies have shown treatment with MT promotes neurite sprouting and elongation when applied to either cortical neurons or retinal ganglion cells immediately after initial plating in culture (R. S. Chung et al, 2003; M. Fitzgerald et al, 2007). Importantly, tethering of MT to an artificial substrate prevents MT-mediated improvement in neurite outgrowth, indicating that uptake of MT is required for this action (R. S. Chung et al, 2008). Neuronal uptake of MT appears have also shown to be mediated through interaction with low-density lipoprotein receptors (LRPs), and in particular LRP2 (also known as megalin) (R. S. Chung et al, 2003; M. Fitzgerald et al, 2007). Hence, siRNA knockdown of megalin expression completely blocks the uptake of exogenous MT into cultured cortical neurons, and subsequently blocks the ability of MT to promote neurite outgrowth (R. S. Chung et al., 2008). However, neurons are known to express a range of LRP receptors, which are known to response to other neuritogenic protein, such as Apo-E (apolipoprotein E) which promotes neurite extension (Z. Qiu et al., 2004). Therefore, whether MT acts via one or more LRP receptors to promote axonal regeneration in the injured CNS *in vivo* remains to be elucidated.

To date, the evidences that MT can promote neural regeneration of cortical neurons is based upon two primary observations; i) injection of MT into the injured cortex promotes substantial regenerative neurite sprouting and ii) in cell culture, exogenous addition of MT into the culture medium promotes regenerative neurite sprouting of axotomised cortical neurons. The goal of this chapter is to investigate whether MT acts directly upon injured cortical neurons to promote their regeneration following injury to the neocortex *in vivo*. This was undertaken by injecting fluorescently-labeled MT into the injured cortex, which allow the visualisation of MT distribution and uptake after injury. Furthermore, the same injury model was also used to study the involvement of megalin in MT-mediated axonal regeneration.

2.2 Materials and Methods

2.2.1 MT Protein Labeling

MT was fluorescently tagged using the AlexFluor® 488 Protein Labeling Kit (Invitrogen). Overall the procedures followed the accompanying instruction manual. In short, 1M of sodium bicarbonate was prepared by adding 1mL of deionised water to the vial provided with the kit (containing sodium bicarbonate), and 50μL of this was then added to a 500μL volume of 2mg/mL MT (Bestenbalt; resuspended in 0.7% saline to final concentraion at 2mg/mL, filter sterilized prior used). The protein mixture was then added to the provided tube containing the reactive dye (warmed up to room temperature prior used) and fully mixed by inverting the vial several times. The vial was then mixed further using the magnetic stirrer bar provided in the kit at room temperature for 1 hour. The separation column was then assembled using a clamp to hold it in upright position. The

purification resin was then mixed thoroughly to ensure homogeneous suspension using the pipette provided in the kit, and the resin was then transferred into the column, ensuring no bubbles were formed as the formation of bubbles may interrupt the flow. The reaction mixture containing the tagged protein was then loaded into the column and the reaction vial rinsed with 100μL of the elution buffer and loaded onto the column. More elution buffer was then continually added into the column until the labeled protein (hereafter referred as 488-MT) had been eluted. During the elution process, all of the eluted fractions were collected including the unincorporated dye (second band that eluted later than the tagged protein, hereafter referred as AlexaFluor-488), which was subsequently used as the vehicle treatment for later experiments.

2.2.2 Focal-cortical Brain Injury in Adult Rat

All animal experimentation was performed under the guidelines stipulated by the University of Tasmania Animal Ethics committee, which is in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Adult Sprague Dawley rats (weight range from 180 - 220g; N=3 per treatments) were first anaesthetised in a gas chamber filled with isoflurane (Attane[TM], Bomac). An anti-inflammatory drug, Meloxicam (150μL Metacam[TM], Boehringer Ingelheim), was then administered intraperitoneally. Following the injection, the scalp of the animal was shaved, and the head immobilised in the stereotactic frame using two ear bars. Continuous anaesthesia was maintained with 2.5% isoflurane at 0.7 lpm using a Kopf gas mask. The shaved scalp was cleaned using alcohol wipes (Webcol[TM], Kendall) and a surgical incision through the skin overlying the skull was then made using a surgical

blade (No.10, Swann-Morton) down the midline. This exposed the skull, and a dental drill (Foredom) was then used to carefully drill through the skull on top of the target region (5mm anterior, 4.5mm lateral from lambda). A Hamilton syringe was used to stereotactically deliver the different treatments (488-MT, 2mg/mL or AlexaFluor-488; Saline, MT 1mg/mL, MT 1mg/mL+RAP (Receptor Associated Protein from Progen;1mg/mL); volume of injection for all treatments was 1μL). The injury was created by lowering the needle 1.5mm from the surface of the cortex, and the needle was left within the injury site for 10 minutes. After the first 5 minutes, 0.5μL of the treatment solution was injected into the injury site. After a further 5 minutes, the needle was raised by 0.5mm and the remaining 0.5μL of the treatment solution was then injected into the cortex. The injury site was closed with Gelfoam (pre-soaked with the appropriate treatment solution). The skin was then pulled back together and sutured (N502 Nylene, Dynek Pty Ltd). Rats were then allowed to recover in their cages. Both the weight and the movement of the rats were monitored for the next 4 days until experimental endpoint. No rats were observed to be in any distress during this period.

(For details of the experimental setup, please refer to Figure 2.1)

2.2.3 Perfusion and Processing on the Cortical Brain Samples

Four days after the injury, the rats were anaesthetised using a gas chamber filled with isoflurane gas, followed by an intraperitoneal injection of Ilium Pentobarbitone (4mg/g of animal weight) to induce deep anaesthesia. Cardiac perfusions were then performed, with 200 mL ice cold PBS (phosphate buffer salin; Medicago, Sweden) followed by 200 mL 4% paraformaldehyde. The brain was then removed from the rats and post-fixed in 4%

paraformaldehyde, 4% sucrose solution for 24 hours, followed by immersion in 10% sucrose for 24 hours and a subsequent 24 hours incubation in 30% sucrose solution. The brains were then embedded in OCT (optimal cutting temperature; Thermo Scientific) and cryosectioning undertaken using Leica CM1850 Cryostat, 16μm sections were then collected onto APTS (3-aminopropyltriethoxysilane)-treated microscopy slides. (For details of the experimental setup, please refer to Figure 2.1)

2.2.4 Immunohistochemistry on Frozen Cortical Brain Sections

The frozen sections were first incubated with PBS for 5 minutes. The PBS was then removed and replaced with primary antibodies (Mouse anti SMI312 from Covance use at 1:1000; Rabbit anti Iba-1 from Wako, use at 1:500; Rabbit anti Ferritin from Abcam, use at 1:1000; Mouse anti MT from Dako use at 1:1000; Rabbit anti LRP1 from Sigma-Aldrich, used at 1:1000; Rabbit anti Megalin from Santa Cruz Biotechnology, used at 1:1000) diluted in immunohistochemistry diluent (PBS + 0.03% Triton-X) and incubated at 4°C overnight. Unbound primary antibodies were then removed with three PBS washes (5 minutes incubation per wash at room temperature). The secondary antibodies (AlexaFluor 594 anti mouse from Invitrogen used at 1:1000; AlexaFluor 594 anti Rabbit from Invitrogen used at 1:1000; AlexaFluor 488 anti mouse from Invitrogen used at 1:1000) were diluted in PBS and applied for 1 hour at room temperature. Unbound secondary antibodies were then removed with three PBS washes (5 minutes incubation per wash at room temperature) and the sections then incubated with Nuclear Yellow (1μg/mL) for 5 minutes at room temperature follow by two PBS washes (5 minutes per wash). The sections were permanently mounted onto glass slides using fluorescent

mounting agent (Dako). Digital images from the sections were then visualised and captured using standard fluorescence microscope (Leica DM LB2) with mounted camera (Olympus Magnifire cooled-CCD camera) and a two-photon confocal microscope (Zeiss LSM 510).

Furthermore, to ensure that the antibody used in these study does not led to any auto-fluorescence, several control were performed. The no secondary antibodies control were performed by incubating the primary antibodies (GFAP, Iba-1 and SMI312) but used PBS instead of secondary antibodies. Using PBS as the primary antibodies and the secondary antibodies (Alexa-Fluor 488 anti mouse and Alexa-Fluor 594 anti rabbit) as the no primary antibodies control (Figure 2.2). All controls undergo same staining procedures as stated previously.

2.2.5 Measurement of Lesion Volume in Brain Sections.

The frozen sections collected from both the AlexaFluor 488 and 488-MT-treated animals (9 sections per treatments) were immunolabeled with SMI312 antibody using protocol stated in Section 2.2.4. The area of the lesion site was then traced and measured using Photoshop CS3. (Abdobe) software. The results were analysed statistically with t-test, where $p < 0.05$ considered as significant.

2.2.6 Quantification of Regenerative Sprouting in Brain sections

The tissue sections were immunolabeled with SMI312 and ferritin antibodies as described in previous sections. Two or three digital images were captured along the

injury site per section (3 sections in total for each treatment, i.e. vehicle, MT, MT+RAP) using a Leica DM LB2 microscope and Olympus magnifire cooled-CCD camera. The lesion site was identified by ferritin labeling, and all SMI-312 immunolabeled processes overlapping with ferritin positive cells were automatically traced using HCA-Vision (CSIRO) neurite tracing software. The sum of all traced neurites was calculated, and for each experimental group is described as a percentage ratio relative to vehicle-treated animals.

2.3 Results

2.3.1 Treatment with 488-MT Leads to a Decrease in Overall Injury Size After Focal Cortical Brain Injury

Injections of 1μl of either 488-MT or AlexaFluor-488 (vehicle treatment) were made at the time of cortical needlestick injury in adult rat brains. Four days after injury, 16μm cryostat sections were prepared and immunohistochemistry staining was undertaken with the SMI-312 antibody (labels phosphorylated neurofilaments). This timepoint was selected for these studies, as it corresponds with the time at which regenerative sprouting can be observed following this type of injury (C. E. King et al., 2001; R. S. Chung et al., 2003). It was found that 488-MT injection resulted in a comparatively smaller injury size compared to the vehicle treatment (marked by white line in Figure 2.3). Quantitative measurements of the lesion site confirmed that MT treatment caused an approximate ten-fold decrease in lesion area in comparison to the vehicle treatment ($2.2 \times 10^4 \mu m^2$ vs $2.1 \times 10^5 \mu m^2$; $p<0.01$). Notably, in the injury site of AlexaFluor-488 treated animals, the appearance of axons within and bordering the injury site was considerably granular and

fragmented, indicative of degenerative processes, in accordance with previously published (C. E. King et al., 2001). However, within the injury site of 488-MT treated animals, numerous regenerative processes (distinguishable by intense SMI-312 labeling and morphology) were observed sprouting into the injury site (Figure 2.3). These results indicate that 488-MT is capable of promoting a neuroregenerative response in the injured cortex comparable to the unlabeled MT protein as has been reported previously (R. S. Chung et al., 2003).

2.3.2 Neuronal Localization of 488-MT Within the Injured Brain

Using high-resolution confocal microscopy, there was a distinct increase in regenerative axonal sprouting caused by 488-MT injection (Figure 2.5) in comparison to the vehicle treatment (Figure 2.4). Furthermore, in AlexaFluor-488 dye injected animals, there appeared to be no neuronal uptake of the fluorescent AlexaFluor-488 (Figure 2.4). However, most of the AlexaFluor-488 appeared to have accumulated in non-neuronal cells present within the injury site (Figure 2.4). Z-stack confocal images were collected through the depth of the tissue section, and used to confirm that there was no co-localisation between the SMI312 staining and the AlexaFluor-488 (indicated by arrow in Figure 2.4).

Noticeably, in 488-MT injected animals, there appeared to be some co-localisation between 488-MT and SMI-312 labelled neuritic processes (Figure 2.5). The intensity of the 594 laser was decreased and increased to two extremes, and this did not alter the fluorescent 488 signal, indicating that there was no cross-over between the two

fluorescent signal responsible for this co-localisation. The presence of 488-MT within SMI-312 labelled neuritic processes was confirmed in the Z-stack confocal images (indicated by arrow in Figure 2.5).

Some sections were also immunolabeled using MT antibodies, which demonstrated that there was no co-localization between the MT labelling with the AlexaFluor 488, However, co-localisation was observed between the MT and the 488-MT. This strongly suggests that most of the green fluorescent signal is 488-MT and not AlexaFluor-488 that has become detached from the MT (Figure 2.6).

2.3.4 MT Promotes Regenerative Axonal Sprouting Via a LRP-Dependent Pathway

Prior *in vitro* studies have reported that MT promotes initial neurite ougrowth of cultured cortical neurons through interaction with megalin (also known as LRP2) (R. S. Chung et al., 2008). To investigate whether interaction with megalin or other LRP receptors is required for MT to promote regenerative sprouting *in vivo*, experiments were undertaken with administration of either saline (vehicle treatment), MT alone (1mg/mL), or MT together with RAP (injected together in one single injection). RAP is a competitive ligand for LRP receptors (including both LRP1 and megalin) that is routinely used experimentally to antagonise and block the activation of LRP receptors (A. Lazic et al., 2006). In this experiment, it was found that injection of MT alone led to a substantial increase in regenerative axonal sprouting after injury (Figure 2.8) in comparison to the saline (Figure 2.7) and MT with RAP (Figure 2.9) treatments. High magnification confocal stack images were collected along the edges of the lesion site (using confocal

microscopy and serial images collected through the z-axis), and revealed that in MT-treated animals, regenerative axonal sprouting was observed in close association with ferritin-immunoreactive inflammatory cells (microglia and/or macrophages; Figure 2.8). This was in contrast with both the vehicle and MT + RAP treatments, in which there was substantially less regenerative axonal sprouting, and limited association between regenerating axons and microglia (Figure 2.7 and 2.9 respectively). This was particularly apparent in animals that received the MT + RAP treatment, with the majority of axons present along the injury site either fragmented or disintegrated (Figure 2.9B-E). The ability of RAP to completely abrogate MT-mediated regenerative axonal sprouting suggests that an interaction with LRP receptors is important. The amount of regenerative sprouting observed along the injury site border was measured and quantitated and the results further support the notion that addition of MT improved regenerative neurite sprouting significantly compared to vehicle treatment (Refer to Figure 2.10).

The uptake of MT by microglia, and the close association between regenerative sprouts and reactive microglia in MT treated animals hint at an unexpected action of MT upon microglia to facilitate wound healing. This will be investigated further in the next chapter.

2.3.5 The Expression Pattern of LRPs in Cortical Neurons In Vivo
Immunohistochemical labelling using antibodies against LRP1 and megalin (LRP2) was performed in vehicle-treated brain sections. The majority of the megalin labeling was found to co-localise with axons (co-labeled with SMI312). However, the majority of

LRP1 labeling was not co-localised with axons but glial cells within the core of the injury site. Notably, LRP-labeled cells bear a similar morphology to microglia as described in a previous section (Refer to Figure 2.11).

2.4 Discussion

Overall this study has demonstrated that after injury, exogenously administered MT accumulates in some neurons, and leads to substantial reduction in lesion volume and improvement in regenerative axonal sprouting into the injury site. Interestingly, most 488-MT appeared to accumulate in Iba-1 labeled inflammatory cells, suggesting that MT might also have effects upon these cells. This will be explored further in the next chapter.

The key finding of this chapter is that exogenously administered 488-MT is internalised by some cortical neurons following needlestick injury, and that co-treatment with RAP is able to completely block MT-mediated regenerative axonal sprouting. In accordance with prior *in vitro* studies, this suggests that exogenous MT interacts directly with neurons through LRP receptors to promote regenerative sprouting in the injured neocortex. Hence, studies have reported the internalisation of MT by cultured cortical neurons via megalin and that this promotes initial neuritie outgrowth after plating (R. S. Chung et al., 2008). One of the limitations of this study is that RAP blocks both LRP1 and megalin receptors, meaning that it is not possible to conclusively determine which LRP receptor (or both) is involved in mediating MT's regenerative action. However, several studies suggest that megalin may be the primary target for MT. Surface Plasmon resonance studies have demonstrated that MT has a 100-fold higher binding affinity to megalin than

LRP1 (M. Ambjorn et al., 2008). Furthermore, specific siRNA knockdown of megalin expression in cultured cortical neurons almost completely blocked MT uptake and MT-mediated promotion of neurite outgrowth *in vitro* (R. S. Chung et al., 2008). Notably, in this chapter, only megalin (and not LRP1) labeling was observed in some neuritic processes along the edge of the injury tract. This suggests that megalin may be primarily responsible for mediating MT-signalling in injured neurons. However, both LRP1 and megalin were expressed in the glial cells within the central lesion cavity, suggesting that these cells may also be responsive to exogenous MT. This will be investigated in the next chapter.

The logical next step to evaluate whether MT promotes cortical neuron regeneration via a LRP- and MAPK-depedent mechanism would be to use purified cortical neuron cultures. These experiments were undertaken concurrently to this PhD thesis by Assoc. Prof. Roger S Chung (who undertook the injury experiments, and post-injury quantitative analysis of regenerative sprouting, and western blotting), with assistance by Jaqueline YK Leung (who prepared the neuronal cultures, and performed the immunostaining). As such, this data has not been included in this thesis, although it is described now as it is pertinent to the discussion of this thesis. Mature cortical neuron cultures were scratch injured and the addition of MT was found to significantly enhance regenerative neurite sprouting after inury. However, pre-treatment with RAP 30 minutes prior to scratch injury completely blocked the MT-mediated improvement in regenerative sprouting (Figure 2.12, Panel A). Quantitation of regenerative neurite sprouting determined that MT treatment resulted in an approximately 3-fold increase in reactive axonal sprouting

after 24 hours and this was also abrogated by the pre-treatment with RAP (Figure 2.12B). Using western blotting performed on the cell lysate collected from the MT treated injured neuronal cultures, it was found that MT treatment led to the elevated level of phosphorylated MAPK (ERK1/2) within one hour of stimulation. The activation of ERK1/2 has also been demonstrated to induce activation of the nuclear transcription factor, CREB, and in subsequent experiments the level of CREB was also elevated within 1 hour of MT treatment (Figure 2.12C).

While both LRP1 and megalin knockout transgenic mice exist, these were not available during this PhD study. However, needlestick injury experiments using the LRP1 and megalin knockout mice would prove insightful in determining the precise molecular pathway involved. It is important to note that there are several caveats to these future experiments. Firstly, both LRP1 and megalin knockout mice have developmental and cognitive deficits, which may confound attempts to observe the regenerative response to brain injury in these mice. Furthermore, LRP1 and megalin are not exclusively expressed by neurons, with expression also reported in microglia (M. P. Marzolo et al., 2000) and astrocytes (M. J. LaDu et al., 2000). Hence, conditional knockout of LRP1 and megalin from cortical neurons prior to brain injury in adult mice would provide an elegant future approach to precisely determining the involvement of LRP receptors in mediating MT induced regenerative sprouting.

Notably, most of the injected MT accumulated in ferritin-immunolabeled cells. This might represent a typical phagocytic response of these inflammatory cells to a foreign

molecule, or it might also indicate that MT has directly interacted with microglia and altered their functional behaviour in the injury site. These possibilities will be investigated in the next chapter.

Overall, this chapter has demonstrated the *in vivo* uptake of exogenously administered and that MT promotes regenerative neural sprouting via a LRP-mediated mechanism.

Chapter 3 Exogenous MT Modulates the Microglial / Macrophage Response After Cortical Brain Injury.

3.1 Introduction

As described in the previous chapter, only a small amount of exogenously administered 488-MT was observed within cortical neurons after cortical needlestick injury. In contrast, majority of the injected 488-MT appeared to accumulate within the non-neuronal cells within the core of the lesion site. These cells are most likely to be the resident microglia or infiltrating macrophages (noted that ferritin is expressed by both microglia and macrophages). The appearance of MT inside these cells, and their reported expression of both LRP1 and megalin (M. P. Marzolo et al., 2000), suggest that exogenous MT may act upon these inflammatory cells to alter their response to brain injury. In this regard, several studies have reported that exogenous MT can alter the response of inflammatory cells to injury. A recent study has shown that exogenous MT significantly reduced the expression of the neurotoxic inflammatory compound quinolinic acid by reactive microglia following cortical brain injury and cytokine activation of microglia *in vitro* (R. S. Chung et al., 2009). Furthermore, X. Yiu et al (2005) have reported that exogenous MT promotes the chemotactic migration of leukocytes, and this response could be specifically blocked by anti-MT antibodies (X. Yiu et al., 2005). In an animal model of multiple sclerosis called experimental autoimmune encephalomyelitis (EAE), the treatment of MT was reported to modulate the inflammatory response of immune cells, which led to a decrease in EAE symptoms observed (M. Penkowa and J.

Hidalgo, 2000). These studies demonstrated that exogenous MT is capable of modulating the inflammatory microglia/macrophages response to injury.

The aim of this chapter was to characterise the uptake of exogenous MT by microglia within the injured brain, and use *in vitro* tissue culture models to evaluate the functional effect of MT upon reactive microglia.

3.2 Materials and Methods

3.2.1 MT protein labeling

MT was fluorescently tagged using the AlexaFluor® 488 Protein Labeling Kit, as described in the previous chapter (Refer to section 2.2.1).

3.2.2 Focal-cortical Injury in Adult Rats

This part of the experiment followed the same protocol as stated in section 2.2.2.

3.2.3 Perfusion and Processing on the Cortical Samples

This part of the experiment followed the same protocol as stated in section 2.2.3.

3.2.4 Immunohistochemistry on Frozen Cortical Sections

This part of the experiment followed the same protocol as stated in section 2.2.4 with slight changes in the use of primary antibody. The sections were incubated with primary antibodies (Rabbit anti Iba-1 from Wako use at 1:500) overnight at 4°C. It should be noted that the Iba-1 primarily labeled both the reactive microglia and macrophages hence

this allowed a better visualization of the microglial and macrophages response after injury. The subsequent procedures followed what has been stated in section 2.2.4 from here onwards.

3.2.5 Preparation of Purified Primary Microglia Cultures

Mixed glial cultures containing astrocytes and microglia were prepared from Sprague Dawley postnatal day 2 rat pups and cultured as mixed glial cultures as stated previously (A. J. Vincent et al., 2005). In short, the pups were first euthanized on ice and beheaded, which the heads were then transferred to cold HBSS (Hanks balanced salt solution; Sigma, the dissecting media), where the skin and the skull were then removed exposing the cortex. Under the dissecting microscope, using forceps, the whole brain was dislodged from the olfactory bulb, the brains collected were then placed on culture media, which is DMEM (Dulbecco's modified eagle medium; Sigma) + 10% fetal calf serum (Gibco) + 1% penicillin-streptomycin-amphotericin B solution (Gibco). Using fine forceps, the two hemispheres were first separated and the cortex were then collected with the meninges removed. The dissected cortex was then collected in HBSS and trypsinised using 0.25% trypsin (Gibco) for 10 minutes on ice. Warmed culture media were then added to the tissues to inactivate the trypsin. The cultures were then triturated and passed through gauze and the tissues collected were then centrifuged for 10 minutes at 500 x G using centrifuge (Allegra^{TM} X-12R, Beckman Coulter). The supernatant was then removed and the pellet was resuspended in 1mL of culture media. The cells were then added into a 75cm^2 tissue culture flask and incubated at 37°C with 5% CO^2 for 1 day.

One day after the initial dissection, culture media were changed and the cultures were allowed to grow to confluence (around 2 weeks after initial plating). Immediately prior the shaking, culture media were first changed to serum free culture media (DMEM + 1% penicillin-streptomycin-amphotericin B solution). The cultures were then shaken at 250rmp at 37°C for 30 minutes, this initial shake will dislodged the microglia present in the cultures. The culture media containing the microglia were then collected and centrifuged at 500 x G for 10 minutes. The pellet containing the microglia cultures were then plated at 8×10^4 cells per well on 1:25 poly-L-lysine coated coverslips (pre-coated overnight with 1:25 poly-L-lysine; Sigma; for co-culture experiments), 2×10^4 cells per well on 1:25 poly-L-lysine coated coverslips (for immunostaining with antibodies against LRP1 and megalin) and 8×10^4 cells per well in 12 well plates precoated with 1:25 poly-L-lysine overnight (for conditioned media collection). The microglia cultures were maintained in serum free media and 10ng/mL of the M-CSF (Recombinant Mouse Macrophage Colony Stimulating Factor from Gibco).

3.2.7 Rat Cortical Neuron Cultures

Cortical neuron cultures were prepared as reported previously from embryonic day 17 Sprague Dawley rats (R. S. Chung et al., 2003). Briefl, the embryos were first be-headed and a portion of the cortex was removed using fine forceps. The tissues were then transfer to cold 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma). The tissue collected were then trypsinized using 0.5% trypsin incubate at 37°C for 15 minutes. The trypsin was then removed using 3 washes with HBSS followed by trituation to dissociate the tissues. The dissociated tissues were then pass through the gauze and the

cell numbers were then determined. The dissociated cells were then plated at a cell density required for the experimental setup, which will be further described in later sections.

3.2.8 Neuron and Microglia Co-Cultures

Microglia cultures were collected and plated onto glass coverslips (8 x 10^4 cells per coverslips) as stated previously and the cultured were allowed to expand for 2 days. The cultures were then treated with MT (1µg/mL) and cytokines (Tumor Necrosis Factor α, TNFα, 10ng/mL for each) for 24 hours. The culture media of the microglia culture were then replaced with the culture media (Neurobasal^{TM} medium (Gibco), supplemented with 0.1% (f/c) B-27 supplement (Gibco), 0.1mM (f/c) L-glutamine (Gibco), and 200U/ml gentamicin (Gibco)) for the cortical neurons, and the cortical neuron cultures were collected as stated previously, and plated on top of the microglia culture at a cell density of 2 x 10^4 cells per well. The co-cultures were then incubated for 24 hours and then fixed with 4% paraformaldehyde for 15 minutes at room temperature follow by three PBS washes (5 minutes incubation per wash at room temperature) and immunolabeled with antibody against SMI-312 (axonal marker) using the protocol stated in the following section.

For each treatment, the neuritis were visualised using fluorescence microcope (Leica DM LB2) and images were captured using the Olympus Magnifier cooled-CCD camera (5 images per coverslips and 4 coverslips per treatment group). The lengths of the neurites were measured using the ImageJ (National Institute of Health) software and the data were

analysed statistically using ANOVA and Bonferroni post-test, with p<0.05 considered as significant.

3.2.9 Immunohistochemistry on Cultured Cells

After fixation, the cultures were washed with PBS three times (5 minutes incubation at room temperature for each wash). The cultures were then incubated overnight at 4 °C with primary antibodies (Mouse anti SMI312 from Covance, used at 1:1000; Rabbit anti Iba-1 from Wako, used at 1:500; Rabbit anti LRP1 from Sigma-Aldrich, used at 1:1000; Rabbit anti Megalin from Santa Cruz Biotechnology, used at 1:1000) diluted in immuno diluent (PBS + 0.03% Triton-X). Following incubation, the cultures were washed three times with PBS follow by 1 hour incubation in secondary antibodies (Alexa-Fluor 594 anti rabbit from Invitrogen, used at 1:1000; Alexa-Fluor 594 anti mouse from Invitrogen, used at 1:1000; Alexa-Fluor 488 anti mouse from Invitrogen, used at 1:1000) at room temperature. The excess secondary antibodies were removed with three PBS washes and the nuclei were labeled using Nuclear Yellow (1μg/mL) for 5 minutes at room temperature. The Nuclear Yellow was then removed by 5 minutes incubation with PBS, the cultures were then mounted on glass slides using the fluorescence mounting agent (Dako) and dried in the dark. The cultures were then visualised on the fluorescence microscope (Leica DM-LB2) and images were taken using Olympus Magnifier cooled-CCD camera.

3.2.10 Collection of the Condition Microglia Media and Neuron Outgrowth Experiments

Microglia were cultured in 12 well culture dishes, coated with 1:25 poly-L-lysine and cells were plated at 1.6×10^5 cells per well. The microglia were maintained for 2 days in serum free culture media with 10ng/mL of MCSF. Half of the culture media were removed from each well prior to the addition of treatments, which are as follow: control (culture media only), TNFα (10ng/mL), TNFα with MT (1μg/mL), TNFα with MT and RAP (0.5μm) pretreatment for 30 minutes prior the addition of MT. The cultures were then incubated with the treatment agents for 24 hours, after which the media were collected and stored at -4°C until used.

Cortical neurons were collected and plated as stated in section 2.2.6. However, instead of using the neuron culture media, the microglia condition media were used with a mix at 1:1 ratio with the neuron culture media in each well prior to the addition of the neurons. The cultures were then incubated for 24 hours, which were then fixed using 4% paraformaldehyde for 15 minutes and immunolabeled with SMI312 antibody as stated in the previous section (refer to section 2.2.7). The neurites of the axons were then visualised under the fluorescence microscope (Leica DM-LB2) where digital images (5 images per coverslips and 4-6 coverslips per treatment) were captured using Olympus Magnifier cooled-CCD camera and the lengths of the axons were measured using the ImageJ (National Institute of Health) software. The data were analysed statistically using ANOVA and Bonferroni post-test, with $p < 0.05$ considered as significant.

3.3 Results

3.3.1 488-MT is Present Within Microglia/Macrophage Within the Injured Brain

As described in the previous chapter, most 488-MT that had been administered directly into the injury site appeared to accumulate in non-neuronal cells within the lesion cavity. Immunohistochemical staining using the Iba-1 antibody (marker for microglia/macrophages) revealed that most 488-MT accumulated in intensely Iba-1 labeled cells, indicative of reactive microglia/macrophages (Figure 3.2). Notably, injected AlexaFluor-488 was also internalized by reactive microglia/macrophages (Figure 3.1). However, high magnification confocal images revealed that there was a marked difference in the morphology of Iba-1 labeled cells that had internalized AlexaFluor-488 or 488-MT (Figure 3.3). Those Iba-1 labeled cells that had taken up 488-MT had a branched morphology, with numerous short branching processes emanating out from the cell body. However, AlexaFluor-488 containing Iba-1 labeled cells displayed a round morphology, with far fewer processes (Figure 3.3).

3.3.2 Exogenous MT Alters the Permissivity of Reactive Microglia or Macrophages to Neurite Outgrowth In Vitro

To test the effect of exogenous MT upon microglial permissivity to neurite outgrowth, neuron-microglia co-cultures were prepared. Cultured microglia were activated by treatment with the pro-inflammatory cytokine TNFα. After 24 hours, cortical neurons were seeded on to the microglial cultures, and neurite outgrowth assessed after 24 hours. TNFα-activated microglia were less permissive to neurite outgrowth than basal, unactivated microglia (receiving vehicle treatment) (Figure 3.4). However, when TNFα-

activated microglia were subsequently treated with exogenous MT for 24 hours prior to seeding of neurons into the culture, this resulted in complete restoration in subsequent neurite outgrowth (Figure 3.4). It should be noted that the result from the ANOVA shown a slight significant different in the neurite lengths between treatments, however, the Bonferroni post-test shown that there were no significant difference between each group. Hence more repeats would have needed to confirm this result.

Despite being plated at relatively high density (8 x 10^4 cells/well), it was noted that there was generally no physical contact between microglia and neurons in the neurite outgrowth assay described above. This suggests that rather than providing a physical barrier to neurite outgrowth, that TNF-α activated microglia might secrete soluble inhibitory factors. To confirm this hypothesis, instead of seeding the cortical neurons directly into microglial cultures, the neurons were plated onto coverslips in the presence of conditioned media collected from microglia recieving different treatment conditions. The treatments were vehicle (culture media only); TNFα; MT + TNFα; MT + TNFα with RAP pretreatment. In accordance with the previous experiments, it was found that the conditioned media collected from the TNFα treated microglia significantly impaired neurite outgrowth in comparison to media from vehicle-treated microglia. However, media from TNFα activated microglia that had been treated with MT was not inhibitory to neurite outgrowth (Figure 3.5).

Previous studies have reported that microglia express LRP receptors (M. P. Marzolo et al., 2000). This was confirmed in this study, with cultured microglia found to express

both of these LRP receptors (Refer to Figure 3.6). This suggests that MT might modulate microglial activity through a LRP-dependent manner. To investigate this, microglial cultures were pre-treated with RAP (a competitive ligand for LRP receptors). Results demonstrated that RAP pre-treatment completely blocked the ability of MT to alter the permissivity of TNF-α activated microglia to neurite outgrowth, suggesting that this action of exogenous MT may signal through an LRP-dependent mechanism in microglia (Refer to Figure 3.5).

3.4 Discussion

This Chapter has demonstrated that the majority of exogenously administered 488-MT and AlexaFluor-488 accumulates primarily within Iba-1-labeled microglia/macrophages within a cortical needlestick injury. The uptake of both 488-MT and AlexaFluor-488 might be in accordance with the well described function of microglia to phagocytose foreign material (R. B. Banati, 2002). However, microglia that had internalised 488-MT had a very different morphological appearance in comparison to those containing AlexaFluor-488, suggesting that 488-MT has altered the functional phenotype of microglia/macrophages. Using the Iba-1 antibody, it is difficult to distinguish between reactive microglia and infiltrating macrophages, as this antibody labels both of these cells. There were no antibodies available that could distinguish between these two subsets of inflammatory cells. However, using both microglia-neuron co-culture model and medium exchange experiments, it was subsequently demonstrated that the addition of TNFα led to a decrease in neurite outgrowth, and that addition of MT had abrogated this effect. Hence, this demonstrated that MT is capable of exerting a specific effect upon

isolated microglia in culture. Furthermore, this action of MT was blocked by the addition of RAP, suggesting the involvement of LRP receptors in the modulation of microglial function by MT.

The uptake of MT by microglia/macrophages after cortical injury *in vivo* and the change in morphology induced by this suggests a potential role of MT in modulating microglia/macrophages response to traumatic brain injury. Previous studies using rats with EAE have shown that intraperitoneal injection of MT during EAE led to a decrease in the inflammatory response by reducing the infiltration of macrophages and T lymphocytes (M. Penkowa and J. Hidalgo, 2000). Furthermore, MT has also been shown both *in vivo* and *in vitro* to reduce the production of quinolinic acid by reactive microglia. Quinolinic acid is a neurotoxic substance known to be produced by microglia during inflammatory response after injury (R. S. Chung et al., 2009). It should be noted that although microglia/macrophages appeared to internalise both the AlexaFluor 488 and the 488-MT, the higher magnification images revealed a clear difference in the morphological appearance of the microglia/macrophages in response to these treatments. The observation that MT treatment alters the morphology of microglia/macrophage within the lesion site would be in accordance with the considerable existing literature describing the link between microglial morphology and function in "resting" vs "trauma" situations. Accordingly, the branched microglial morphology that was observed within the injury site of 488-MT treated animals resembled that of "resting" microglia, in comparison to the ameboid morphology observed followed the injection of Alexa-Fluor dye, which correlates with an "activated microglia" phenotype (R. B. Banati, 2002). It is important to note that the AlexaFluor 488 itself was internalized by microglia is possibly

reflective of the well-characterized phagocytic behavior ("activated") of these cells (J. Brockhaus et al., 1996).

The results collected from the *in vivo* part of this study were followed up in a series of *in vitro* experiments to allow further investigation into the potential mechanism underlying how MT influences the inflammatory response (reactive microglia in particular) to brain injury. The results collected from these *in vitro* studies demonstrate that MT might potentially modulate the permissivity of activated microglia to regenerative neurite sprouting. In the co-culture model, the inflammatory signaling in microglia was simulated using the pro-inflammatory cytokine, TNFα, a well described activator of an inflammatory phenotype in microglia (U. K. Hanisch and H. Kettenmann, 2007; J. Mikita et al., 2011). Accordingly, TNFα treatment caused microglia to become significantly less permissive to neurite outgrowth in comparison to vehicle treatment. However, treatment with MT reversed this effect, causing microglia to become significantly more permissive to neurite outgrowth. Notably, in this co-culture set-up, there was minimal physical contact between neurons and microglia, suggesting that the soluble factors presence in the culture might be the factors influencing the outgrowth of neurites. This was supported by the conditioned media experiments, where the conditioned media from TNFα-treated microglia impaired neurite outgrowth in comparison to both control and TNFα-MT-treatment. Interestingly, pre-treatment with RAP (a competitive ligand of LRP receptors) completely abrogated the effect of MT. This result suggests that MT might potentially modulate the secretion of soluble inhibitory factors by reactive microglia. The usage of

RAP also had further demonstrated that it is possibly that MT acts through a LRP-receptor dependent manner to exert its effect on microglia.

Another interesting phenomenon observed from the *in vivo* studies was the alteration in microglia/macrophages morphology caused by MT treatment in comparison to the vehicle treatment. A number of studies have focused upon the relationship between microglial morphology and its function in the injured CNS. Hanish and Kettenmann (2007) have proposed that the "resting" microglia should actually be referred to as "alternatively activated" microglia instead, as these microglia have been shown to exert neuroprotective properties in contrast to "activated" microglia which have an inflammatory and neurotoxic action in response to CNS injury (U. K. Hanisch and H. Kettenmann, 2007). For example, microglia have been shown to release inflammatory mediators in response to phagocytosing bacteria but not myelin debris, which stimulated secretion of anti-inflammatory factors instead. This demonstrated that microglia are able to interchange between the two different functional phenotypes, based mainly upon the type of stimulus present in the extracellular environment (as reviewed by U. K. Hanisch and H. Kettenmann, 2007). Further studies have recently characterized the two functional phenotypes of microglia in response to injury in the CNS. The "activated" microglia have also been referred to as M1 cells, with a functional phenotype associated with secreting pro-inflammatory factors as well as being inhibitory to axonal growth. The "alternatively activated" microglia are referred to as M2 cells, which have been shown to promote axonal growth as well as being neurotogenic compare to M1 (K. A. Kigerl et al., 2009). The finding that MT treatment induces a striking morphological change in microglia

suggests that MT may alter the functional phenotype of the microglia, most likely from M1 to M2. Unfortunately attempts to specifically immunolabel M2 microglia with the RELMα antibody were not successful in this study, with no RELMα labeling observed in the injury site in any experimental condition (results not shown). RELMα has been reported to be a marker of the M2 phenotype (G. Raes et al., 2002).

Notably, other studies from this laboratory have shown that MTKO mice have a lower M2:M1 ratio compared to that of wild type mice both before and after brain injury (M. W. Pankhurst, 2011). Furthermore, a previous study has also reported that conditioned media collected from M1 microglia contains soluble inhibitory factors which impair neurite extension of cultured neurons (K. A. Kigerl et al., 2009). This potentially explains the results collected from the condition media experiments in this Chapter, where the addition of TNFα led to a decrease in neurite outgrowth compared to vehicle treatment. While microglia are known to secrete a range of soluble factors that might impede neurite outgrowth, study has also implicated nitric oxide and its derivatives as a key inhibitory mediator (G. Münch et al., 2003).

Overall, the *in vitro* part of this study has shown that exogenous MT can altered the function of activated microglia and make them more permissive to neurite outgrowth, which is in accordance with a number of studies showing that the neuroinflammatory response after injury can be modulated by MT as well as the finding from the *in vivo* part of this study. This modulation effect by MT could possibly act through altering the ratio of M1:M2 present after the brain injury. However, further studies are still warranted in

order to identify the histological changes in the microglia after MT treatment both *in vivo* and *in vitro* using specific M2 marker such as RELMα. Furthermore, the expression of M2 specific RNA (ribonucleic acid) specific transcripts such as Ym1 could also be used to analyse the RNA samples collected from microglia culture with or without MT treatment to allow the confirmation of the role of MT in modulating the M1:M2 ratio towards a M2 bias.

Chapter 4: Metallothionein Promotes Regenerative Axonal Sprouting in Dorsal Root Ganglion Neurons After Physical Axotomy.

4.1 Introduction

As shown in previous chapters and in several recently published studies, MT directly promotes survival and regeneration of injured neurons through a specific molecular pathway (R. S. Chung et al., 2008). In several studies, it has been reported that the megalin receptor (also known as LRP2) is directly involved in the molecular signaling pathway underlying the neuro-protective effect of MT (M. Fitzgerald et al., 2007; R. S. Chung et al., 2008). Chung et al (2008) reported that the megalin receptor is responsible for the uptake of MT into cultured cortical neurons. Furthermore, siRNA-mediated knockdown of megalin expression blocked the ability of MT to promote initial neurite outgrowth of cultured cortical neurons (R. S. Chung et al., 2008). These results are similar to those published by Fitzerald et al (2007), in which they reported that following intravitreal injection of fluorescently labeled MT, there was only uptake of MT into retinal ganglion cells, which were the only cells in the eye expressing megalin. They also showed that pre-treatment of cultured retinal ganglion cells with an antibody against megalin blocked the ability of MT to stimulate neurite extension (M. Fitzgerald et al., 2007). More recently, an artificial peptide comprised of a small section of the native β-domain of MT-II, named Emtin B, has been shown to promote neurite outgrowth in primary cerebellar granule neuron cultures in a similar manner to native MT via interaction with the megalin receptor (M. Ambjorn et al., 2008). Overall studies using different neuronal models have suggested that megalin is an important component in the

signaling pathway through which MT (or the synthetic MT-analogue Emtin B) is able to promote regenerative outgrowth of CNS neurons.

Interestingly, megalin has also recently been reported to be involved in the internalisation of another neuritogenic protein, transthyretin (C. E. Fleming et al., 2009). Fleming et al (2009) reported that megalin is expressed in dorsal root ganglion (DRG) neurons, a type of peripheral neurons, *in vivo* through both immunohistochemistry staining as well as RT-PCR (Reverse transcription polymerase chain reaction) (C. E. Fleming et al., 2009). Since megalin is expressed by DRG neurons, it is possible that MT may act neuritogenically upon DRG neurons after injury in a similar manner to its action upon CNS neurons. Therefore, the first part of this study was to investigate whether MT is able to promote axonal sprouting of injured DRG neurons, and whether this involves a megalin-dependent mechanism. Notably, up until now there is very limited published literature describing the potential role of MT in the PNS. The only report described that MT has an important role in peripheral nerve regeneration after injury, which has demonstrated that a shorter regenerative distance of the peripheral nerve was found in the MT-knock out mice after sciatic nerve crush injury (D. Ceballos et al., 2003). Hence this part of the study utilized cultured DRG neurons as an experimental model of peripheral neurons to investigate whether MT promotes regeneration of PNS neurons.

4.2 Material and Methods

4.2.1 Primary Dorsal Root Ganglion Explants Cultures

All animal experimentation was performed under the guidelines stipulated by the University of Tasmania Animal Ethics Committee, which is in accordance with the Australian code of practice for the care and use of animals for scientific purposes. The adult female mice (with 13.5 embryonic day mice; wild type svj129 mice) were first euthanized using a high percentage of CO^2 for 10 minutes. The embryos were then collected in petri dishes containing the dissecting media, HBSS. Under the dissecting microscope, spinal cords were slowly dislodged and removed aseptically from decapitated embryonic day 13.5 mice, leaving the DRG intact between each vertebrate. Fine forceps were then used to severe the DRGs from the roots and they were then placed onto the coverslips, pre-coated with poly-ornithine (Sigma) overnight followed by laminin (Invitrogen) 1 hour prior used. The explants were incubated in culture media, comprised of Dulbecco's modified eagle medium-F12 (DMEM F-12, Invitrogen) with 10% Nerve growth factor 7S (NGF, Sigma), 1% N2 supplement 100X (Invitrogen) and 1% Gentamicin reagent solution (Gibco), and maintained at 37°C, 5% CO2 for 1 week.

At 1 week *in vitro*, rabbit MT was added to the culture media at 1µg/mL concentration immediately before the scratch injury, which was performed using a pulled glass capillary under the dissecting microscope. Using this approach, it was possible to ensure that the injury was only applied to axons, and not nearby cell bodies. As a vehicle treatment, culture media was added instead. The cultures were then fixed with 4% paraformaldehyde for 20 mins, 16 hours after injury.

4.2.2 Immunohistochemistry on Dorsal Root Ganglion Explant Culture

The DRG explant cultures were immunolabeled using the same protocol as described in the previous chapter (for detailed protocol, refer to section 3.2.8). Briefly, the cultures were first wash three times with PBS followed by an overnight incubation with primary antibodies (mouse anti-SMI312 from Covance, used at 1:1000; rabbit anti-megalin from Santa Cruz Biotechnology, used at 1:1000) at 4°C. The next day the cultures were then incubated with secondary antibodies (AlexaFluor 488 anti-mouse from Invitrogen, used at 1:1000; Alexa Fluor 594 anti-rabbit from Invitrogen, used at 1:1000) for 1 hour at room temperature follow by 5 minutes incubation with nuclei yellow. The cultures were then mounted on coverslips were visualized using the fluorescence microscope (Olympus BX50) and the images were captured using the CoolSNAP HQ2 camera (Photometrics).

4.2.3 Dissociated DRG Cultures for Outgrowth Assay

The DRGs were removed as stated in previous sections (Section 4.2.1), and collected into a small petri dish containing the dissecting media. The collected tissues were then dissociated using 0.125% trypsin and incubated at 37°C, 5% CO_2 for 20 mins. Cultures were then triturated, counted and plated (1×10^5 cells/well) on poly-ornithine and laminin coated coverslips in wells containing either the culture medium alone (same as described previously for the explant culture), or culture medium containing either 1μg/mL or 2μg/mL of MT. The cultures were incubated for 24 hours and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Cultures were then immunolabelled with SMI312 antibodies using the protocol described previously (for detailed protocol, refer to section 3.2.8). Digital images (5 images per coverslips and 3

coverslips per treatment) were visualized and captured using fluorescence microscope (Olympus BX50) and the camera (CoolSNAP HQ^2 camera), and the length of the axons was measured using ImageJ (National Institute of Health) software. The data were then analyzed statistically using ANOVA and Bonferroni post-test, with $p < 0.05$ considered as significant.

4.2.4 Addition of Inhibitors to DRG Explants and Dissociated Culture

To identify the cellular mechanisms in which the MT exerts its effect, inhibitors against the MAPK (PD98059, Sigma, final concentration at 100nM) and the antagonist for the LRP receptors (RAP, final concentration at 1μM) were used. For the DRG explant experiment, PD98059 or RAP were added 30 minutes prior the addition of MT (1μg/mL) the scratch injury was performed which followed as described previously (Section 4.2.1). For the dissociated cultures (outgrowth assay), immediate after the plating of the cells, both of the inhibitors were added into the culture wells for 30 minutes followed by the addition of MT (1μg/mL) the protocol then followed as described previously (Section 4.2.3).

4.2.5 Compartmentalised In Vitro DRG Neuron Injury Model

To set up the *in vitro* Campenot chamber injury model, circular Campenot chambers (Telfon inserts, Tyler Research) divided into three equal chambers were first adhered onto collagen-coated (Purecol®, Advanced Biometrix) plastic dishes using high vacuum grease (Dow Corning®). Dissociated DRG neurons (collected as stated previously) were then plated into the left compartment and the cultures were maintained at 37°C, 5% CO2

in culture media consisting of Minimum essential medium (MEM, Invitrogen) and N3 supplements for 3 weeks until DRG axons had fully extended through to the other two compartments. Scratch injury was then made specifically to axons within the middle compartment (Refer to Figure 5A&B) using a pulled glass capillary, with 1μg/mL of MT being added immediately prior to injury. Culture medium alone was applied as the vehicle treatment. Cultures were then incubated for 16 hours after injury and then fixed with 4% paraformaldehyde for 30 mins.

After fixation, the cells were first washed with 0.1% Triton followed by four PBS washes (5 minutes incubation per wash) and then blocked with 3% goat serum in PBS for 1 hour. Cultures were then incubated overnight with primary antibodies (mouse anti-metallothionein from Dako, used at 1:1000; chicken anti-neurofilament from Millipore, used at 1:1000; rabbit anti-megalin, used at 1:1000). Secondary antibodies were then applied for one hour followed by 5 minutes incubation with Nuclear yellow. Between each step of the staining process, PBS was used to wash the cultures three times (5 minutes each). The Campenot Chambers were then removed and the cultures were mounted using Vectashield Mounting Media (Vector Laboratories). These immunolabelled cultures were then visualized and images were captured using two-photon confocal microscope (LSM 510, Zeiss). The length of axons was measured using ImageJ software and the data were analyzed statistically using t-test, with $p < 0.05$ considered as significant.

4.3 Results

4.3.1 Metallothionein Promotes Axonal Sprouting and Outgrowth of Injured DRG Neurons

To determine whether MT promotes axonal sprouting in DRG neurons after injury, the axons of DRG explants were scratched injured using a fine glass capillary, and the cultures were fixed 16 hours after injury. A neurofilament marker, SMI312, was then used to immunolabel axons within the DRG explants. It was found that MT treatment led to extensive regenerative neurite sprouting observed in the injured DRG explants, compared to explants receiving vehicle treatment (Figure 4.1). This difference was very clear, as the injury tract in vehicle-treated explants (indicated by arrow in Figures 4.1A and B) was substantially larger in comparison to MT treated DRG explants (indicated by arrow in Figures 4.1C and D). In this explant model, it is difficult to accurately track and quantitate the length of individual, sprouting axons entering the injury tract. To overcome this issue, a DRG outgrowth assay was established using dissociated DRG neurons. The DRG neurons used in this assay were isolated from E13.5 to E14 embryos, with the dissociated neurons plated immediately into either culture media only (vehicle treatment) or culture media with MT ($1\mu g/mL$ or $2\mu g/mL$). After 24 hours, the cells were fixed and immunolabeled with the neurofilament marker SMI312. There was a statistically significant increase (t-test, $p < 0.05$) in the length of regenerative neurite growth when the cultures were plated in media containing $1\mu g/mL$ or $2\mu g/mL$ MT (2.38-fold and 2.63-fold increase respectively) when compared to the vehicle treatment (Figure 4.2).

Overall these results demonstrated that application of exogenous MT led to significantly enhanced neurite regeneration after injury in DRG neurons *in vitro*, and this involves a direct interaction between MT and DRG neurons.

4.3.2 Metallothionein Promotes Regenerative Sprouting of Injured DRG Neurons Via MAPK and LRP receptors-dependent Signaling Pathway

Previous studies have revealed that ligand-interactions with megalin activate a MAPK-dependent pathway of neurite outgrowth in CNS neurons (J. W. Asmussen et al., 2009). The LRP receptors (especially megalin, LRP2) have also shown to have important roles in mediating the promotive effect of MT in CNS neurons after injury (M. Ambjorn et al., 2008). Therefore, this part of the study investigated whether MT acts through a similar pathway in DRG neurons. The established DRG explants were first treated with both the MAPK signaling inhibitor (PD98059) or RAP (antagonist for LRP receptors) 30 minutes prior to the addition of MT, which occurred immediately after axonal injury was performed. Results demonstrated that both PD98059 and RAP inhibit the axonal sprouting response that was observed from the MT treatment alone (Refer to Figure 4.1 E and F).

To confirm this result in a quantitative manner, dissociated DRG neurons were pre-treated with PD98059 or RAP for 30 minutes prior to the addition of MT. It was found that PD98059 itself had no effect upon neurite outgrowth of dissociated DRG neurons (Figure 4.3). However, PD98059 completely abrogated the effect of MT in promoting neurite outgrowth of DRG neurons significantly (Figure 4.3). The addition of either RAP

alone or RAP with MT also led to decreased neurite outgrowth. This indicates that MT acts through MAPK- and LRP-dependent signaling pathways to promote neurite outgrowth, as have shown in previous study using CNS neurons (R. S. Chung et al., 2008; J. W. Asmussen et al., 2009). However, due to the fact that RAP binds to both LRP1 receptors and megalin, it is not possible to conclude that MT acts specifically through megalin.

4.3.3 Expression of Megalin in DRG Neurons In Vitro

Dorsal root ganglion neurons have recently been reported to express megalin *in vivo* (C. E. Fleming et al., 2009), therefore it was investigated whether DRG explants express megalin *in vitro*. Immunohistochemical staining using a megalin antibody in DRG explant cultures (7 DIV) revealed that the majority of megalin expression appeared to be specifically localised within the soma of DRG neurons, with minimal staining observed in the neurofilament stained axons (Figure 4.4). Nuclei staining of the DRG explants revealed the presence of non-neuronal cells (SMI312-negative) cells underlying the axons of the DRG neurons (Indicated by arrow in figure 4.4C and 4.4D), however these cells were not found to contain megalin.

4.3.4 MT Only Promotes Axonal Sprouting When Delivered to the Soma of Injured DRG Neurons

According to the previous results, megalin was predominantly expressed in the soma of DRG neurons, suggesting that MT may only exert its regenerative effect upon certain cellular compartments of DRG neurons. To investigate this, an *in vitro* model of axonal

injury was established in which DRG neurons were cultured within Campenot chambers, allowing the separation of neuronal cell bodies and axons in separate fluid-sealed compartments (Figures 4.5 A and B). The cultures were grown for 21 days to allow the axons to extend from the soma compartment into the axonal compartment, and then scratch injuries were made using a fine glass capillary within the middle compartment (axon-only compartment) and MT or vehicle treatment were then applied immediately to either the middle axonal chamber or the side soma compartment prior the injury. At 16 hours post-injury, the cultures were fixed and immunolabeled.

It was found that MT treatment led to enhance regenerative neurite sprouting after injury when added either to axons or cell bodies (Figure 4.5C). However, the action of MT-IIA was most effective when delivered to the soma compartment of DRG neurons, resulting in almost four-fold enhancement of regenerative sprouting (Figure 4.5D). The intensity of neurofilament staining was substantially elevated in newly sprouting axons, an indicative marker of regenerative sprouting (Figure 4.5C). Restricted delivery of MT to only the fluid-tight compartment with the injured axons of DRG neurons also promoted some regenerative sprouting, although to a far lesser degree in comparison to MT that was delivered to neuronal cell bodies (Figure 4.5D).

4.4 Discussion

Overall this study has demonstrated that MT can promote regenerative sprouting of DRG neurons after scratch injury. This is the first report demonstrating the ability of MT to promote regeneration of injured neurons in the PNS. Fleming et al (2009) have recently

shown that megalin is expressed in DRGs *in vivo* using both RT-PCR and immunohistochemistry. These observations have been confirmed in this study, with megalin expression noted in DRG neurons *in vitro*. Interestingly, megalin expression was localised primarily to the soma of DRG neurons, with very little immunostaining found within the axons of the DRG explants.

To investigate in greater detail the site of action of MT upon injured DRG neurons, Campenot chambers were used to create separate, fluid-tight sub-cellular compartmentalisation of DRG neurons (i.e. soma, and axons). Campenot chambers have been utilized in a similar manner to study Wallerian degeneration in myelinated axons after localised axotomy caused by a razor blade (A. D. Guertin et al., 2005). In this study, a slightly different chamber arrangement was used whereby the DRGs were plated into one of the lateral compartments instead of the mid-compartment, facilitating unidirectional axonal extension through to the other two compartments. A pulled fine-glass capillary was used to cause scratch injury to the axons. The advantage of this system is that it allowed precisely localised injury to axons and not cell bodies, and also restricted the exogenous application of MT specifically to either neuronal soma or injured axons. It was found that MT significantly enhanced regenerative sprouting when applied directly to the soma of injured DRG neurons, and to a much-lesser extent when applied directly to injured axons. Notably, this site-specific action of MT appears to correlate with the regional distribution of megalin receptors within DRG neurons, since it was found that megalin expression was localized primarily to the soma of DRG neurons and not in axons. These results correlate with recent studies in the injured optic nerve, where

it was reported that there was a significant increase in axonal sprouting after injury when MT was injected into the vitreous humor (where MT was internalized by the cell bodies of the retinal ganglion cells that project their axons into the optic nerve) following complete surgical axotomy of the optic nerve, and not when MT was applied directly at the site of injury in the optic nerve (R. S. Chung et al., 2008).

Moreover, the RAP (LRP-receptors antagonists) and PD98059 (MAPK-inhibitors), both abrogated the promotive effect of MT. Hence suggested that MT exerts its effect through MAPK-dependent signaling as well as via interaction with LRP receptors. Notably, several studies have reported that LRP receptor-binding activates a MAPK-dependent signaling pathway. One of the lmitations of using RAP is that it can bind to a number of LRP receptors including LRP1 and megalin (LRP2). Hence the specific receptor that MT acts through cannot be determined. However, as published previously in CNS neurons, MT has a 100-fold higher binding affinity to megalin in comparison to LRP1 (M. Ambjorn et al., 2008). Therefore, it is plausible that MT might primarily act through megalin receptors in promoting axonal sprouting in DRG neurons.

In conclusion, this study has demonstrated that exogenous administration of MT led to an increase in regenerative sprouting after axotomy in several different *in vitro* models of DRG neuronal injury. This regenerative action of MT correlated with the localisation of megalin expression in DRG neurons, and it was found that MT acts via both LRP- and MAPK-dependent signaling pathway.

Chapter 5: Conclusion

Summary of findings

This study has shown that exogenous MT exerts pro-regenerative effects upon both central and peripheral neurons after injury. In accordance with previously published studies, exogenous MT was found to improve neuronal sprouting after injury in a LRP-dependent manner in cortical neurons *in vivo*. Notably, in addition to acting directly upon neurons, exogenous MT also modified the microglial response to traumatic brain injury *in vivo*. The direct administration of MT into the site of cortical brain injury caused a substantial change in microglial morphology, as well as an increased physical association between reactive microglia and regeneratively sprouting axons. This effect of exogenous MT was characterised through a series of *in vitro* experiments using neuron-microglia co-cultures, which also confirmed the involvement of an LRP-mediated mechanism.

The second part of this study provides the first evaluation of the potential ability of exogenous MT to promote nerve regeneration of injured neurons from the PNS. This study found that exogenous MT promote both regenerative sprouting and outgrowth in DRG neurons after injury *in vitro*.

In summary, these studies suggested that MT can promote regenerative sprouting of both injured CNS and PNS neurons, and might represent a potential therapeutic molecule for improving nerve regeneration following injury.

Role of MT in CNS

The first part of this study investigated the effect of exogenously administered MT within the injured CNS by characterising the distribution of MT following injection into the injured neocortex. It was found that some MT was internalised by neurons surrounding the injury site, and this is in accordance with several *in vitro* studies demonstrating the uptake of exogenous MT by cultured neurons, and that this promotes regenerative neurite sprouting after injury.

Notably, the majority of the exogenous MT was observed in reactive microglia. However, due to the inherent complexities of the *in vivo* brain injury model, it is difficult to precisely determine whether this has functional effects upon the behaviour of microglia in the injured brain, or whether it represents the phagocytic uptake of exogenously administered MT. Therefore, an *in vitro* model was developed to specifically investigate the potential actions of exogenous MT have upon cultured microglia. These *in vitro* experiments established an experimental model of reactive microglia, whereby treatment with TNFα made cultured microglia inhibitory to neurite outgrowth. Notably, treatment with MT made TNFα activated microglia significantly more permissive to neurite outgrowth. Using a series of media-exchange experiments, it was found that the inhibitory action of TNFα activated microglia was through the secretion of inhibitory factors into the culture media. This coincided with previous publications where the cultured microglia, activated with either cytokines or LPS, have shown to induced neurite retraction as well as the production of CSPGs (major components of glial scar) (G. Münch et al., 2003; J. Yin et al., 2009). These results suggest that exogenous MT is

capable of modulating the microglial inflammatory response to cytokines, which might contribute towards a more permissive environment to promote and support regenerative axonal sprouting following brain injury.

Recent studies have revealed that reactive microglia may not be present as a single functional phenotype that is inhibitory to nerve regeneration. In fact, it appears that reactive microglia (and perivascular macrophages) may actually be present in the injured brain as two distinct functional populations – defined broadly as regeneration inhibitory (M1) and regeneration promoting (M2). However, the precise molecular signals that trigger the activation of these different phenotypes remain unclear. Based upon the morphological and functional characteristics of MT-treated microglia identified in this thesis, it is possible that MT represents a candidate molecule that might promote microglia towards a M2 phenotype. Supporting this is in a recent study, which has indentified that MT-deficient mice have a lower level of M2 macrophage/microglia activation following cortical cryolesion (M Pankhurst, manuscript in review). Future studies should evaluate whether MT can induce a M2 phenotype in microglia or macrophages. Two experimental approaches to do this might be to: i) use RT-PCR to determine the expression of M2 specific markers, such as YM-1, in MT-treated and non-treated microglial cultures, and ii) inject MT systemically into the blood of mice and collect the macrophages in the blood where the presence of the YM-1 can be quantitated using RT-PCR.

In summary, these studies demonstrate the potential therapeutic value of MT as a molecule that can promote axonal regeneration after injury, potentially through two distinct mechanisms: i) via direct interaction with neurons, and ii) the indirect modulation of the extracellular environment by altering the reactive response of microglia to improve permissiveness to regenerative sprouting. Notably, both of these actions appear to be LRP-dependent, suggesting that this receptor family may also represent a potential target for therapeutic intervention to promote nerve regeneration. This is in accordance with a growing literature describing the involvement of LRP receptors in promoting neurite outgrowth (Z. Qiu et al., 2004; C. E. Fleming et al., 2009).

It is well described that the primary site of MT expression / production in the brain is astrocytes, and particularly reactive astrocytes in response to external stress or injury. Notably, it has been suggested that reactive astrocytes may also secrete MT under physiologically stressful situations. In an experimental context, it is plausible to consider that the exogenous administration of MT performed in this study might mimic the secretion of MT by reactive astrocytes. However, so far studies have shown that in physiological conditions, astrocytes would not be able to secrete as high concentration as what was injected (i.e. 1μg). Briefly, in this study, assume that the injected MT diffused in a 1mm diameter sphere, which the concentration of the MT injected presence in this parameter would be 0.239 μg/mm^3. In comparison to what had published previously in homogenized brain tissue samples, the physiological concentraion of MT presence was estimated to be about 0.0257μg/mm^3 (Y. Suzuki and M. G. Cherian, 2000). In this regard, it is possible that in response to injury, a physiological response of reactive astrocyte

might be to secrete MT, which subsequently modulates the response of microglia and neurons to traumatic brain injury (Refer to Figure 5.1). This model is in accordance with studies reporting that MTKO mice have a significant deficiency in wound healing, resulting in alterations in non-astrocytic cells (ie: cells that do not express MT endogenously, but which have been demonstrated in this thesis to be responsive to extracellular MT) leading to increased neuronal regeneration as well as an altered microglial response to traumatic brain injury.

It is important to note that for many years reactive astrocytes have been considered as being detrimental to wound healing after CNS injury. However, recent studies utilising the selective ablation of reactive astrocytes has revealed unexpected and important roles for reactive astrocytes in promoting neuronal survival and regeneration after injury. This thesis contributes to this important field by presenting one feasible protective action of reactive astrocytes, through the production and secretion of MT in response to injury, which can directly and beneficially modulate the microglial and neuronal response to injury.

The role of MT in PNS

The DRG neurons (peripheral neurons) have been recently reported to express the putative MT receptor megalin (LRP2). This formed the basis of the aims of the second part of this study, which was to determine whether exogenous MT promotes regenerative sprouting of DRG neurons through LRP receptors (and especially megalin). Using several different experimental DRG neuron culture models, it was found that treatment

with MT led to a significant improvement in axonal sprouting after injury. Furthermore, the utilization of a specialized compartmentalised culture model allowed the identification of neuron compartment-specific action of MT, whereby the ability of exogenous MT to promote regenerative axonal sprouting of injured DRG neurons co-incided with the highly localized expression of megalin in the soma of DRG neurons. MT-mediated regenerative sprouting of injured DRG neurons could be inhibited by both RAP- and PD98059, providing further evidence for an LRP-dependent mechanism.

From a therapeutic point of view, it will be important to evaluate whether MT can promote regenerative sprouting of DRG axons *in vivo*. This might involve experiments utilising sciatic nerve crush/cut injury in rats or mice, followed by administration of MT to the site of injury to see if this improves regenerative sprouting. These types of injury models were not available during this thesis.

It is also possible that a physiological role of MT in the PNS may be to promote regenerative sprouting. Indeed a report by D. Ceballos et al (2003) describes that MT-3 knockout mice have enhanced regenerative sprouting of peripheral nerves following facial nerve axotomy, in accordance with the inhibitory actions of MT-3 upon nerve regeneration (R. S. Chung et al, 2003). To determine whether MT might contribute to DRG nerve regeneration after injury, a logical first series of future experiments would be to perform sciatic nerve crush/cut injuries in MTKO and wildtype mice, and evaluate whether a deficiency in MT leads to impaired regenerative sprouting.

<u>Conclusion</u>

The main focus of this study is the difference in the regenerative capacity between CNS and PNS after injury. This study had demonstrated the protective effect of MT in both CNS and PNS. However, further study, especially the action of MT in PNS should be further charaterized. Including the location of megalin (MT receptors) *in vivo*. Another factor warrant further investigation could be the cellular source of MT presence in the PNS, as well as the endogenous level of MT presence. These were the important factors to consider when comparing the factors that contribute to different regenerative capacity between the CNS and PNS.

Overall the findings of this study provide a clearer understanding of the molecular mechanisms through which exogenous MT can promote nerve regeneration in both peripheral and central neurons after injury.

Figure 5.1
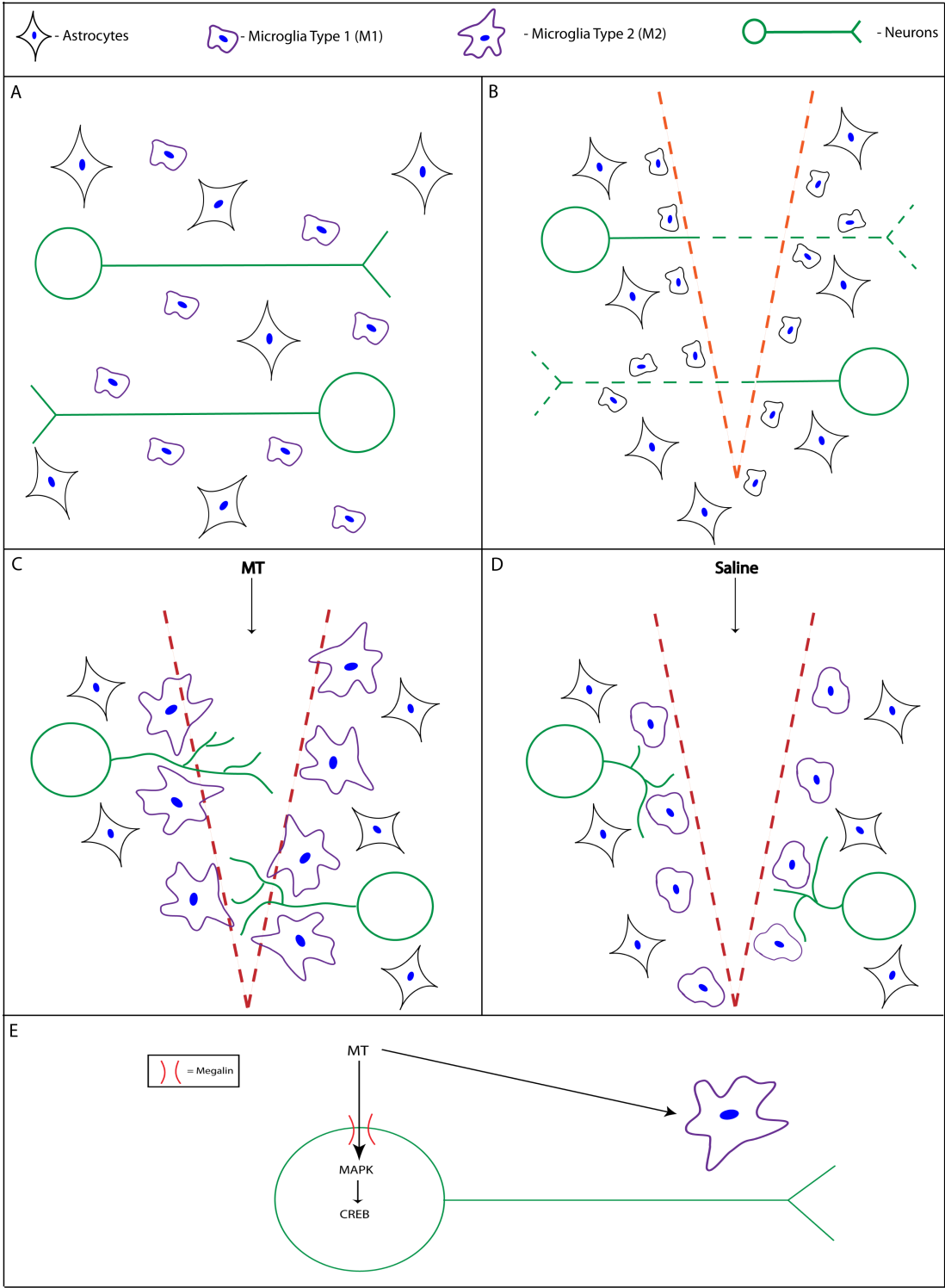
Figure 5.1. Schematic diagram demonstrating the overall action of MT in promoting regeneration after injury in the CNS.

(A) In uninjured bran, both the astrocytes and microglia maintain a close relationship with neurons

(B) After injury, both the astrocytes and microglia become activated and lined along the injury site which associate in containing the injury site

(C) The addition of MT into injury lead to a change in microglia morphology as well as an increase axonal sprouting, furthermore, the regenerative sprouting also appear to maintain a close association with the microglia

(D) The addition of Saline (vehicle treatment) lead to a comparatively less axonal sprouting compare to the MT treatment, the microglia also appear to exhibit a different morphology as well

(E) MT exert both intrinsic and extrinsic effect on cortical neurons, by interacting directly with megalin receptors on neurons to promote neuronal sprouting. MT also shown to potentially modulate the microglia response to injury, hence providing a more permissive extracellular environment, which favor the axonal to sprout through the injury site.

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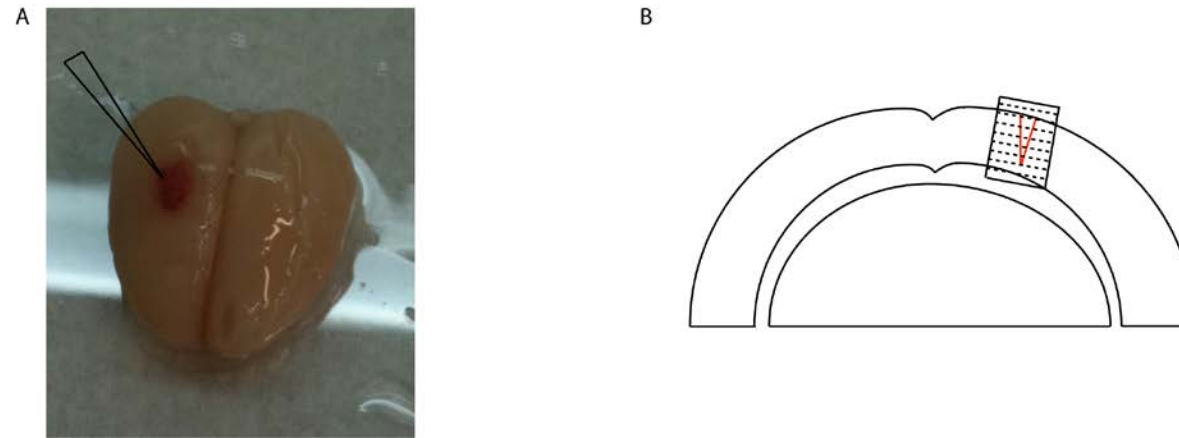
Figure 2.1 Schematic diagram demonstrating the experimental setup of the focal cortical injury

The injury was performed using a Hamilton syringe (represented by arrow in figure A) filled with the treatment solution at the particular sterotactic coordinates (5mm anterior, 4.5mm lateral from lambda). The needle was lowered by 1.5mm from the surface of the brain (marked in B, a schematic diagram from a coronal viewpoint). The animal was then perfused at 4 days after injury and the cortical tissue containing the injury site (marked by the solid line in B) was then removed. The tissues were then sectioned in the horizontal plane as marked by the dotted lines.

Figure 2.2 Control immunohistochemical staining of brain sections

No Secondary Antibody Control
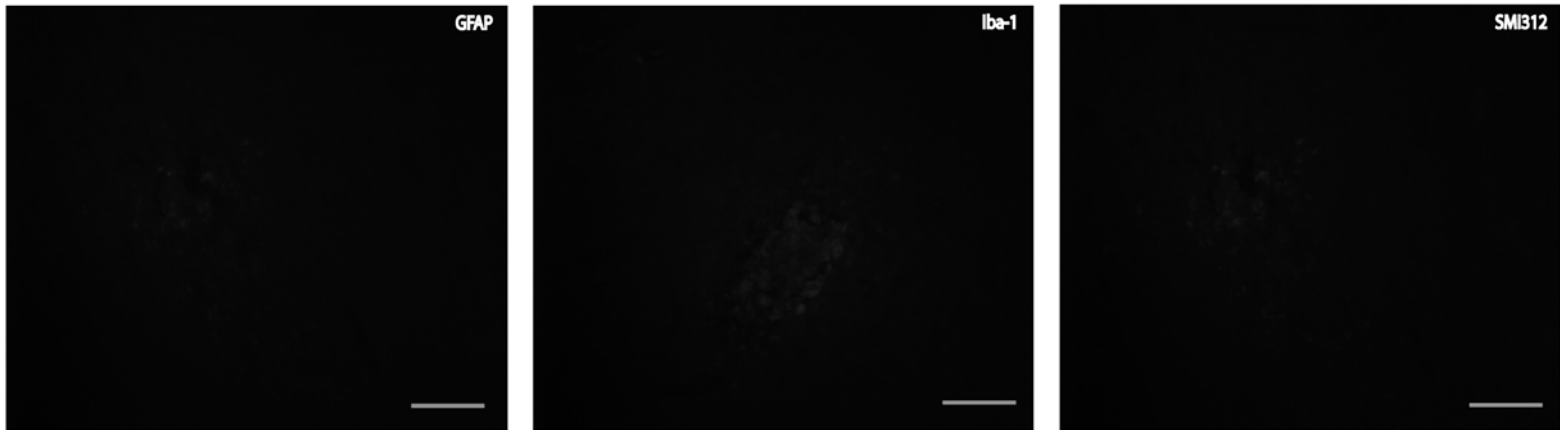
No Primary antibody control
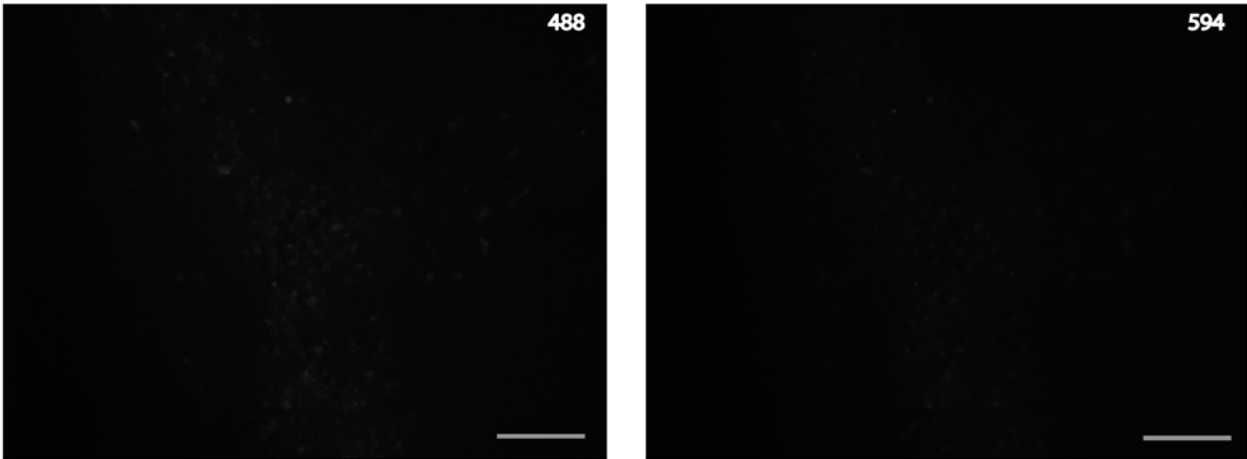
Figure 2.2 Control immunohistochemical staining of brain sections

To ensure that there was no non-specific fluorescent signal or autofluorescent signal present in the brain injury sections, several different staining controls were performed. This involved either immunostaining with primary antibody and no secondary antibody, and only secondary antibody combinations. In all conditions, there was minimal fluorescence observed.

Figure 2.3 Injection of 488-MT leads to an overall reduction in lesion volume after focal cortical brain injury.
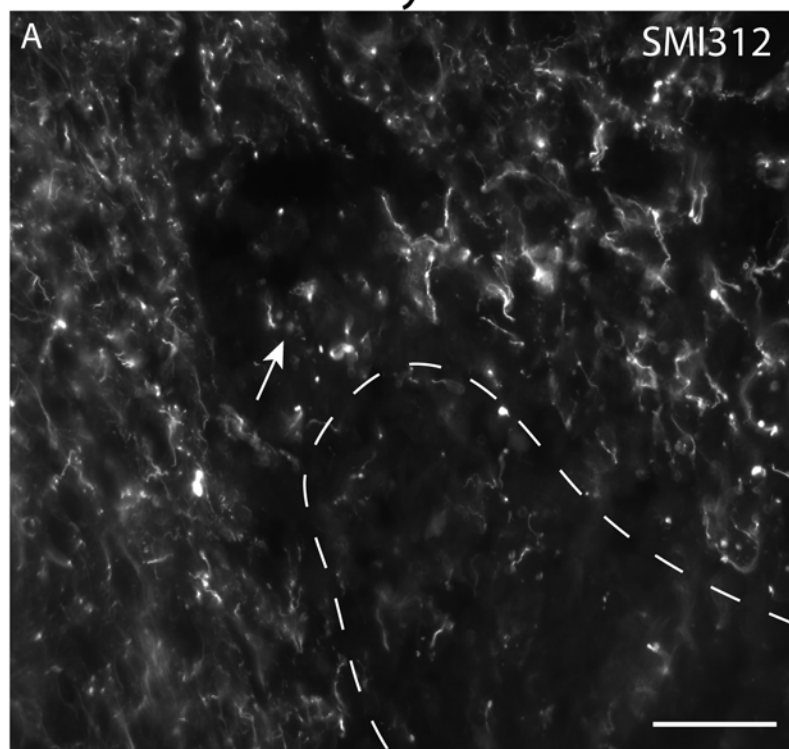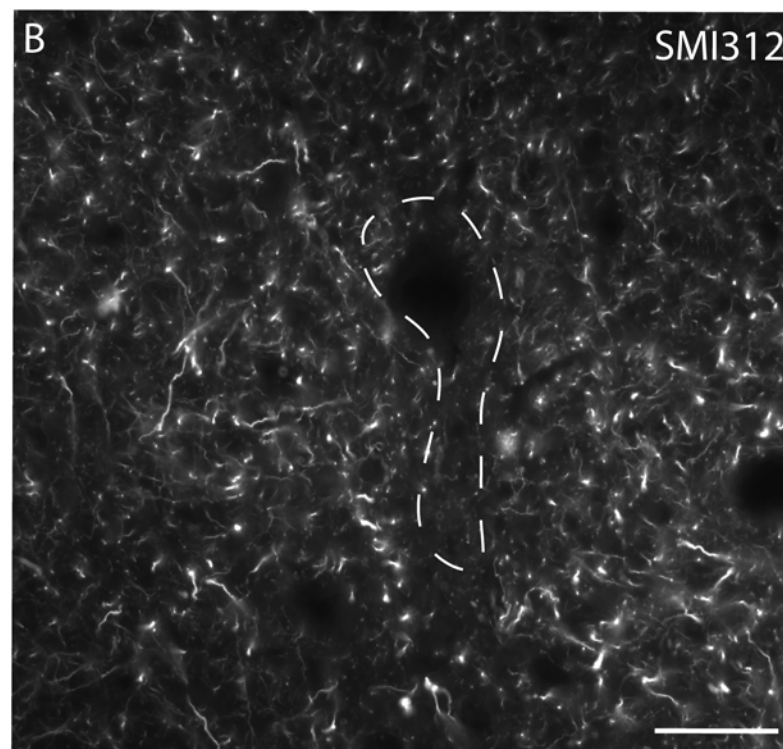
Figure 2.3 Injection of 488-MT leads to an overall reduction in lesion volume after focal cortical brain injury.

Neurofilament labeling using the SMI312 antibody was performed upon cryostat cortical brain sections collected 4 days after focal cortical injury with injection of either 488-MT or AlexaFluor 488 into the injury site. Injection of 488-MT clearly resulted in reduced lesion volume in comparison to injection with AlexaFluor-488 alone (central lesion area marked by the dotted line in A and B). Furthermore, SMI312 labeled neuritic processes in AlexaFluor-488 treated animals was fragmented and granular, which indicate the degeneration of processes (indicated by arrow in A). This was in contrast to 488-MT treatment, in which neuritic processes were brightly labeled and had a smooth appearance (B).

(Scale Bar = 50µm)

Figure 2.4 Cellular distribution of AlexaFluor-488 following injection into the site of focal cortical injury *in vivo*.
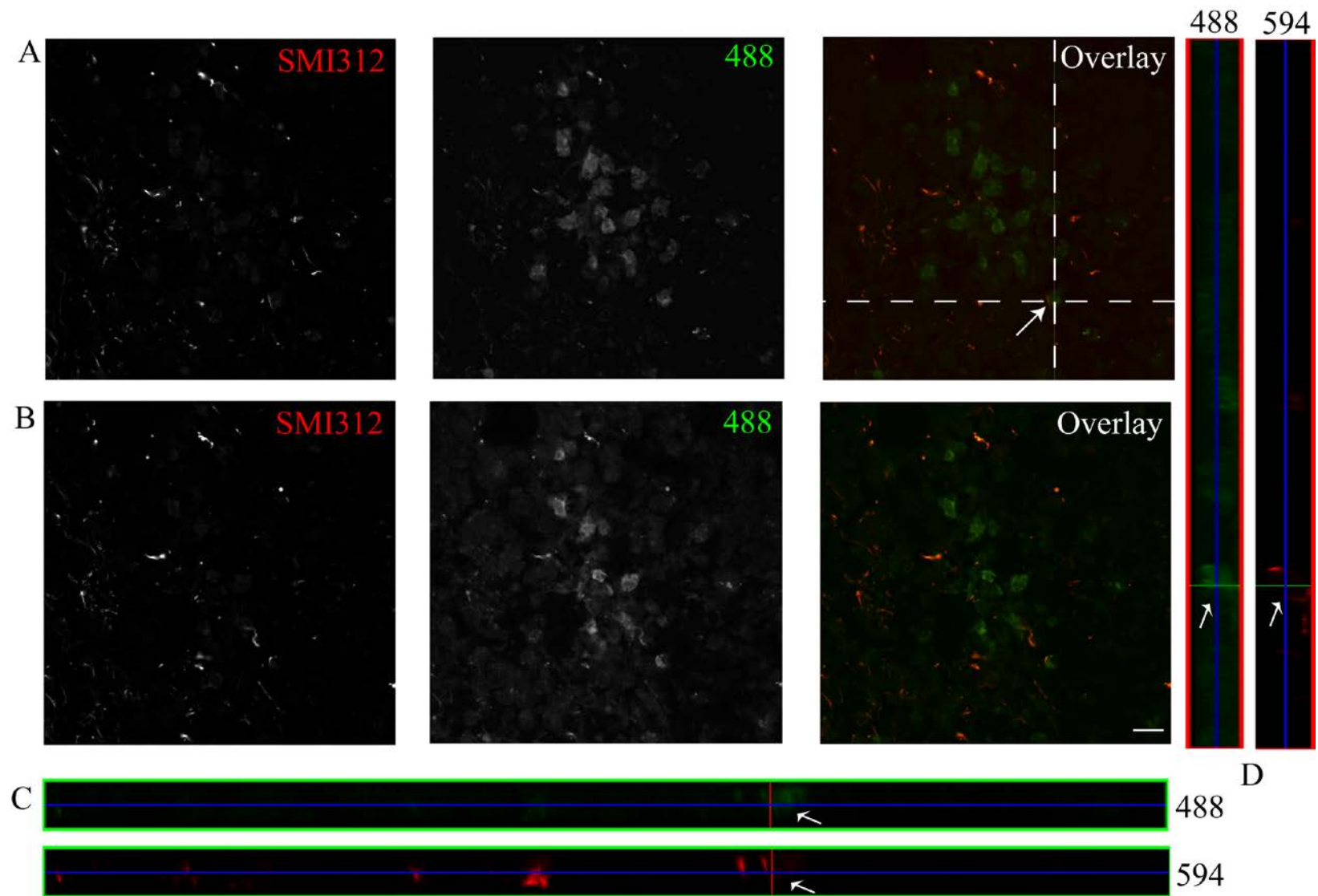
Figure 2.4 Cellular distribution of AlexaFluor-488 following injection into the site of focal cortical injury *in vivo*.

Cryostat brain sections collected from the injured cortex of rats receiving injection of the Alexa-Fluor 488 dye (green) were immunolabeled with SMI312 antibody (red). High magnification confocal stack images (63X) were captured near the lesion site, which clearly showed that the AlexaFluor-488 was found exclusively in non-neuronal cells (SMI312 negative) (Panel A-B). The Z-axis confocal stack collected from the position indicated by the dotted lines confirmed that there is no colocalization between the SMI312 staining and the Alexa-Fluor 488 dye (Panel C-D). (Scale Bar = 16μm)

Figure 2.5 Cellular distribution of 488-MT following injection into the site of focal cortical injury *in vivo*.
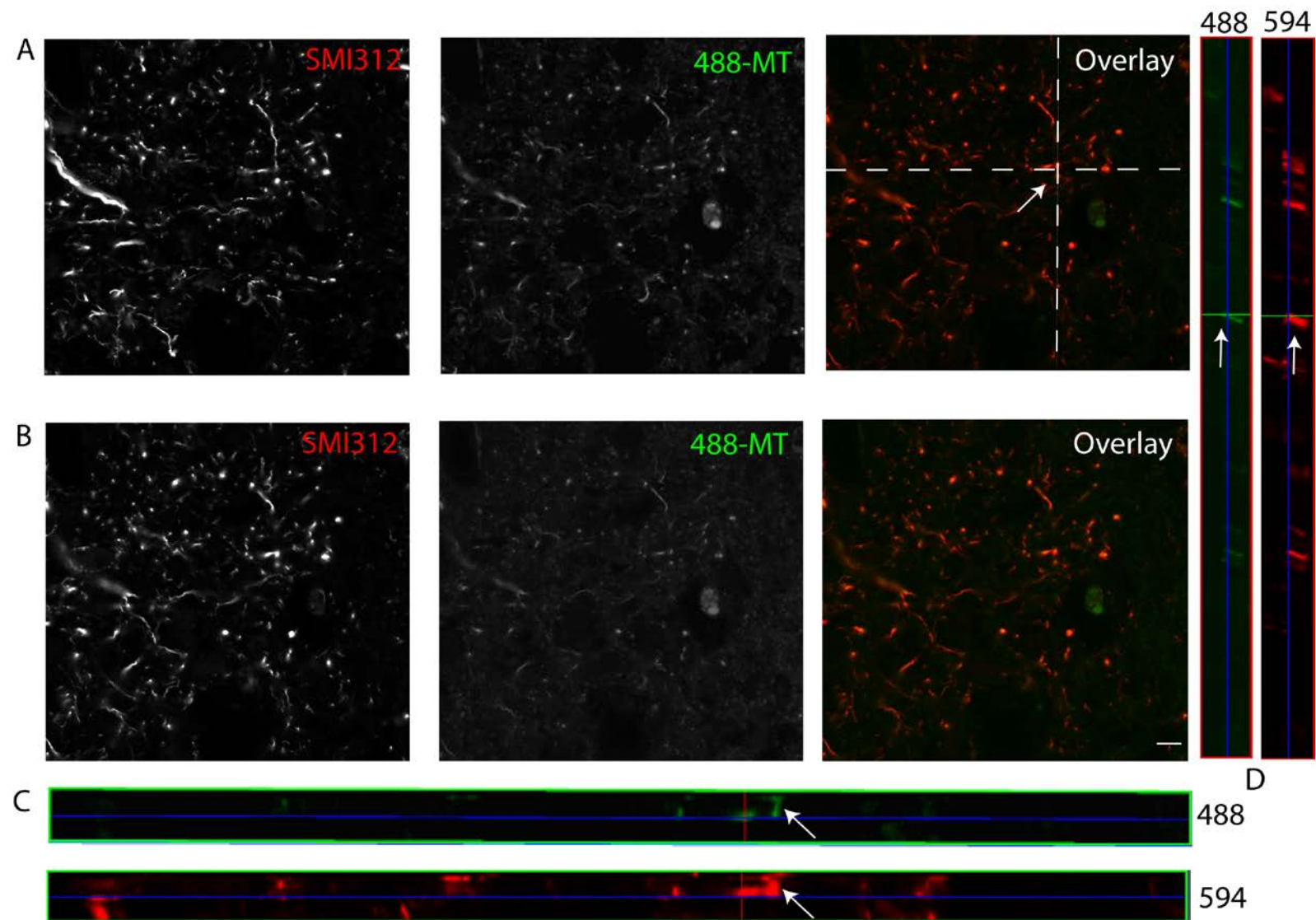
Figure 2.5 Cellular distribution of 488-MT following injection into the site of focal cortical injury *in vivo*.

Cryostat brain sections collected from the injured cortex of rats receiving injection of 488-MT (green) were immunolabelled with SMI312 antibody (red). The high magnification stack images (63X) demonstrate that some neurons and neuritic processes (labeled with SMI312) contained 488-MT (Panel A-B). The Z-axis confocal stack confirmed the colocalization between MT and some SMI312 labelled neurons (Panel C-D). (Scale Bar = 16μm)

Figure 2.6 MT immunolabeling co-localises with the distribution of injected 488-MT, but not the AlexaFluor 488 in the injured brain.
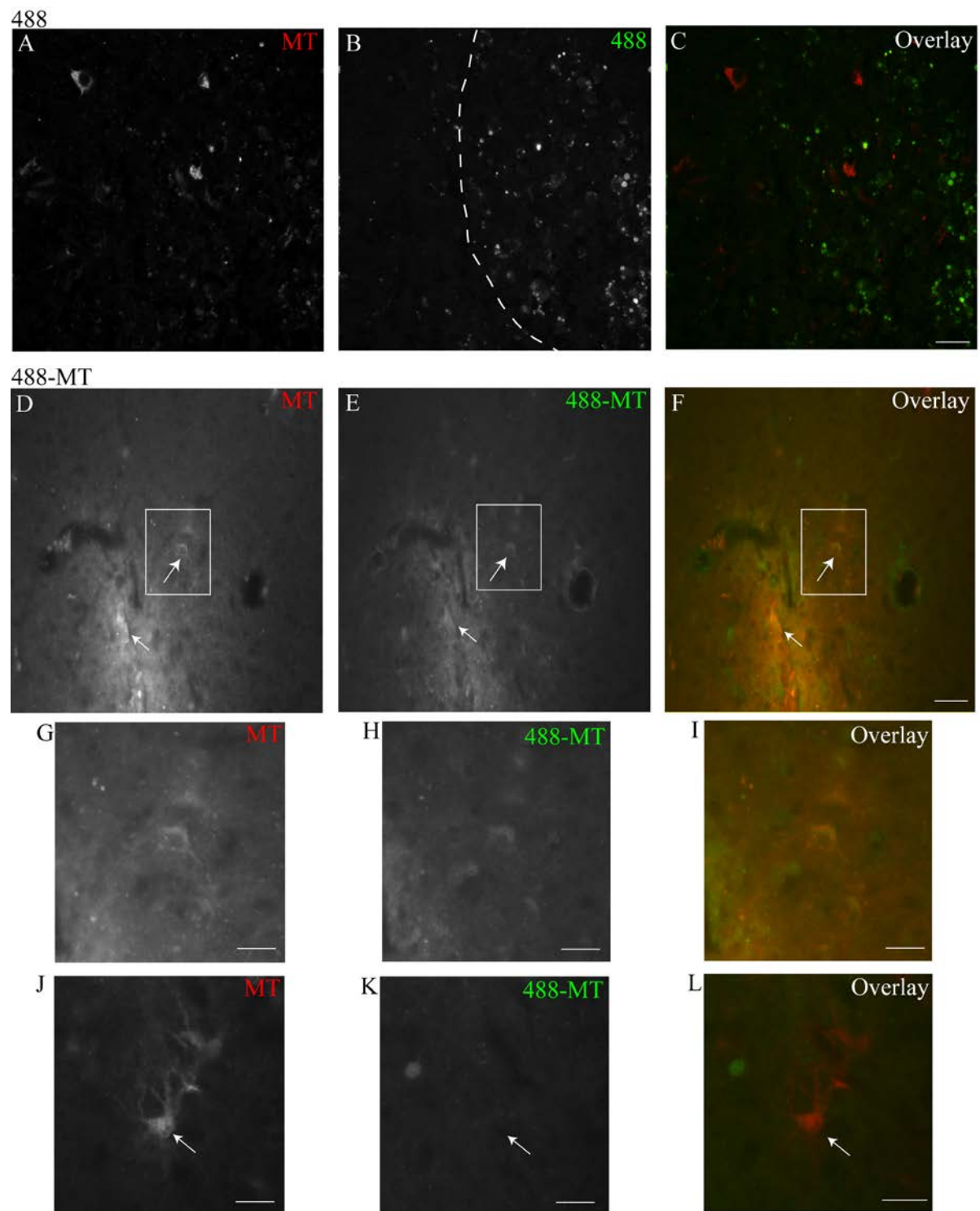
Figure 2.6 MT immunolabeling co-localises with the distribution of injected 488-MT, but not the AlexaFluor 488 in the injured brain.

Immunohistochemical labeling using a MT antibody was performed upon tissue sections from either AlexaFluor-488 (vehicle) or the 488-MT injected animals. The MT labeling in the cortex receiving vehicle treatment did not co-localised with the AlexaFluor-488 (A-C). The majority of the AlexaFluor-488 was observed primarily in the inflammatory cells located in the core of the injury site (marked by dotted line in panel B). In contrast, MT immunolabeling appeared to co-localise with some 488-MT (marked by arrow in D-F). Higher magnification images of the area marked in panels D-F clearly demonstrate this co-localisation (G-I). Notably, some of the cells located further away from the injury site are positively labeled with the MT antibody, however do not have 488-MT (marked by arrow in J-L). The morphology of these cells resembles reactive astrocytes (J-L), which are the major cell type that expresses MT in the brain following injury (Chung et al, 2003).

 (A-F: Scale Bar = 20μm; G-L: Scale Bar = 40μm)

Figure 2.7 Regenerative axonal sprouting and accumulation of microglia/macrophage at four days after cortical needle injury following vehicle treatment (saline).
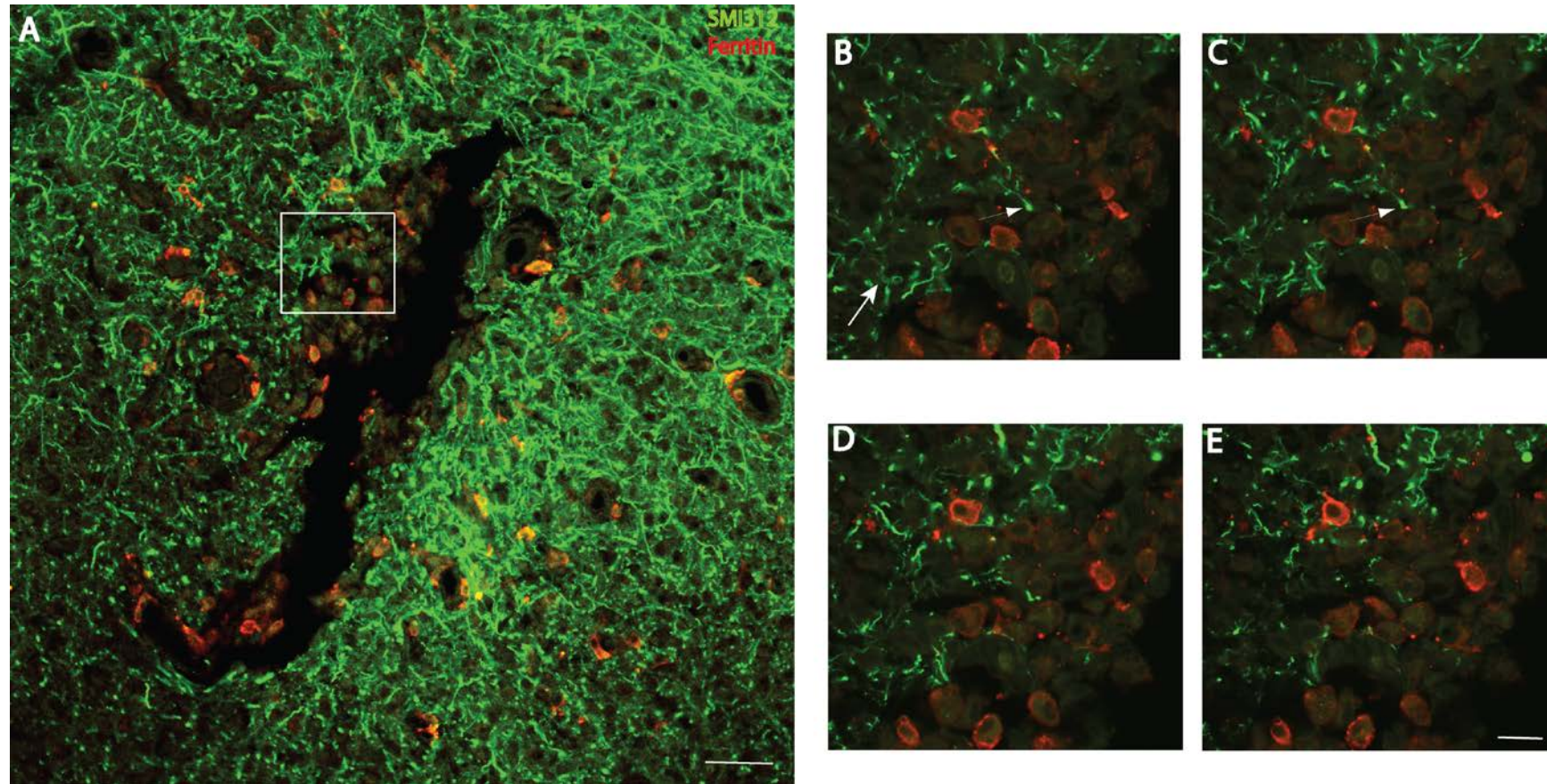
Figure 2.7 Regenerative axonal sprouting and accumulation of microglia/macrophage at four days after cortical needle injury following vehicle treatment (saline).

Panel A displays the overall injury site (labeled with neurofilament marker, SMI312, in green), with numerous regenerative sprouts observed entering the lesion site. A confocal stack (at high magnification) captured from different Z-axis depths (Panel B-E) along the injury edge shows some of the regenerative sprouts possess growth cone-like structures (marked by the arrowhead in B&C). There was very little physical association between regenerative sprouts (marked by arrows at B) and ferritin immunolabelled inflammatory cells (red) (Scale Bar = 50μm in A and 16μm in B-E).

Figure 2.8 Regenerative axonal sprouting and microglia/macrophage accumulation at four days after cortical needle injury following MT treatment.
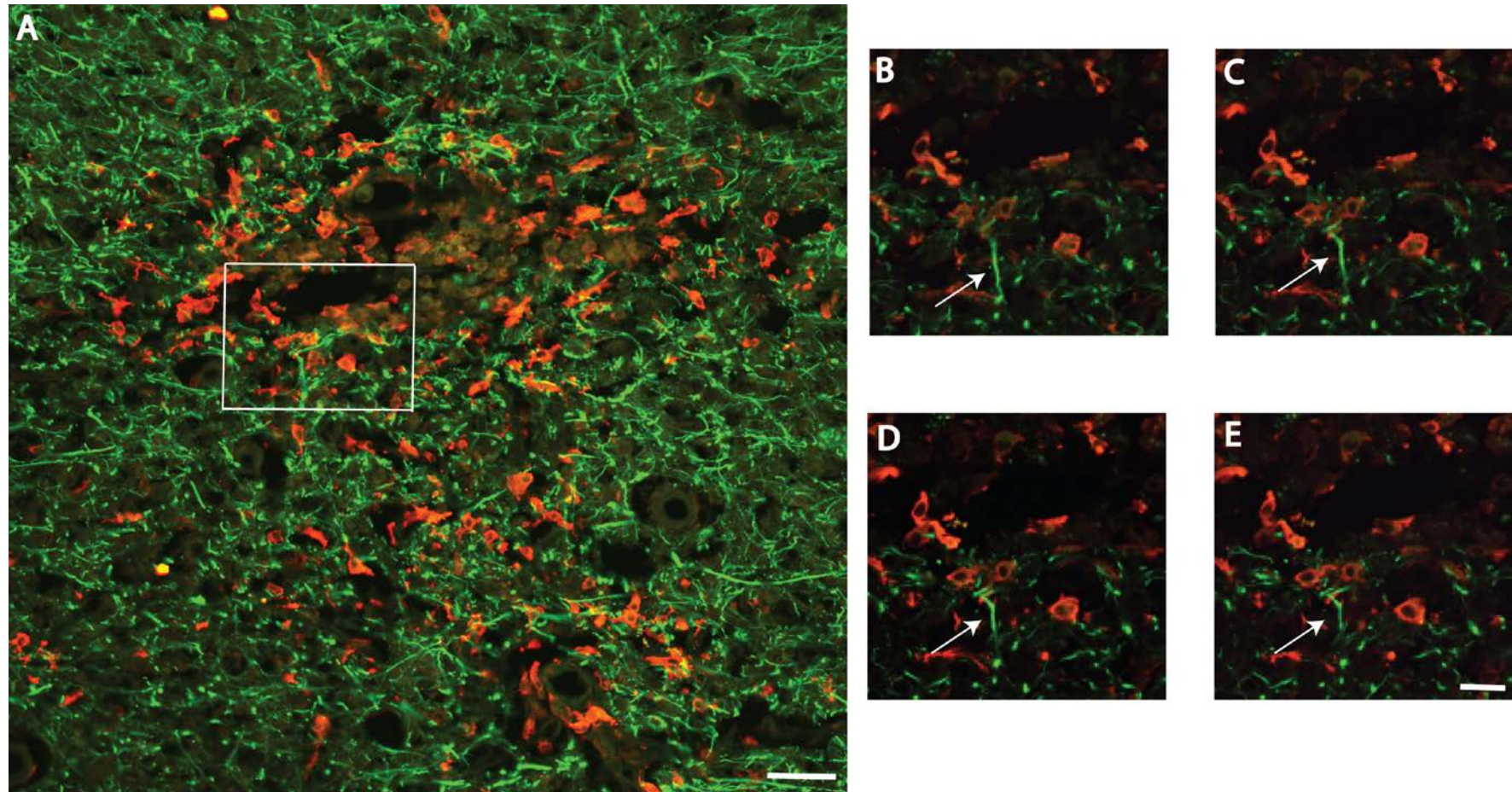
Figure 2.8 Regenerative axonal sprouting and microglia/macrophage accumulation at four days after cortical needle injury following MT treatment.

The overall lesion volume was comparably smaller in MT-treated animals than vehicle treatment (A). The high magnification z-stack image captured along the border of the injury site demonstrates that many SMI312 immunolabeled regenerative axons (in green) extend a considerable distance into the mass of ferritin-labeled inflammatory cells (red, marked by arrow in B-E) (Scale Bar = 50μm in A and 16μm in B-E).

Figure 2.9 Regenerative axonal sprouting and microglia/macrophage accumulation at four days after cortical needle injury following MT + RAP treatment.
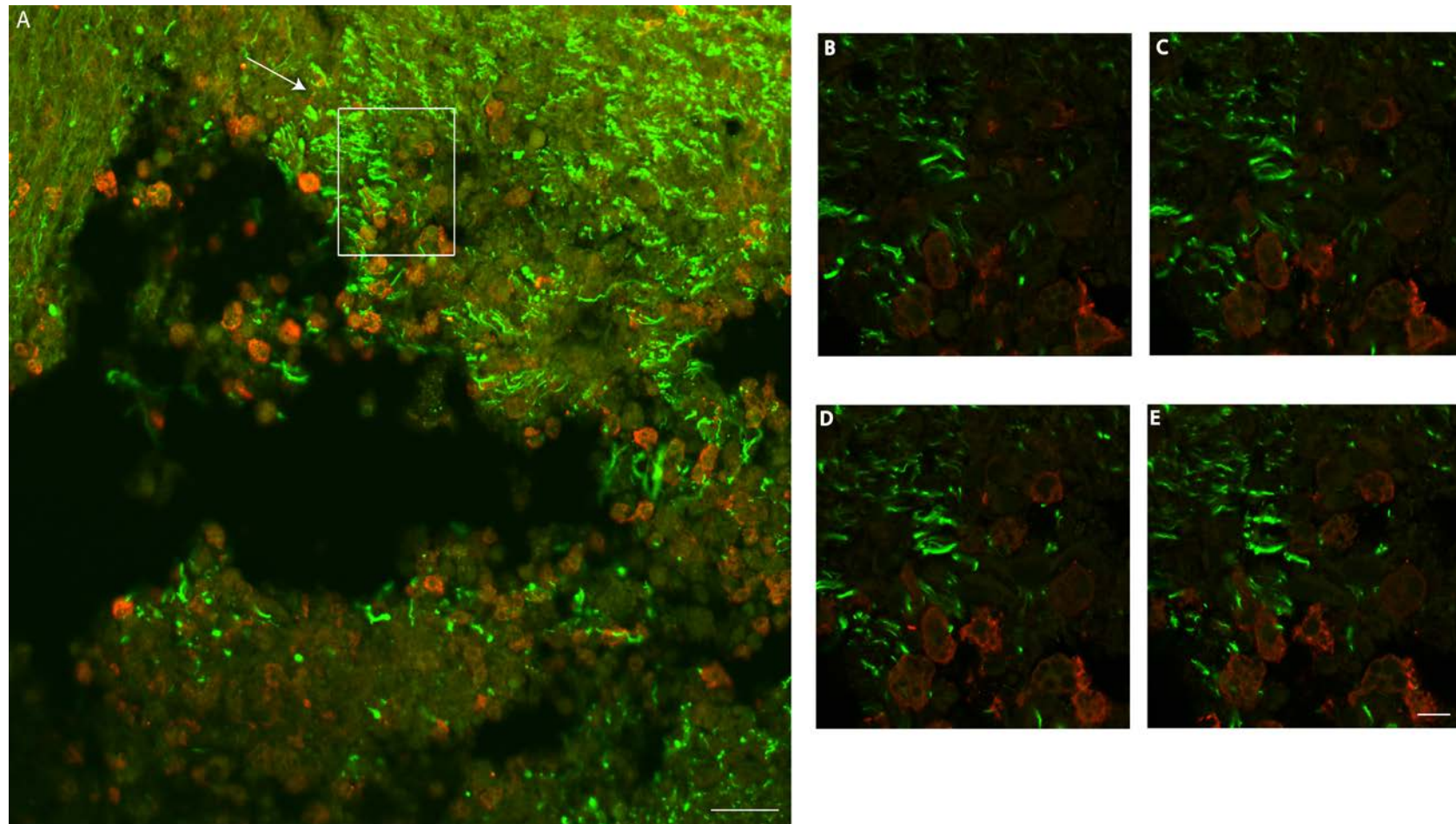
Figure 2.9 Regenerative axonal sprouting and microglia/macrophage accumulation at four days after cortical needle injury following MT + RAP treatment.

In comparison to both the vehicle (Figure 2.7) and MT (Figure 2.8) treatment, the MT + RAP treatment resulted in a considerably larger lesion volume (A). The axons (green) bordering the injury site were often fragmented (marked by arrow in A). The high magnification z-stack image collected bordering the injury site indicates that there is comparatively less regenerative sprouting into the injury site (B-E) (Scale Bar = 50μm in A and 16μm in B-E).
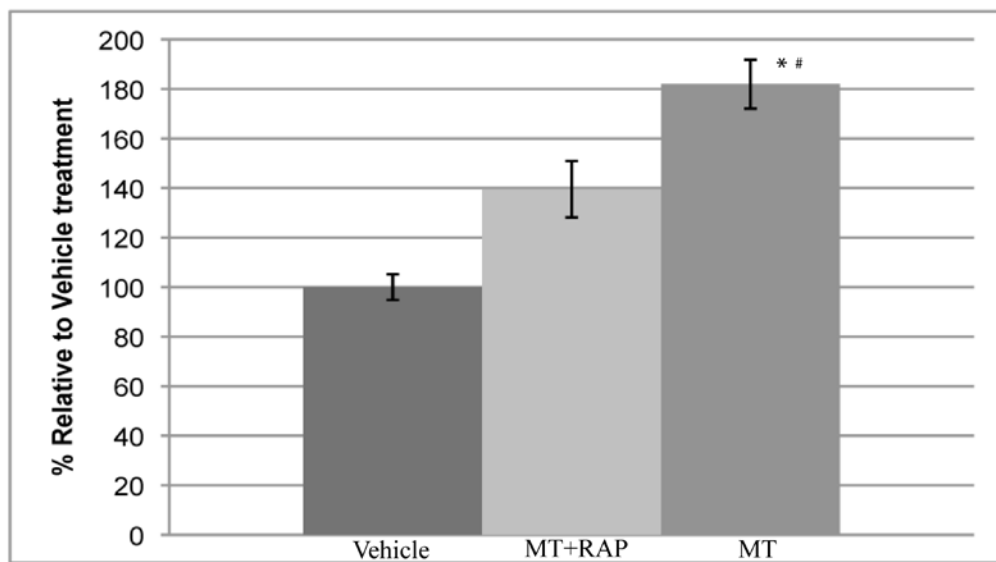
Figure 2.10

Figure 2.10 Quantitation of regenerative sprouting along the border of the lesion site in response to cortical brain injury.

The degree of regenerative sprouting present along the injury site was automatically traced using HCA-Vision software, and quantitatively determined. It was found that MT treatment led to a significant increase in regenerative sprouting compared to vehicle treatment ($p<0.05$). Co-treatment with RAP significantly reduced the pro-regenerative effect of MT ($p > 0.05$). (* $p < 0.05$ compare to vehicle treatment; # $p<0.05$ compare to MT+RAP)

Figure 2.11 Expression of megalin (LRP2) and LRP1 in the injured neocortex.
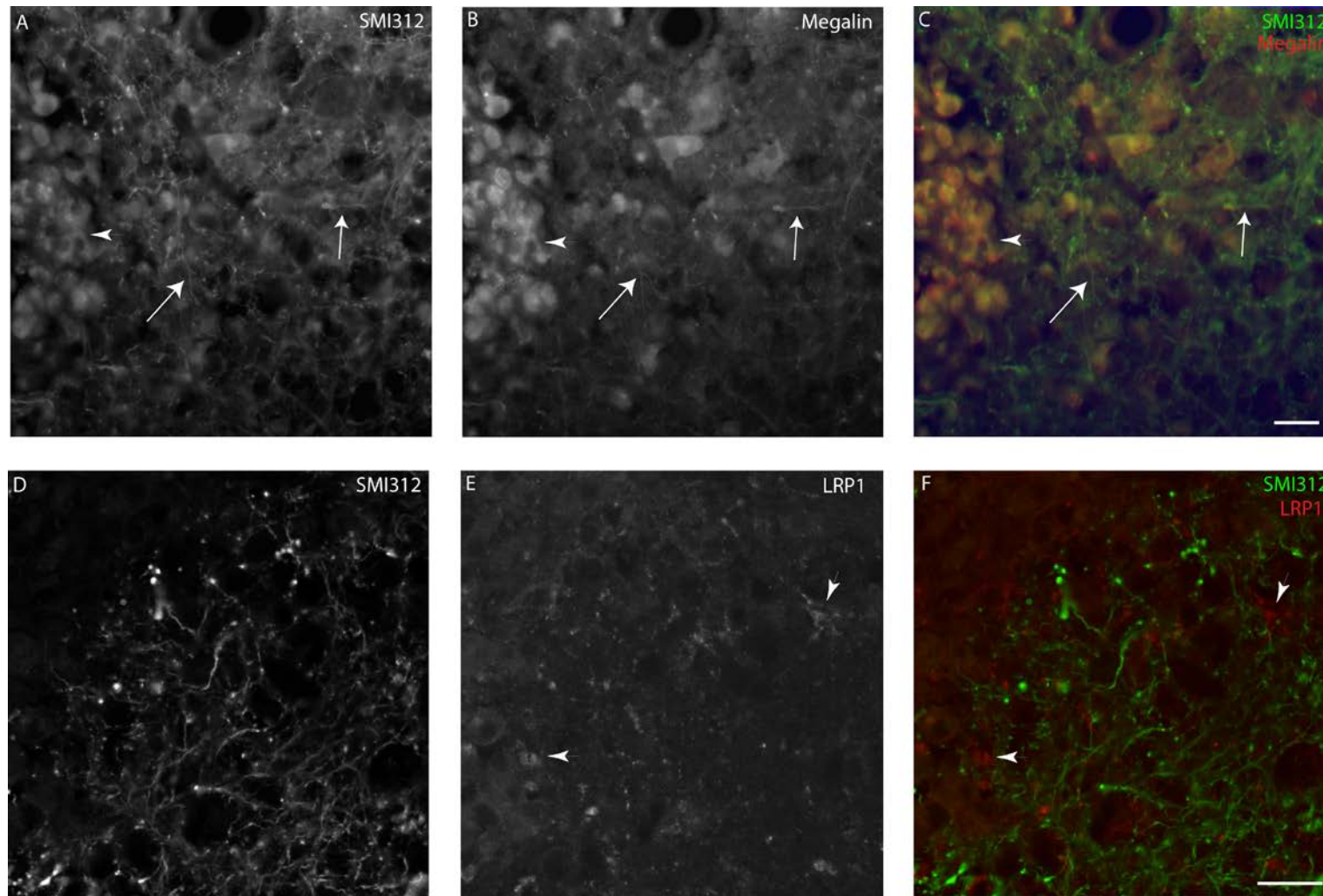
Figure 2.11 Expression of megalin (LRP2) and LRP1 in the injured neocortex.

Brain sections were labeled with antibodies against LRP1 (red) and megalin (LRP2, red), together with SMI312 (green). Some megalin labeling was observed in SMI312-immunoreactive axons (Arrows in A, B and C). Furthermore, megalin was also observed within inflammatory cells in the central region of the lesion site (Arrowheads in A, B and C). However, dual labeling with antibody against both megalin and ferritin (microglia marker) could not be performed as both of these antibodies were raised against rabbit. The distribution of LRP1 in the injured brain was quite different, appearing in a distinct punctate distribution that was not co-localized with SMI312 labeling (Arrowhead in E and F). Some LRP1 expression was noted in inflammatory cells within the centre of the lesion site, but the level of immunolabeling was significantly less than that observed for LRP2 (Scale Bar = 20μm)
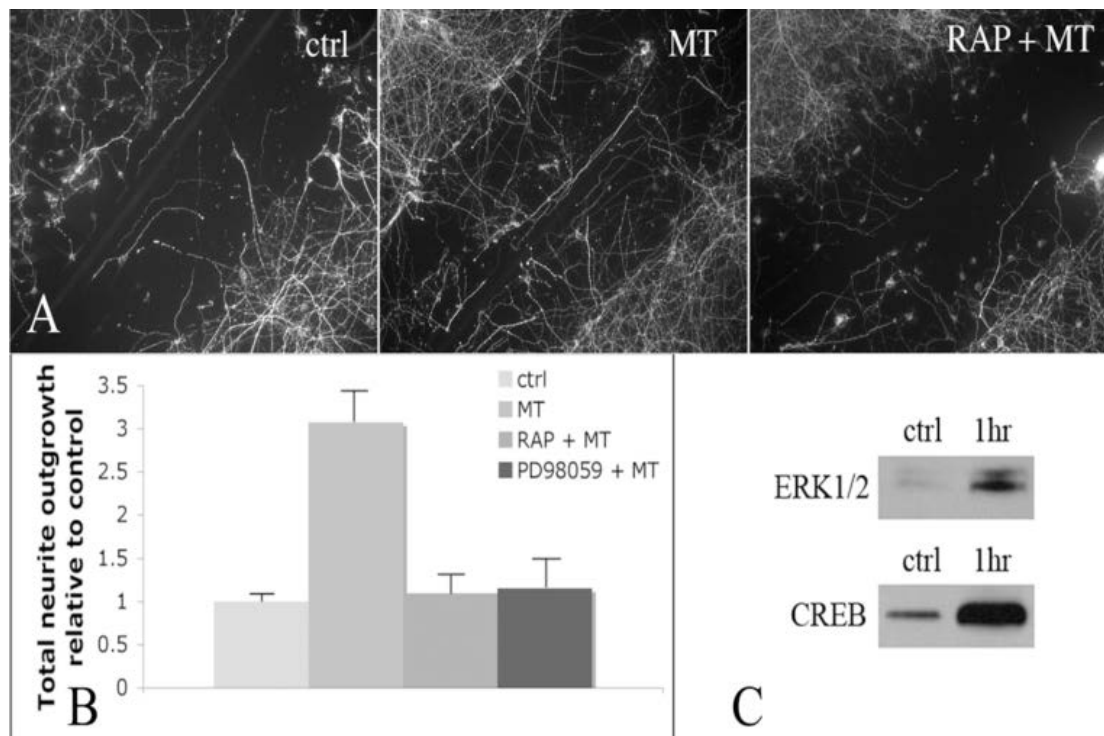
Figure 2.12

Figure 2.12 MT promotes regenerative axonal sprouting via a LRP/MAPK-dependent pathway.

A scratch injury was performed upon 14 day *in vitro* in cortical neuron cultures using a microscalpel, and regenerating axons observed sprouting into the injury site by SMI-312 imunolabelling. In the presence of 10μg/ml MT, there were significantly more regenerating axons present 24 hours post-injury, and the effect of MT-IIA was blocked by pre-treatment with RAP (A). Using HCA-Vision, all regenerating axons were traced and the total amount of axonal regeneration quantitatively determined (B). MT-IIA treatment resulted in a 3-fold increase in axonal regeneration, which was blocked by both RAP and PD98059 (B). Experiments were repeated in triplicate, and error bars represent standard error values across the three experiments. MT treatment led to elevated levels of the phosphorylated MAPK (ERK1/2) and the transcription factor CREB within 1 hour of stimulation (C).

Scale bar = 20μm (A) 10μm (D)

Figure 3.1 Internalisation of AlexaFluor-488 by microglia following focal cortical brain injury.
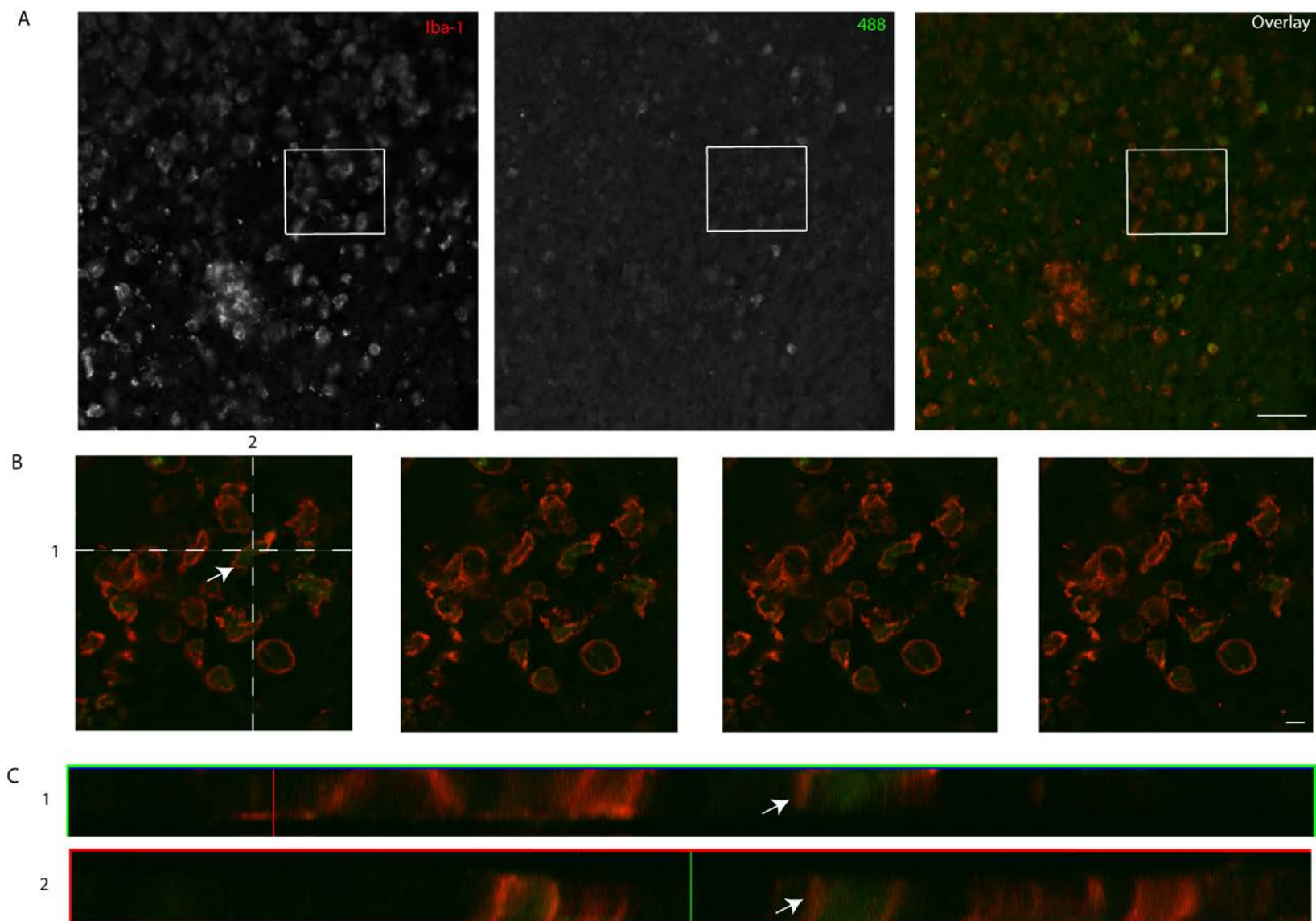
Figure 3.1. Internalisation of AlexaFluor-488 by microglia following focal cortical brain injury.

Immunohistochemical staining for Iba-1 was performed following AlexaFluor-488 injection directly into the site of focal cortical brain injury. It was found that at 4 days post-injury most AlexaFluor-488 was internalised by Iba-1-labeled cells (red) within the lesion zone. High magnification Z-stack confocal images clearly indicate the internalisation of dye within round-shaped Iba1-labelled cells (arrows in Panel C).

(Scale Bar in Panel A = 50μm; Panel B = 6μm)

Figure 3.2 Microglial/macrophage uptake of 488-MT in the injured cortex.
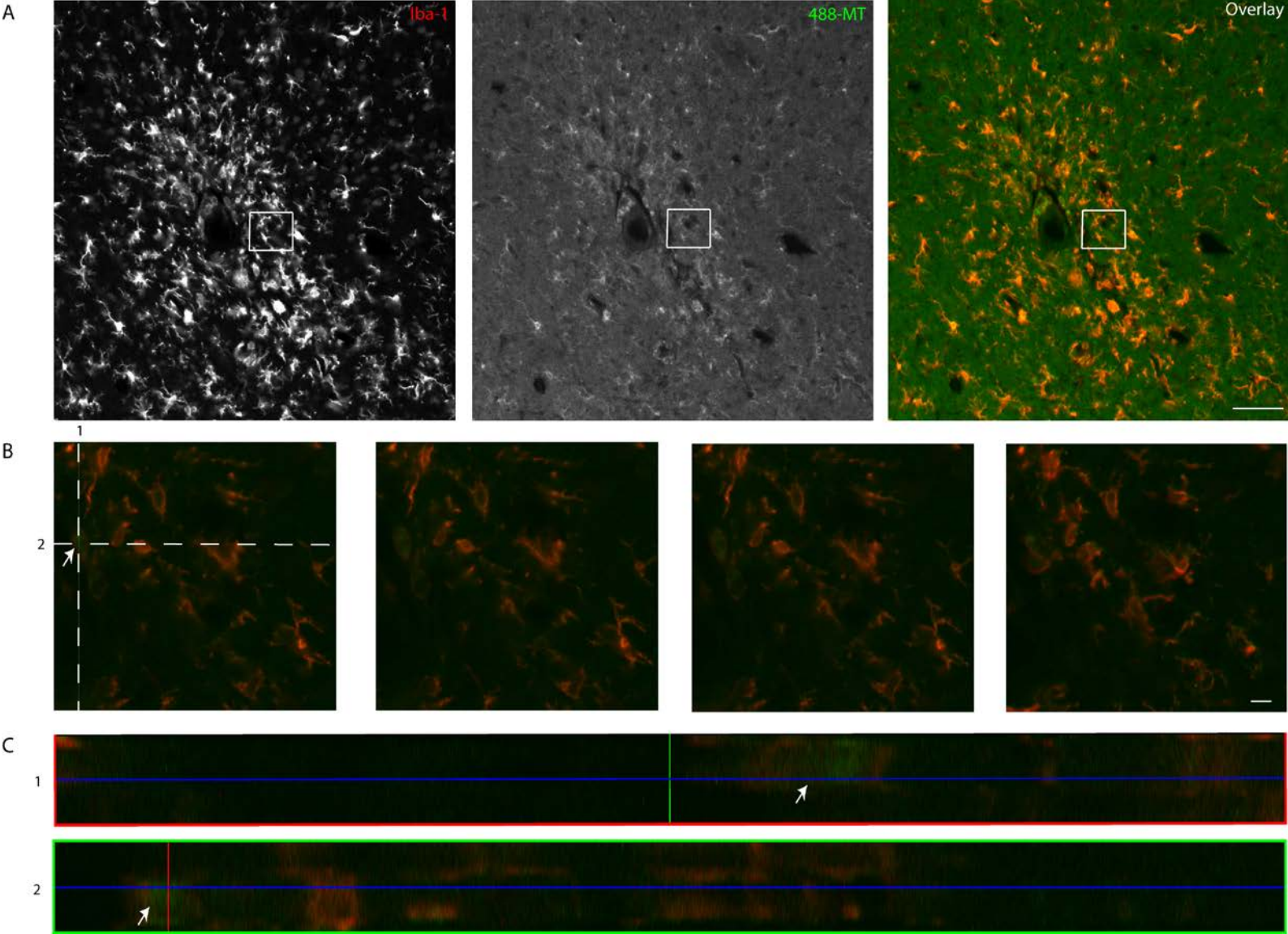
Figure 3.2. Microglial/macrophage uptake of 488-MT in the injured cortex.

At 4 days post-injury, it was found that most 488-MT was presenced in Iba1-labeled cells within the lesion site. High magnification confocal z-stack compilations captured along the border of the injury site clearly show the presence of 488-MT inside Iba-1-labeled cells (red) that have a primarily branched morphology (indicated by arrow in Panel C).

(Scale Bar in Panel A = 50μm; Panel B = 6μm)
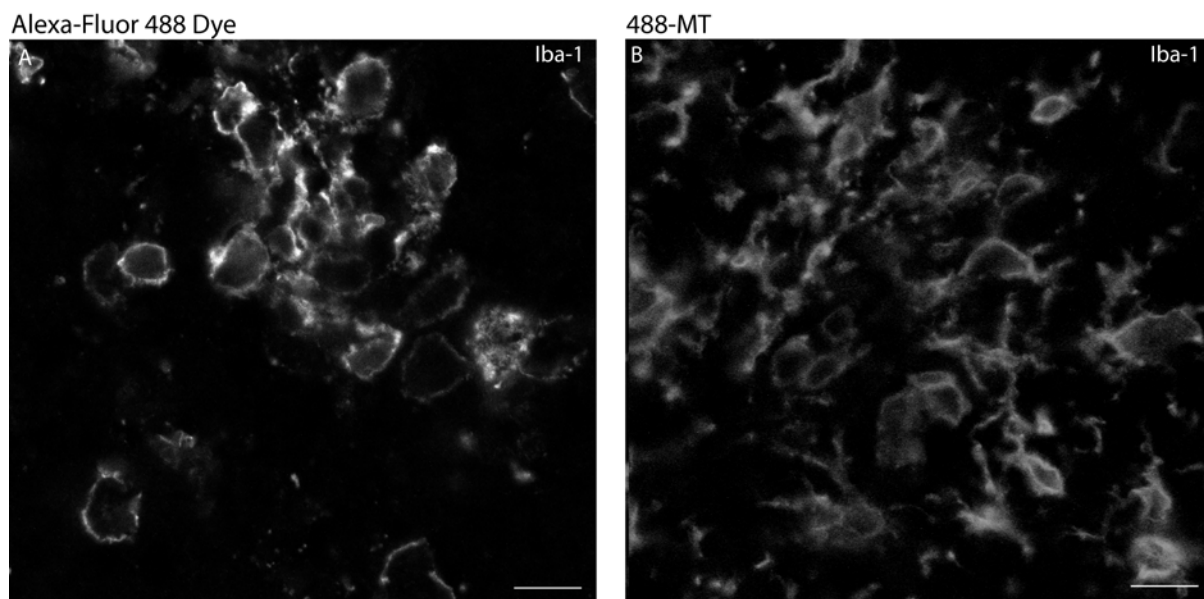
Figure 3.3

Figure 3.3: The morphology of microglia/macrophages within the lesion site are different in response to AlexaFluor 488 and 488-MT treatments.

Iba-1 labeled cells in the injured brain had a difference morphological appearance in response to either the AlexaFluor-488 or 488-MT treatment. Microglia/macrophages appeared to have ameboid morphology in response to the AlexaFluor-488 treatment, however, the microglia/macrophages appeared to have a branched morphology in response to 488-MT.

(Scale Bar = 10μm)
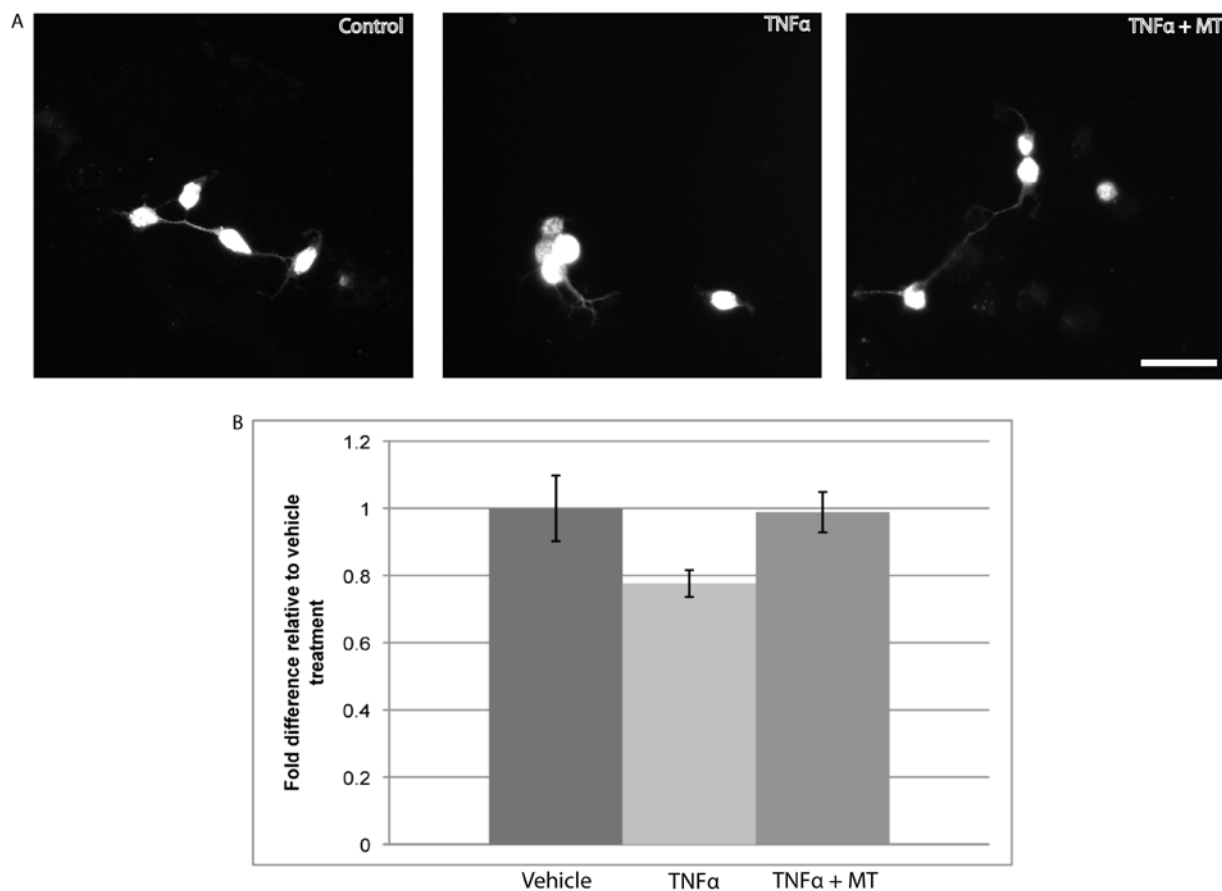
Figure 3.4

Figure 3.4: Cytokine-treated microglia inhibit neurite outgrowth, which can be blocked by exogenous MT

Cortical neurons were co-cultured with microglia that had been pre-treated with TNFα. After 24 hours, cells were fixed and neurons were immunolabeled with an SMI312 antibody. It was found that TNF-α treated microglia impede neurite outgrowth (A), by approximately 20% in comparison to vehicle-treated microglia (B). However, treatment of the TNF-α activated microglia with MT for 24 hours prior to seeding of neurons into the culture completely reversed TNF-α induced inhibition (A). Quantitative analysis confirmed these observations (B).

(Scale Bar = 25um)

Figure 3.5
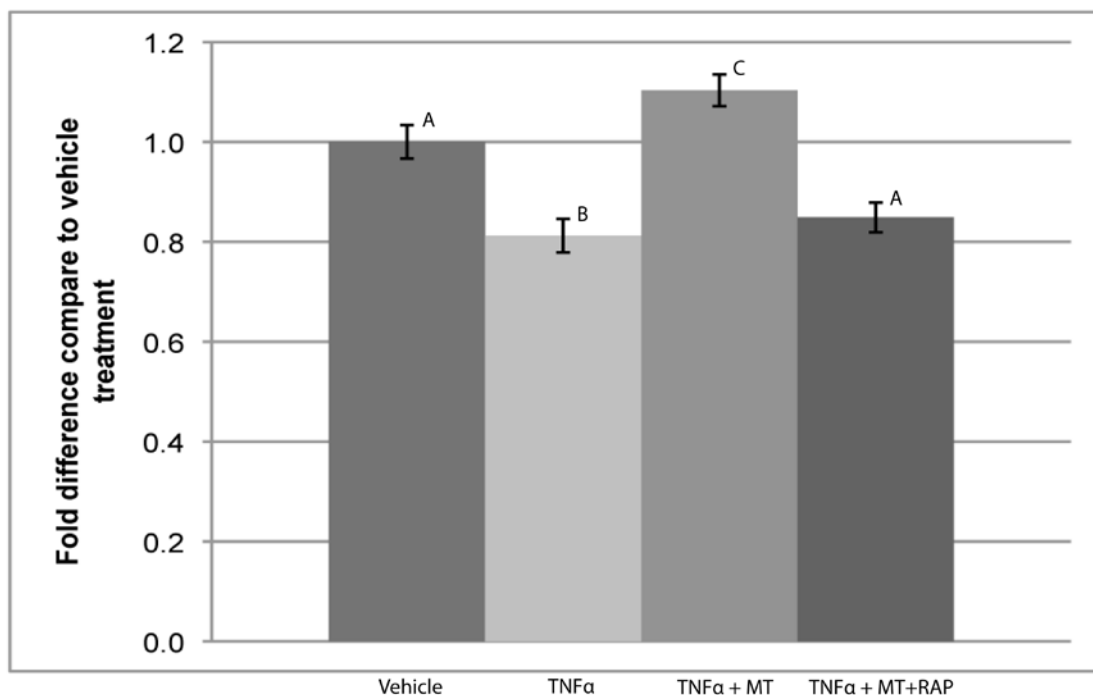
Figure 3.5 Inhibition of neurite outgrowth by cultured microglia is mediated by the secretion of soluble inhibitory factors

Conditioned medium was collected from cultured microglia, into which cortical neurons were directly plated. After 24 hours neurite outgrowth was measured. It was found that conditioned medium from TNFα treated microglia was significantly ($p < 0.05$) inhibitory to neurite outgrowth. However, conditioned medium from TNFα activated microglia that had been treated with MT was not inhibitory to neurite outgrowth, rather led to significantly ($p < 0.05$) growth when compare to vehicle. Furthermore, pre-treatment of TNF-α activated microglia with RAP completely blocked the ability of MT to improve the permissivity of microglia conditioned medium to neurite outgrowth.
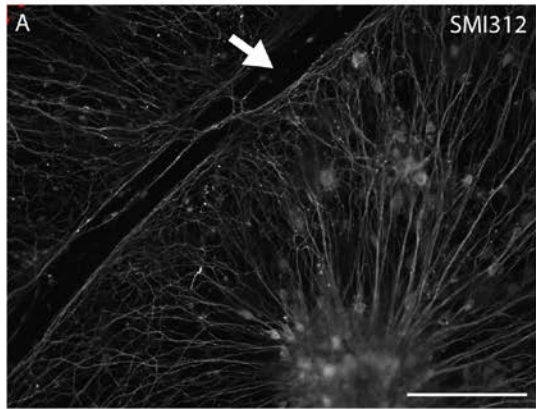
Figure 3.6
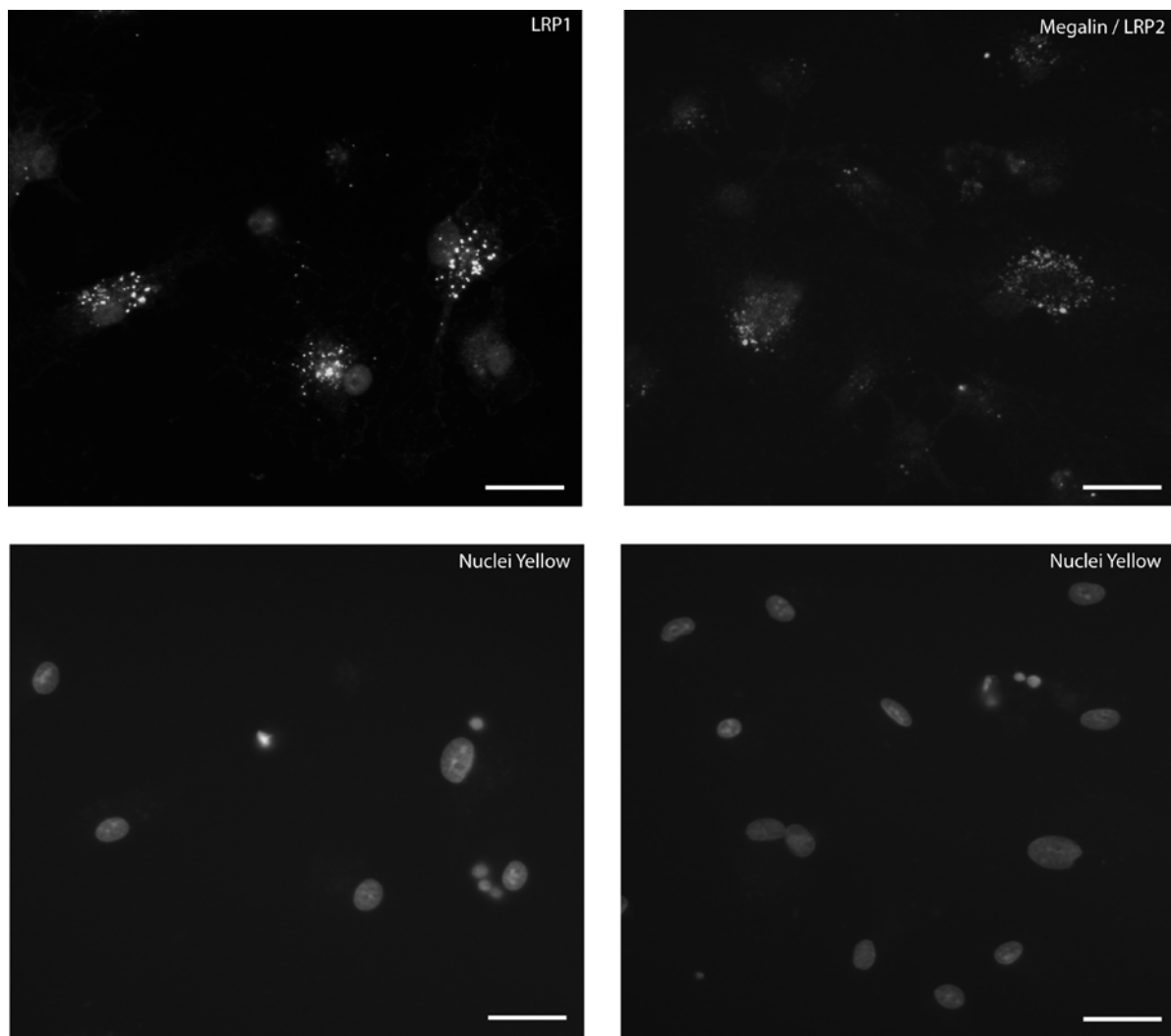
**Figure 3.6 Microglia express both LRP1 and megalin (LRP2)** *in vitro*

Immunohistochemical staining using antibodies against both LRP1 and megalin (LRP2) show a distinct staining pattern in cultured microglia. Both LRP1 and megalin staining appeared to be punctate and perinuclear. However, not all cells appeared to express LRP1 or megalin. Unfortunately, the co-labelling between the LRP receptors and the microglia marker was not possible as these antibodies are from the same origin (Rabbit), however, the morphology of the cells suggested that these were microglia. (Scale Bar = 25μm)

Figure 4.1 MT promotes regenerative sprouting of scratch injured DRG explants *in vitro*.
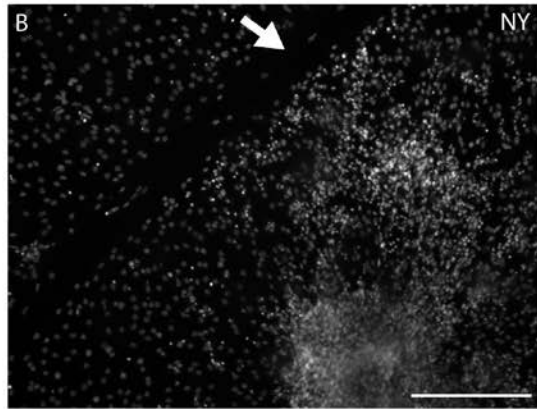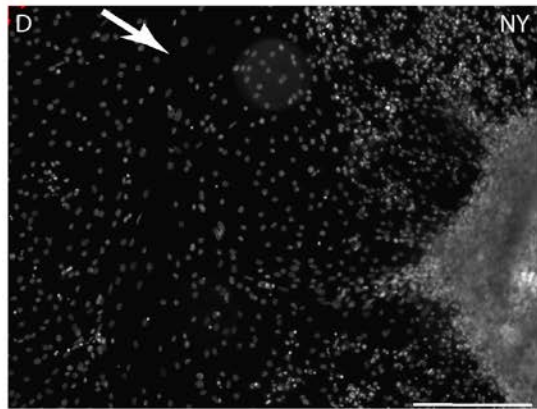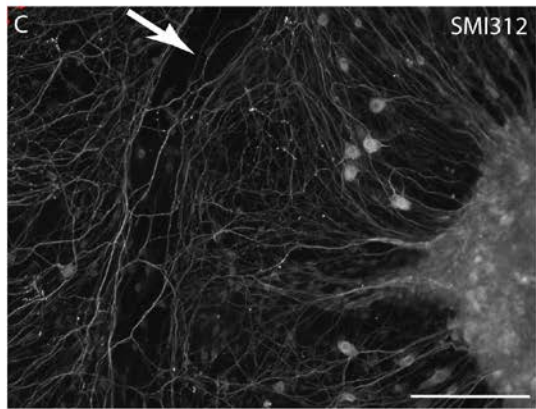
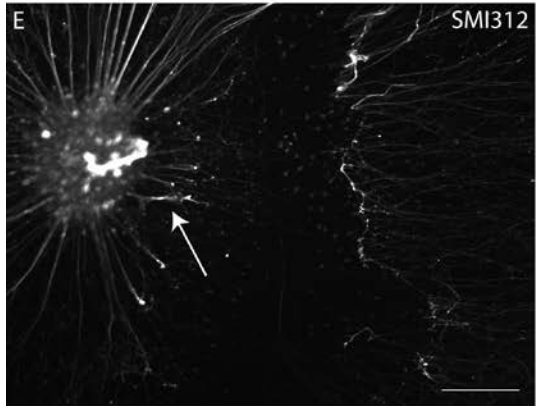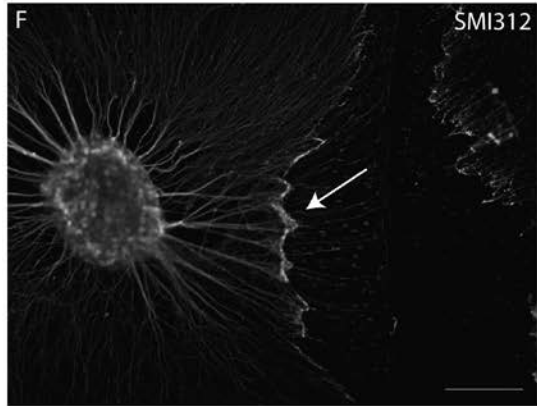**Figure 4.1 MT promotes regenerative sprouting of scratch injured DRG explants *in vitro*.**

Immunostaining of DRG explants 16 hours after scratch injury was performed. The neuronal cytoskeleton of DRG neurons was labeled using the neurofilament antibody SMI312 (A, B, C and D), while Nuclear yellow was used to identify nuclei (C and D). Results indicate that a more extensive regenerative axonal sprouting was observed in the explants receiving MT treatment (C and D) compared to that receiving vehicle treatment (A and B). The injury tract (indicated by arrow in A-D) also appeared to be substantially wider in the explants receiving vehicle treatment compared to those receiving MT treatment. In some DRG explant cultures, it was found that 30 minute pre-treatment with PD98059 (inhibitor of MAPK signaling pathways) or RAP (antagonist of LRP receptors) blocked the pro-regenerative effect of MT (marked by arrow in E and F).

(Scale Bar = 200μm)

Figure 4.2
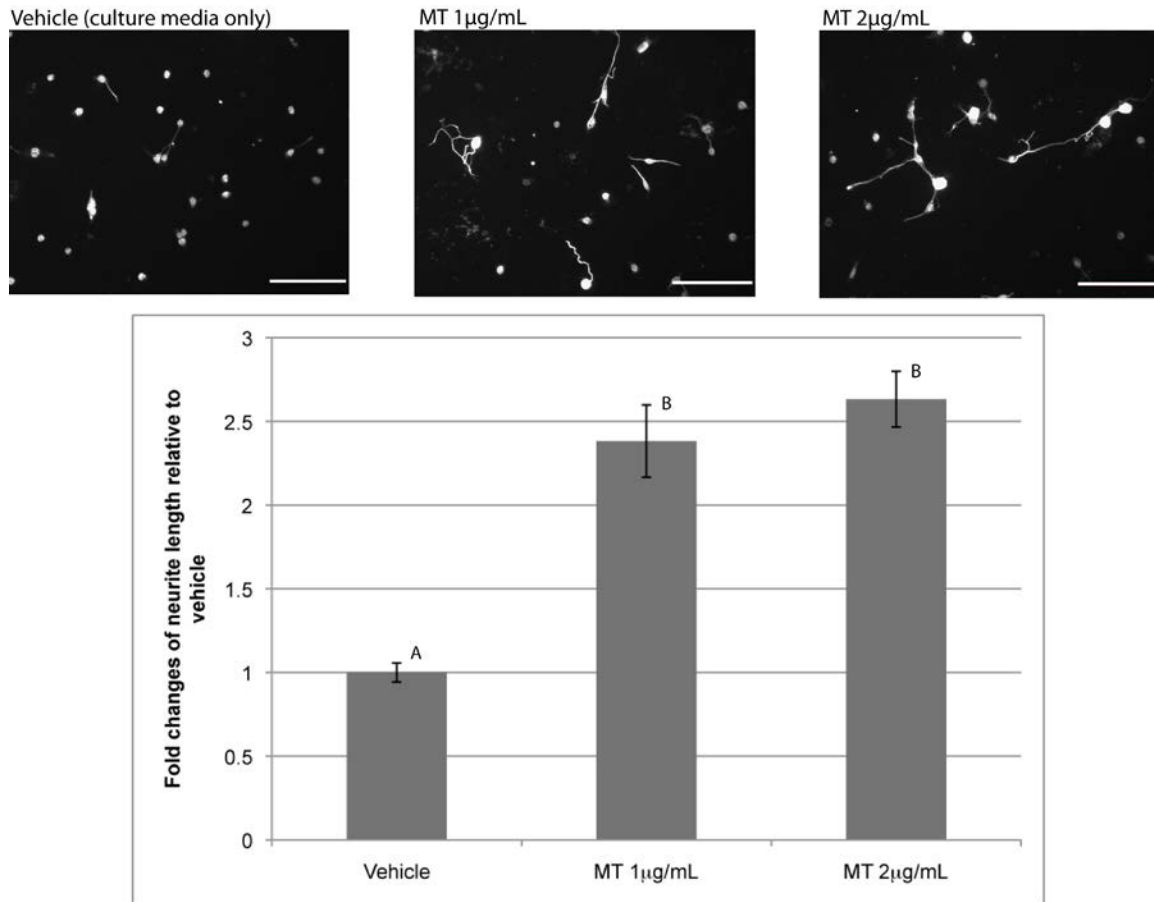
Figure 4.2 MT promotes neurite outgrowth of DRG *in vitro*.

Dissociated cultures of DRG neurons were immunolabeled with the neurofilament marker SMI312, 24 hours after plating. Results demonstrated that MT, at both concentrations tested (1-2µg/ml), promoted neurite outgrowth compared to vehicle treatment. Quantitative analysis confirmed that MT treatments at both concentrations led to a significant (p<0.05; N=15) increase in axonal length compare to the vehicle treatment.

Error Bars = Standard errors of mean fold changes (Scale Bar = 100µm)

Figure 4.3
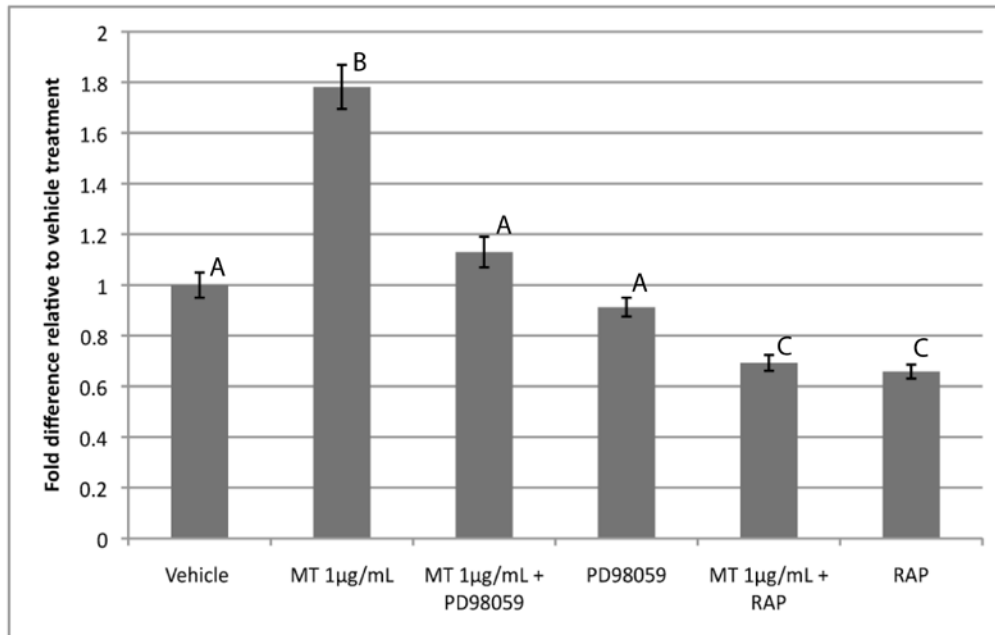
Figure 4.3 MT-IIA promotes neurite outgrowth through both LRP- and MAPK- - dependent pathway

Dissociated DRG neurons were treated with PD98059 or RAP 30 minutes prior to treatment with MT. Quantification of neurite length 24 hours after MT treatment found that both RAP and PD98059 block the neuritogenic effect of MT. Notably, RAP treatment with and without MT also significantly ($p<0.05$) inhibited neurite outgrowth. (N = 20-30 per treatment groups. Error Bars = Standard errors of mean fold changes)
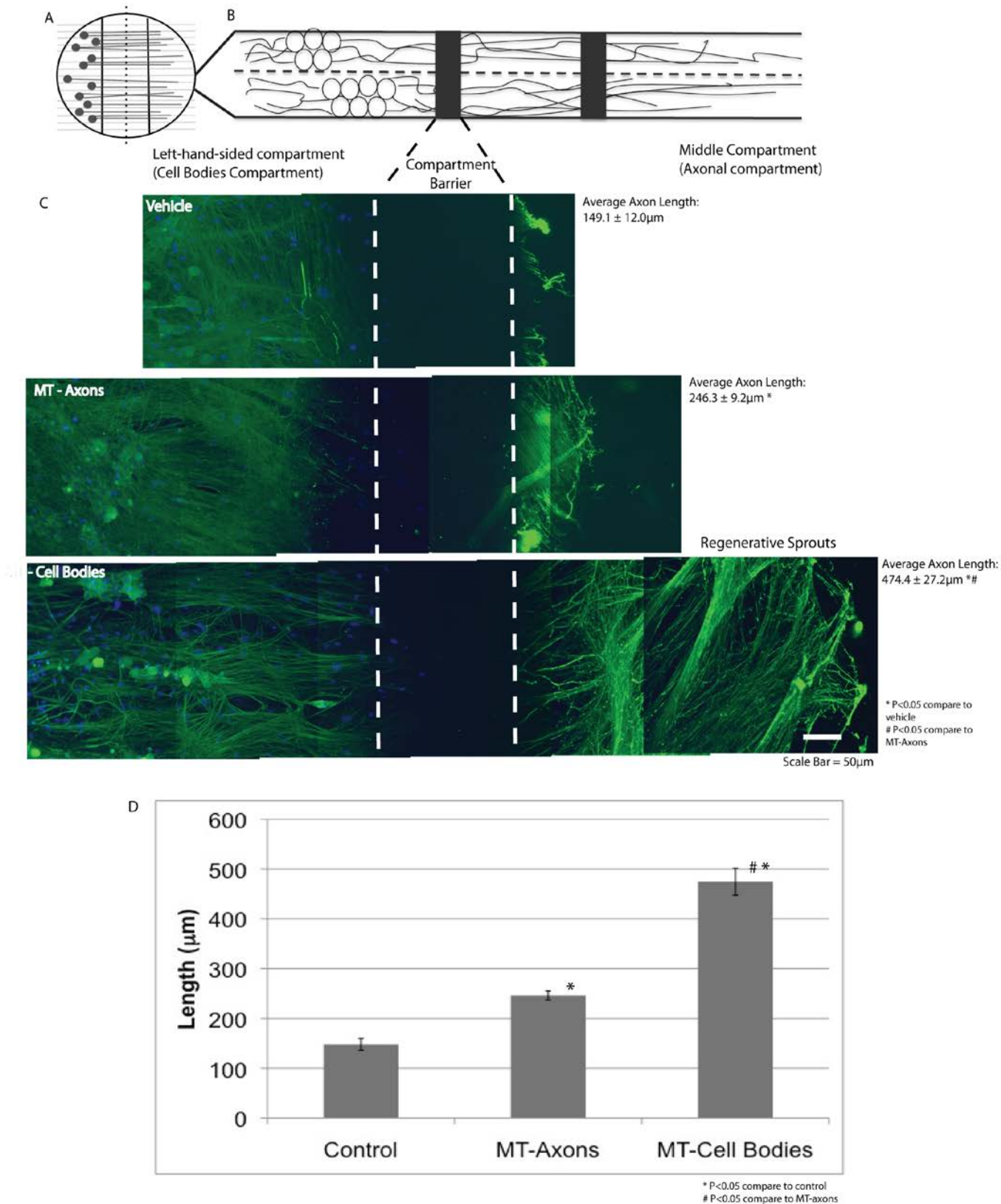
Figure 4.4
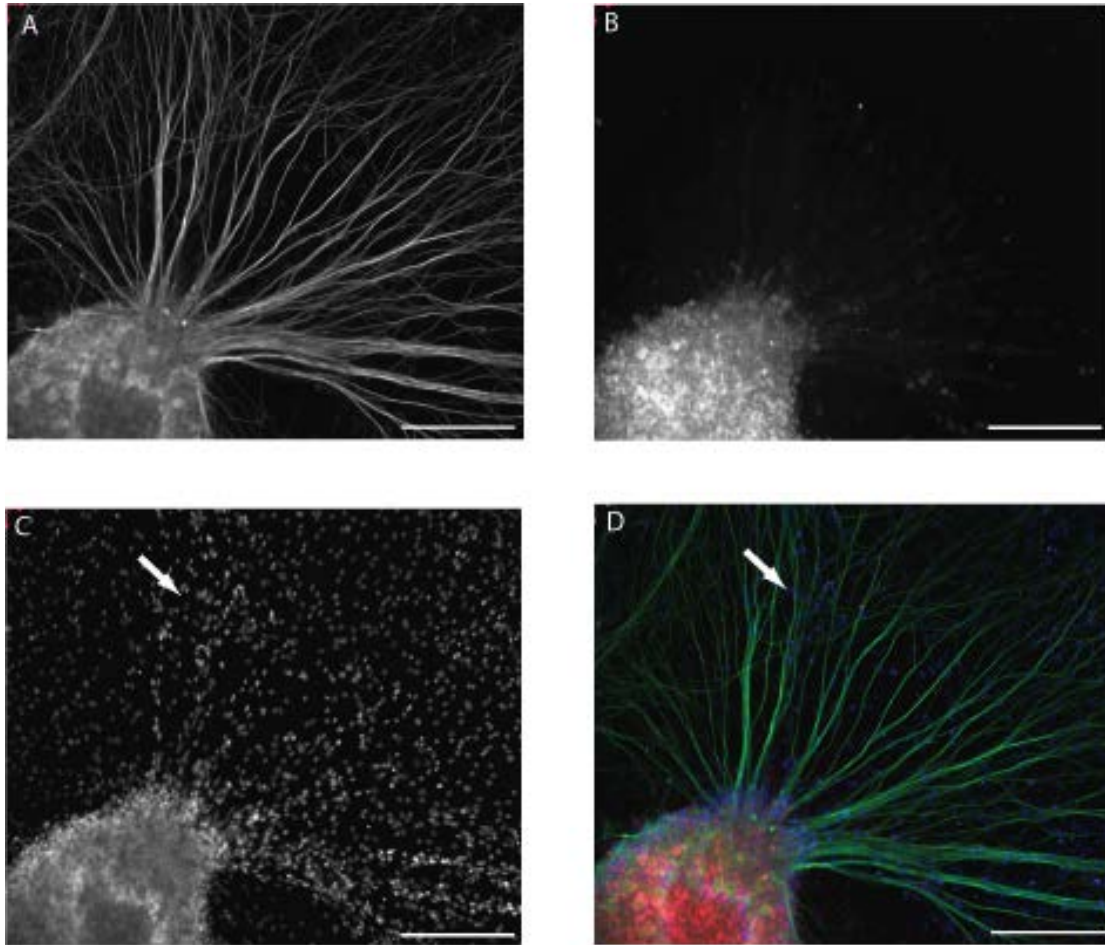
Figure 4.4 The expression of megalin in DRG explants *in vitro*.

Immunohistochemical staining of DRG explant cultures for megalin (red) and neurofilaments (SMI312; green), revealed that megalin staining was punctate and restricted primarily to the soma of DRG neurons, with limited expression in axons. Nuclei staining (C) indicate the presence of non-neuronal cells (SMI-negative cells) within the culture (arrow in panel C and D); however these did not express megalin. Therefore, megalin is specifically expressed by neurons in the DRG explant cultures.

(Scale Bar = 200μm)

Figure 4.5

A

B Left-hand-sided compartment
(Cell Bodies Compartment)

Compartment
Barrier

Middle Compartment
(Axonal compartment)

C Vehicle
Average Axon Length:
149.1 ± 12.0μm

MT - Axons
Average Axon Length:
246.3 ± 9.2μm *

Regenerative Sprouts

- Cell Bodies
Average Axon Length:
474.4 ± 27.2μm *#

* P<0.05 compare to
vehicle
# P<0.05 compare to
MT-Axons

Scale Bar = 50μm

D

* P<0.05 compare to control
# P<0.05 compare to MT-axons

Figure 4.5 MT promotes more extensive regenerative response when added selectively to cell bodies of injured DRG neurons.

DRG neurons were grown in Campenot chambers, allowing the separation of neuronal soma and axons into separate fluid tight compartments (A and B). Comparison of regenerative neurite outgrowth 24 hours after scratch injury between the three treatment conditions (C), revealed that MT applied specifically at the soma of injured neurons led to significantly longer regenerative neurite outgrowth after injury than when MT was applied directly to injured axons. (Scale Bar = 50μm). This difference was confirmed when the average length of regenerative sprouts was measured (D).

(* $P < 0.05$ compare to control, [#] $P < 0.05$ compare to MT - Axons. N = 9 photos per treatment groups. Error Bars = Standard errors of mean fold changes)