MATRIX METALLOPROTEINASES AND EXPERIMENTAL DIABETIC NEUROPATHY

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Table of Contents

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List of Figures

Chapter One

Chapter Two

List of Tables

Abbreviations

AGEs	Advanced glycation end-products
AG	Aminoguidiane
ALE	Advanced lipoxidation end-product
α2M	α2-Macroglobulin
AP-1	Activator protein-1
AR	Aldose reductase
ARI	Aldose reductase inhibitor
ATF-3	Activating transcription factor-3
BACE	Bovine aortic endothelial cells
BBB	Blood brain barrier
BDNF	Brain derived growth factor
BNB	Blood nerve barrier
BRB	Blood retinal barrier
BSA	Bovine serum albumin
BS medium	Bottenstein and Sato's medium
CAMs	Cellular adhesion molecules
ChABC	Chondroitinase ABC
CEL	Carboxyethyllysine
CGRP	Calcitonin gene-related peptide
CML	N^{ε}-(carboxymethyl) lysine
CNS	Central nervous system
COLL	Collagen
CS	Chondroitin sulphate
CSPGs	Chondroitin sulphate proteoglycans
CT	Cycle threshold
Cy3	Cyanine-3
Cys	Cysteine
DCCT	Diabetes Control and Complications Trial Research Group
DRG	Dorsal root ganglia
DSPN	Distal sensory polyneuropathy

3-DG	3-deoxyglucose
ECM	Extracellular matrix
EEM	Extracted extracellular matrix
ERK	Extracellular signal-regulated kinase
F-6-P	Fructose-6-phosphate
FGF	Fibroblast growth factor
FL	Fructosllysine
FITC	Fluorescein isothiocyanate
FN	Fibronectin
HNE	4-hydroxynonenal
HRP	Horseradish peroxidase
HSPGs	Heparin sulphate proteoglycans
GAG	Glycosaminoglycan
GAP-43	Growth associated protein
GDNF	Glial line-derived growth factor
GFOGER	Glycyl-phenylalanyl-hydroxypropyl-glycyl-glutamic acid-arginine
GH-1	Methylglyoxal - Nδ-(5-hydro-4-imidazolon-2-yl)ornithine
GOLD	Bis(lysyl) crosslink derived from glyoxal
IB4	Isolectin B4
IENF	Intraepidermal nerve fibres
IL-1	Interleukin-1
IL-6	Interleukin-6
i,p.	Intraperitoneal
IR	Immunoreactivity
JNK	Jun N-terminal kinase
KO	Knock-out
L4/5	Lumbar 4/5
LM	Laminin
LPS	Lipopolysaccaride
LRP-1	LDL receptor-related protein-1
MAPK	Mitogen-activated protein kinase
MAG	Myelin associated glycoprotein

MBP	Myelin basic protein
MG	Methylgloxal
MG-H1	Methylglyoxal-derived Hydroimidazo-1
MMP	Matrix metalloproteinase
MOLD	Bis(lysyl) crosslink derived from methylglyoxal
MT-MMP	Membrane-type MMP
NCV	Nerve conduction velocity
NDS	Normal donkey serum
NF200	Neurofilament 200
NF-H	Heavy neurofilament protein
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NO	Nitric oxide
NT-3	Neurotrophin-3
NROS	Reactive nitrogen-oxygen species
OS	Oxidative stress
PB	Phosphate buffer
PC12	Pheochromocytoma
PDL	poly-D-lysine
PCR	Polymerase chain reaction
PG	Proteoglycans
PGP9.5	Protein gene product 9.5
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PM	Pyridoxamine
PNS	Peripheral nervous system
RAGE	Receptor of advanced glycation end-product
RGD	Arginyl-glycyl-aspartic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
STZ	Streptozotocin

T1DM	Type 1 diabetic mellitus
T2DM	Type 2 diabetic mellitus
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of matrix metalloproteinase
TN	Tenascin
TNFα	Tumor necrosis factor alpha
Trk	Tropomyosin-related kinase receptors
UDP-GlcNAc	Uridine Diphosphate-N-acetylhexosamine
VEGF	Vascular endothelial growth factor

Abstract

Diabetic symmetrical polyneuropathy is the most common secondary complication of diabetes, with no effective treatment, apart from maintaining tight glycemic control. It is therefore essential to understand the mechanisms underlying the pathogenesis of the disease in order to develop new therapeutic strategies. Biochemical and structural changes are observed in the extracellular matrix (ECM) of the peripheral nerve in diabetes: including increased endoneurial collagen; reduplication of basement membranes around endoneurial capillaries; a thickening of basal lamina; and accumulation of advanced glycation end-products (AGEs). In normal nerves, ischaemic or other damage to distal axons provokes a regenerative response; in diabetes this is abortive and failure of axonal regeneration is a hallmark of clinical and experimental diabetic neuropathy. Matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteolytic enzymes that cleave the protein components of the ECM. MMP-2 and MMP-9 play a central role in Wallerian degeneration and regeneration following nerve injury. This thesis investigates whether MMP-2 and -9 expression and/or activity were altered in the peripheral nerve in diabetes, and could contribute to regenerative failure in diabetic neuropathy. Using an experimental model of diabetes, we have demonstrated that MMP-2, but not MMP-9, is upregulated at gene, protein and activity levels in the rat sciatic nerve 8 weeks post-streptozotocin (STZ). This upregulation was not maintained at later time-points of diabetes. *In vitro* sciatic nerve cryoculture studies showed that peripheral nerve from STZ-diabetic rats was less supportive for neurite outgrowth from dissociated adult rat sensory neurons than nerve obtained from age-matched control rats. Cyrocultures were pre-treated with either MMP-2 or chondroitinase ABC, remodelling the peripheral nerve ECM, via the removal of inhibitory chondroitin sulfate proteoglycans from the sciatic nerve, and significantly enhanced its ability to support axonal regeneration, and partially restored the diabetes-associated regenerative deficit. However, exogenous MMP-2 or MMP-9 did not directly affect neurite outgrowth of dissociated adult rat sensory neurons. Finally, we assessed the neuroprotective effects of the AGE inhibitors LR90 and pyridoxamine in experimental diabetes, using a number of electrophysiological, behavioural and biochemical endpoints. These inhibitors were effective at preventing the development of some of the functional deficits observed in STZ-diabetes. Sensory nerve conduction velocity deficits and lipid peroxidation in the sciatic nerve were prevented by both LR90 and pyridoxamine. These agents have potential for the treatment of diabetic neuropathy.

Declaration and Disclaimer

I, Heather E Brooke, declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

All of the work described here was conducted by me except for the following:

1) Dr Sumia Ali performed quantitative real time PCR and MMP-2 and s100 co-localisation in Chapter 2, and ELISAs in Chapter 4.
2) Dr Natalie Gardiner performed the NCV measurements in Chapter 2 and 4.
3) Laura Smith and Dr Ian Millar performed thermal and mechanical behaviour testing in Chapter 4.

Copyright Statement

Autobiographical Statement

I obtained my undergraduate degree in Biochemistry with Industrial Experience (2:1 Class) from the University of Manchester in 2006. During my degree I spent a year working at Nationwide Laboratories, an accredited Veterinary Diagnostic and Clinical Pathology Laboratory based in Poutlon-Le-Fylde. My final year undergraduate project was entitled *'The advance of current purification techniques of the Breast Cancer Resistance Protein (BCRP)'*, which I completed under the supervision of Professor Robert Ford. In October 2006 I commenced my PhD under the supervision of Dr Natalie Gardiner and Professor Dame Nancy Rothwell. I have been investigating the role of matrix metalloproteinases in experimental diabetic neuropathy.

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Next, I have to say a big thank you to my family. You have always been hugely supportive throughout my life, always happy to take random phone calls asking for spelling advice and always encouraging me to push that little harder when things got tough. Thank you for always understanding, whenever I've been too busy to see you as often as I would have liked. Your never ending love has helped me through all the good and bad times over the years. Thank you so much, I love you guys more than I can say!

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This thesis is dedicated to my shiny penny.

"I offer the world my genius, all I ask in return is the world cover my expenses" – Robert Rankin

1.1. Introduction

Diabetes mellitus is a disease of global epidemic proportions which is increasing at an alarming rate. Diabetic symmetrical polyneuropathy is the most common secondary complications of diabetes, with no effective treatment, apart from maintenance of tight glycemic control. The Diabetes Control and Complications Trial research group confirmed that both the incidence and severity of neuropathy in patients with type 1 diabetes were worse in patients who experienced periods of sustained hyperglycaemia, or regular episodes of hyperglycaemia, thus indicating glucose as a primary cause in the pathogenesis of diabetic neuropathy. Hyperglycaemia drives multiple secondary biochemical changes including formation of reactive oxygen species and advanced glycation endproducts (AGEs). These biochemical changes contribute to many of the structural alterations and functional deficits observed in experimental and clinical diabetic neuropathy, such as reduced nerve conduction velocity and failed axonal regeneration

This thesis investigates the expression and regulation of matrix metalloproteinase (MMP)-2 and MMP-9 in the peripheral nervous system (PNS) in an experimental model of diabetes. MMPs constitute a large family of calcium-dependent proteolytic enzymes which are best known for their ability to remodel the extracellular matrix (ECM). MMPs play a crucial role in Wallerian degeneration following nerve injury, mediating blood-nerve barrier breakdown, removal of myelin debris and damaged axons and degradation of ECM components, and enabling nerve regeneration. The therapeutic potential of the AGE inhibitors, LR-90 and pyridoxamine in treating experimental diabetic neuropathy is also examined.

This introductory chapter will first introduce the PNS, and describe the basic anatomy of the PNS, nerve fibre structure, and nerve function before introducing the ECM of the peripheral nerve. The second section of this introduction will introduce diabetes mellitus, associated biochemical abnormalities and functional consequences of diabetic neuropathy. The third section will introduce the reader to MMPs; their structure, function and biology, largely focussing on their role in Wallerian degeneration and nerve regeneration, their altered expression in diabetes and finally the observations that lead us to focus our research on their expression in the PNS of streptozotocin-induced diabetic rats. The final section

will present the main hypotheses of this thesis, and describe the structure of this alternative format thesis. The specific aims and objectives of each result chapter will then be presented.

1.2. Peripheral nervous system

The peripheral nervous system (PNS) can be divided into three parts: the somatic, autonomic and enteric systems (Longstaff, 2005). The somatic divisions of the PNS consist of the sensory and motor nervous systems (Delcomyn, 1998;Hammond, 1996) and provide the link between the central nervous system (CNS) and peripheral tissues (FitzGerald et al., 2007). Sensory neurons are pseudo-unipolar, meaning they extend one axon which branches into two: a centrally-directed axon towards CNS and a peripherally-directed axon, which terminates in target tissues (Snell, 2006). Primary afferent nerves are involved with transmitting sensory information such as movement, pain and temperature from receptors within the peripheral organs and tissue of the body to the CNS (Kiernan, 2005). Whilst the motor efferent nerves carry nerve impulses from the CNS to effectors such as muscles (Martin and Jessell, 1993a).

The cell bodies of the sensory neurons lie within dorsal root ganglia (DRG; Figure 1.1), which are structures on the posterior root of the spinal nerves, and are located in protective cavities of the vertebral column (Kiernan, 2005;Longstaff, 2005). Each ganglia is surrounded by a layer of connective tissue that is continuous with the epineurium and perineurium of the peripheral nerve (see Figure 1.2) (Snell, 2006). There are 43 pairs of DRG in total (31 spinal nerves and 12 cranial nerves), which are named according to their position along the length of the spinal column, namely cervical, thoracic, lumbar (L) and sacral (Longstaff, 2005).

Sensory neurons in L4/5 DRG mainly project axons along the sciatic nerve, the largest and longest nerve in a mammal's body (Gardner and Bunge, 1993). The sciatic nerve innervates the skin, joints and muscle of the leg and foot via its articular and muscular branches. The articular branch (*rami articulares*) arises from the upper part of the nerve and supplies the hip-joint, perforating the posterior part of its capsule. Whilst the muscular branch (*rami musculares*) firstly distributes the muscles of the thigh, and in the lower third of the thigh, separates into its two components: the tibial and common peroneal nerves (Gardner and Bunge, 1993). Both these branches innervate the skin and muscles of the lower leg with the tibial nerve going on to innervate all muscles of the foot (Gardner and Bunge, 1993). The cell bodies of motor neurons lie within the ventral horn of the grey

matter of the spinal cord (Figure 1.1) (Longstaff, 2005) and extend myelinated efferent fibers along the ventral root and peripheral nerve, terminating in specialised neuromuscular junctions.

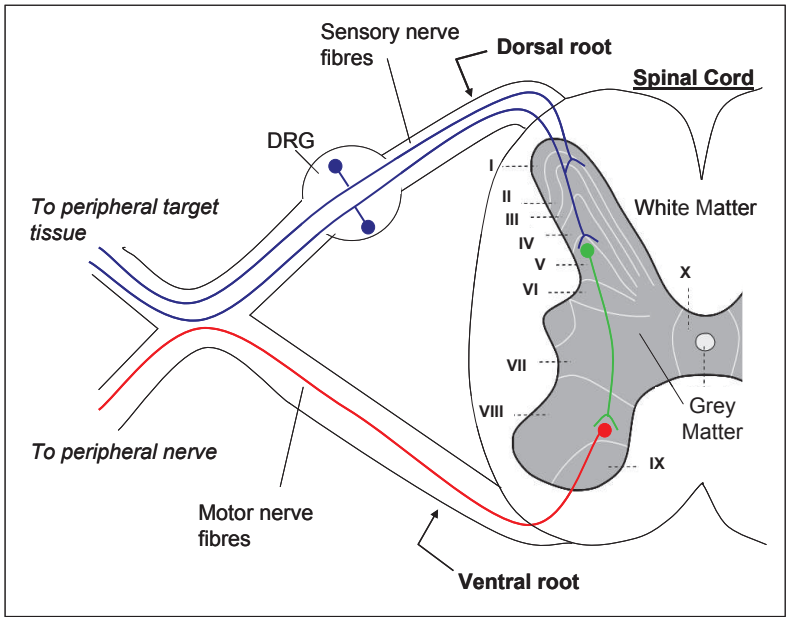

Figure 1.1: **Anatomy of the sensory and motor nervous system.** The spinal cord is connected to the peripheral organs via the ventral and dorsal roots, containing the motor efferent and sensory afferent nerves respectively. The nerves from both roots unite a short distance from the spinal cord forming the mixed population peripheral nerve. Primary afferent fibres synapse with dorsal horn neurons in specific laminae of the grey matter of the dorsal horn of the spinal cord (Adapted from (Snell, 1997)).

1.2.1. Sensory neurons are a mixed population

The DRG contain subpopulations of heterogeneous sensory neurons (Kiernan, 2005;Tucker et al., 2006), which can be categorised according to their neurochemistry, morphology, trophic requirements and sensory modalities into three major cell groups (Table 1.1; (Tucker et al., 2006)).

The first group comprise of the medium-large diameter neurons (>40µm). These neurons express the low affinity binding neurotrophic receptor $p75^{(NTR)}$, and the tropomyosin-related kinase receptors (Trk) A, B and C (Averill et al., 1995;Priestley et al., 2002). Consequently, they are able to respond to neurotrophins, such as neurotrophin-3 (NT-3) and brain derived growth factor (BDNF), and to a lesser extent, nerve growth factor (NGF)

(Tucker et al., 2006). These neurons are often identified based on their expression of heavy neurofilament protein (NF-H, NF200). The two other groups of small-medium diameter (<40μm) sensory neurons can be divided into the peptidergic neurons, which express calcitonin gene-related peptide (CGRP) and substance P, high levels of $p75^{NTR}$ and TrkA; thus, preferentially responding to NGF (Tucker et al., 2006) and non-peptidergic neurons, which bind isolectin B4 (IB4) (Averill et al., 1995;Priestley et al., 2002) and express P2X3, the receptor tyrosine kinase RET and growth factor receptor-α1 subunit. Therefore, they are responsive to glial-derived neurotrophic factor (GDNF), and largely unresponsive to NGF, NT-3 and BDNF (Molliver et al., 1997;Kashiba et al., 2001;Bennett et al., 1998).

	Axon	Diameter	Neurotrophic factor receptor	Respond to
Large- and medium- neurons (NF200-positive)	Myelinated	>40μm	$p75^{NTR}$ Trk-A, -B and -C	NT-3 NGF BDNF
peptidergic unmyelinated neurons (CGRP-positive)	Unmyelinated	<40μm	$p75^{NTR}$ TrkA	NGF
non-peptidergic unmyelinated neurons (IB4-positive)	Unmyelinated	<40μm	tyrosine kinase RET growth factor receptor-α1	GDNF

Table 1.1: **Subpopulation of the sensory neurons and their neurotrophic receptors.** Different subpopulations of sensory neurons respond to different neurotrophic factors via their expression of specific neurotrophic receptors. Some overlap does exist between sensory neuron subtypes and neurotrophic responses (Adapted from (Tucker et al., 2006)).

1.2.2. Peripheal-directed axons

The axons contained in the peripheral nerve can also be classified into subpopulations according to a number of criteria (Table 1.2).

The axons of the peripheral nerve terminate in specialised receptors according to their sensory function. There are four main receptor types in the periphery: mechanoreceptors, which respond to mechanical force; proprioceptors, which sense movement and where body parts are in relation to one another; thermoreceptors, which transduce changes in

temperature; and nociceptors, which respond to high threshold noxious, pain-producing stimuli (mechanical, thermal or chemical) (Longstaff, 2005).

Fiber	Axon Diameter (μm)	Mean NCV (m/sec)	Function
Aα	15	100	Motor neurons
Aβ	8	50	Skin touch afferents
Aγ	5	20	Motor to muscle spindles
Aδ	4	15	Skin touch and temperature afferents
C	1	1	Unmyelinated pain and temperature afferents

Table 1.2: **Classification of peripheral nerve fibres.** Axons can be classified (Erlanger/Gasser ref) into sub-populations according to their diameter and nerve conduction velocity (NCV) and function (Adapted from (Longstaff, 2005)). Axons of the PNS vary in calibre between 1-15μm, NCV reflects a number of parameters including axon diameter, myelin thickness, internodal distance and structural integrity at the nodes of Ranvier (Waxman, 1977).

Generally, there is a correlation between the type of sensory modality detected and the type of afferent fibres involved. The large myelinated Aα and Aβ nerve fibres are predominantly associated with mechanoreceptor and proprioceptors, terminating in muscle spindles and Golgi tendon organs in muscle, joints, and deep cutaneous layers of the skin (Martin and Jessell, 1993b). There are four main types of mechanoreceptors in the skin of humans: Pacinian corpuscles, Meissner's corpuscles, Merkel's discs and Ruffini corpuscles (Martin and Jessell, 1993b). Thermoreceptors are naked terminals of small-diameter afferent nerves that innervate various tissues including the skin (as cutaneous receptors), cornea and bladder in mammals. Warm thermoreceptors respond to increases in skin temperature between 29-45^{o}C via slow conducting unmyelinated C fibres. Cool thermoreceptors, which are sensitive to temperatures between 5-40^{o}C, respond via myelinated, faster conducting Aδ fibres (Longstaff, 2005;Martin and Jessell, 1993b). Nociceptors are the bare endings of small-diameter high-threshold afferent nerves that sense noxious, pain-producing stimuli. They are found in any area of the body that can sense pain, either externally or internally. The nociceptors are classified by what excites them; mechanical nociceptors are stimulated by intense mechanical forces via Aδ-fibres; thermal nociceptors are excited by changes in temperature via Aδ- and C-fibres; whilst

polymodal nociceptors respond to a puncture and a variety of noxious stimuli likely to cause tissue damage via the slower C-fibres (Longstaff, 2005;Martin and Jessell, 1993b).

1.2.3. Centrally-directed axons

Sensory afferent nerves interact with second-order neurons in the dorsal horn of the grey matter of the spinal cord (Figure 1.1). The second-order neurons act as a relay between the periphery and the midbrain and the cerebellum, sending out an axon along the ascending tracts to the CNS. There are ten distinct regions within the grey matter of the spinal cord, which are referred to as Rexed laminae (Figure 1.1). Each laminae has distinctive input-output relations, which reflect a measure of its functional specialisation (Longstaff, 2005;Martin and Jessell, 1993b), demonstrated by the fact that the different subpopulation of sensory neurons synapse with distinct laminae (Martin and Jessell, 1993b). Different laminae regions are also associated with different sensations, with laminae I-IV concerned with exteroceptive sensations and laminae V and VI primarily concerned with proprioceptive sensation (Longstaff, 2005;Martin and Jessell, 1993b). The small diameter nociceptive unmyelinated C-fibre and thinly myelinated Aδ-fibre afferents synapse with second order neurons in laminae I and II, with the Aδ afferents forming synapses in laminae V (Jinks and Carstens, 2000). Even though they innervate similar peripheral tissue, peptidergic C-fibres innervate laminae I and II (outer) and non-peptidergic C-fibres terminate almost exclusively in laminae II (inner) (Hunt and Mantyh, 2001). The larger myelinated Aβ fibres connect with laminae I, III & V. The motor neuronal cell bodies, which synapse with skeletal muscles, lie in laminae VIII and IX of the ventral horn of the grey matter (Figure 1.1; (Martin and Jessell, 1993a)).

Surrounding the grey matter in the spinal cord is the white matter, which largely contains axons and associated glia (Martin and Jessell, 1993a). The afferent Aδ- and C-fibres of the sensory nervous system branch and enter the ascending and descending tracts of the Lissauer's tracts before terminating at the second-order neurons in laminae I, II IV-VI regions in the dorsal horn of the grey matter (Martin and Jessell, 1993a). The axon of these second-order neurons cross the midline and ascends in the anterolateral quadrant of the contralateral half of the spinal cord, where they join the spinothalamic tract. There are two main parts of the spinothalamic tract: the lateral spinothalamic tract, which transmits pain

and temperature and the anterior spinothalamic tract, which transmits crude touch (Longstaff, 2005). After entering the spinothalamic tract, the axons travel up the length of the spinal cord into the brainstem where the neurons ultimately synapse with third-order neurons in several nuclei of the thalamus, including the medial dorsal, ventral posterior lateral, and ventral medial posterior nuclei (Martin and Jessell, 1993a). The interneurons within laminae VIII and IX form the final motor pathway to initiate and modulate motor activity via α and γ motor neurons, which innervate striated muscle (Longstaff, 2005). All visceral motor neurons are located in laminae VII and innervate neurons in autonomic ganglia (Longstaff, 2005).

1.2.4. Peripheral nerve structure

The majority of the peripheral nerve contains bundles of nerve fibres called fascicles (Figure 1.2A). Each fascicle is encased by another connective sheath called the perineurium, which consists of several layers of flattened epithelium cells. Within each fascicle, the individual nerve fibres are covered by a network of reticular collagenous fibers called the endoneurium (FitzGerald et al., 2007;Kiernan, 2005), and the space between each fascicle is filled by interfascicular collagen (COLL) perineurium (Figure 1.2B) (Kiernan, 2005). The outmost layer of the peripheral nerve structure is the epineurium, which is composed of connective tissue and the blood vessels supplying the nerve (Kiernan, 2005).

Schwann cells may encase the axon with a myelin sheath, which electrically insulates the axon (Figure 1.2A and 2B). The Schwann cells are aligned discontinuously along the axon, and the myelin sheath is interrupted at regular intervals by nodes of Ranvier (Figure 1.2A). The Schwann cells associate with the basal lamina, which consists of extracellular matrix (ECM) components such as COLL type-IV, laminin (LM), tenascin (TN) and fibronectin (FN) (Platt et al., 2003). The basal lamina extends as an uninterrupted sleeve along the length of the whole axon (Bunge, 2007). External to the basal lamina is the ECM of the endoneurium, which occupies the extracellular space between each of the axon-Schwann cell units (Delcomyn, 1998;Hammond, 1996).

Unmyelinated axons are grouped within Remak bundles (see Figures 1.2A & 1.2B) (Murinson and Griffin, 2004). Each Remak bundle is comprised of one or more

unmyelinated axons ensheathed by Schwann cells and arranged within a single basal lamina (Murinson and Griffin, 2004). The Schwann cell of non-myelinating axons is involved in the maintenance of axons and is crucial for neuronal survival (Murinson and Griffin, 2004).

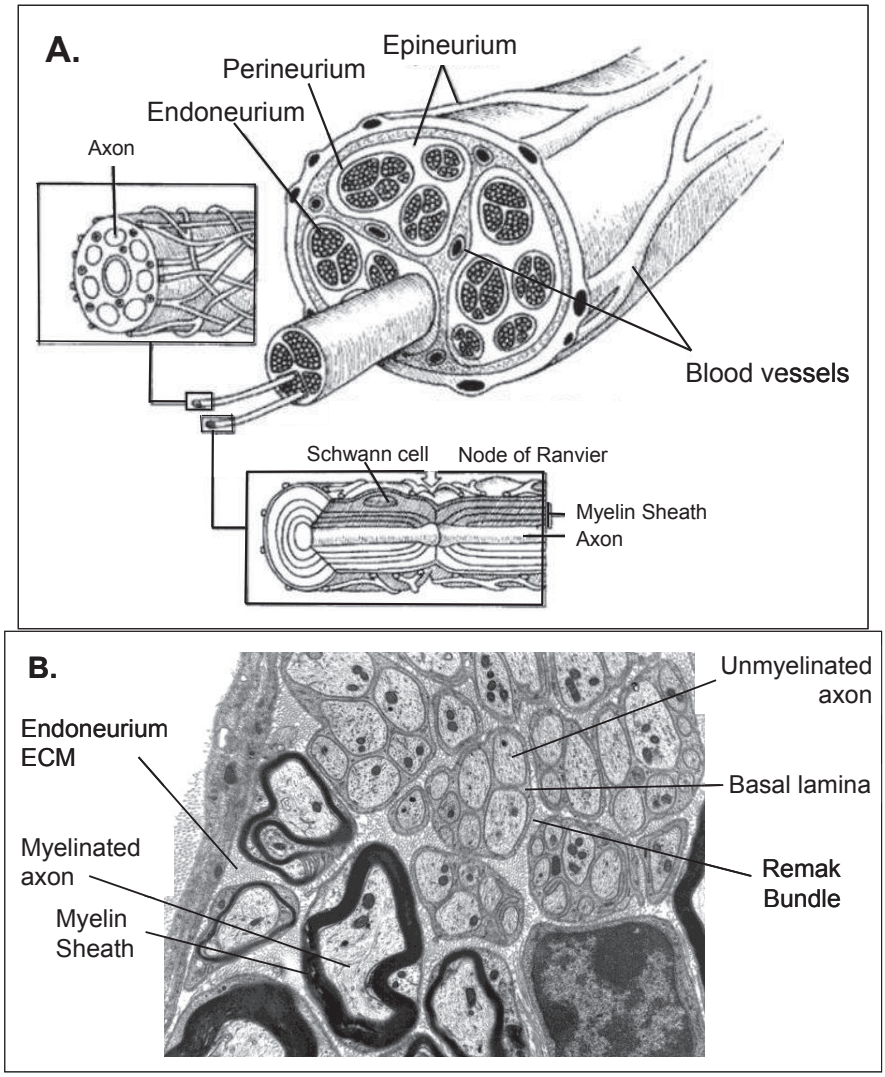

Figure 1.2: **An illustration of the different components of the peripheral nerve.** ***(A) Nerve fibre structure.*** The epineurium, a connective tissue sheath, encases the peripheral nerve with each fascicle encapsulated by another connective tissue shear, the perineurium. Axons are insulated by Schwann cells, which are the myelin-producing cells of the PNS. The nodes of Ranvier are unmyelinated gaps between myelin sheaths (Stroncek and Reichert, 2008). ***(B) Individual nerve facicle.*** This high powered image shows individual myelinated nerve fibres and Remak bundle structures, which contain groups of unmyelinated axons. The Schwann cell basal lamina surrounds the nerve fibres and between each of the fascicles are interfascicular endoneurial collagen fibres (Adapted from image by David Tomlinson).

The main blood vessels of the peripheral nerve are found in the epineurium and perineurium and give rise to a network of capillary vessels in the endoneurium of each individual fascicle (Figure 1.2A) (McManis et al., 1993). All blood vessels that enter and terminate within the nerve are termed the *vasa nervorum*; they arise from nutrient arteries, which branch from main arteries in the limbs and provide a direct conduit of blood to the nerve (McManis et al., 1993). The perineurium of the nerve acts as a protective barrier to the nerve fibres by shielding it from the blood-borne substances of the endoneurial capillaries. This blood-nerve barrier (BNB) property of the perineurium is provided by a layer of epithelial cells that encircle the nerve fascicles. These epithelial cells have closed contact and tight junctions between the endothelial cells of the capillaries providing an impermeable barrier (Seneviratne, 1972;Gerhart and Drewes, 1990). Proteins that are known to play a pivotal role in the generation of these tight junctions include occludin and claudin (Furuse et al., 1993;Furuse et al., 1998). The BNB regulates the endoneurial microenvironment by resisting the passage of ions, water soluble non-electrolytes and macromolecules (Gerhart and Drewes, 1990). The presence of a glucose transporter within the perineurium of the peripheral nerve enables the BNB to also provide the nerve fibres with an essential nutrient delivery system to the peripheral nerve metabolism (Gerhart and Drewes, 1990). This suggests that glucose is supplied to the nerve fibres via the extrafascicular network of blood vessels and endoneurial capillaries (Gerhart and Drewes, 1990). This barrier function of the perineurium is not static, meaning it alters in response to changes in the surrounding environment, including changes in oxygen tension, by inflammatory processes or in response to nerve injury (Seneviratne, 1972).

1.2.5. Peripheral nerve extracellular matrix

The major component of the peripheral nerve is the ECM, an intrinsic network of macromolecules that fills the extracellular space that surrounds all cells (Alberts et al., 2009). The ECM is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organised meshwork in close association with the surface of the cell that produced them (Alberts et al., 2009). It was once thought that the sole function of the ECM was to provide an inert scaffold that simply offered the tissue structural stability. However, it is now known that the ECM plays a far more active and complex role in regulating the behaviour of the cells that it contacts.

The most important function of the ECM is the regulation of cell adhesion to the basement membrane of neighbouring cells, and it is this cell-cell attachment that is critical for the maintenance of tissue integrity (Gumbiner, 1996). Cell-to-ECM adhesion is regulated by specific cell surface cellular adhesion molecules (CAMs), including a family of cell surface proteoglycans called syndecans and a large family of heterodimeric transmembrane proteins called integrins (Gumbiner, 1996;Taipale and Keski-Oja, 1997). Another function of the ECM is its ability to sequester and release a wide range of cellular growth factors and to supply the cells with growth factors in the absence of new protein synthesis. It does this by releasing growth factors 'buried' within the ECM via matrix degradation or cell movement (Taipale and Keski-Oja, 1997). The ECM of the peripheral nerve has four main categories of components: fibrous proteins, glycoproteins, glycosaminoglycans and proteoglycans (Hill, 2009).

1.2.5.1. Fibrous proteins

COLL is the most abundant of all the fibrous proteins of the ECM, with 49% of total protein in the whole nerve composed of COLL- I and -III (Bunge, 2007). There are many sub-types of COLL present in the peripheral nerve ECM (Table 1.3); however, their function in nerve development and regeneration varies widely.

	Epineurium	Perineurium	Endoneurium
Collagen Type-I	+	+	+
Collagen Type-III	+	+	+
Collagen Type-IV		+	+
Collagen Type-V		+	+

Table 1.3: **Distribution of collagen in the mature peripheral nerve.** COLL sub-types are expressed in different regions of the peripheral nerve (Tonge et al., 1997;Bradley et al., 2000).

COLL-I and COLL-III are the main components of collagen fibrils, with COLL-I also being the major COLL constituent in the peripheral nerve (Bradley et al., 2000), neither play a role in the support of axonal elongation during regeneration (Tonge et al., 1997) suggesting that the main function in the nerve is a structural one. COLL-IV is the major

structural component of all basement membranes and is thought to act as an ECM organiser by providing a scaffold onto which the rest of the basement membrane is built (Hill and Williams, 2002). COLL-IV also has an important positive effect in terms of axonal regeneration, with its presence shown to support axonal elongation (Tonge et al., 1997).

Laminin (LM) is the most abundant non-collagenous component of the peripheral nerve basement membrane (Hill and Williams, 2002). Seven LM types have been shown to be expressed in the mature peripheral nerve (Table 1.4). LM has been shown to play an important role in PNS development where is it vital in the proliferation, differentiation and subsequent survival of Schwann cells during normal development (Chen and Strickland, 2003) and the development of neuromuscular junctions (Feltri and Wrabetz, 2005). In the mature peripheral nerve, LM influences an important positive effect on axonal elongation (Chen et al., 2007;Hill, 2009) and is involved in intracellular signalling via its interaction with cellular receptors, such as integrins ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha7\beta1$ and $\alpha6\beta4$), as well as dystroglycans and some non-fibrillary COLLs (Feltri and Wrabetz, 2005).

	DRG	Perineurium	Schwann cell basement membrane	Node of Ranvier	Neuromuscular Junctions
Laminin-2	+		+	+	
Laminin-4					+
Laminin-8	+		+		
Laminin-9		+			+
Laminin-10				+	
Laminin-11		+			+
Laminin-12			+		

Table 1.4: **Distribution of laminin in the mature peripheral nerve.** LM sub-types expression in the different regions of the peripheral nervous system (Feltri and Wrabetz, 2005).

Tenascin (TN) is another glycoprotein, whose expression levels are only high in early development, where it acts as a mediator of neuron-glia interactions (Hill, 2009;Matsumoto et al., 2002). However, following maturity of the nervous system, tenascin expression is low but is re-expressed in wound healing and nerve regeneration (Jones and Jones, 2000). There are five members of the TN family (TN-C, TN- R, TN-W, TN-X and TN-Y), which are tightly regulated by growth factors, cytokines, ECM protein and other biomechanical

factors (Matsumoto et al., 2002). All the TN family members, except TN-W, are expressed in the PNS, Table 1.5 shows the distribution of TN in the peripheral nerve. TN is generally classified as an anti-adhesive protein since cells do not adhere to it, and if they do, they do not spread. Whereas, sensory neurons and growth cones adhere well to TN and a TN substrate can support neurite extension (Wehrle-Haller and Chiquet, 1993;Wehrle and Chiquet, 1990). TN has been shown to interact with other extracellular proteins, including fibronectin (FN), and also bind with high affinity to a class of extracellular chondroitin sulfate proteoglycans (CSPGs) as well as CAMs of the immunoglobulin super-family (Jones and Jones, 2000). Furthermore, TNs interact with and are cleaved by serine proteinases and matrix metalloproteinases (MMPs) (Jones and Jones, 2000).

	Spinal root / DRG	Perineurium	Endoneurium	Node of Ranvier
Tenascin-C		+		+
Tenascin -R				
Tenascin-X		+	+	
Tenascin -Y	+			

Table 1.5: **Distribution of tenascin in the mature peripheral nerve.** TN sub-types expression in the different regions of the peripheral nervous system (Matsumoto et al., 2002).

FN is a fibrous protein of the peripheral ECM that is predominately expressed in the perineurium, and also present in the endoneurium of the sciatic nerve (Palm and Furcht, 1983;Bunge, 2007). FN plays a role in both neuron development and axonal regeneration in the mature nervous system. However, the FN growth-promoting response is generally observed to be weaker than that of LM (Gardiner et al., 2007;Bunge, 2007). FN provides a permissive substrate for cell migration and is also thought to be involved in the neuronal-Schwann cell interaction (Palm and Furcht, 1983). FN's ability to enhance neurite outgrowth is mediated through integrins, $\alpha 5\beta 1$ and $\alpha 4\beta 1$, (Gardiner et al., 2007) and its involvement in the adhesion of various cells to COLL, especially COLL-I and COLL-III (Palm and Furcht, 1983).

1.2.5.2. Proteoglycans

Proteoglycans are heavily glycosylated protein which have a core protein with one or more covalently attached glycosaminoglycan (GAG) chain(s); these GAG chains attach at the serine side chain on the core protein. The chains are long, linear polysaccharides consisting of repeated disaccharide units, which are negatively charged under physiological conditions due to the occurrence of sulfate and uronic acid groups. The GAGs present in the peripheral nerve ECM are chondroitin sulphate, dermatan sulphate, heparin and heparan sulphate, and keratan sulfate (Alberts et al., 2009). They form large complexes, both with other proteoglycans, hyaluronan and with fibrous proteins of the ECM, including COLL, FN and LM (Ruoslahti, 1988). Proteoglycans have been shown to affect the activity and stability of proteins and signalling molecules within the matrix (Tonge et al., 1997).

Important proteoglycans include chondroitin sulphate proteoglycans (CSPGs) and heparin sulphate proteoglycans (HSPGs). CSPGs are expressed on the surface of most cells and in the ECM of most tissues, and are important regulators of many biological processes, such as cell migration, cell recognition and bone development (Properzi and Fawcett, 2004). CSPGs are inhibitory to both nerve development and regeneration. During development, CSPGs play an important role in facilitating axon guidance by their association with boundary regions that block axon extension during development (Hynds and Snow, 1999;Katoh-Semba et al., 1995;Snow et al., 1996). Following nervous system injury, and in diseases of aging, CSPGs are associated with axon devoid regions and degenerating nerve cells and block successful regeneration (Snow et al., 2001). CSPGs inhibitory function occurs via its binding to, and the consequential blocking of, the neurite-promoting properties of the LM in the endoneurial basal lamina (Zuo et al., 1998a). Removal of GAG sidechains with chondroitinase ABC (ChABC) removes this inhibitory function. ChABC has been shown to enhance neurite outgrowth of embryonic and adult sensory neurons plated on sciatic nerve section (Zuo et al., 1998a;Zuo et al., 1998b) as well as improves axonal regeneration and functional recovery following spinal cord injury *in vivo* (Galtrey and Fawcett, 2007). HSPGs are another proteoglycan found in the ECM of the peripheral nerve. HSPGs are known to enhance outgrowth of embryonic neurons (Tonge et al., 1997). However, HSPGs function in adult sensory neuron regeneration is less well understood. It is known that HSPGs bind to the globular region of LM thereby suggesting its inhibition of

growth-promoting function of LM (Tonge et al., 1997). However, the addition of HSPG to matrigel does not alter the neurite outgrowth capability of adult mice sensory neurons (Tonge et al., 1997).

1.3. Diabetes mellitus

Diabetes mellitus is a chronic life-threatening disease characterised by hyperglycaemia and/or hypoinsulinemia. The disease has reached epidemic proportions in the Western world and approximately 2.6 million people have been diagnosed with it in the UK; moreover, there is an estimated half a million undiagnosed people who have the disease. This equates to roughly 1 in 20 people in England. Diabetes mellitus has a massive impact on the NHS with approximately 10% of its budget being spent on the disease (Based on 2007/2009 budget); this works out at approximately £9 billion a year. Diabetes mellitus also affects health and social care services as people with the disease are twice as likely to require hospital care; to experience prolonged stays in hospital; and to incur social care service costs as they require residential and nursing care (Diabetes UK, 2010). Most health experts predict a huge increase in the number of people with diabetes in the UK. Since 1996, the number of people diagnosed with the condition has nearly doubled from 1.4 million and by 2025, it is estimated that over 4 million people in the UK will have the disease. Globally, it is thought that the impact of diabetes will be even greater, with an estimated 150% rise in the condition by 2030. Most of these cases will be Type 2 diabetes due to the ageing population and the rapidly rising number of overweight and obese people (Diabetes UK, 2010). Other factors in this global rise include the predicted explosive increase of diabetes mellitus in developing countries (Rolo and Palmeira, 2006).

There are two defined types of diabetes mellitus: insulin dependent Type 1 diabetes mellitus M (T1DM) and insulin independent Type 2 diabetes mellitus (T2DM). T1DM is an autoimmune disease that develops due to the body's own destruction or dysfunction of the β-cells of the pancreas. T1DM usually appears before the age of forty and is a multi-factorial polygenetic disease, which results from the interaction between environmental and genetic factors (Nosikov and Seregin, 2008). Multiple studies have concluded that whilst there is a low family risk, T1DM does depend on genetic factors to a substantial extent, with over 20 loci reported to influence susceptibility to T1DM (Davies et al., 1994;Concannon et al., 1998). It is now commonly accepted that the autoimmune process that causes T1DM develops when the immune tolerance mechanism is distorted and a destructive immune response to the body's own protein arises after a virus infection or another immune stress (Nosikov and Seregin, 2008).

T2DM accounts for 90% of diabetes mellitus cases and usually develops in people over the age of 40. However, the incidence and prevalence of T2DM in children and young adults is increasing (American Diabetes Association, 2000). In the UK, children as young as 7 have been diagnosed with T2DM, with the first case being diagnosed in 2000 (Diabetes UK, 2010). T2DM develops when the body is no longer able to regulate the amount of glucose in the blood due to the development of insulin resistance and/or β-cell secretary defects (Flier, 1983). There are multiple factors that can potentially give rise to or exacerbate T2DM, including obesity, hypertension and elevated cholesterol (American Diabetes Association, 2000). There is also a strong genetic link in T2DM development, with people of certain ethnic origins being more at risk of developing the disease than others (American Diabetes Association, 2000).

Diabetes mellitus contributes to the development of several secondary complications including retinopathy, nephropathy and neuropathy (Brownlee, 2001;McManis et al., 1993) resulting in patients developing blindness, end stage renal disease and multiple debilitating neuropathies respectively. The development of these secondary complications is a major cause of morbidity and mortality in diabetes mellitus, and is an ever-increasing burden to health care authorities in both developed and developing nations (Jakus and Rietbrock, 2004).

1.3.1. Diabetic neuropathy

Diabetic neuropathy is the most common secondary complication of diabetes, occurring in approximately 50% of T1DM patients, and is typically associated with long-term diabetes (> 25 years) and poor glycaemic control (The Diabetes Control and Complications Trial Research Group, 1993). The predicted global increase in the incidence of early-onset T2DM means the numbers of T2DM patients presenting with diabetic neuropathy is likely to rise (Feldman, 2003).

Diabetic neuropathy is an overall term given to a number of complex and heterogeneous disorders that affect multiple components of the nervous system. There are three broad types of neuropathy: sensory, motor and autonomic (Table 1.6), with distal sensory

polyneuropathy (DSPN) being the most common of the peripheral nerve disorders associated with diabetes (Thomas et al., 2005).

Neuropathy Related Terms	Nerves Affected	Symptoms
Sensory Neuropathy		
Distal neuropathy	Sensory	Pain
Femoral neuropathy		Numbness / Tingling in extremities
		Loss of protective sensation
Autonomic Neuropathy		
Gastroparesis	Autonomic	Impotence in men
Diabetic diarrhea		Bladder nerve dysfunction
Bladder neuropathy		Diabetic diarrhea
Postural hypotension		Bloated stomach
Charcot joint		
Unilateral foot drop		
Impotence		
Motor Neuropathy		
Diabetic amyotrophy	Motor	Muscle weakness

Table 1.6: Diabetic neuropathies and their symptoms. Sensory neuropathy is the most common of the neuropathy conditions (Adapted from (Thomas et al., 2005)).

DSPN is associated with distal axonopathy and die-back of the peripheral nerve fibres in a length-dependent manner. DSPN particularly affects the longest axons in the PNS, i.e. those supplying hands and feet, and is often described as having a 'glove and stocking' distribution. The loss of peripheral protective sensation, combined with poor wound healing (a feature of poorly controlled diabetes (The Diabetes Control and Complications Trial Research Group, 1993)), often leads to tissue damage, foot ulceration, gangrene and increased risk of amputation (Tomlinson and Gardiner, 2008;Feldman, 2003).

Currently, no treatment exists for diabetic neuropathy apart from careful glycaemic control. In 1993, a 9 year multicentre trial run by the Diabetes Control and Complication Trial Research Group (The Diabetes Control and Complications Trial Research Group, 1993)

showed that the incidence and severity of neuropathy in patients with T1DM was worsened by poor glycaemic control; thus, indicating glucose as a primary causative factor. Sustained hyperglycaemia, or regular hyperglycaemic episodes, has been shown to drive secondary biochemical changes and these, along with oxidative stress, in turn, impact upon cellular phenotype, causing functional and structural changes that can alter normal function. Whilst early metabolic anomalies can be reversed, the chronic progression of neuropathy ultimately leads to changes that are refractory to glycaemic control and to damage that is likely to become irreversible. Prolonged hyperglycaemia results in the development of structural changes in the nerve (including Schwann cell pathology; endoneurial microangiopathy; axonal degeneration; paranodal demyelination; and distal dying-back of myelinated and unmyelinated fibres) and the accessory electrophysiological defects that are thought to coincide with more chronic biochemical changes (Bisby and Chen, 1990;Ekstrom and Tomlinson, 1989;Kennedy and Zochodne, 2000;Bradley et al., 2000;Giannini and Dyck, 1995;Hill and Williams, 2002;Terada et al., 1998a).

1.3.2. Experimental models of diabetes

Several experimental models of diabetic neuropathy have been developed in rodents in order to study the underlying changes that may contribute to the development and pathology of diabetic neuropathy.

Alloxan, a cyclic urea analogue, or more commonly, the methylnitrosurea analogue of alloxan, streptozotocin (STZ), are compounds commonly used to produce experimental, insulin-dependent diabetes in laboratory animals (Rerup, 1970). STZ-induced diabetes is the most commonly used T1DM model in rodents. STZ accumulates in the β-cells of the pancreas by competing with glucose for uptake via the Glut-2 glucose transporter (Schnedl et al., 1994). STZ decomposes spontaneously, producing highly cytotoxic compounds including methyldiazohydroxide, which further decompose to form a highly reactive carbonium ion; this alkylkates many cellular components including DNA and proteins (Rerup, 1970). STZ administration results in β-cell death and long lasting hyperglycaemia, usually within 3 days of administration.

Other models of T1DM exist, including BB/W, a genetically-modified rat model in which spontaneous diabetic neuopathy occurs in 30-50% of animals between 60 and 120 days old (Seemayer et al., 1982). Immune-mediated destruction of the pancreatic β-cells means BB/W rats require daily insulin therapy treatment to survive (Seemayer et al., 1982;Sima and Sugimoto, 1999). Non-obesity diabetic mice also are susceptible to spontaneous development of T1DM in 60-80% of females and 20-30% of males. Insulitis (leukocytic infiltrate of the pancreatic islets) occurs in the mice at 4-5 weeks old, followed by subclinical β-cell destruction and decreasing circulating insulin concentrations (Rees and Alcolado, 2005). T1DM is rarely mild in these animals; however, the affected ones can survive for weeks without the administration of insulin (Rees and Alcolado, 2005). Mice heterozygous for the $Ins2^{Akita}$ mutation exhibit severe insulin deficiency despite co-expression of normal insulin molecules. Akita mice have a mutation of the insulin-2 gene whereby the mutation of a cysteine (Cys96), critical for the formation of an intermolecular disulfide bond, into a tyrosine results in protein mis-folding (Breyer et al., 2006) and therefore, the disordered architecture of secretary path organelles in the β-cells (Breyer et al., 2006).

Experimental models of T2DM include: the db/db mouse, which contains a single gene mutation in the leptin receptor; WKY/Ncp, which is known as the fatty Wister diabetic rat; WBN/Kob, which is a spontaneously diabetic model that develops chronic pancreatitis and non-insulin requiring diabetes; and the BB/ZDR, which possesses a defective leptin receptor gene (Sima and Sugimoto, 1999). The ob/ob, or obese mouse, is a mutant mouse that eats excessively and becomes profoundly obese because of a gene mutation in the leptin hormone, which is important in the control of appetite. At 8 weeks old, the ob/ob mouse weighs at least twice as much as wild-type controls and has hyperinsulinemia and hyperglycemia.

As diabetic neuropathy is a disease associated with long-term diabetes, the use of T1DM animal models has long been considered the most appropriate for the study of the pathogenesis of the disease. Althought T2DM models also show diabetic neuopathy (db/db, ob/ob and Zucker rats) (Drel et al., 2006;Brussee et al., 2008). STZ-diabetic rats show similar features to those observed in clinical diabetic neuropathy, such as: neurotrophin deficit (Sima and Sugimoto, 1999;Tomlinson et al., 1996); structural

abnormalities of the peripheral nerve such as distal axonal atrophy and a reduction in axonal calibre (Mizisin et al., 1999); and abnormalities of the nodal and paranodal region in nerves similar to those observed in diabetic neuropathy patients (Brismar et al., 1987), as summarised in Table 1.7.

	STZ rat	BB/W rat
Axonal atrophy	++	+++
Axoglial dysjunction	++	+++
Paranodel demyelination	++	+++
Segmental demyelination	+	+
Wallerian degeneration	+	+
Fibre loss	-	++

Table 1.7: **Features of neuropathy present in experimental models of the disease**. Comparision of functional deficits seen in the STZ and BB/W models of T1DM (+ mild, ++ moderate, +++ severe) (Adapted from (Sima and Sugimoto, 1999)). In addition, STZ and BB/W rats display increased polyol pathway flux(Greene and Lattimer, 1983), decreased endoneurial blood flow (Sima and Sugimoto, 1999), impaired neural Na^+/K^+-ATPase (Greene and Lattimer, 1983;Krishnan et al., 2008), nitric oxide synthase activities (Sima and Sugimoto, 1999) and impaired nerve regeneration (Bisby, 1980).

STZ-induced diabetic rats also display an altered pain threshold (Fox et al., 1999). STZ rats display tactile allodynia and mechanical hyperalgesia with a reduction in the nociceptive threshold of 30-40% (Wuarin-Bierman et al., 1987;Courteix et al., 1994;Calcutt et al., 1996;Courteix et al., 1993b;Courteix et al., 1993d); hypersenstivity to noxious chemical stimuli; and increased responses to intraplantar formalin injection (Calcutt et al., 1996;Calcutt et al., 1994;Courteix et al., 1993a;Malmberg et al., 1993). Conflicting results are described regarding thermal nociceptive thresholds; both thermal hyperalgesia and hypoalgesia (Calcutt, 2004;Courteix et al., 1993c;Forman et al., 1986) have been reported whilst other groups report no change in thermal thresholds (Raz et al., 1988) in experimental diabetic rats. In the clinic loss of thermal sensation has been reported (Akunne and Soliman, 1987;Ueno et al., 1996).

The STZ model has been criticised due to concerns regarding STZ neurotoxicity and loss of condition (Fox et al., 1999). Concerns as to whether the observed changes in pain thresholds may be attributed simply to the diabetic rats' ill-health rather than neuropathy

per se. STZ rats show signs of a poor general condition when they become very low in bodyweight, which results in greatly reduced general activity, extreme lethargy and thus, reduced exploratory behaviour (Fox et al., 1999;Hoybergs et al., 2008). However, insulin treatment of STZ rats prevents these alterations in pain thresholds and NCV deficits (Calcutt, 2004;Jakobsen and Lundbaek, 1976) . Thereby linking these deficits to presence of hyperglycaemia and/or hypoinsulinaemia and not to the animals' 'loss' of health (Greene et al., 1975;Jakobsen, 1979).

1.3.3. Functional Defects in diabetic neuropathy

1.3.3.1. Nerve conduction velocity abnormalities

Neurons are excitable cells, meaning they can be stimulated by either the axon terminal within its sensory organ or by synaptic inputs onto their dendrites or cell body. This stimulation results in the generation of an action potential, which rapidly conducts information along the nerve fibres, resulting in potential changes to the neuron's cell body (Longstaff, 2005). The NCV of a nerve fibre is affected by a number of factors including: axon diameter; myelin thickness; internodal distance; and structural integrity at the nodes of Ranvier (Waxman, 1977). NCV is reduced in rats as early as 2 to 3 weeks post STZ (Eliasson, 1964;Greene et al., 1975), and deficits are maintained (Price et al., 2004). This deficit is regarded as a good functional marker for the presence and progression of diabetic neuropathy in both experimental animals and in the clinic (Albers et al., 1996;Gregersen, 1968). At the early stages of the disease, preventing hyperglycaemia with strict insulin treatment means that NCV deficits are readily reversible (Greene et al., 1975), however at later stages NCV defcicts are not readily reversaible (Greene et al., 1975).

1.3.3.2. Altered nerve blood flow

Diabetes mellitus is associated with macrovascular diseases, hypertension, raised triglyceride levels and a high body-mass index (Tesfaye et al., 2005). The reduction in nerve blood flow velocity in clinical and experimental diabetes has been attributed to alterations in the nerve vasculature (Tuck et al., 1984;McManis et al., 1993). Using electron microscopy, the thickening and hyalinisation of the vessel walls and reduplication

of the basement membranes around the capillaries of endoneurial blood vessels have been observed in patients diagnosed with diabetes (Giannini and Dyck, 1995;Williams et al., 1980;McManis et al., 1993). This thickening of the diabetic blood vessel is due to a disturbance in the balance between the deposition and removal of fibrin (Williams et al., 1980) and the accumulation of other ECM molecules including COLL-I and –II in the basement membrane (Ban and Twigg, 2008). The thickening of the vessel wall results in impaired BNB function (Seneviratne, 1972). Endoneurial pressure increases, causing the disruption of the endothelial cells via cell damage, resulting in further vascular leakage and the seepage of haematological constituents into the vessel wall (Williams et al., 1980;Ashton, 1974). This progressive thickening results in nerve blood flow reduction and also tissue hypoxia because of the decreased rate of oxygen diffusion to the tissue (Tuck et al., 1984;Seneviratne, 1972). Hence, BNB breakdown and the subsequent reduced blood flow is thought to contribute to biochemical and morphological abnormalities seen in the diabetic peripheral nerve (Tuck et al., 1984;Seneviratne, 1972). Early BNB changes are thought to precede axonal degeneration or demyelination (Ohi et al., 1985).

1.3.3.3. Alteration in peripheral sensation

Chronic painful sensory neuropathy is estimated to affect about 1 in 6 people with diabetes in the UK, compared to 1 in 20 in an age and sex matched control group (Diabetes UK, 2010).

Patients with painful DSPN may experience paresthesia, dysthesia, hyperalgesia and allodynia (Calcutt, 2004). Paresthesia refers to abnormal skin sensations such as itching, burning and tickling without an apparent physical cause. Dysthesia is the impairment of senses and includes sensations similar to pins and needles. Hyperalgesia is a heightened response to painful stimuli and allodynia is the feeling of pain from a normally non-noxious stimulus (Calcutt, 2004). Pain is commonly worse at night and most frequently felt in the feet and lower limbs (Boulton et al., 2005). In addition, distal loss of afferent fibres leads to an altered perception of thermal, tactile and vibratory stimuli and can lead to many patients experiencing loss of sensation in their hands and feet (Beiswenger et al., 2008b;Said, 2007), and these patients are at high risk of insensate injury to their feet (Boulton et al., 2005).

The skin is innervated by low-threshold mechanoreceptors, thermoreceptors and nociceptors, their myelinated (A-fibres) and unmyelinated axons (C-fibres) (Ebenezer et al., 2007). Intraepidermal nerve fibres (IENF) are predominantly unmyelinated C-fibres involved in the detection of thermal nociceptive pain (Malmberg et al., 2004). These IENF form subepidermal bundles in the papillary dermis which are immediately adjacent to the stratum basale of the epidermis. The individual fibres lose their Schwann cell ensheathment as the axons cross the dermal-epidermal junction and run through the keratinocytes of the epidermis (Lauria and Devigili, 2007). The epidermal nerve endings can be divided into subsets - peptidergic and non-peptidergic. The peptidergic neurons are NGF-responsive and express calcitonin gene-related peptide (CGRP), substance P and TrkA receptor; whilst non-peptidergic are GDNF-response and express $GDNFR_{\alpha}$ and the P_2X_3 receptors (Snider and McMahon, 1998).

In control conditions, cutaneous nerves have a vigorous ability to reinnervate the skin following damage, either by regeneration of degenerated axons or by collateral branching of undamaged axons (Beiswenger et al., 2008b;Beiswenger et al., 2008a). Collateral sprouting of axons is a branching outgrowth of new axon terminals from uninjured axons, leading to an expansion of their receptive field. Collateral sprouting is stimulated by the production of NGF from skin cells (Murray and Thompson, 1957).

Both epidermal and dermal nerve fibres show structural abnormalities in patients with diabetic neuropathy including segmented degeneration and swelling (Yasuda et al., 2003). IENF density is reduced in both clinical T1DM and T2DM and experimental animal models (Beiswenger et al., 2008a;Lauria and Devigili, 2007). This depletion in IENF represents loss of peripheral terminals of C-fibres, and is seen as an early index of diabetic neuropathy (Ebenezer et al., 2007;Beiswenger et al., 2008b). It is this loss of IENF that is thought to contribute to the development of neuropathic pain, with IENF density lower in patients that present with pain compared with those presenting without such pain (Ebenezer et al., 2007). The mechanisms underlying the progressive degeneration of the IENF in diabetic neuropathy are not fully understood. However, the loss of neurotrophin support and reduction in the rate of regeneration are thought to be contributing factors.

1.3.3.4. Axonal regeneration failure

The progressive nerve fibre loss found in human diabetic neuropathy as well as rodent models, could be due, in part, to an impaired ability of the diabetic nerve to regenerate in response to the distal degenerative process (Sima et al., 1988). In early stages of the disease both degenerating and regenerating axons can be seen in peripheral nerve biopsies from human patients diagnosed with diabetes mellitus, however the number of regenerating axons decreasing with disease progression (Sima et al., 1988).

Under normal conditions, during Wallerian degeneration, myelin and axonal debris is removed by resident Schwann cells and invading macrophages. This leaves an empty Schwann cell basal lamina that normally ensheaths the axons. The basal lamina quickly become filled with proliferating Schwann cells, which provide positive guidance for the regenerating axons (Stoll and Muller, 1999). Successful Wallerian degeneration is a pre-requisite for normal regeneration following injury to the peripheral nerve. Studies using STZ-induced diabetes in rats and C57BL/Wd mice have shown that axonal degeneration during Wallerian degeneration is delayed in diabetes (Terada et al., 1998a;Terada et al., 1998b). Delayed axonal breakdown decreases Schwann cell response and invasion of non-resident macrophages following nerve transection in STZ-induced diabetic animals compared to control groups (Bisby and Chen, 1990).

Regeneration of the peripheral nerve after nerve injury has also been shown to be impaired in STZ-induced diabetes in rodents (Bisby, 1980;Kennedy and Zochodne, 2000;Ekstrom and Tomlinson, 1989). Defects have been seen in a number of steps in the regeneration process including delayed start of regeneration, decrease in the number of regenerating nerve fibres (Bisby, 1980), reduction in the rate of regeneration (Bisby, 1980;Kennedy and Zochodne, 2000;Ekstrom and Tomlinson, 1989); and impaired maturation of regenerated nerve fibres (Maxfield et al., 1995). IENF regeneration is reduced in diabetic subjects, and precedes the development of other symptoms of neuropathy (McArthur et al., 1998;Beiswenger et al., 2008a).

Recently, ARIs, sorbinil and tolrestat have been reported to not only improve clinical symptoms and neurophysiologic function but also to increase nerve regeneration in human diabetic neuropathy (Sima et al., 1988;Yasuda et al., 2003).

The factors underlying nerve regeneration failure in diabetes are complex and are undoubtedly a consequence of multiple factors. This makes treating diabetic neuropathy with multiple targeting complicated as the treatment would need to not only prevent the progression of the neuropathic symptoms, nerve dysfunction and degeneration but also promote the regeneration of the degenerated nerve fibres.

1.3.3.5. Neurotrophic factor deficits

Neurotrophins are growth factors that promote the survival of neurons. Neurotrophins are secreted by target tissues and act by stimulating the associated neuron to regenerate. Target-derived neurotrophins bind to their associated Trk receptors at neuron terminals inducing both a local signalling at the axon terminals and the retrograde transport back of receptor complexes to the DRG (Leinninger et al., 2004(Howe and Mobley, 2005;Campenot and MacInnis, 2004)). This retrograde transport of neurotrophic factors to the DRG regulates transcription and cell function, promoting, amongst other things, cell survival, neurite outgrowth and axonal remodelling (Howe and Mobley, 2005;Campenot and MacInnis, 2004).

NGF shows trophic effects on the small- and medium-diameter sensory neurons in the DRG and also sympathetic neurons (Crowley et al., 1994;Smeyne et al., 1994). NGF is produced by the target tissues including skeletal muscle and skin (Yasuda et al., 2003), where it is incorporated with its high affinity receptor, TrkA. In adults, NGF is not necessary for survival of the neurons but maintains neuropeptide levels such as substance P and CGRP (Yasuda et al., 2003). However, after injury, NGF is released by infiltrating macrophages in the distal stump (Heumann et al., 1987b;Heumann et al., 1987a;Lindholm et al., 1987) where it stimulates collateral sprouting from uninjured neurons but not regeneration (Diamond et al., 1992).

Altered neurotrophin expression and uptake and retrograde transport of NGF to DRG is thought to result in neuronal vulnerability and may contribute to pathogenesis of diabetic neuropathy (Tomlinson et al., 1996;Leinninger et al., 2004;Jakobsen et al., 1981;Hellweg et al., 1994;Christianson et al., 2007). In diabetic patients, serum and skin NGF levels are reported to be decreased (Tomlinson et al., 1996). This decrease in NGF levels has also been shown in experimental models, with NGF mRNA and protein reported to be reduced in the skin, DRG and sciatic nerve of STZ-induced diabetic rats (Fernyhough et al., 1995;Hanaoka et al., 1992) and genetically diabetic mice (Kasayama and Oka, 1989). Insulin treatment improves decreased NGF mRNA levels in the skin of diabetic rats (Fernyhough et al., 1994), indicating the role of hyperglycemia and/or hypoinsulinemia in this deficict. Experimental diabetes has also been treated with human recombinant NGF to try and prevent/reverse the symptoms of the disease (Unger et al., 1998;Maeda et al., 1996;Elias et al., 1998). However, observations in NGF-treated diabetic rodent models were not replicated in clinical trials (Apfel et al., 1998;Apfel et al., 2000).

There is conflicting evidence as to whether the expression of the high-affinity NGF-receptor, TrkA is also altered in diabetes. Some groups report no difference in TrkA immunoreactivity in the DRG of 12 weeks post STZ-induced rats compared to non-diabetic controls (Unger et al., 1998), whilst other groups report decreased expression of TrkA (Tomlinson et al., 1996).

Neurotrophin (NT)-3 supports large neurons of the DRGs that mediate proprioception. NT-3 binds to its high affinity receptor TrkC and promotes peripheral nerve regeneration (Sterne et al., 1997). In STZ-induced diabetic rats, NT-3 protein and mRNA are reduced in hind-limb skeletal muscles and sciatic nerve (Fernyhough et al., 1998;Tomlinson et al., 1996;Fernyhough et al., 1995). However, NT-3 protein levels have been resported to be increased in the dorsal root ganglia (Leinninger et al., 2004;Unger et al., 1998). These conflicting reports are mirrored in the sural nerve with some groups show an increase (Leinninger et al., 2004;Unger et al., 1998;Cai et al., 1999) and some a decrease (Rodriguez-Pena et al., 1995). The expression of NT-3 associated high-affinity receptor, TrkC is decreased in the DRG of STZ-diabetic rats (Fernyhough et al., 1995;Rodriguez-Pena et al., 1995). Combined, these observations suggest that TrkC-postive sensory neurons may be receiving reduced levels of neurotrophins in experimental diabetes

(Fernyhough et al., 1995). It is thought that increased NT-3 expression in the DRG may be part of a compensatory response that occurs due to loss of NT-3 in the target-tissue, which would normally be retrogradely transported back to the DRG cell body (Leinninger et al., 2004).

Brain-derived neurotrophic factor (BDNF) supports medium-sized DRG neurons via its high affinity receptor TrkB. BDNF is produced by target tissue as well as by the neurons themselves and transported anterogradely (Zhou et al., 1996). BDNF mRNA levels are increased in the DRG and skeletal muscles of rats with experimental diabetes (Fernyhough et al., 1995;Leinninger et al., 2004). Whilst interestingly, TrkB receptor levels are seen to be decreased in the sciatic nerve of STZ-rats along with reduced anterograde transport of BDNF (Rodriguez-Pena et al., 1995). These data are supported by the observation in the sciatic nerve of diabetic patients, where increased BDNF levels were seen in connection with decreased TrkB levels (Leinninger et al., 2004). It is thought that up-regulated BDNF expression occurs in response to diminished TrkB receptor levels, resulting in the saturation of the neurotrophin system with BDNF to compensate for its receptor deficiency (Leinninger et al., 2004).

It has not been established whether glial-derived growth factor (GDNF) production in diabetes is impaired; however, treatment of experimental diabetes with exogenous GDNF has been shown to normalise cutaneous innervation in STZ-diabetic mice (Christianson et al., 2003;Christianson et al., 2007;Leinninger et al., 2004).

1.3.4. Biochemical changes in diabetic neuropathy

It is generally accepted that hyperglycaemia plays a key role in the pathogenesis of diabetes and that it is the major cause of secondary complication development (Bonnefont-Rousselot et al., 2000;West, 2000;The Diabetes Control and Complications Trial Research Group, 1993). Hyperglycaemia induces oxidative stress through multiple pathways (Figure 1.3) including enhanced aldose reductase (AR) activity of the polyol pathway (Gugliucci, 2000;Brownlee, 2001); increased advanced glycation end product (AGE) formation (Taylor and Agius, 1988;Bonnefont-Rousselot, 2002); and altered protein kinase C (PKC) activity (Brownlee, 2001;Tomlinson, 1999).

The neurons of the PNS have a continuous high glucose uptake demand. Their glucose uptake is insulin-independent and is dependent on the extracellular concentration of glucose (Patel et al., 1994). This method of glucose uptake leaves neurons vulnerable to damage during periods of hyperglycaemia, which is seen as a result of unmanaged/undiagnosed or poorly managed diabetes (Tomlinson and Gardiner, 2008).

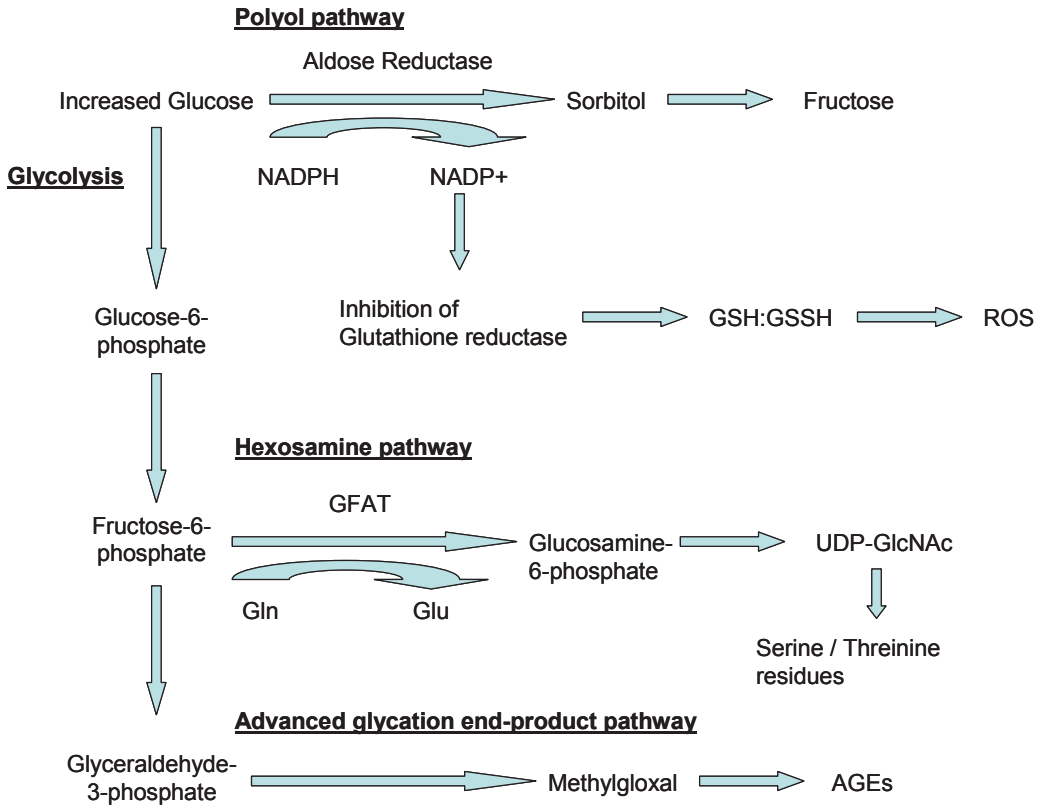

Figure 1.3: **Hyperglycaemia increased flux through numerous metabolic pathways.** Glucose normally enters the glycolysis pathway via phosphorylation by hexokinase. However, in high glucose, hexokinase becomes saturated and glucose is diverted into the polyol pathway, leading to increased ROS production via a build-up of H_2O_2 as a result of comprised GSSG recycling, which is due to increased NADPH consumption. Increased flux through the glycolysis pathway also has it's consequences. A build-up of F-6-P leads to the synthesis of UDP-GlcNAc, which can combine with serine/threonine residues of protein. Whilst G-3-P can be converted to highly reactive methylglyoxal, which forms advanced glycation end-products on proteins and other macromolecules. Both UDP-GlcNAc and AGEs attachment of protein may compromise the proteins' function.

Hyperglycaemia, as a result of diabetes, causes the normal route of glucose processing, hexokinase and entry into the glycolysis pathway, to be saturated. This causes glucose influx into the polyol pathway via the enzyme aldose reductase, as well as activation of the hexosamine pathway and the advanced glycation end-product pathway via accumulation of downstream products of glycolysis (Tomlinson and Gardiner, 2008). Other mechanisms stimulated by high glucose induce glucose-driven oxidative stress in the neuronal mitochondria and cytosol NADPH oxidase; activation of MAP kinases; abnormal PKC activity; and indirect effects through accessory cells, resulting in neurotrophin deficits and reduction in growth-related gene expression (Tomlinson and Gardiner, 2008).

1.3.4.1. Increased polyol pathway flux

The normal method of glucose processing is via the glycolysis pathway. The first enzyme in this pathway is hexokinase, which phosphorylates glucose and enters it into the glycolyisis pathway. In a hyperglycaemic environment, the glycolysis pathway becomes saturated and glucose fluxes through the polyol pathway via the enzyme, aldose reductase (AR). AR has a low affinity (high Km) for glucose, resulting in a low flux level through the polyol pathway in non-diabetic individuals (Brownlee, 2001). Increased flux through the polyol pathway results in the intracellular accumulation of sorbitol; decreased ATPase activity; increase in cytosolic NADH/NAD^+; and depletion of NADPH levels (Brownlee, 2001).

Sorbitol has a low plasma membrane permeability, resulting in its intracellular accumulation; this leads to tissue swelling through an increase of osmotic effect (Brownlee, 2001). Activity of AR is associated with changes in NADH/NAD^+ ratio via the oxidisation of sorbitol by NAD^+ and the inhibition of glutathione regeneration, resulting in a compromised conversion of hydrogen peroxide to water, which ultimately results in superhydroxyl radical production (via the Fenton reaction) (Obrosova et al., 2005;Obrosova et al., 2002). Hence, increased polyol pathway flux, as a result of hyperglycaemia, leads to a compromised antioxidant defence mechanism and induces intracellular oxidative stress (Brownlee, 2001).

1.3.4.2. Hexosamine pathway flux

A second consequence of increased glucose through the glycolysis pathway is the accumulation of glycolytic intermediates, such as fructose-6-phosphate (F-6-P). F-6-P drives the hexosamine pathway, resulting in the generation of uridine diphosphate-N-acetylhexosamine (UDP-GlcNAc), which combines with serine and threonine residues of many proteins, leading to changes in both gene expression and protein function (Brownlee, 2001). Many other proteins are known to be modified by GlcNAc, including the cytokines TGF-α and TGF-β, and it is also known to play a role in proteoglycan synthesis and formation of O-linked glycoproteins (Brownlee, 2001).

1.3.4.3. Increased oxidative stress production

Oxidative stress is the imbalance between reactive oxygen species (ROS) production and antioxidant defences. ROS are small, free radicals that are highly reactive due to the presence of unpaired valence shell electrons (Bonnefont-Rousselot, 2002). ROS are traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins and DNA (Thannickal and Fanburg, 2000). However, cells possess several antioxidant enzymes such as superoxide dismutase, which reduces $O_2^{-}\cdot$ to H_2O_2), catalase and glutathione peroxidise (which reduces H_2O_2 to H_2O). ROS play an essential role in cell signalling and regulation, such as nitric oxide (NO) mediated vasodilation and cytokine-activated signalling (Thannickal and Fanburg, 2000).

In addition to causing a drop in enzymatic and non-enzymatic cell antioxidant defences, hyperglycaemia also drives the production of ROS and reactive nitrogen-oxygen species (NROS) via several mechanisms (Bonnefont-Rousselot et al., 2000). Increased oxidative stress has been shown in the peripheral nerve, dorsal root and sympathetic ganglia of diabetic animal models (Pop-Busui et al., 2006). ROS stimulate COLL accumulation (Yamagishi et al., 2001), contribute to activation of mitogen-activated protein kinases (MAPKs) and promote the generation of intracellular AGEs (Bonnefont-Rousselot, 2002). AGE production is also associated with further ROS production via interactions and activation of the AGE receptors (RAGE), which in turn, can activate NFκB (Bonnefont-Rousselot, 2002). These ROS-induced altered pathways contribute to nerve blood flow and conduction deficits; impaired neurotrophic support; changes in signal transduction and

metabolism; and morphological abnormalities, which are all characteristic of diabetic neuropathy (Pop-Busui et al., 2006;Obrosova, 2009).

1.3.4.4. Altered intracellular signalling

MAPKs, PKC and phosphoinositide 3-kinase (PI3K) are all intracellular signal transduction mediators that transduce signals from the cell surface to the nucleus where they activate specific transcriptional factors, gene expression and eventually initiate a cellular response (Cobb and Goldsmith, 1995).

PKC promotes neurite outgrowth by promoting integrin interaction with LM (Yasuda et al., 2003) and is involved in the phosphorylation of GAP-43, which is strongly expressed in developing and regenerating axons (Sheu et al., 1990). PKC inhibitor (chelerythrine) reduces PKC anterograde and retrograde axonal transport and prevents neurite outgrowth in cultured mouse DRG neurons. This suggest that PKC activity is required to maintain axonal transport and thereby, neurite outgrowth (Hiruma et al., 1999). The effect of hyperglycaemia on PKC is thought to be tissue and isoform specific, several isoforms are present in the peripheral nerve (α, βI, βII, γ, δ, ε, η, ζ, θ, ξ, λ, and μ) (Kawano et al., 1997;Roberts and McLean, 1997). The activity of PKC is altered in the peripheral nerve in diabetes, with no alteration in PKC levels. A PKC-β inhibitor improves both NCV and nerve blood flow deficits in STZ-induced diabetic rats, however, no differences in PKC levels could be detected between the treated and non-treated groups (Nakamura et al., 1999). Other groups have also shown there to be no differences at protein level of any PKC isoform in diabetic nerves (Roberts and McLean, 1997). This suggests that this improvement may be obtained through improving nerve blood flow, via inhibition of PKC activity of endoneurial microvasculature rather than as a direct-inhibition of neuronal PKC levels (Nakamura et al., 1999).

There are three main groups of MAPK: extracellular signal regulated kinases (ERK); c-Jun N-terminal kinases (JNK); and the p38 kinases. ERK is activated by membrane depolarisation and calcium influx and is known to be involved in multiple and varied intracellular signalling pathways; eg. neuronal plasticity, such as long-term potentiation learning and memory (Obata and Noguchi, 2004). Whilst p38 and JNK are stress-activated

protein kinases that participate in injury response and cell death (Ip and Davis, 1998;Han et al., 1994). Both are known to act as transducers for cellular responses and their activation may arise from hypertonicity, oxidative stress, RAGE activation or a combination of these stimuli (Tomlinson, 1999). Altered expression and/or signalling of the MAPKs have been implicated in the development of diabetic neuropathy. In several studies, increases in the phosphorylation of ERK, p38 and JNK have been reported in the motoneurons (Price et al., 2004) and microglial of the spinal cord (Daulhac et al., 2006) and DRG neurons (Purves et al., 2001;Daulhac et al., 2006) of STZ-diabetic rats. With increases in total p38 and JNK protein levels observed in the sural nerves of T1DM and T2DM human patients (Purves et al., 2001). In STZ rats, increased phosphorylation of MAPKs was correlated with the development of mechanical hyperalgesia (Daulhac et al., 2006) and thermal hyperalgesia (Obata and Noguchi, 2004).

Phosphorylated ERK can be seen in spinal dorsal horn neurons and the primary afferent neurons on the DRG in response to acute noxious stimulation of the peripheral tissue, such as formalin or capsaicin (Obata and Noguchi, 2004). Phosphorylation of ERK at the spinal level is also seen after peripheral nerve damage (Daulhac et al., 2006). p38 activation has been reported in both the spinal microglia and DRG of nociceptive neurons following spinal nerve ligation as a consequence of peripheral inflammation (Jin et al., 2003). Long-term JNK activation is seen in the DRG neurons following peripheral axotomy. Inhibition of both the p38 and ERK activation attenuates thermal hyperalgesia, whilst p38 and JNK inhibition reduces mechanical allodynia (Obata and Noguchi, 2004). Inhibition of both p38 and JNK MAPKs suppresses mechanical hyperalgesia and decreases phosphorylation in STZ-diabetic rats (Daulhac et al., 2006). Altered MAPK, specifically p38, have also been shown to play a role in the development of reduced NCV, which is classically associated with diabetic neuropathy (Price et al., 2004). In STZ rats, treatment with a specific inhibitor of p38 prevented reductions in both motor and sensory NCV (Price et al., 2004).

Diabetes-induced activation of MAPK has been shown to occur by both the direct effects of glucose and via glucose-induced oxidative stress (Purves et al., 2001). Adult rat cultures display different response in MAPK activation following treatment with high glucose or oxidative stress (DEM or Hydrogen peroxide), suggesting that these two stimuli work using alternative mechanisms, resulting in the MAPK activation (Purves et al., 2001). It is

thought that the glucose-dependent activation of JNK/p38 occurs as a result of a direct effect of glucose itself, as well as elevated polyol pathway intermediates or transcription-dependent induction of activators of JNK/p38 (Purves et al., 2001). In culture, JNK/p38 activation has no effect on cell damage and did not contribute to oxidative stress development (Purves et al., 2001). Whilst oxidative stress activated ERK and p38 MAPK in primary neuron cultures, resulting in cellular damage and reduced cell viability (Purves et al., 2001). These observations suggest MAPKs may act as transducers, linking raised glucose and secondary metabolic stress to cellular damage and diabetic neuropathy (Purves et al., 2001).

1.3.4.5. AGE formation

Glycation (or non-enzymatic glycosylation) is the result of a sugar molecule, such as fructose or glucose, bonding to a protein or lipid molecule without the controlling action of an enzyme. Fructose or glucose is covalently linked to lysine and *N*-terminal amino acid residues of a protein. Early stage reactions lead to the formation of glycation adducts; fructosyl-lysine (FL) and N-terminal amino acid residue-derived fructosamines. These glycation adducts undergo a slow, spontaneous rearrangement (the Amadori rearrangement) to form stable AGEs (Singh et al., 2001;Jakus and Rietbrock, 2004). Summarised in Figure 1.4.

Reactive dicarbonyl and intermediates are formed from the degradation of glycated proteins, glycolytic intermediates and lipid peroxidation (Thornalley, 2008), such as glyoxal and methylglyoxal (MG). These highly reactive dicarbonyl compounds react with proteins to form AGE residues directly and relatively rapidly. AGEs from glyoxal and MG include carboxyethyllysine (CEL), carboxymethyl-lysine (CML), and methylglyoxal-derived hydroimidazo-1 (MG-H1), methylglyoxal - Nδ-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), bis(lysyl) crosslink derived from glyoxal (GOLD) and bis(lysyl) crosslink derived from methylglyoxal (MOLD) (Thornalley, 2008;Duran-Jimenez et al., 2009).

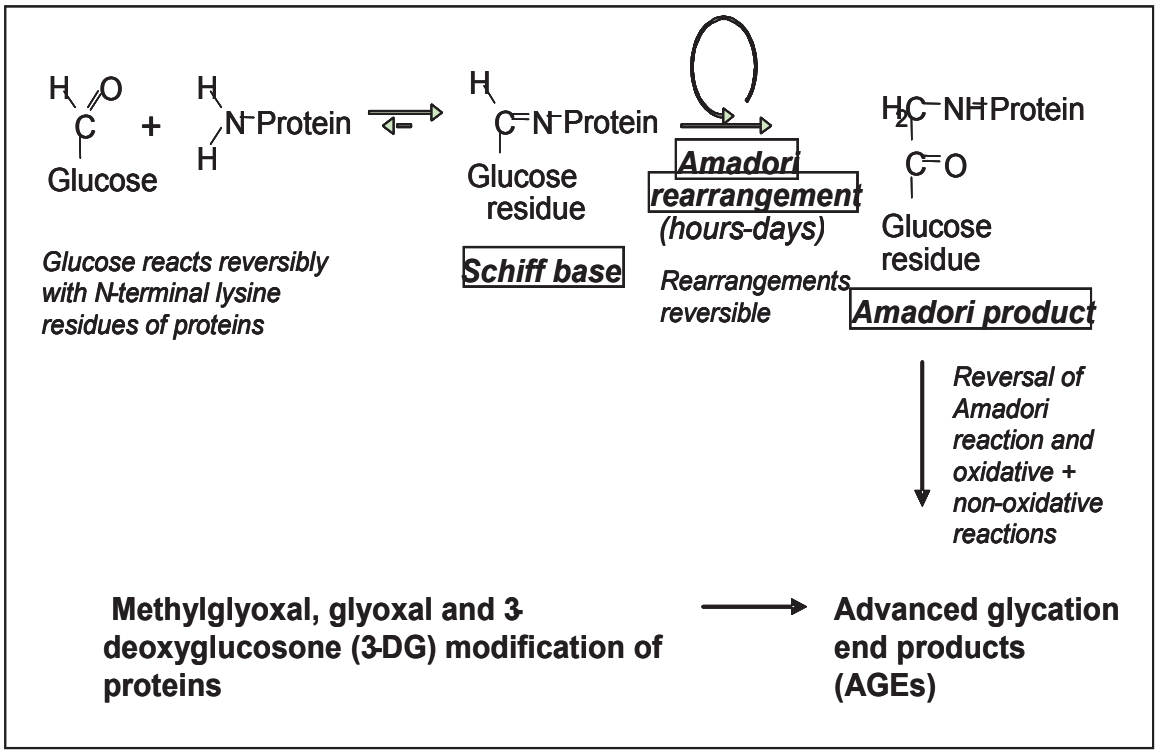

Figure 1.4: **Mechanism for the formation of AGEs.** Flowchart showing the stages involved in the formation of AGEs (Adapted from Tomlinson, unpublished). Irreversible AGE occur as part of normal ageing process. However, in hyperglycemic conditions, this process of accelerated AGE formation is further enhanced by the formation of reactive intermediates including methylglyoxal, glyoxal and 3-deoxyglucosae (Thornalley, 2008).

Reactive dicarbonyl intermediates are formed from the degradation of glycated proteins, glycolytic intermediates and lipid peroxidation (Thornalley, 2008), such as glyoxal and methylglyoxal (MG). These highly reactive dicarbonyl compounds react with proteins to form AGE residues directly and relatively rapidly. AGEs from glyoxal and MG include carboxyethyllysine (CEL), carboxymethyl-lysine (CML), and methylglyoxal-derived hydroimidazo-1 (MG-H1), methylglyoxal - Nδ-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), bis(lysyl) crosslink derived from glyoxal (GOLD) and bis(lysyl) crosslink derived from methylglyoxal (MOLD) (Thornalley, 2008;Duran-Jimenez et al., 2009).

AGEs are naturally occurring and are formed slowly throughout life. Chemically stable AGEs (such as CML and CEL) form on long-lived proteins such as COLL. AGEs are ingested in the diet (particularly in cooked food), although, few dietary AGEs are absorbed and most are excreted, and therefore, do not particularly impact on cell/tissue function (Thornalley, 2008).

Under certain pathologic conditions, AGE formation is increased, AGE residues accumulate in both intracellular and extracellular proteins, and in tissues including the retina, kidney, heart and nerve, in both clinical (Sugimoto et al., 1997;Ryle and Donaghy, 1995) and experimental diabetes (Thornalley et al., 2003;Duran-Jimenez et al., 2009). This accelerated AGE formation occurs as a consequence of hyperglycaemia and the subsequent accelerated formation of the highly reactive intermediate carbonyl groups, such as 3-deoxyglucosone, glyoxal and MG (King, 2001;Brownlee, 2001;Bonnefont-Rousselot, 2002), as well as increased free radical production and oxidative stress (Inoguchi et al., 2000). Increased AGE is associated with the development of diabetic complications, especially renal, retinal and cardiovascular disease (Degenhardt et al., 2002;Figarola et al., 2003;Figarola et al., 2008;Stitt et al., 2002;Bhatwadekar et al., 2008) with increased AGE accumulation thought to contribute to the aetiology of diabetic neuropathy (Pop-Busui et al., 2006;Brownlee, 2001;Bonnefont-Rousselot et al., 2000;Bonnefont-Rousselot, 2002). The most widely studied glycated proteins are the long-lived ECM molecules, especially those of the renal basal lamina. The increase in glycation adduct levels in the plasma has been shown to be associated with changes in the appearance and biophysical properties of the glomerular basement membrane (Osterby, 1986;Kverneland et al., 1986;Feldt-Rasmussen et al., 1989).

The glycation of proteins can cause structural changes that alter cellular behaviour via modification of protein function (Tanaka et al., 1988;Monnier et al., 1984), production of intramolecular cross-linking causing structural distortion (Tanaka et al., 1988) and activation of cellular pathways (Bonnefont-Rousselot et al., 2000), and resistance to proteolysis (Lubec et al., 1982;Lubec and Pollak, 1980;Pollak et al., 1982).

X-ray diffraction studies have shown differences in the crystalline structure of native COLL and COLL glycated by aldoses. Formation of AGEs on COLL results in an expansion of three-dimensional structure of COLL due to an increase in the intramolecular spacing of COLL (Tanaka et al., 1988) and formation of one or more specific cross-links between glycated residues in the protein, resulting in the distortion of COLL's three-dimensional structure (Tanaka et al., 1988). AGEs form on the NC1 domain of COLL-IV (Tsilibary et al., 1988), which is important in the regulation of the assembly of COLL into fibrils via its

interaction with the inhibitory molecule, HSPGs (Tsilibary et al., 1988). Therefore, AGE formation could alter COLL-IV's self-assembly regulatory mechanism by blocking HSPG binding at the NC1 domain. LM function has also been shown to be affected by glycation, with AGE-LM showing decreased polymer self-assembly and heparin binding (Luo et al., 2002).

The cross-linking of molecules is also thought to alter the properties of the ECM. Generally, changes observed *in vitro* include reduced excitability of protein; reduction in hydrophobicity and viscosity; reduction in susceptibility to thermal rupture; and increase in tensile strength (Tanaka et al., 1988) . This is thought to be a result of decreased binding of AGE-proteins to other major components of the ECM (Haitoglou et al., 1992). Glycation of LM and COLL-IV alters adhesion and endothelial cell migration (Haitoglou et al., 1992). This altered function is likely due to the modification of functionally important arginine residues of arginyl-glycyl-aspartic acid
(RGD) and glycyl-phenylalanyl-hydroxypropyl-glycyl-glutamic acid-arginine (GFOGER) motifs by the AGE formation on ECM proteins inhibiting binding of substrates to their receptors (Ahmed and Thornalley, 2002). Integrin binding to cell surface receptors is thought to be altered by AGE formation. RGD and GFOGER motif are modified following exposure to glycation intermediates, which leads to decreased binding affinity of integrins and as a consequence, reduces cell detachment (Ahmed and Thornalley, 2002). Non-enzymatic glycation reduction in integrin-mediated signaling pathways would result in decreased tyrosine phosphorylation and MAPK phosphorylation, which lay downstream of integrin-receptor binding (Hasegawa et al., 1995;Rittie et al., 1999). This would result in altered integrin-mediated cell signaling, which may affect transcriptional activators, including AP-1 (McLennan et al., 2002). Matrix glycation also interferes with intracellular signalling, alters gene expression and contributes to the development of diabetic complications (Bonnefont-Rousselot et al., 2000).

1.3.5. Diabetes and Inflammation

In the last two decades, the activation of innate immunity with the subsequent development of a chronic low-grade inflammatory response has become a recognised contributor to the pathogenesis of diabetes mellitus and diabetic complications.

The activation of the inflammatory cascade and the production of pro- inflammatory cytokines have been linked with the development of insulin resistance in type-2 diabetes and β-cell dysfunction in both type-1 and type-2 diabetes (Goldberg, 2009). In type-2 diabetes, in particular, the certain biomarkers of inflammation may have independent predictive value for the diagnosis of diabetes mellitus (Goldberg, 2009). Expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, has been shown to be increased in activated macrophages in adipose tissue of obese individuals (Suganami et al., 2005;Rajala and Scherer, 2003). Administration of recombinant TNF-α to cultured cells or to whole animals has been shown to impair insulin action, and obese mice lacking functional TNF-α , or TNF receptors have improved insulin sensitivity compared with wild-type counterparts (Hotamisligil et al., 1993). These observations show that overproduction of pro-inflammatory cytokines in adipose tissue is an important feature of obesity, which contributes significantly to insulin resistance. Therefore, inhibiting inflammation is currently a potential new target for intervention.

Perhaps more interesting, however, is the involvement of pro-inflammatory cytokines, and their downstream mediators, in the development of secondary complications of diabetes. The involvement of pro-inflammatory cytokines has been most extensively studied in the development of diabetic nephropathy. Hasegawa et al, (1991) demonstrated that macrophages cultured with glomerular basement membranes from diabetic rats produced significantly higher amounts of the inflammatory cytokines TNF-α and IL-1 than macrophages cultured with glomerular basement membranes from normal rats (Hasegawa et al., 1991). In both diabetic patients (Navarro and Mora, 2006) and experimental diabetes (Tanaka et al., 1992), significant elevation in urinary TNFα levels have been observed, this increase in production is the result of intra-renal production (Navarro and Mora, 2006). Proinflammatory cytokines have been associated with significant renal effects which play a critical role in the development of renal injury in type 2 diabetes (Navarro-Gonzalez et al., 2009). These effects include altered expression of, and synthesis of, adhesion molecules, selectins, prostaglandins and fibronectin; intraglomerular hemodynamic abnormalities; alteration of the extracellular matrix and glomerular basement membrane; alteration of endothelial permeability and oxidative stress (Navarro and Mora, 2006;Pfeilschifter et al., 1989;Ruef et al., 1990;Koike et al., 2007).

The role pro-inflammatory cytokines may play in diabetic neuropathy is poorly understood. In the peripheral nerve, cytokines are known to play a key role following nerve injury. Following nerve injury, cytokines are produced from resident and recruited macrophages, Schwann cells and probably neurons (Yasuda et al., 2003). TNF-α induces Wallerian degeneration (Yasuda et al., 2003), whilst IL-1β helps overcome the inhibitory nature of myelin associated glycoproteins (MAGs), promotes axonal and myelin debris cleavage by scavenger macrophages and enhances nerve regeneration (Temporin et al., 2008).

However, the role of cytokines in the in pathogenesis of diabetic neuropathy is unclear. In the early stages of experimental diabetic neuropathy, IL-1β co-localises with infiltrating macrophages and along nerve fibres. By 5 weeks post-STZ, macrophage clearance was completed and IL-1β became undetectable. No alteration of TNF-α was observed in the peripheral nerve of diabetic mice (Tanaka et al., 1992). However, TNF-α production has been shown to be increased in serum and plasma in diabetic patients (Navarro and Mora, 2006;Gordin et al., 2008) and experimental diabetes (Tanaka et al., 1992). This information does not give a clear picture as to whether the expression of cytokines is altered and/or has a functional effect in diabetic neuropathy. However, a number of the structural alteration observed in diabetic nephropathy including alteration of the extracellular matrix and thickening of the glomerular basement membrane, alteration of endothelial permeability and oxidative stress, which has been shown to be the result of overproduction of cytokines, are also observed in the peripheral nerve of diabetic individuals (described previously in this chapter). Another interesting concept to consider is whether these cytokine effects occur directly and/or via activation of downstream molecules, such as MMPs (Cawston and Wilson, 2006;Hijova, 2005), which are cytokine-activated molecules known to involved in matrix alterations and successful nerve repair (Hijova, 2005).

1.4. Matrix Metalloproteinases

MMPs are a sub-family of the metzincin super-family of proteases, which constitute one of several metalloendopeptidase families (Cawston and Wilson, 2006). The metzincins share a conserved structural topology. They all contain a consensus motif within the catalytic domain, which contains three His that provide a zinc binding site and a conserved 'Met-turn' motif that lay beneath the active site zinc ion (Cawston and Wilson, 2006;Yong, 2005). The metzincins are divided into 4 sub-families: the serralysins, adamalysins, astracins and matrixins, also known as MMPs (Cawston and Wilson, 2006).

MMPs play a central role in tissue remodelling due to their ability to cleave the protein components of the ECM (Hijova, 2005). The MMPs are divided into 8 groups based on their structure (see Figure 1.5). All MMPs have a secretry signal sequence (pre-domain), the function of which is to maintain latency of the MMP until the signal for activation is given, and a pro-peptide domain, whose proteolytic cleavage is required for MMP activation (Becker et al., 1995;Van Wart and Birkedal-Hansen, 1990). This pro-peptide domain is followed by a catalytic domain that contains the consensus zinc-binding motif HEBXHXBGBXH (where X is a variable residue and B is bulky hydrophobic residue). Most of the MMP groups also contain a haemopexin-like domain, which contains four repeats that have weak homology to haemopexin. The function of the haemopexin domain is not completely clear but is thought to influence substrate specificity (Cawston and Wilson, 2006;Hijova, 2005).

Regulation of MMP activity occurs in at least three ways: transcription regulation; proteolytic activation of latent MMPs; and inhibition of the active enzyme by endogenous inhibitors (tissue inhibitors of MMPs; TIMPs). Most MMPs are expressed at low levels, or not at all, in resting-state adult tissue (Nagase and Woessner, Jr., 1999). However, MMP expression can be rapidly induced by various stimulatory factors, including cytokines, growth factors and physical cellular interactions (Cawston and Wilson, 2006;Hijova, 2005).

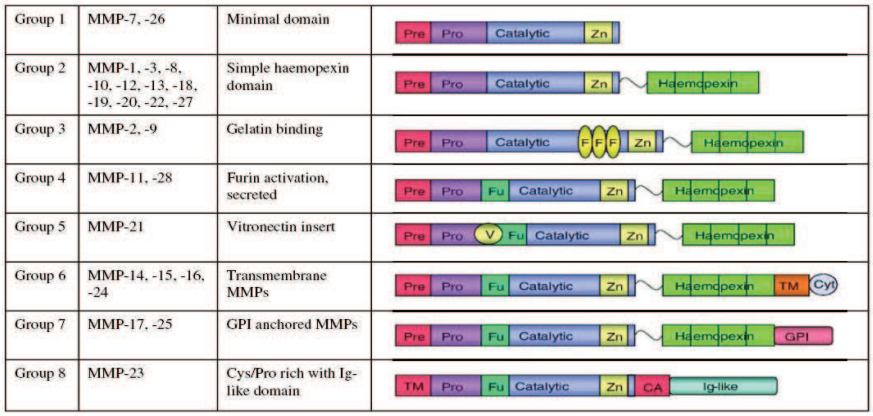

Figure 1.5: **Domain structure of the MMP families.** Classification of MMP family members on functional domain structure. All MMPs have a catalytic, zinc-binding (Zn), pre and pro-domain. Additional features include furin recognition motif (Fu); haemopexin domain; fibronectin-like domain (F); vitronectine-like domain (V); cytoplasmic tail (Cyt); glycosyliphosphatidyl inositol (GPI) anchor; cysteine array (CA); and immunoglobin-like domain (Ig-like) (Adapted from (Cawston and Wilson, 2006)).

MMP gene expression is tightly regulated by numerous stimulatory and suppressive factors that influence multiple signalling pathways (Fini et al., 1998). MMP expression is regulated by several cytokines and growth factors, including interleukins, interferon, EGF, KGF, NGF, basic FGF, VEGF, PDGF, TNF-α, TGF-β (Fini et al., 1998). Many of these extracellular stimuli induce MMP gene expression via the activation of c-fos and c-jun proto-oncogene products, which heterodimerise and bind activator protein-1 (AP-1) sites found at approximately -70bp in the promoter region of each inducible MMP gene (Yoon et al., 2003;Sternlicht and Werb, 2001;Chakraborti et al., 2003). Although AP-1 complexes play a critical role in the regulation of several MMP genes, other factors are also involved. This is demonstrated by the ability of one signal to co-ordinately regulate some MMP genes and differentially regulate others (Fini et al., 1998). An example of this is TGF-β, which suppresses the transcription of MMP-1 and MMP-3 but induces the expression of MMP-13 (Uria et al., 1998).

Following protein translation, all MMPs are produced as zymogens, either in secreted or membrane-bound form (Chakraborti et al., 2003;VanSaun and Matrisian, 2006). The propeptide domain maintains the MMP in its latent form until a signal for activation is given (Woessner, Jr., 1994). The proMMP structure is maintained by an unpaired cysteine (Cys) sulfhydryl group, which is located near the carboxyl end within the distinctive

consensus PRCGXPDV motif (Sternlicht and Werb, 2001;Woessner, Jr., 1994). The sulfhydryl acts as a fourth ligand for the active site zinc ion, forming what is referred to as the Cys-zinc switch (Sternlicht and Werb, 2001;Woessner, Jr., 1994). The Cys residue by itself is not able to maintain latency, other residues within the pro-peptide region bind to the area around the active site and thereby, hold the Cys in place (Woessner, Jr., 1994). MMP activation requires the interruption of this Cys-to-zinc interaction, resulting in the 'opening' of the switch and allowing the autocatalytic or proteolytic cleavage of the COOH-terminal of the PRCGXPDV motif (Nagase and Woessner, Jr., 1999;Sternlicht and Werb, 2001).

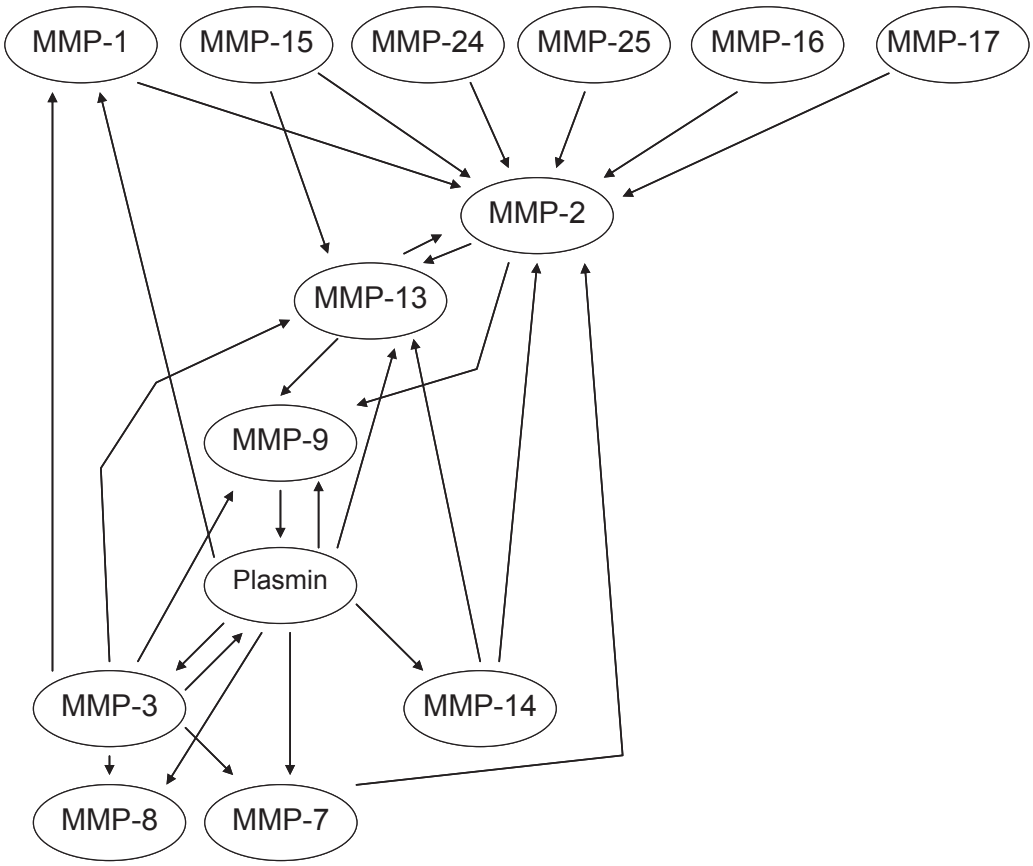

Figure 1.6: **Mutual MMP activation.** Flowchart displaying the activated MMPs and other proteinases to activate pro-MMPs (Adapted from (Chakraborti et al., 2003)*)*.

Exceptions to this latent zymogen model are MMP-11, MMP-27 and MT-MMPs, which contain furin-like enzyme recognition motif in their pro-peptide domain (Coussens and Werb, 1996). These furin-containing MMPs are activated intracellularly by a group of calcium-dependent transmembrane serine proteinases of the subtilisin group (Pei and Weiss, 1995;Sternlicht and Werb, 2001). The extracellular activation of most proMMPs

can also be initiated by other already activated MMPs or other serine proteinases, these interaction are summarised in Figure 1.6 (Coussens and Werb, 1996;Woessner, Jr., 1994;Chakraborti et al., 2003).

Once the zymogen is activated, the major point of control is the proteinase inhibitors of the extracellular matrix. These are α_2-macroglobulin (α2M) and members of the tissue inhibitor of metalloproteinases (TIMPs) (Woessner, Jr., 1994). α2M is a large plasma protein found on the phagocytes, which is able to inactivate an enormous variety of proteinases including MMPs; however, the mechanism is unknown. α2M binds to and removes the active forms of the gelatinase (MMP-2 and MMP-9) from the circulation (Borth, 1992). TIMPs are a family of secretory proteins that reversibly inhibit MMPs though non-covalent binding to either a pro- or active form of MMPs in a 1:1 ratio (Cawston and Wilson, 2006;VanSaun and Matrisian, 2006). TIMP binding inhibits either the autocatalytic activation of the latent enzymes or the proteolytic capacity of the active MMP (Coussens and Werb, 1996). All TIMPs contains two domains; the N-domain reacts with the active centre of most MMPs and the C-domain is involved in the binding to other components, such as the C-domain of the gelatinases MMPs (MMP-2 and MMP-9) (Murphy et al., 1992;Sternlicht and Werb, 2001). The various types of TIMPs can inhibit all of the MMPs; however, there are clear differences in the binding affinity of each type of TIMP for the various MMPs, as shown in Table 1.8 (Woessner, Jr., 1994).

	Molecular weight (kDa)	Target MMP
TIMP-1	28.5	MMP-1, MMP-9 > MMP-2 >>> MT-MMP-1
TIMP-2	21	MMP-2 > MMP-3, MMP-9, MT-MMP-1
TIMP-3	24-25	MMP-2 = MMP-9 > MT-MMP-1

Table 1.8: **TIMP and their MMP binding specificity.** TIMPs bind either proMMPs or active MMPs, thereby inhibiting the autocatalytic activation of latent enzymes as well as the proteolytic capacity of active proteinases (Adapted from (Coussens and Werb, 1996)).

1.4.1. MMP-2 and MMP-9 in the peripheral nervous system

MMP-2 and MMP-9 belong to the group 3 gelatin-binding family of MMPs. The gelatinase MMPs contain a haemopexin-like domain connected to the catalytic domain by hinge or

linker region, and fibronectin-like domain within their catalytic domain (Cawston and Wilson, 2006). The haemopexin domain is involved in specific TIMP binding, the binding of certain substrates, membrane activation and some proteolytic activities. The fibronectin inserts resemble the COLL-binding type II repeats of fibronectin and are required to bind and cleave COLL and elastin (Murphy et al., 1992;Sternlicht and Werb, 2001;Cawston and Wilson, 2006).

The best-known substrates of MMP-2 and MMP-9 are shown in Table 1.9 (Coussens and Werb, 1996). As well as these ECM substrates, MMP activity has been linked to proteolytic cleavage and activation of cytokines and growth factors (e.g. IL-1β, TNFα, ErbB2). Less is known about these non-ECM substrates but evidence from proteomics/degradomics shows a large number of MMP interactions (Butler and Overall, 2009).

	Other names	Molecular weight (kDa)	Substrates	
			Matrix	Non-Matrix
MMP-2	Gelatinase A / 72kDa type IV collagenase	Pro; 72kDa Active; 69kDa	Gelatins Collagens I, IV, V, VII and X Fibronectin Elastin Procollagenase-3 (MMP-13)	Decorin Pro-TGF-β2 Pro-IL1-β MCP-3 IGF BP-3/5 Pro-TNFα FGF-R1 Pro-MMP-1, 2, 13
MMP-9	Gelatinase B / 92kDa type IV collagenase	Pro; 92kDa Active; 88kDa	Gelatins Collagens IV and V Elastin	Pro-TGF-β2 Pro-IL1-β Cell-surface bound IL1-Ra Plasminogen α1-proteinase inhibitor Pro-TNFα

Table 1.9: **MMP-2 and MMP-9 substrates** (Coussens and Werb, 1996;Butler and Overall, 2009).

1.4.1.1. MMP-9 regulation

MMP-9 is barely detectable at mRNA, protein and activity level in uninjured adult peripheral nerve (Shubayev et al., 2006). However, MMP-9 mRNA and protein is significantly up-regulated at site of injury within hours of nerve injury, and is rapidly converted to active form (Demestre et al., 2004;Ferguson and Muir, 2000;Shubayev et al., 2006). The up-regulation of MMP-9 occurs for a shorter time-period than the other MMPs,

with down-regulation by 10 days, suggesting that MMP-9 may play a role in the Wallarian degeneration (Demestre et al., 2005;Hughes et al., 2002). MMP-9 immunoreactivity is increased in Schwann cells, blood vessels and ED-1 positive/activated macrophages (Demestre et al., 2005).

MMP-9 production by macrophages (Shubayev et al., 2006), Schwann cells (Chattopadhyay et al., 2007) and myelinating Schwann cells (Chattopadhyay et al., 2007) in the peripheral nerve following injury are all primarily controlled by pro-inflammotry cytokines TNF-α and IL-1β. The role of TNFα, and IL-1β in the induction of MMP-9 gene expression have been extensively researched. The direct injection of TNFα or IL-1β to the site of injury following a sciatic nerve crush has been shown to result in a significant increase in MMP-9 mRNA (Chattopadhyay et al., 2007). Work with TNF-α receptor and TNF-α KO mice has shown reduced MMP-9 production following nerve crush injury (Shubayev et al., 2006;Chattopadhyay et al., 2007). Both TNFα and IL-1β stimulate MMP-9 up-regulation via the intracellular activation of p38, JNK and ERK MAPKs. All these MAPKs regulate NF-κB production, whilst ERK 1/2 also regulates AP-1 production, which promotes transcription of MMPs (Moon et al., 2004;Arai et al., 2003;Ehrlichman et al., 2009).

tPA, a serine protease found on endothelial cells, is another regulator of MMP-9 expression (Zou et al., 2006). The administration of exogenous tPA or tPA/plasminogen 7 days after nerve crush promotes axonal regeneration and remyelination in C57Bl/6J mice (Zou et al., 2006). The improvement in nerve regeneration ability is due to increases in fibrin clearance via tPA activation of MMP-9-postive macrophages and tPA induced recovery of the BNB (Zou et al., 2006;Adams et al., 2004). tPA-induced MMP-9 expression has been shown to occur via the Raf/MEK/ERK MAPK pathway in MC7 cell line (Kim et al., 2009).

Lipopolysaccharide (LPS) is another regulator of MMP expression. LPS is a complex glycolipid that stimulates a strong inflammatory response via toll-like receptors by inducing both upregulation and release of cytokines. LPS stimulation of human peripheral blood monocytes/macrophages *in vitro* induces MMP-9 secretion (Lai et al., 2003). This LPS-mediated MMP production is stimulated mainly through the ERK1/2 pathway as treatment

of cultures with MAPK 1/2-specific inhibitors inhibits MMP-9 production (Lai et al., 2003). LPS-induced activation of macrophages causes peripheral nerve disturbances including gait abnormalities, proprioceptive loss, and to a lesser extent, hind limb weakness and sensory deficits (Brown et al., 1999;Brown et al., 1997) as well as conduction block in the sciatic nerves of rats (Brown et al., 1999). However, the direct involvement of MMP-9 in the development of these peripheral deficits following LPS treatment has not been investigated.

1.4.1.2. MMP-2 regulation

Realtively little is known about MMP-2 regulation in the PNS. In contrast to most other MMPs, MMP-2 is widely expressed by most cell types and appears to not be as tightly regulated at transcription level (Sternlicht and Werb, 2001). Similar to MMP-9, pro-inflammatory cytokine-stimulation of MAPKs has been implicated in MMP-2 up-regulation at both gene and protein levels. TGF-β promotes MMP-2 transcription and post-transcriptional levels (Overall et al., 1991) whilst reducing levels of MT1-MMP mRNA and increasing TIMP mRNA in human fibroblasts (Overall et al., 1991). Whilst TNF-α stimulates NF-κB activation in fibroblasts, which induces MT1-MMP gene expression and subsequently activates proMMP-2 in isolated human dermal fibroblasts from skin (Han et al., 2001). In carcinoma cells, ERK activation via PKC stimulates MMP-2 protein expression. ERK inhibition by myricetin results in reduced ERK phosphorylation, translocation of PKCα to membrane and c-Jun protein expression with a reduction in MMP-2 protein expression and enzyme activity (Ko et al., 2005). Other studies using human retinal epithelial cells have also shown a role for p38 MAPK in the up-regulation of MMP-2 mRNA and protein expression (Hou et al., 2009). Pro-inflammatory cytokines have the ability to alter the balance between the activation and inhibition of MMP-2 and therefore, regulate its activation (Han et al., 2001;Overall et al., 1991). MMP-2 is resistant to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP-2, which is summarised in Figure 1.7 (Strongin et al., 1995). First, a cell surface MT-MMP binds to and is inhibited by the N-terminal domain of TIMP-2, and the C-terminal domain of the bound TIMP-2 acts as a receptor for the hexopexin domain of proMMP-2. Then, an adjacent uninhibited MT-MMP cleaves and activates the tethered proMMP-2, releasing a catalytically inactive

proMMP-2 intermediate (Strongin et al., 1995). To be fully activated, this proMMP-2 intermediate must be further cleaved by an already activated MMP-2 (Deryugina et al., 2001). MT1-MMP is particularly efficient at MMP-2 activation (Sternlicht and Werb, 2001). TIMP-2 at low-to-moderate levels promotes the activation of MMP-2, whilst at higher levels, TIMP-2 saturates the free MT-MMPs; thereby, inhibiting MMP-2 activation (Sternlicht and Werb, 2001).

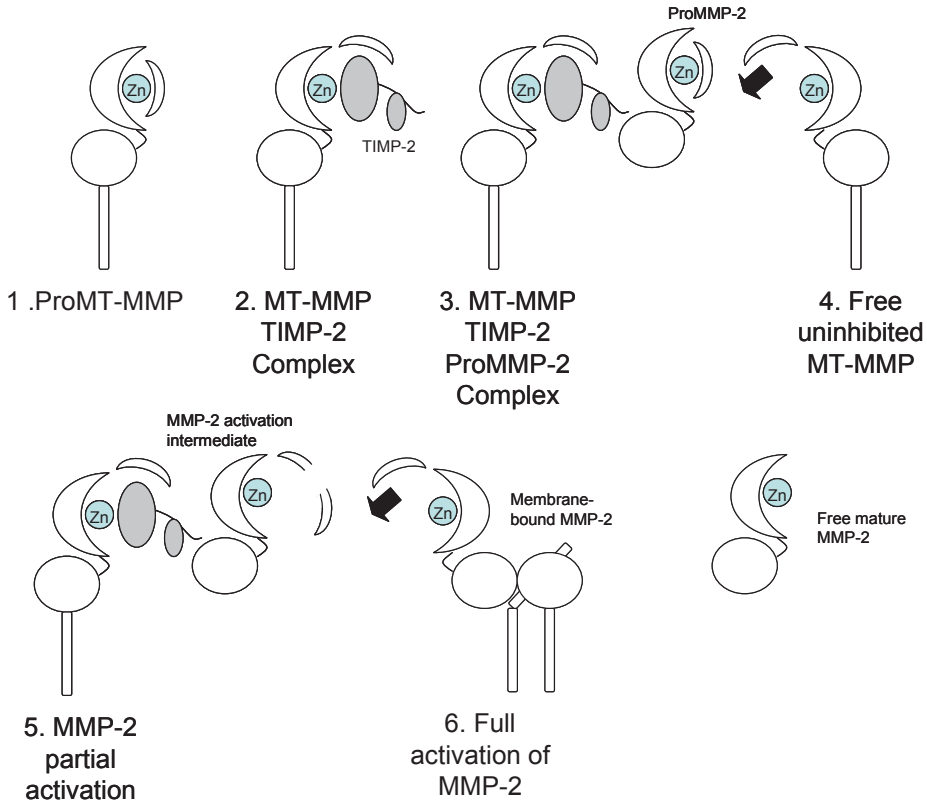

Figure 1.7: **Cell surface activation of MMP-2 via the formation of trimolecular complex with activated transmembrane MT-MMP and TIMP-2.** (1) Membrane bound proMT-MMP is activated by an intracellular furin-like serine proteinase. (2) Activated MT-MMP is then inhibited by TIMP-2. (3) Forms a trimolecular complex with proMMP-2. (4) An uninhibited MT-MMP then partially activates the proMMP-2 by removing part of the MMP-2 propeptide. (5). (6) The remaining proppetide is removed by a separate MMP-2 molecule at the cell surface to yield fully active mature MMP-2 (Adapted from (Sternlicht and Werb, 2001)).

Other factors can influence MMP-2 activation either directly or via alteration in the production of its inducer and/or activator. COLL-IV, a MMP-2 substrate, is thought to be able to induce MMP-2 activation by stimulating TIMP-2 degradation (Maquoi et al., 2000). The presence of COLL-IV results in no alteration in mRNA levels of MMP-2, MT1-MMP and TIMP-2 but TIMP-2 protein levels are reduced and MMP-2 activation enhanced

(Maquoi et al., 2000). This suggests that the local accumulation of COLL-IV may trigger its own degradation by lowering TIMP-2 levels, resulting in MMP-2 activation and ECM remodelling (Maquoi et al., 2000).

Interestingly, MMP-2 may require cell surface localisation to potentiate proteolytic activation (Brooks et al., 1996). MMP-2 has been seen in its proteolytically active form on the cell surface of a variety of cells both in vitro and in vivo (Emonard et al., 1992). Active MMP-2 has been shown to bind directly to integrin αvβ3 via its C-terminus domain of invasive cells, including endothelial cells undergoing angiogenesis and melanoma cells (Brooks et al., 1996). Co-localisation of MMP-2 and integrin αvβ3 allows cell-mediated COLL degradation and motility (Brooks et al., 1996).

MMP-2 is expressed constitutively in normal nerve and is up-regulated rapidly and converted from the latent to the active form after injury (Ferguson and Muir, 2000). mRNA levels of MMP-2 peaked by 5-10 days in adult male rats and remained up-regulated until 40-63 days post-nerve crush (Demestre et al., 2004), whilst MMP-2 protein and activity levels are at their highest between days 3-14 (Ferguson and Muir, 2000). This pattern of expression and activation suggests the MMP-2 plays a role in both the nerve degeneration and regeneration processes (Demestre et al., 2004). Pro and active MMP-2 was localised to the site of injury and showed distal progression from the site of injury (Ferguson and Muir, 2000). However, no change in MMP-2 protein levels was detected in the DRG and dorsal spinal cord of the injured rats, suggesting a localised expression of MMP-2 to the site of injury (Ferguson and Muir, 2000). Early immunoreactivity of MMP-2 following nerve trauma is by the Schwann cells (Demestre et al., 2004;La et al., 1996;Muir, 1994), endoneurial cells and the axons (Demestre et al., 2004); whilst at later timepoints MMP-2 was increased predominatly within the regenerating axons (Demestre et al., 2004). The role of MMP-2 in axonal regeneration has been studied in chick, embryonic sensory neuron cultures, where MMP-2 immunoreactivity was localised in neuronal cell bodies, cell bodies, neurites, and growth cones, including filopodia (Zuo et al., 1998a).

1.4.2. MMPs and nerve degeneration

Injury to the peripheral nerve is followed by Wallerian degeneration in the denervated nerve. Wallerian degeneration involves the degradation and phagocytosis of axons and myelin sheaths, the recruitment of macrophages and non-resident Schwann cells, and the proliferation of Schwann cells forming the basal lamina column (Hughes et al., 2002;Muir and Manthorpe, 1992;Ide and Kato, 1990;Ide, 1996). Successful nerve regeneration is dependent on the remodelling of endoneurial and basal lamina to an environment favourable to axonal regeneration following the completion of Wallerian degeneration (Ide, 1996;Stoll and Muller, 1999). The regenerating axons access the LM-rich basal lamina column of the degenerating nerve stump elongate along the basal lamina via attachment to positive signal molecules including LM, COLL IV and FN (Vogelezang et al., 1999;McKerracher et al., 1996;Venstrom and Reichardt, 1993;Tonge et al., 1997;Stevens and Jacobs, 2002). The success of peripheral nerve regeneration has been linked to changes in the microenvironment of the peripheral nerve, particularly changes of ECM components. The distribution of ECM components, MMPs and TIMPs, during Wallerian degeneration and nerve regeneration has a major effect on the success of nerve repair following injury (Gantus et al., 2006).

1.4.2.1. Degradation of blood nerve barrier

The breakdown of the BNB is an important event in axonal degeneration in the PNS. The BNB is the barrier between the perineurium of the peripheral nerve and the endothelium of endoneurial capillaries. The BNB is a selectivity permeabile barrier that maintains the homeostasis of the PNS, controlling the movement of molecules into and out of the peripheral nerve. In the sciatic nerve, the BNB disruption occurs rapidly following sciatic nerve crush. A key mediator in BNBb reakdown is TNFα, which is increased rapidly at the site of injury at the perineurial-endoneurial junction via its release from endoneurial and Schwann cell stores (Shubayev and Myers, 2002). MMP-2 activates the perineurial vessels and perineurial-endoneurial junction of the sciatic nerve, resulting in increased vascular permeability of the BNB via degradation of sub-endothelial COLL-IV (Fujimura et al., 1999;Rosenberg et al., 1992;Shubayev and Myers, 2002;Woessner, Jr., 1994). Activated MMP-9 is released from resident macrophages and contributes to further BNB disruption,

the formation of edema (Shubayev and Myers, 2002) and subsequent macrophage migration into the endoneurium (Leppert et al., 1995).

1.4.2.2. Wallerian degeneration

During peripheral nerve degeneration, axon and myelin debris is phagocytosed by Schwann cells and resident and haematogenous macrophages (Myers et al., 2006;Kobayashi et al., 2008). In the immediate early response, Schwann cells alone mediate the initial myelin degradation (Fernandez-Valle et al., 1995;Kobayashi et al., 2008); this is closely followed by activation of resident macrophages (Chattopadhyay et al., 2007;Kawasaki et al., 2008;Shubayev and Myers, 2002;Shubayev et al., 2006). Following the breakdown of the BNB, infiltration of haematogenous macrophages (Kobayashi et al., 2008;Stoll et al., 1989;Shubayev et al., 2006) join the process of myelin breakdown.

MMP-9 is important in the recruitment of haematogenous macrophages into the injured peripheral nerve. Studies using mice with delayed Wallarian degeneration (Wld^S), (characterised by reduced macrophage recruitment to the injured nerve), show reduced MMP-9 and TNFα expression at the lesion site (Shubayev et al., 2006). MMP-9 mRNA and protein levels rapidly increase in Schwann cells following nerve injury (Chattopadhyay et al., 2007;Kobayashi et al., 2008) and plays a direct role in the initial Schwann cell response to injury and mediates degradation of myelin basic protein (MBP) (Kobayashi et al., 2008). The importance of MMP-9 in Wallerian degeneration was confirmed in nerve injury experiments in MMP-9 knockout mice (Chattopadhyay et al., 2007) and in mice treated with the general MMP inhibitor, Ilamostat, where MBP degradation, macrophage influx and Schwann cell activation was attenuated following nerve crush injury (Kobayashi et al., 2008). In MMP-9 KO mice, preserved myelin thickness is evidence due to failure of MBP degradation; thus, demonstrating the role of MMP-9 in the demyelination process (Chattopadhyay et al., 2007).

1.4.2.3. Formation of the Schwann cell column

Schwann cell migration and proliferation play a critical role in the successful regeneration of the peripheral nerve. Following Wallarian degeneration of the distal nerve axons, Schwann cells migrate towards axonal signals. Here, they differentiate and ensheath the undamaged basal lamina tubes of the degenerated nerve forming the Schwann cell column, which forms a framework for nerve regeneration (Hughes et al., 2002;Ide, 1996;Johnson et al., 2005;Mantuano et al., 2008).

MMP-9 promotes Schwann cell migration via activation of LDL receptor-related protein (LRP-1) (Mantuano et al., 2008). LRP-1 is a regulator of cell signalling in response to specific ligands, including tPA, ApoE and activated α-macroglobulin (Mantuano et al., 2008), known to be involved in the Schwann cell migration process. Schwann cell expression of LRP-1 (Mantuano et al., 2008;Campana et al., 2006) and MMP-9 (La et al., 1996;Shubayev and Myers, 2002) is substantially increased in PNS injury. Studies have demonstrated that LRP-1 is essential for MMP-9-initated cell signalling and consequential promotion of Schwann cell migration (Mantuano et al., 2008). MMP-9 ability to activate cell signalling and migration can be blocked by using receptor-associated protein, which inhibits MMP-9 binding to LRP-1 (Mantuano et al., 2008) and by blocking the cell-signalling response (Mantuano et al., 2008).

MMP-9 bind directly to LRP-1 to trigger this cell signalling response, activation does not require the MMP-9 active-site but instead, is mediated by the binding of the hemopexin domain (MMP-9 PEX) to LRP-1 (Mantuano et al., 2008). MMP-9 PEX activates Schwann cell migration *in vitro* and *in vivo* via activation of extracellular signal-regulated kinase (ERK-1/2) pathway (Mantuano et al., 2008). MMP-9 PEX is only functional in the injured peripheral nerve when Schwann cells express abundant LRP-1 (Mantuano et al., 2008;Campana et al., 2006) and adopt a migration phenotype (Ide, 1996;Mantuano et al., 2008;Torigoe et al., 1996).

1.4.3. MMPs and nerve regeneration

The adult peripheral nerve ECM environment restricts nerve regeneration due to the presence of inhibitory factors including CSPGs and HSPGs (Zuo et al., 1998b;Venstrom

and Reichardt, 1993). This non-permissive environment serves to inhibit nerve sprouting during periods of non-injury. However, following nerve injury, the peripheral nerve degenerates and this denervated nerve is more favourable to axonal growth than the normal nerve (Ferguson and Muir, 2000) due to changes in nerve morphology, including differential expression of positive and negative factors including ECM proteins, and altered expression of MMPs and their associated TIMPs (Gantus et al., 2006).

CSPGs are proteoglycans that are expressed on the surface of most cells and in the ECM of most tissues. CSPG is a known inhibitory factor that plays important roles in CNS and PNS development as well as nerve regeneration following injury (Hynds and Snow, 1999;Katoh-Semba et al., 1995;Snow et al., 1996;Snow et al., 1990;Smith-Thomas et al., 1995). Following nerve injury, CSPG levels increase in the distal nerve section (Zuo et al., 1998b). CSPGs in uninjured nerves form slender bands surrounding the axon-Schwann cell units and within the nodes of Ranvier; this changes to a more intense staining in the distal nerve and within the endoneurial tissue surrounding the Schwann cell basal lamina following nerve injury (Zuo et al., 1998b;Braunewell et al., 1995).

MMP-2 plays a role in the conversion of a non-permissive environment to an environment favourable for axonal growth via cleavage of CSPG, resulting in the unmasking of LM in peripheral nerve (Zuo et al., 1998b;Zuo et al., 1998a). Studies using embryonic sensory neuron cultures have been used to demonstrate the important role of MMP-2 in axonal outgrowth. Embryonic sensory neurons seeded on longitudinal nerve sections extend neurites along the exposed surfaces of the Schwann cell basal lamina. The length of the axons increased by 50% when nerve sections were pre-treated with recombinant MMP-2, with similar results achieved with pre-treatment with Chondroitinase ABC (ChABC) (Goldberg and Barres, 2000). ChABC is a bacterial enzyme that disrupts the normal organisation of the ECM via the removal of inhibitory chondroitin sulphate GAG chains (Crespo et al., 2007). With both treatments, neurite outgrowth stays closely associated with the basal lamina of the nerve sections, indicating that MMP-2, like ChABC, degrades the inhibitory factor CSPG (Zuo et al., 1998a;Ferguson and Muir, 2000) with the unmasking of LM of the basal lamina promoting neurite outgrowth. The addition of function-blocking antibodies against LM resulted in similar neurite outgrowth to non-treated cultures (Zuo et al., 1998a). The ‘poor’ regenerative growth on untreated nerve was further impaired by

inclusion of a general MMP inhibitor (GM6001, Ilamostat) (Goldberg and Barres, 2000). It is thought that the sensory neurons themselves secrete matrix-degrading enzymes, including MMPs and plasminogen (Zuo et al., 1998a;Muir, 1994). MMP-2 and MMP-9 are secreted *in vitro* in NGF-dependent manner by PC12 cell line and embryonic sensory neurons (Shubayev and Myers, 2004;Muir, 1994). MMP-2 is transported to the growth tip of the elongating neurites and secreted suggesting local remodelling of the peripheral ECM at the neurite growth tip, thereby generating an environment favourable to regeneration (Zuo et al., 1998a). MMP-9 enhances NGF-induced neuite elongation in PC12 cells (Shubayev and Myers, 2004).

1.4.4. MMPs role in the development of neuropathic pain

Increased levels of MMP-2 and MMP-9 have also been implicated in the development of early- and late-phase neuropathic pain in rats and mice following nerve injury (Kawasaki et al., 2008). Inhibition of MMP-9 by intrathecal route inhibits the development of mechanical allodynia following spinal nerve ligation (SNL), whilst inhibition of MMP-2 or TIMP-2 has been shown to reverse SNL-induced late phase allodynia (Kawasaki et al., 2008). Furthermore, intrathecal administration of MMP-2 or MMP-9 to animals without SNL was sufficient to produce neuropathic pain symptoms in uninjured rats (Kawasaki et al., 2008). MMP-9 involved in early stage pain development was further assessed by using MMP-9 KO mice. MMP-9 KO mice with SNL had reduced levels of early-phase allodynia. However, MMP-9 deficient mice developed late-stage allodynia, suggesting MMP-2 compensation (Kawasaki et al., 2008). Treatment of MMP-9 KO with MMP-2 inhibitor produced a more profound anti-allodynic effect (Kawasaki et al., 2008). MMP-9 involvement in the development of mechanical allodynia has been shown to involve both the activation of p38 MAPK and the cleavage of IL-1β. p38 inhibition reverses both MMP-9 and SNL-induced allodynia. Whilst MMP-9 treatment increased both the levels of IL-1β as well as the cleavage of IL-1β in the DRG, with SNL-induced IL-1β, cleavage was reduced in MMP-9 null mice (Kawasaki et al., 2008). MMP-2 mediated-maintenance of neuropathic pain involves both IL-1β cleavage and ERK activation in astroglia (Kawasaki et al., 2008).

1.4.5. MMPs and diabetes

Several clinical studies have described elevated levels of MMPs and TIMPs in blood samples from both type 1 (Maxwell et al., 2001;Jacqueminet et al., 2006) and type 2 diabetic patients (Lee et al., 2005;Derosa et al., 2007) and STZ rats (Uemura et al., 2001). At 8 weeks post-STZ induced diabetes, STZ-rats had significantly increased MMP-9 plasma levels when compared to age-matched controls, with no change in MMP-2 levels (Uemura et al., 2001). Whilst in clinical diabetes, higher MMP-2, MMP-9 and TIMP-1 are present in the plasma of type-1 (Maxwell et al., 2001;Jacqueminet et al., 2006) and type-2 patients (Derosa et al., 2007;Lee et al., 2005). However, these circulating values do not provide information on tissue localisation or activity.

Further supporting evidence of altered MMP expression in diabetic subjects comes from upregulation of AP-1 and TGF-β under hyperglycaemic conditions (Chen et al., 2003;Srinivasan et al., 2004). Both MMP and TIMP genes possess response elements for a number of transcription factors, including AP-1 and TGF-β. Therefore, hyperglycaemic-induced expression of these transcriptional regulators could result in altered expression of MMPs and their associated TIMPs. This hyperglycaemia-induced alteration in MMP and TIMP via altered expression of transcription factors and growth factors has been seen in several *in vitro* cultures in several cell types. Hyperglycaemia, in combination with IL-6, has been shown to induce MMP-1 expression in U937 mononuclear cell-lines via ERK and c-Jun activation. The same observation has been seen in human primary monocytes (Li et al., 2010). As circulating levels of IL-6 are elevated years before the onset of T2DM, IL-6 levels are used as a predictor for the development of T2DM. Youths with T1DM have also been shown to have higher levels of circulating cytokine compared to youths without diabetes of a similar age, especially IL-6 and fibrinogen (Snell-Bergeon et al., 2010). These increased circulating cytokines levels are thought to contribute to the development of diabetic complications, especially atherosclerosis, via increased MMP expression and matrix alterations; however, little work has been done in this area. In endothelial cell cultures, the presence of hyperglycaemia results in a direct modulation of the regulation, expression and activity of a number of MMPs and TIMPs. Bovine aortic endothelial cells (BACE) grown in culture do not express MMP-9 constitutively. However, in the presence of hyperglycaemia, BACE have increased MMP-9 promotor activity and increased MMP-9

mRNA and protein expression and gelatinase activity levels (Uemura et al., 2001). Whilst in humans, umbilical vein endothelail cells see increased MMP-1 and MMP-2 activated in the presence of hyperglycaemia and increased MMP-9 in monocytes-derived macrophage cultures following exposure to high glucose (Death et al., 2003).

1.4.5.1. MMP dysfunction and diabetic neuropathy?

In diabetic neuropathy, structural changes are observed in the peripheral nerve including increased COLL type-IV levels in the endoneurium and increased deposition of types IV and V COLL in the perineurium in the diabetic nerves (Bradley et al., 2000); reduplication of basal lamina around the endoneurial capillaries, including increases in COLL types IV, V and VI (Bradley et al., 2000;Giannini and Dyck, 1995); and thickening of the basal lamina of epineurial cells (Bradley et al., 2000;Giannini and Dyck, 1995). MMPs are well known for their ability to remodel the ECM (Hijova, 2005), with COLL the main substrate of MMP-2 and MMP-9 degradation (Coussens and Werb, 1996;Butler and Overall, 2009). Therefore, the accumulation of COLL in the peripheral nerve could be due to downregulation or inhibition of MMP-2 and/or -9 in diabetes.

Impaired axonal regeneration is also observed in experimental and clinical diabetic neuropathy. Distal nerve fibres degenerate, and regeneration attempts by the damaged fibres, although initially vigorous, are short lived and the numerous regenerative sprouts fail to survive (King, 2001;Bradley et al., 1995). This failure of axonal regeneration has been shown to be inversely proportional to duration of diabetes (Bisby, 1980;Ekstrom and Tomlinson, 1989;Kennedy and Zochodne, 2000). Both MMP-2 and MMP-9 are significantly upregulated in the rat sciatic nerve immediately after nerve crush (Demestre et al., 2004;Ferguson and Muir, 2000;La et al., 1996;Shubayev et al., 2006;Gantus et al., 2006;Hughes et al., 2002); MMP-2 and MMP-9 expression following injury are increased in Schwann cells (Demestre et al., 2004;La et al., 1996;Muir, 1994) (Chattopadhyay et al., 2007;Chattopadhyay and Shubayev, 2009), whilst MMP-2 expression is also increased in the axons themselves (Demestre et al., 2004) and MMP-9 is increased in resident and invading macrophages of the peripheral nerve (Shubayev et al., 2006). MMP-2 and MMP-9 play multiple roles in the peripheral nerve following injury; for example, mediating the breakdown and subsequent repair of the blood-nerve-barrier (Shubayev and Myers, 2002),

macrophage recruitment (Kobayashi et al., 2008;Shubayev et al., 2006), Schwann cell migration and proliferation (Mantuano et al., 2008) and myelin degradation (Kobayashi et al., 2008) during Wallerian degeneration. *In vitro* studies have also suggested a pro-regenerative role for MMP-2 and MMP-9, in modulating axonal regeneration. MMP-9 enhances NGF-induced neurite elongation and inhibition of MMP-9 by either a MMP-9 neutralising antibody or a broad-spectrum MMP inhibitor blocks NGF-mediated axonal elongation and sprouting (Shubayev and Myers, 2004). MMP-2 plays a role in remodelling of the matrix during axonal elongation (Krekoski et al., 2002;Ferguson and Muir, 2000;Zuo et al., 1998a), via the degradation and inactivation of neurite growth-inhibiting CSPG (Ferguson and Muir, 2000;Zuo et al., 1998a;Krekoski et al., 2001). Therefore altered expression or activity of MMP-2 and MMP-9 in diabetes may also contribute to nerve regeneration failure, either directly or via impaired Wallerian degradation and nerve regeneration.

We, and other groups, have shown that accumulation of AGEs on intracellular and extracellular proteins collected from STZ-induced diabetic rats, occurs as early as 3 weeks post-STZ (Duran-Jimenez et al., 2009;Thornalley et al., 2003). *In vitro* work has established that ECM glycation dramatically impairs neuronal outgrowth and regeneration. Glycation of the COLL, LM and FN proteins hinder the extension of axonal sprouts from dorsal root ganglia explants from mice (Ozturk et al., 2006) and from dissociated primary sensory neuron cultures of neonatal and adult rats (Duran-Jimenez et al., 2009;Luo et al., 2002). Glycation of integrin binding domains in ECM proteins could contribute to nerve regeneration failure. Glycation of the RGD domains of COLL-IV inhibits $\alpha v\beta 3$ integrin binding and renal and retinal endothelial cell adhesion (Dobler et al., 2006;Pedchenko et al., 2005). Similarly, glycation of FN results in reduced retinal endothelial cell attachment and spreading (Bhatwadekar et al., 2008;McDonald et al., 2009). As our laboratory has previously shown that RGD domain of fibronectin is important for $\alpha 5\beta 1$ integrin binding and subsequent neurite outgrowth, this suggests that glycation of RGD domain may be responsible for impaired neurite outgrowth on glycated fibronectin. Therefore, these observations suggest that glycation of ECM proteins may result in the modification of functionally important integrin-binding sites, resulting in reduced integrin activation and neurite outgrowth. Another consequence of ECM glycation is reduced proteolyis and

degradation of ECM proteins. A high glucose environment results in reduced ECM degradation due to the diminished expression of ECM-degrading enzymes, specifically MMPs, (McLennan et al., 2007;Rittie et al., 1999) via altered integrin-mediated cell signalling (Hasegawa et al., 1995;Rittie et al., 1999). Reduced MMP production has been shown in mesangial cell cultures; conditioned media collected from cells grown on glycated mesangium matrix have a reduced degradation capability compared to media from cells grown on control matrix (McLennan et al., 2002). The reduced degradation ability seen in the glycated mesangial cell was thought to occur in two ways: by the direct reduction in MMP mRNA and protein expression, as seen with MMP-7 (McLennan et al., 2007), or by reduction in the expression of its activator, as seen with MT1-MMP levels, which would result in an altered activator-inhibitor balance, resulting in reduced MMP-2 activation (McLennan et al., 2002;Rittie et al., 1999). This AGE-induced reduction in MMP proteolysis could further contribute to ECM protein accumulation in the basal lamina (McLennan et al., 2002;McLennan et al., 2007). Areas of glycated ECM proteins could act as a physical barrier to elongation of axonal sprouts, via inhibition of axonal attachment, integrin activation and failure to convert the ECM from a non-permissive environment to a growth-permissive environment. All these processes could contribute to axonal regeneration failure, and subsequent loss of peripheral sensation in diabetic neuropathy.

1.5. Research Aims

MMP-2, MMP-9 and TIMP-1 concentrations are elevated in serum and plasma samples from type 1 (Maxwell et al., 2001;Jacqueminet et al., 2006) and type 2 diabetic patients (Lee et al., 2005;Derosa et al., 2007) and also STZ-diabetic rats (Uemura et al., 2001). Furthermore, there is an increasingly large body of evidence which suggests that MMP dysregulation may contribute to the pathophysiology of diabetic complications, including retinopathy (Giebel et al., 2005;Jacqueminet et al., 2006;Noda et al., 2003), chronic non-healing diabetic ulcers (Lobmann et al., 2006;Medina et al., 2005), coronary and peripheral arterial disease (Tayebjee et al., 2005a;Galis and Khatri, 2002;Tayebjee et al., 2005b) and nephropathy (Thrailkill et al., 2007). However, there is no information to date about the expression or activity of MMPs in diabetic neuropathy. Therefore the main aim of this thesis was to characterise and compare MMP-2 and MMP-9 expression and activity in the peripheral nervous system of control and STZ-induced diabetic rats. We used sybr-green PCR, Western blotting, immunocytochemistry and zymography to examine changes in MMP-2 and -9 gene, protein and activity levels in the DRG and the sciatic nerve, and primary cultures of adult rat sensory neurons to examine direct effects of MMP-2 and MMP-9 on neurite outgrowth. Since, MMP-2 and -9 play crucial roles in Wallerian degeneration and nerve regeneration we hypothesise that the regenerative failure in diabetic neuropathy may be associated with MMP dysregulation. We have previously demonstrated that AGE residues accumulate in ECM proteins of sciatic nerve endoneurium and that exogenous glycation of laminin and fibronectin reduces neurite outgrowth from sensory neurons. In this thesis we investigate whether 'endogenous glycation' of the peripheral nerve in diabetes is also inhibitory to neurite outgrowth from sensory neurons, and test the ability of the AGE inhibitors LR-90 and pyridoxamine to correct regenerative failure and nerve deficits using a number of electrophysiological, behavioural and biochemical endpoints in an experimental model of diabetes.

1.5.1. Thesis structure

This thesis is written as an alternative format thesis according to the guideline stipulated by the University of Manchester. The alternative format allows for thesis data chapters to be written in a format suitable for publication in a peer reviewed journal, whether solely or

jointed authored. This thesis comprises a general introduction (Chapter One), three independent data Chapters (Chapters Two, Three and Four) each written as manuscript for publications and a final discussion (Chapter Five). References for the general introduction and final discussion chapters are combined and are found at the rear of this thesis (Chapter Six).

1.5.2. Chapter Objectives

1.5.2.1. Chapter 2: "Matrix Metalloproteinase-2 is upregulated in Experimental Diabetic Neuropathy"

This chapter was written as a manuscript intended for submission to the *Journal of the Peripheral Nervous System*. Experiments in this paper were designed to test the hypothesis that MMP-2 and MMP-9 levels or activity may be altered in the peripheral nervous systems of experimental diabetic rats and as such could influence the development of diabetic neuropathy.

The specific aims of this paper were to:

- Establish the baseline expression of MMP-2 and -9 gene and protein expression, and activity levels in the DRG and sciatic nerve of control rats.
- Determine whether MMP-2 and -9 gene and protein expression and activity levels in the DRG and sciatic nerve are changed in STZ-diabetic rats compared to age-matched controls.
- Characterise the cell types that express/upregulate/downregulate MMP-2 and/or MMP-9 in DRG and sciatic nerve in STZ-diabetic rats.

This paper was a collaborative effort; all work presented was carried out by me with the exception of:

- The quantitative real time PCR (Figures 2.5, 2.6 & Supplementary Figure 3) was carried out by Dr Sumia Ali
- MMP-2 and s100 co-localisation (Figures 2.2 & 2.9) and the subsequent confocal microscopy were carried out by Dr Sumia Ali

- The Nerve Conduction Velocity measurements (Supplementary Figure 1) were conducted by Dr Natalie Gardiner.

1.5.2.2. Chapter 3: "Modification of the extracellular matrix of sciatic nerve in diabetic rats contributes to the pathogenesis of diabetic neuropathy"

This chapter has been written as a manuscript intended for submission to the *Journal of the Peripheral Nervous System*, and was designed as a follow up study to the data presented in Chapter 2, where increased MMP-2 expression was observed in Schwann cell and axons of the sciatic nerve in STZ-induced diabetic rats. Experiments in this paper were designed to determine whether MMP-2 and -9 directly modulate neurite outgrowth in sensory neuron cultures and to test the hypothesis that the ECM of the sciatic nerve from diabetic animals is less supportive for regenerating axons, and that increased levels of MMP-2 may be an attempt by the peripheral nerve to re-model its ECM to allow successful regeneration to occur.

The specific aims of this paper were to:

- Establish a sensory-neuron/peripheral nerve cryoculture system to determine whether the extracellular matrix of diabetic peripheral nerve is less supportive for sensory neuron regeneration than sciatic nerve obtained from age-matched controls.
- Determine whether MMP-2 / ChABC treatment improves the ability of the diabetic peripheral nerve to support nerve regeneration.
- Establish the role of MMP-2 and -9 in primary cultures of adult rat dissociated sensory neuron cultures (NGF-dependent collateral sprouting model), and pre-conditioned (sciatic nerve injury, 7 days prior to culture) models).

1.5.2.3. Chapter 4: "Neuroprotective effects of the advanced glycation end-product inhibitors, LR90 and pyridoxamine in experimental diabetes"

This chapter has been written as a manuscript intended for submission to *Diabetologia*. Experiment in this paper were designed to test the hypothesis that glycation of the peripheral nerve ECM may be a potential mechanism for the failure of collateral sprouting and axonal regeneration observed in diabetic neuropathy.

The specific aims of this paper were to:

- Investigate whether AGE inhibitors (LR90 and PM) are able to normalise NCV deficits.
- Determine the effects of LR90 and PM treatment on sensory behaviour (tactile allodynia, mechanical hypersensitivity and thermal hyperalgesia) of STZ-rats.
- Investigate changes in cutaneous IENF fibre profile in AGE inhibitor treated STZ-rats.
- Investigate whether alterations of the extracellular matrix of diabetic peripheral nerve which render the ECM less supportive to axonal regeneration can be normalised by AGE inhibitor treatment.

This paper was a collaborative effort, therefore all work presented in this paper was carried out by me with exception of:

- Nerve Conduction Velocity (NCV) measurements (Figure 4.2) were carried out by Dr Natalie Gardiner
- Thermal and mechanical behaviour testing (Figure 4.4 and 4.5) were carried out with the help of Dr Ian Millar and Laura Smith
- All ELISAs (Figure 4.1 and 4.6) were conducted by Dr Sumia Ali

Matrix metalloproteinase-2 is upregulated in experimental diabetic neuropathy

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2.1. Abstract

Dysregulation of matrix metalloproteinases (MMPs) contributes to the development of a number of diabetic complications including: retinopathy, nephropathy and coronary and peripheral arterial disease. The aim of this study was to investigate whether levels or activity of MMP-2 or MMP-9 change in the peripheral nervous system of streptozotocin (STZ)-induced diabetic rats.

MMP-2 and MMP-9 gene, protein and activity levels were measured in the dorsal root ganglia (DRG) and sciatic nerve at 4, 8 and 12 weeks following induction of diabetes in adult male Wistar rats. MMP-2 mRNA and protein were expressed constitutively in the DRG and sciatic nerve of control adult rats. The induction of diabetes had no significant effect on MMP-2 mRNA or protein levels in the DRG. However, MMP-2 mRNA, protein and activity were upregulated in the sciatic nerve 8 weeks post-STZ induction of diabetes. MMP-2 immunoreactivity was localised to the Schwann cells and axons of the peripheral nerve of both control and diabetic rats.

This study demonstrated a diabetes-associated upregulation of MMP-2 gene and protein expression in the sciatic nerve, with conversion of proMMP-2 to its active form in a discrete population of diabetic rats. The functional consequence of this increase in MMP-2 expression and whether it contributes to the development of diabetic neuropathy warrants further investigation.

Key Words: Diabetes, Diabetic Neuropathy, Peripheral Nervous System, Matrix Metalloproteinase.

2.2. Introduction

Diabetic neuropathy is the most common secondary complication of diabetes, occurring in 50% of type 1 diabetic patients. The development of diabetic neuropathy is typically associated with long-term diabetes (25 years or more) and poor glycemic control (The Diabetes Control and Complications Trial Research Group, 1993). Diabetic neuropathy is characterised by the loss of the protective sensation in the patient's extremities resulting from the distal 'die-back' of the peripheral sensory nerve terminals. Distal nerve fibres degenerate and regeneration attempts by the damaged fibres, although initially vigorous, are short lived and the numerous regenerative sprouts fail to survive (King, 2001;Bradley et al., 1995).

The factors underlying axonal degeneration and regeneration failure in diabetes are complex. Structural abnormalities of the peripheral nerve in diabetes include Schwann cell pathology (King et al., 1989), endoneurial microangiopathy (Thrainsdottir et al., 2003;Zochodne and Nguyen, 1999), axonal degeneration (King, 2001;Bradley et al., 1995), demyelination of the paranodal (axonal-glial) junction and nerve fibres (Hill and Williams, 2004) as well as increased endoneurial extracellular matrix (ECM) deposition (Bradley et al., 2000;Giannini and Dyck, 1995), glycation of intracellular and extracellular proteins of the peripheral nerve (Duran-Jimenez et al., 2009;Thornalley et al., 2003) and the thickening of the basement membranes of perineurium and epineurium (Hill and Williams, 2002;Hill and Williams, 2004). All these structural components of the peripheral nerve play important roles in nerve degeneration and regeneration: therefore, their alteration, as a result of diabetes, could contribute to the development of neuropathy.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that are best known for their role in the breakdown and turnover of the ECM (Hijova, 2005). They play crucial roles in Wallerian degeneration (Chattopadhyay et al., 2007;Kobayashi et al., 2008) and subsequent axonal regeneration following nerve injury (Zuo et al., 1998b;Zuo et al., 1998a). MMPs have recently been proposed as being more than simply modulators of extracellular matrix turnover. Non-matrix MMP substrates have been discovered, which suggest that MMPs also play a role in growth factor release, cytokine activation and receptor cleavage and play a part in altered cell growth, regulation of

apoptosis, cell migration and cell-cell communication (Butler and Overall, 2009;McCawley and Matrisian, 2001).

The gelatin-binding family of MMPs (MMP-2 and MMP-9) are particularly involved in axonal regeneration following injury. MMP-2 and MMP-9 are significantly upregulated in the rat sciatic nerve immediately after nerve crush (Demestre et al., 2004;Ferguson and Muir, 2000;La et al., 1996;Shubayev et al., 2006). MMP-2 is expressed by Schwann cells (Demestre et al., 2004;La et al., 1996;Muir, 1994), endoneurial cells and axons (Demestre et al., 2004), whilst MMP-9 is expressed by Schwann cells (Chattopadhyay et al., 2007;Chattopadhyay and Shubayev, 2009) and macrophages (Shubayev et al., 2006) following nerve injury. MMP-2 and MMP-9 play multiple roles in the peripheral nerve following injury; for example, mediating the breakdown and subsequent repair of the blood-nerve-barrier (Shubayev and Myers, 2002), macrophage recruitment (Kobayashi et al., 2008;Shubayev et al., 2006), Schwann cell migration and proliferation (Mantuano et al., 2008) and myelin degradation (Kobayashi et al., 2008) during Wallerian degeneration. In addition, MMP-2 is also involved in the removal of inhibitory ECM components such as chondroitin sulfate proteoglycans (CSPGs) allowing successful axonal regeneration (Zuo et al., 1998b;Zuo et al., 1998a).

MMPs are tightly regulated at three levels: transcriptional regulation, activation of pro-peptide and endogenous inactivation of MMPs by tissue inhibitor of MMPs (TIMPs). MMP gene expression can be induced by a number of factors including growth factors such as nerve growth factor (NGF), proinflammatory cytokines, reactive oxygen species and protein kinase C, whilst their expression is suppressed by glucocorticoids and interferons (Pizzi and Crowe, 2007). Many of these stimuli alter MMP gene expression via activation of the MAPK cascade, specifically ERK-1/2, p38 kinases and JNK (Arai et al., 2003;Ehrlichman et al., 2009;Moon et al., 2004). These signalling pathways activate MMP gene expression through Fos and Jun family members that bind to the activator protein-1 binding site located in the promoter region of each inducible MMP (Pizzi and Crowe, 2007;Cawston and Wilson, 2006). Many of these MMP activators are increased in the peripheral nerve in clinical and experimental diabetes (Haorah et al., 2007;Haorah et al., 2008;Bonnefont-Rousselot, 2002;Purves et al., 2001).

MMP-2, MMP-9 and TIMP-1 concentrations are elevated in serum and plasma samples from type 1 (Maxwell et al., 2001;Jacqueminet et al., 2006) and plasma samples from type 2 diabetic patients (Lee et al., 2005;Derosa et al., 2007) and also STZ-diabetic rats (Uemura et al., 2001). Furthermore, dysregulation of MMP activity has been implicated in the pathophysiology of other diabetic complications and dysfunctions including diabetic retinopathy (Giebel et al., 2005;Jacqueminet et al., 2006;Noda et al., 2003), chronic non-healing diabetic ulcers (Lobmann et al., 2006;Medina et al., 2005), coronary and peripheral arterial disease (Tayebjee et al., 2005a;Galis and Khatri, 2002;Tayebjee et al., 2005b) and, more recently, diabetic nephropathy (Thrailkill et al., 2007). However, there is no information to date about the expression or activity of MMPs in diabetic neuropathy.

The aim of this study was to investigate whether MMP-2 and/or MMP-9 expression is altered in diabetic neuropathy as reduplication of collaganase in the basement membrane of the peripheral nerve of diabetic patients could be the result of diabetes-induced down-regulation of MMP-2 or -9. Alternatively, the increased expression of many MMP activators in the peripheral nerve of diabetic rats suggests that MMP production could be increased. This study describes the spatial and temporal expression of MMP-2 in the dorsal root ganglia (DRG) and sciatic nerve of control and streptozotocin (STZ)-induced diabetic rats. We demonstrate that MMP-2 mRNA, protein and activity levels are upregulated in the sciatic nerve 8 weeks post-induction of diabetes compared to age-matched controls.

2.3. Materials and methods

2.3.1. Animal studies and tissue harvest

All experiments were conducted in accordance with the UK Animals Scientific Procedures Act (1986) and institutional regulations. Adult male Wistar rats (Charles River, UK) with a starting weight of 250-300g were given a single intraperitoneal injection of STZ (Sigma, UK, Cat No. S0130) at a dose of 55mg/kg in 0.9% NaCl, which was administered the morning after an overnight fast. Three days later, blood glucose levels were monitored using a strip-operated reflector photometer (MediSense Optimum Plus; MediSense, UK). Animals with blood glucose less than 15mmol/l were rejected from the study. All rats were provided with standard rodent chow (Beekay International) and tap water, and were housed at a constant temperature of 21°C with 12:12 hour light/dark cycle. Animals were weighed and checked biweekly. Age-matched rats were used as non-diabetic controls. Animals were maintained for 4, 8 or 12 weeks post-STZ.

2.3.1.1. Nerve conduction velocity measurements

Motor NCV and sensory NCVs were measured at the end of the trials (4, 8 and 12 weeks post-STZ). Rats were anesthetised with isofluorane (2% in oxygen). The sciatic nerve was stimulated via insertion of fine percutaneous needles electrodes firstly at the sciatic notch and then at the Achilles tendon. Stimulation, comprising 0.1ms pulses of varying amplitude was delivered using Neurolog stimulus isolator, a pulse generator and an AC/DC amplifier. Evoked electromyograms from the interosseous foot muscles were recorded using ABI Scope version 3.6.8 for Powerlab 4 software. Motor NCVs were measured from latencies of compound M waves, and sensory NCVs were measured from H reflex latencies from the same stimulation sites. H reflexes were considered genuine if they appeared at a lower stimulus voltage than M waves. Nerve length from sciatic notch to Achilles tendon was measured *ex vivo*. The latency difference between the two sets of H reflexes was calculated and related to the nerve length separating the two stimulus points to calculate sensory NCV. M wave latency differences were used similarly to calculate motor NCV.

After NCVs were measured, rats were terminally anaesthetised using isoflurane (2% in oxygen) and either perfused transcardially with 0.9% heparinised saline (0.9% NaCl and 50 units/ml heparin) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB), or killed by exsanguination from the right ventricle. The sciatic nerve and lumbar (L)4/5 DRG were removed and either snap-frozen on dry-ice and stored at -80°C, or post-fixed for 2 hours at 4°C then cryoprotected in 10% sucrose in 0.1M PB overnight at 4°C, followed by 30% sucrose in 0.1M PB overnight at 4°C. The sciatic nerve and DRG were mounted in OCT embedding medium (VWR, UK, Cat No. LAMB/OCT), frozen on dry ice and stored at -80°C.

2.3.2. RNA extraction and PCR

RNA was extracted from the L4/5 DRG and sciatic nerve from age-matched controls and 4, 8 and 12 week diabetic rats using the RNAeasy mini kit (Qiagen, Cat No. 74104) or RNeasy Lipid Tissue kit (Qiagen, Cat No. 74804) respectively, according to the manufacturer's instructions.

The sciatic nerves were homogenised in 1ml QIAzol lysis reagent followed by the addition of chloroform to separate the homogenate into aqueous and organic phases by centrifugation. DRGs were placed in 600 µl of the provided RLT solution and homogenised. The DRG were further homogenised by transfer of lysate to a Qia-shredder and spun. Following generation of lysate, both tissue types were treated the same. One volume of 70% ethanol was added to the flow-through to precipitate the RNA, which was then centrifuged through an RNAeasy column to bind the RNA to the column. The RNA bound to the RNAeasy column was washed once with RW1 and twice with RPE buffer. The RNA was eluted using 30 µl of RNase-free water.

RNA was DNase treated using the DNA free kit (Applied Biosystems UK, Cat No. AM1906) and total RNA concentration was quantified using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop technologies, Wilmington, USA). 1 µg RNA was used to synthesise cDNA using the Taqman Reverse Transcription Reagent Kit (Applied Biosystems UK, Cat No. N8989234). Multiscribe reverse transcriptase was used to

synthesise cDNA using the following thermal cycling: 25 °C for 10 minutes, followed by 48 °C for 30 minutes and then inactivation of the enzyme at 95 °C for 5 minutes.

cDNA samples were used to quantitatively analyse the copy number of specific genes using real time PCR. A master mix of SYBER green with a ROX reference dye (DyNAmo^{TM}HS SYBER Green, Finnzymes, Finnland, Cat No. F410) was used in the real time PCR with the following thermal cycling: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 1 minute at 60 °C. Each sample was assayed in duplicate and a no-template control for each primer set was also included in each analysis. All reactions were performed in an ABIPrism^{TM} 7700 sequence detector system (Applied Biosystems Ltd).

All primer pairs were optimised at an annealing temperature of 60 °C using traditional PCR prior to use in real time PCR. Table 2.1 displays the primer sequences used in the real time PCR reactions. Following thermal cycling, a cycle threshold (CT) value was generated for each sample (the cycle number at which a defined fluorescence threshold was reached). A dissociation curve was also generated for each sample, indicating the melting temperature of the PCR product. A single peak indicated that a single product was generated from the PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actin	GAAGGCGACAGCAGTTGGTT	ACGCGACCATCCTCCTCTTA
MMP 2	ATGGCATTGCTCAGATCCGT	AGCCTTCTCTTCCTGTGGGG
MMP 9	CTGCGTATTTCCATTCATCTTCG	CGAGTTGCCCCCAGTTACAG
MT1-MMP (MMP-14)	AGTCAGGGTCACCCACAAAG	GGGTATCCGTCCATCACTTG
TIMP 1	TGGGGTGTGCACAGTGTTTC	CGCTCTGGTAGCCCTTCTCA
TIMP 2	CGGAAGGAGATGGCAAGATG	CCATCCAGAGGCACTCATCC
TIMP 3	ACTCCGACATCGTGATCCGG	TGTCAGGAGGTACTGGTATT

Table 2.1: **Forward and reverse primer sequences (5'-3').** Primers were designed using Primer 3.0 and blast primer programmes following Pubmed identification of gene sequence of interest. Actin was used as reference (house-keeping) gene.

2.3.2.1. Statistical analysis

The data generated from the real time PCR were analysed using Microsoft Excel. The average of two CT measurements per animal per primer was used and then normalised to actin expression to give a measurement of RNA expression. Statistical analysis was conducted using GraphPad PRISM 4 (GraphPad Software Inc). Data were analysed using an unpaired t-test. Data are shown as mean ± standard deviation.

2.3.3. Western blots

Desheathed sciatic nerves and DRG were homogenised in a freshly prepared ice cold lysis buffer (25mM Tris HCl pH 7.4, 15mM NaCl, 10mM NaF, 10mM Na Pyrophospahte, 2mM EDTA, 0.2mM Na_4Ov_3, 1mM PMSF, Protease Inhibitor Cocktail (1:200, Sigma, Cat No. P8340) and 1% (w/v) Nonidet P40) for 30-60 seconds using a hand-held homogeniser (Omni International μH, Camlab). The tissue homogenates were centrifuged (9,000g, 30 minutes, 4°C) and the supernatant transferred to a clean Eppendorf tube. Proteins were denatured with a SDS sample buffer (0.25M Tris pH 6.8, 20% glycerol, 0.01% bromophenol blue, 2% SDS and 2% mercaptoethanol) by boiling for 5 minutes at 95°C. Protein levels were quantified according to the colorimetric Bramhall assay with albumin used as a standard. 20μg of total protein were resolved through 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in electrophoresis buffer (25mM Tris-base, 192mM Glycine and 0.1% (w/v) SDS). The gel was run at 100V until samples were in a concentrated line in the stacking gel and then increased to 120V until the samples had run to the required distance. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL, GE Healthcare) at 80V in transfer buffer (25mM Tris-base, 192mM Glycine and 20% (w/v) Methanol). The even loading and transfer of protein was confirmed by staining the membrane for 30 seconds with 10% Ponceau S (Sigma) followed by washing with blotting buffer (10mM Tris-base, 100mM NaCl and 0.1% (w/v) Tween 20) until Ponceau staining was removed.

Non-specific binding was blocked by incubating the membranes in 10% non-fat dry milk (Marvel, Premier International food Ltd) in blotting buffer either overnight at 4°C or for 2 hours at room temperature with constant agitation. The membrane was then incubated

overnight at 4°C with constant agitation with primary antibodies (details listed in Table 2.2) in 1% bovine serum albumin (BSA; Fluka, Cat No. 05473) in blotting buffer.

	Dilution	**Company**
Rabbit Anti-Rat MMP-2 [Gelatinase A; 72kDa Type IV Collaganase]	1:2000	Millipore (Cat No. AB19015)
Rabbit Anti-Rat MMP-9 [Gelatinase B; 92kDa Type IV Collaganase]	1:3000	Millipore (Cat No. AB19016)
Rabbit anti-TIMP-1	1:5000	Millipore (Cat No. AB80050)
Rabbit anti-TIMP-2	1:2500	Millipore (Cat No. AB80150)
Rabbit anti-TIMP-3	1:5000	Millipore (Cat No. AB80250)
p44/42 MAP kinase antibody	1:5000	Cell Signalling Technologies (Cat No. 9102)

Table 2.2: **Details of antibodies used for Western blotting.**

The following day, the membranes were washed with blotting buffer and then incubated for 1 hour at room temperature in anti-rabbit horseradish peroxidase (HRP)-linked IgG antibody (1:5000; Cell Signalling Technology) diluted in 5% non-fat dry milk in blotting buffer. The membranes were washed three times for 15 minutes with blotting buffer. Proteins were visualised using LumiGLO^{TM} and peroxidase reagent (Cell Signalling Technology, Cat No.7003) according to the manufacturer's instruction. Membranes were exposed to Hyperfilm ECL (Amersham BioSciences, Cat No. 28-9068-37) for between 30 seconds and 1 hour depending on signal strength.

2.3.4. Gel zymography

Desheathed sciatic nerves and DRGs were homogenised in freshly prepared ice cold lysis buffer (0.5M Tris pH 7.6, 0.2M NaCl, 10mM $CaCl_2$ and 1% Triton X-100) and centrifuged (9,000g, 30 minutes, 4°C). The supernatant was transferred to a clean Eppendorf containing sample buffer (400mM Tris pH 6.8, 10% SDS, 50% glycerol and 0.025% bromophenol blue).

10µg of proteins were separated on 0.2% gelatin / 8% polyacrylamide gel by SDS-PAGE in electrophoresis buffer. Gels were run at 60V until samples were in a concentrated line in

the stacking gel and then increased to 100V. Gels were washed overnight at room temperature in wash buffer (50mM Tris pH 7.5, 5mM $CaCl_2$, 1μM $ZnCl_2$ and 2.5% Triton X-100) with constant agitation to renature proteins by removing the SDS from the gel. The gels were then washed with distilled water twice for 10 minutes, then incubated in activity buffer (50mM Tris pH 7.5, 5mM $CaCl_2$ and 1μM $ZnCl_2$) for 24 hours at 37°C with constant agitation to activate protease. Followed by two washes in distilled water for 10 minutes. Proteins within the gel were visualised by incubation in development solution (40% methanol, 10% acetic acid and 0.1% Coomassie Brilliant blue) for 1 hour at room temperature. Finally, the gel was placed in destain solution (10% acetic acid and 10% methanol) until bands appeared on a uniformly stained background. Areas of gelatinase activity (i.e., MMP-2 and MMP-9) appeared as white bands on a blue background. The gels were dried using DryEase Mini-Gel Drying System (Invitrogen).

2.3.4.1. Analysis and statistics

Westerns films and zymograph gels were scanned using a flatbed scanner with back lighting (HP Scanjet 2400). The average intensity of each band were measured using SigmaScan Pro5 software (SPSS, UK). An average of three recordings per band were used to measure average intensities of total protein expression or protein activity. Background levels and total ERK levels were measured as described above. If either were found to be significantly different in their average intensities across the gel, then experimental samples were normalised to the levels of either background or total ERK, as appropriate. Statistical analysis was conducted using GraphPad PRISM 4 (GraphPad Software Inc). Data were analysed using an unpaired t-test and shown as mean ± standard deviation.

2.3.5. Immunohistochemistry

Transverse sections of sciatic nerve and DRG were cut at 12μm using a Bright cryostat (Instrument Company Ltd), mounted onto Superfrost Plus slides (VWR, UK, Cat No. 631-0108) and dried overnight at room temperature. The next day, slides were washed in PBS (Sigma, Cat No. P4417) and then incubated in blocking solution (10% normal donkey serum (NDS; Sigma, UK, Cat No. D9663) in 0.2% Triton X-100 in PBS) at room

temperature for 2 hours. Blocking solution was replaced with a combination of primary antibodies (details listed in table 2.3), or without primary antibody as a negative control, and incubated overnight at 4°C.

	Dilution	**Company**
Rabbit anti-MMP-2	1:2000	Abcam plc, UK (ab37150)
Mouse anti-S-100 (β subunit)	1:500	Sigma (S2532)
Anti-calcitonin gene related peptide (CGRP) raised in mouse	1:500	Sigma (C7113)
Monoclonal Anti-Neurofilament 200 (NF200) antibody, produced in mouse	1:400	Sigma (N0142)
FITC-conjugated isolectin B4 (IB4) from *Griffonia simplicifolia*	20μg/ml	Vector Labs (B-1205)

Table 2.3: **Concentration of antibodies or lectin used for immunocytochemistry.**

Slides were then washed three times in 0.2% Triton X-100 in PBS and then incubated in cyanine-3 (Cy3)-conjugated anti-rabbit (1:250, Jackson ImmunoResearch, Cat No. 711-166-152) and/or fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse secondary antibodies (1:500, Jackson ImmunoResearch, Cat No. 715-097-003) in 0.2% Triton X-100 in PBS for 1 hour at room temp, protected from light. Slides were then washed 3 times in 0.2% Triton X-100 in PBS and mounted using Vectorshield containing DAPI (Vector Labs, Cat No. H-1200). Immunofluorescence was visualised using a Leica MPS60 DMR fluorescent microscope and representative images acquired using a Hamamatsu camera and HC Image Imaging Software (Hamamatsu, US).

2.3.6. In-situ gelatin zymography

Longitudinal sections of fresh frozen sciatic nerve were cut at 14μm using a Bright Cryostat, mounted onto Superfrost Plus slides and dried at room temperature overnight before being stored at -20°C. Sections were washed three times in PBS and then incubated for 18 hours at 37°C in reaction buffer (50mM Tris-HCl, pH 7.6, 5mM $CaCl_2$ and 0.2mM sodium azide) containing 40μg/ml quenched, fluorescein-labelled gelatin substrate

(Molecular probes, Eugene, OR, Cat No. G-13187). As a negative control, 1mM of broad-spectrum metalloproteinase inhibitor (Ilomastat, Millipore (Chemicon), Cat No. CC1100) was added to the reaction buffer. Sections were washed three times in PBS then mounted in Vectorshield containing DAPI.

Gelatinase activity generated fluorescein-linked gelatin peptides that were detected and localised by fluorescent microscopy. Images were acquired using a Leica MPS60 DMR fluorescent microscope and representative images acquired using a Hamamatsu camera and HC Image Imaging Software (Hamamatsu, US).

2.3.6.1. Image and statistical analysis

The size distribution of neuronal profiles in L4/5 DRG was determined using SigmaScan Pro Software. Cell profiles were traced and feret diameter and average intensity were calculated automatically. The threshold of high MMP-2 immunoreactivity (IR) was determined by calculating the mean intensity of 5 neurons per animal that were subjectively deemed to be positively stained (Sigma Scan Pro, SPSS). The percentage of MMP-2 IR neurons was calculated by counting the number of MMP-2 IR positive profiles from a minimum of four randomly selected sections of L4/5 DRG (each section at least 100µm apart) from control (n=3) and 8 week post-STZ (n=4) rats. A minimum of 500 neurons was measured per animal. The profile-based analysis results were bias because of an over-estimation of large diameter neurons, due to the increased probability of being included in a section, and over-estimation of small-diameter neurons due to large cells being cut (Rose and Rohrlich, 1988). The method of recursive translation was applied to correct the bias and generate a size-distribution of the sensory neurons. Statistical analysis was conducted using GraphPad PRISM 4 (Graphpad Software Inc). Data were analysed using an unpaired t-test and shown as mean ± standard deviation.

2.4. Results

2.4.1. Basal expression of MMP-2 and MMP-9 in the rat peripheral nervous system

We have firstly characterised the constitutive expression of MMP-2 and MMP-9 in the L4/5 DRG and sciatic nerve of control adult male Wistar rats. As MMP expression and activity is regulated at three levels – transcriptional, activation of pro-peptide and active MMP inhibition, a combination of conventional PCR, Western blot, immunocytochemistry and gelatin zymography was used to investigate mRNA and protein expression and activity levels.

MMP-2 mRNA and protein were expressed constitutively in the DRG and in the sciatic nerve (Figure 2.1A & B). Furthermore, gelatin-zymography shows MMP-2 is active in protein samples obtained from the DRG and sciatic nerve (Figure 2.1C). In contrast, MMP-9 was undetectable in DRG and sciatic nerve samples at all three levels (Figure 2.1A & C).

In order to characterise the cell types that express MMP-2 in L4/5 DRG and the sciatic nerve, immunocytochemical analysis was conducted. MMP-2-IR was expressed in both Schwann cells (white arrows, Figure 2.2A & B) and axon profiles (arrowheads, Figure 2.2A, B, D-F) within the sciatic nerve. To confirm the cell types that express MMP-2-IR in the sciatic nerve, dual immunofluorescence was performed using antibodies against MMP-2 and s100 (marker for Schwann cells; Figure 2.2D-F). MMP-2-IR co-localised with s100-IR (arrows, Figure 2.2E - F) and can clearly be seen within the axon profiles (colocalised with β-III-tubulin (data not shown)).

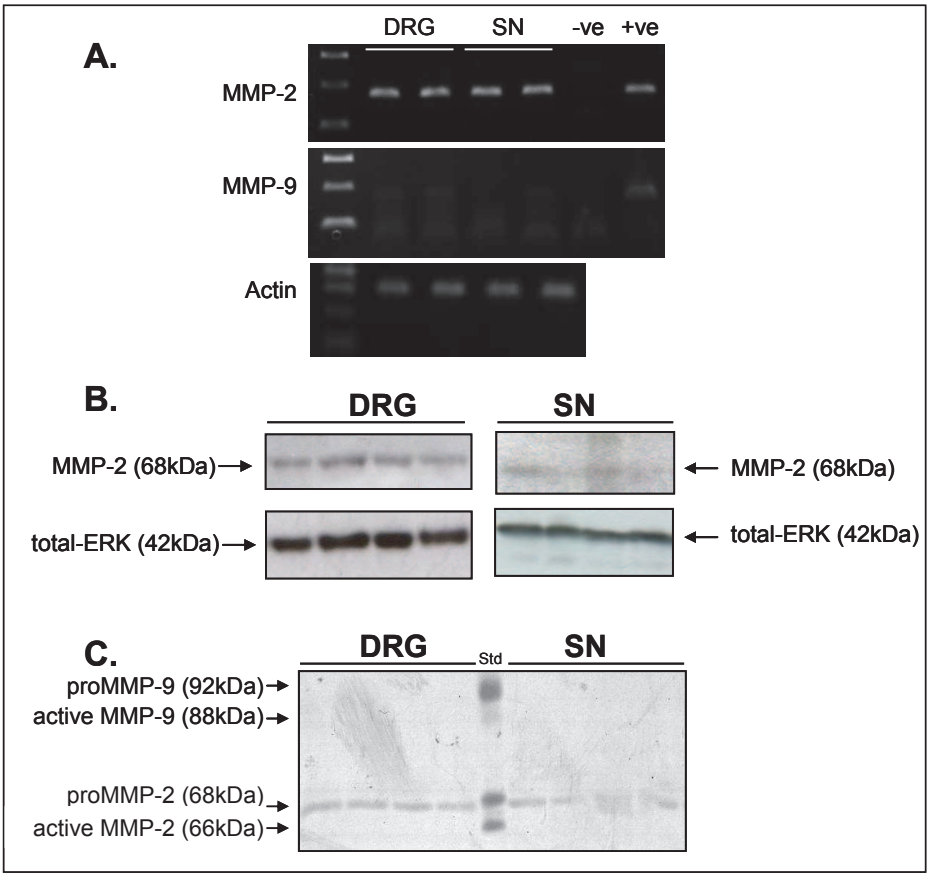


Figure 2.1: **MMP-2, but not MMP-9, is expressed constitutively in the lumbar DRG and sciatic nerve of control adult rats.** A) mRNA expression of MMP-2 and MMP-9 was determined by conventional PCR in the DRG (n=2) and sciatic nerve (n=2) of control adult Wistar rats (+ve control was liver; -ve control water). B) Western blots showing MMP-2 in the DRG (n=4) and sciatic nerve (n=4) of control adult rats. Total ERK acted as a loading control. C) Gelatin zymography shows proMMP-2, but the absence of proMMP-9, activity in DRG (n=4) and sciatic nerve (n=4) in control adult rats. Each band represents a sample obtained from an individual animal.

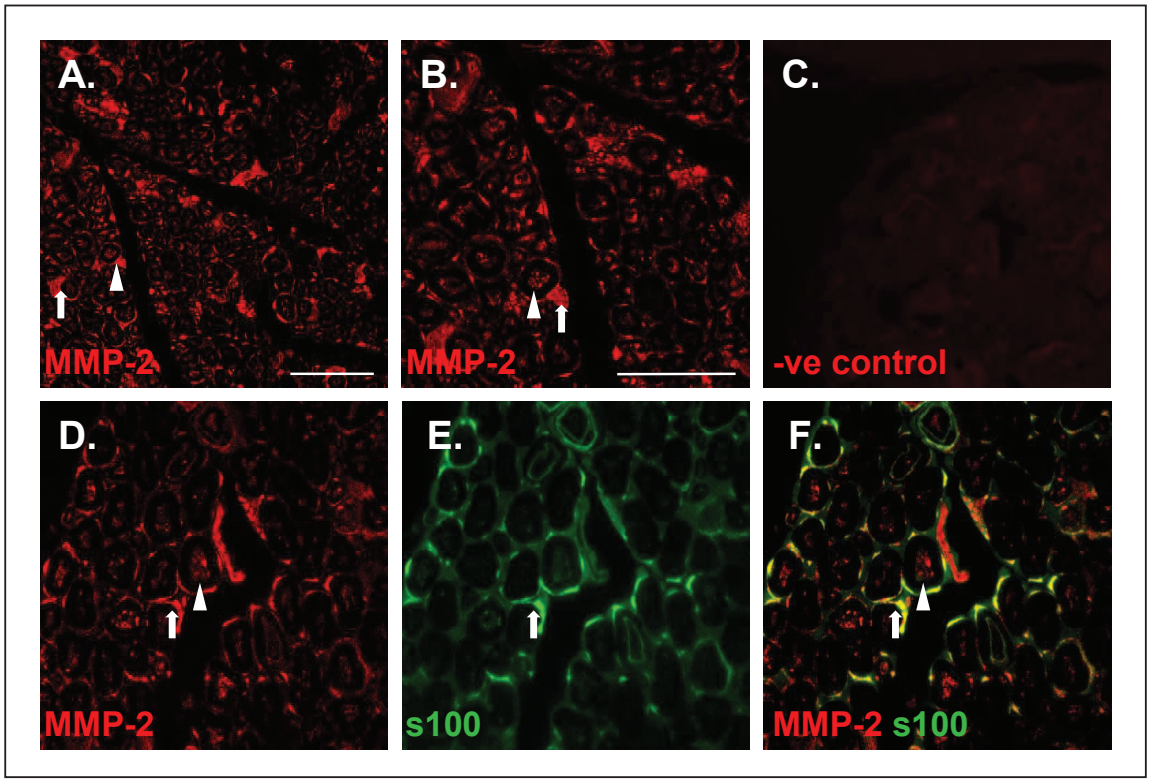


Figure 2.2: **MMP-2 is constitutively expressed in axons and Schwann cells of the sciatic nerve in control adult rats.** Sciatic nerves of naïve adult rats were immunofluorescently stained for MMP-2 (A, B & D; red stain) and co-stained with s100 (E & F; marker for Schwann cell; green stain). White arrowheads indicate areas of axonal immunostaining whilst full arrows indicate areas of Schwann cell staining. C) Low levels of background staining were observed with negative control where neurons were stained with secondary antibody only. Scale bars = 50µm.

MMP-2- IR was expressed in discrete populations of sensory neurons (clear arrowheads, Figure 2.3A & B), but not glia, within the DRG. Sensory neurons are a heterogeneous population, which can be categorised into three cell groups according to their neurochemistry, morphology, neurotrophic requirements and sensory modalities (Tucker et al., 2006). In order to classify and characterise MMP-2 expression in L4/5 DRG, we used cell size analysis and dual-labelling with neuronal markers to identify these three different populations of sensory neurons. The phenotypic markers used were: Fluorescein-conjugated IB4, (a lectin that binds small diameter nociceptive neurons, which are glial line-derived growth factor (GDNF) responsive), and antibodies against CGRP (a marker of small-medium diameter neurons, which are NGF-dependent)) or neurofilament 200 (NF200, a marker of large diameter neurons, which are Neurotrophin (NT)-3 responsive). MMP-2-IR was expressed by 48% of sensory neurons within L4/5 DRG (Fig 2.4C), mainly

in small to medium-diameter neurons (less than 40µm; Figure 2.3 & 2.4D) that either bind IB4 (Figure 2.3E & F) or express CGRP-IR (Figure 2.3H & I). MMP-2 IR co-localisation with NF200 is also visible in the L4/5 DRG (Figure 2.3L).

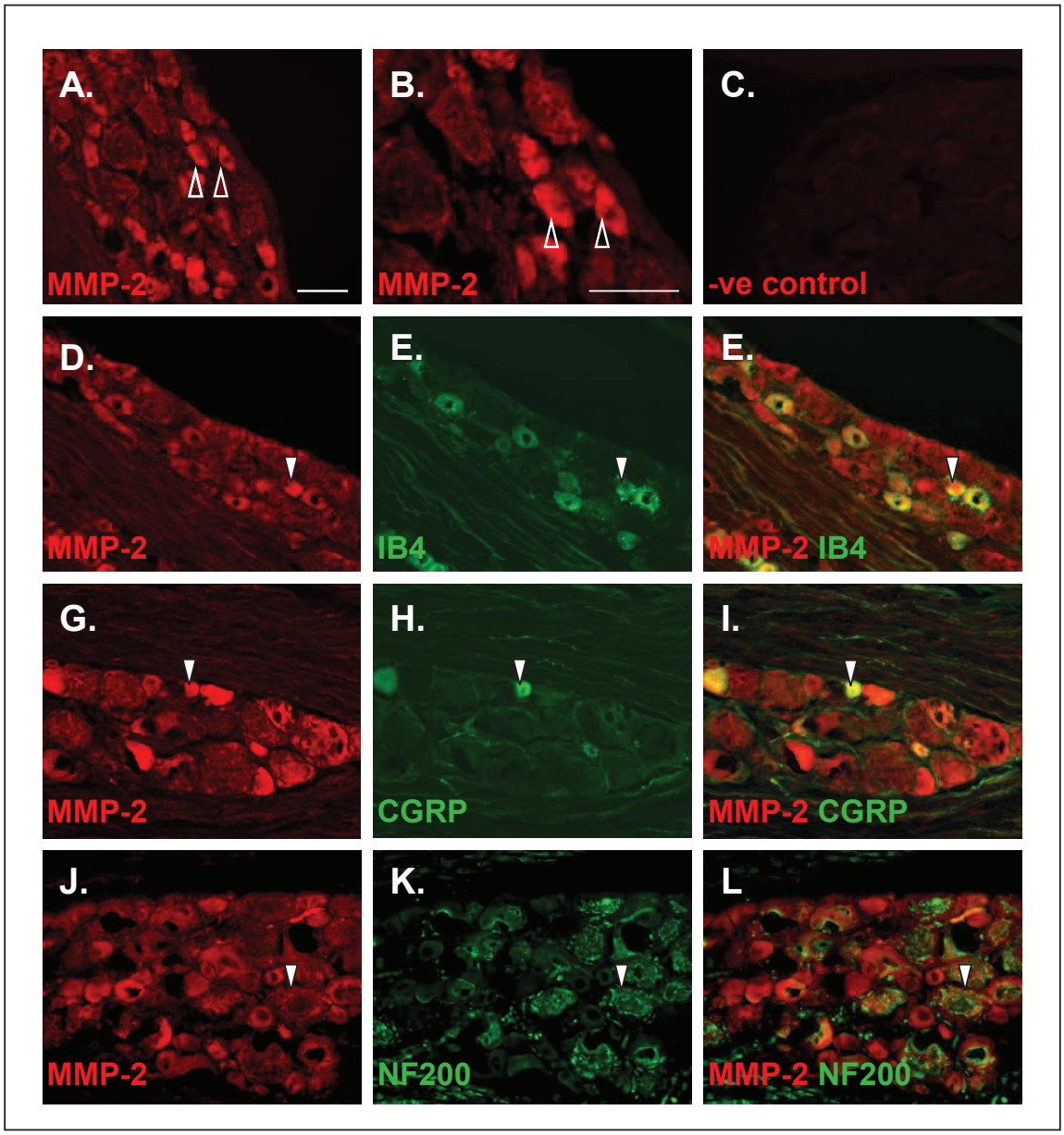


Figure 2.3: **MMP-2 is expressed in the small-to-medium diameter sensory neurons of lumbar DRG of control adult rats.** L4/5 DRG of control adult Wistar rats were immunofluorscencetly labeled with MMP-2 (A, B, D & G; red stain). C) Low levels of background staining were observed with negative control where neurons were stained with secondary antibody only. Clear white arrowheads indicate MMP-2 expression in a discrete population of sensory neurons. MMP-2 was dual labeled with IB4 (E &F: marker for GDNF-dependent small unmyelinated neurons; green stain), with CGRP (H & I; marker for mainly NGF-dependent small-to-medium neurons; green stain) and with NF200 (K & L; marker for large diameter neurons; green stain). White arrowheads indicate examples of sections that co-express MMP-2 IR with the phenotypic markers IB4, CGRP or NF200. Scale bars = 50μm.

2.4.2. Does MMP-2 expression change in the peripheral nervous system in diabetes?

STZ-induced diabetes resulted in metabolic dysfunction (hyperglycaemia and reduced weight gain compared to age-matched control rats; Supplementary Table 1A,) and a reduction in sensory and motor nerve conduction velocity in the sciatic nerve (Supplementary Table 1B), indicating the presence of diabetic neuropathy.

The distribution of MMP-2-IR in L4/5 DRG obtained from diabetic rats was compared to age-matched controls; diabetes had no effect on the proportion of immunopositive MMP-2 neurons in L4/5 DRG or on the cell-size distribution of MMP-2-IR sensory neurons (Figure 2.4C & D). As with the control rats, MMP-2-IR mainly colocalised with IB4 and CGRP with less intense MMP-2-IR in the medium-to-large diameter neurons that express NF200 (Data not shown; Control <40μm 84% ± 1.4; >40μm 17% ± 1.4 (*n=3*) vs Diabetic <40μm 85% ± 2.5; >40μm 15% ± 2.5 (*n=4*) arbitrary units). Since immunocytochemistry is not quantifiable, we assessed MMP-2 mRNA and protein expression in L4/5 DRG obtained from age-matched control and STZ-diabetic adult male rats (4, 8 or 12 weeks post-STZ) using real-time PCR and Western blotting.

The induction of diabetes had no significant effect on either MMP-2 mRNA (Figure 2.5A) or protein levels (Figure 2.5B & C) in L4/5 DRG compared to age-matched control rats at any time-point tested (Figure 2.5A & C). In addition, the expression of MMP-9 mRNA

and protein levels were also assayed but were undetectable, indicating that MMP-9 was not upregulated in STZ-induced experimental diabetes at any time-point tested (data not shown).

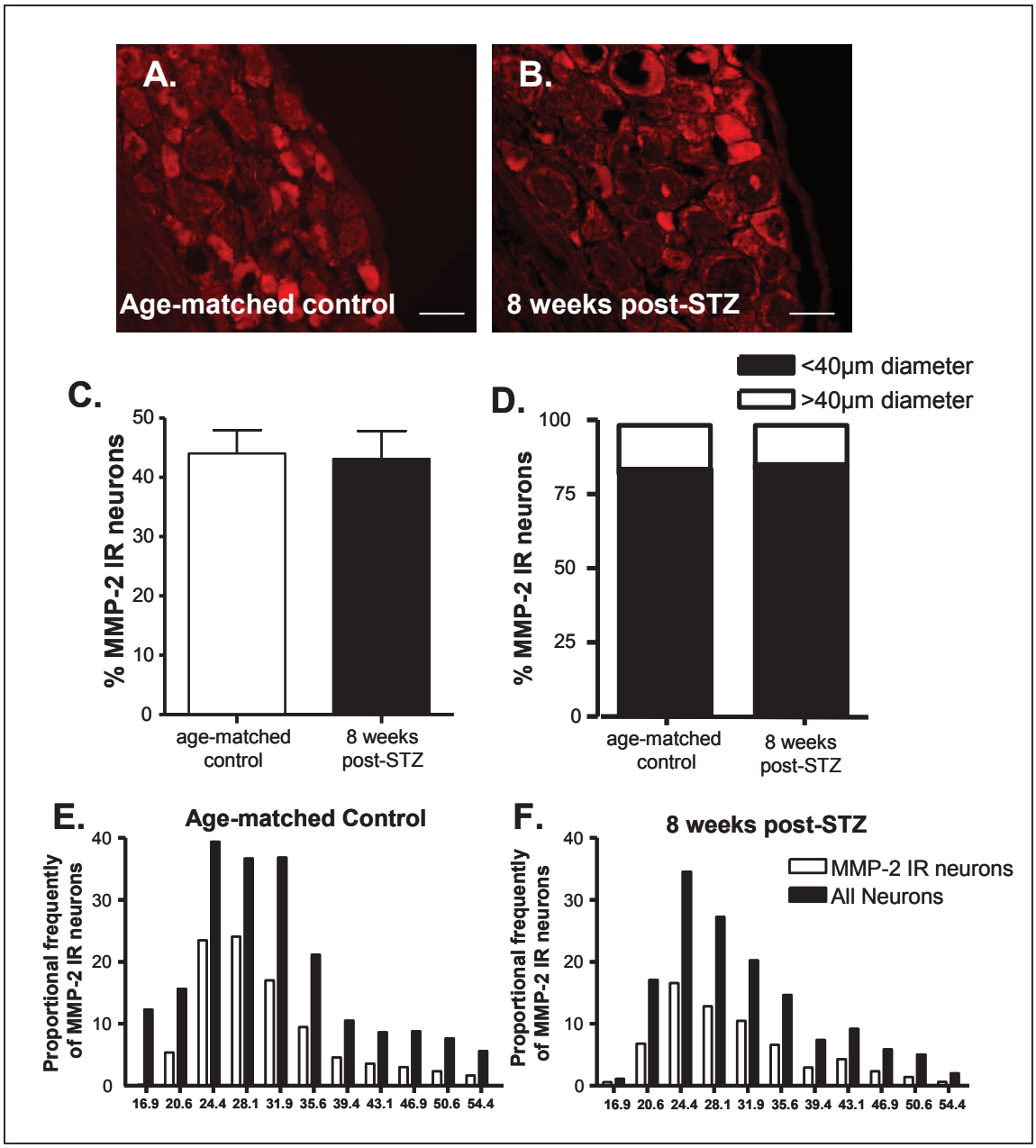


Figure 2.4: **Expression profile analysis of MMP-2 immunoreactivity in the lumbar DRG is unaltered in experimental diabetes compared to age-matched control.** L4/5 DRG from age-matched controls (*n=3*) and experimental diabetic adult Wistar rats (n=4) were labeled for MMP-2 (red stain; A and B respectively). C) The percentage of sensory neurons that express MMP-2 and D) the size distribution of these MMP-2 expression neurons are unaltered in 8 weeks post-STZ induced diabetic rats compared to age-matched controls. E &F) Morphometric analysis for the number of cells positive for MMP-2 IR relative to DRG sensory neuron cell diameter (in μm) shows there is no alteration in the cell size distribution of MMP-2 IR neurons in age-matched controls and 8 weeks post-STZ diabetic rats, respectively.

Quantitative real-time PCR showed that experimental diabetes resulted in significant upregulation of MMP-2 mRNA in the sciatic nerve 8 weeks post-STZ induction (Control 0.15 ± 0.02 (*n=6*) vs Diabetic 0.22 ± 0.02 (*n=12*) arbitrary units, * $p<0.05$, Figure 2.6). Western blot analysis showed that this upregulation of MMP-2 mRNA was also translated into a significant upregulation in MMP-2 protein levels (Control 58.5 ± 2.9 (n=7) vs Diabetic 96.5 ± 5.1 (n=12) arbitrary units, *** $p<0.001$, Figure 2.7) and activity (Figure 2.8). Gelatin zymography demonstrated a significant increase in proMMP-2 activity (Control 131.5 ± 3.4 (n=7) vs Diabetic 141.8 ± 2.2 (n=12) arbitrary units, $p<0.05$; Figure 2.8). Interestingly, as well as the proMMP-2, four out of twelve diabetic rats tested also expressed the cleaved form of active MMP-2 in the sciatic nerve (Figure 2.8A, indicated by black full arrows, and C). As with the MMP-2 mRNA time-course, significant increases in MMP-2 protein and activity levels were only seen at the 8 week time-point (Figure 2.7C and 2.8D, respectively). The increase in MMP-2 activity levels in the 8 weeks post-STZ diabetic sciatic nerve was confirmed using in-situ gelatin zymography (Supplementary figure 2A & B, respectively), where increased fluorescence indicates increased gelatinase activity. Inclusion of the pan MMP inhibitor (Ilomastat) abolished the fluorescence, indicating the gelatinase activity to be MMP-driven (Supplementary Figure 2C & D).

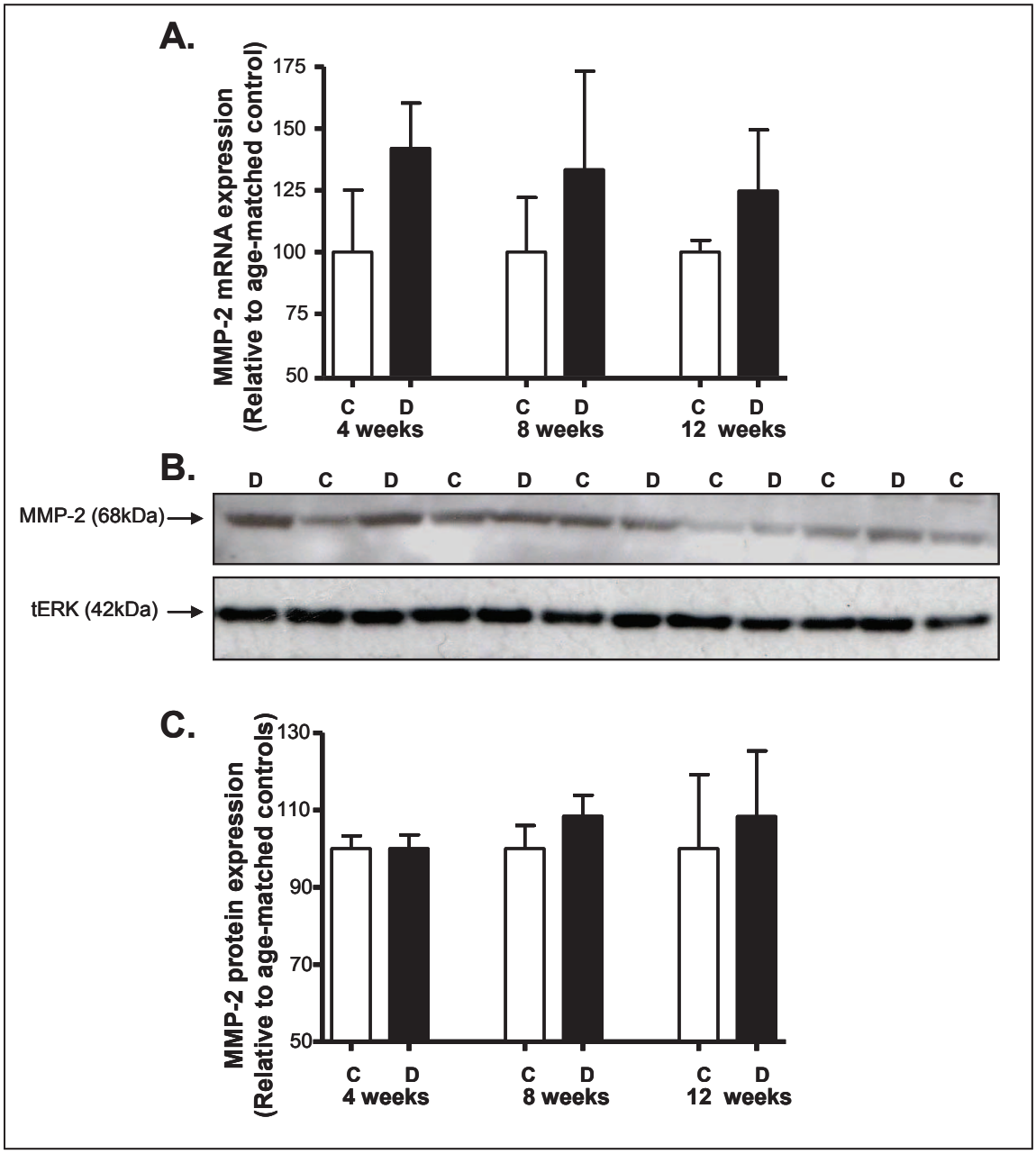


Figure 2.5: **MMP-2 expression is unaltered in the lumbar DRG in experimental diabetic adult rats.** A) MMP-2 mRNA expression levels increase in L4/5 DRG, but not significantly at 4, 8 and 12 weeks post-STZ. B) Western blots showing MMP-2 protein expression in DRG from age-matched controls (C; *n=6*) and 8 weeks post-STZ (D; *n=8*). Each band represents a sample from an individual animal. C) Densitometric analysis shows that MMP-2 protein expression levels were unaltered at 4, 8 and 12 weeks post-STZ.

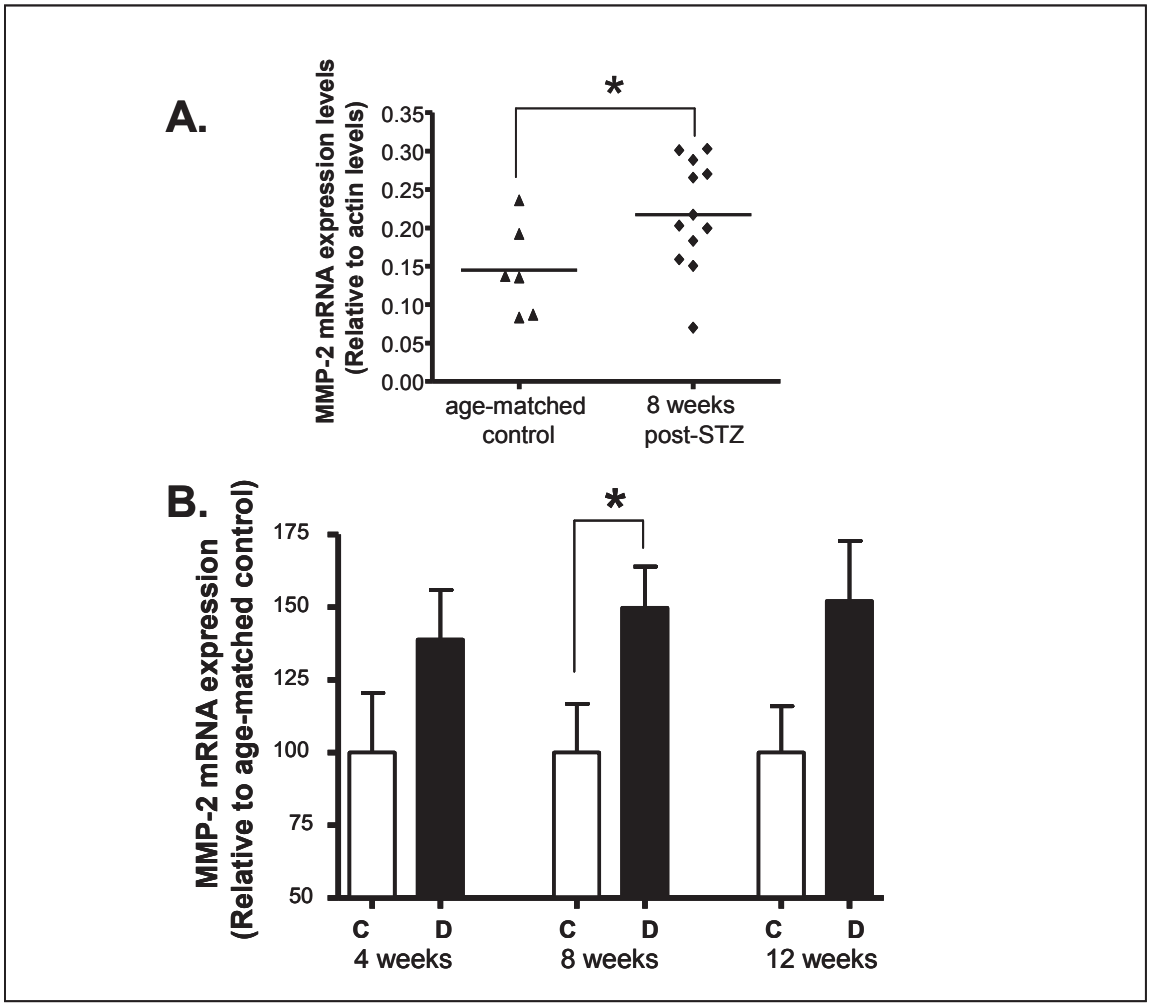


Figure 2.6: **MMP-2 mRNA expression is significantly increased in sciatic nerve of diabetic rats.** A) Graphical representation of the relative expression of MMP-2 mRNA at 8 weeks post-STZ (n=12) rats compared to age-matched controls (n=6), showing a significant increase in MMP-2 mRNA. B) A time-course study of MMP-2 mRNA expression levels at 4, 8 and 12 weeks post-STZ show a trend to an increase in MMP-2 mRNA expression, however only the increase at 8 weeks post-STZ significant (* $p<0.05$).

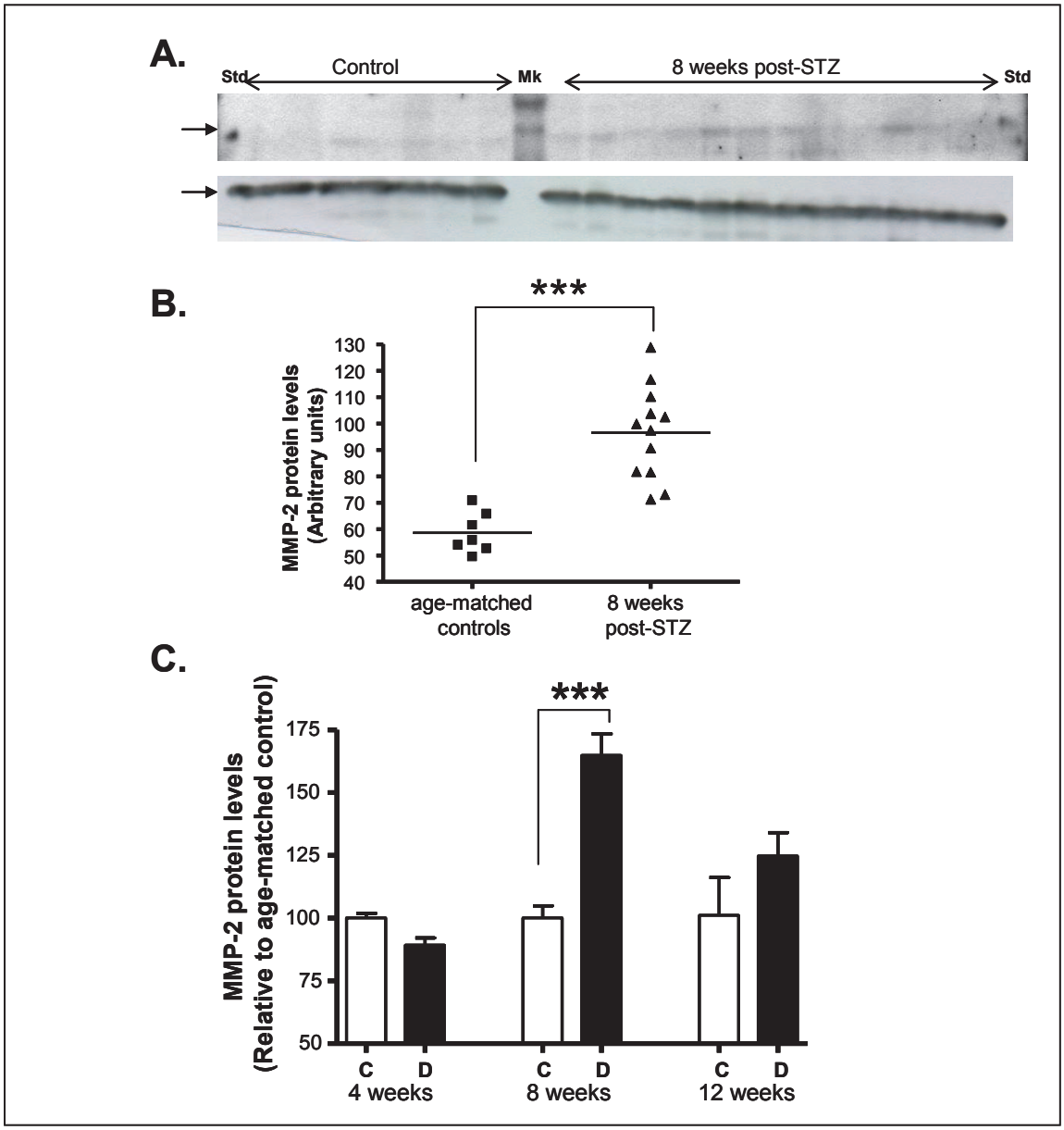


Figure 2.7: **MMP-2 protein expression is significantly increased in sciatic nerve of diabetic rats compared to age-matched controls.** A) Western blots displaying MMP-2 protein expression in sciatic nerve from control and STZ-diabetic rats (8 weeks post-STZ). Each band represents a sample from an individual animal. B) Western blots were quantified using densitometry, and show a significant increase in total MMP-2 protein levels in sciatic nerve from 8 weeks post-STZ rats compared to age-matched controls(*** $p<0.0001$). C) Results from timecourse study of MMP-2 protein expression in sciatic nerve at 4, 8 and 12 weeks post-STZ.

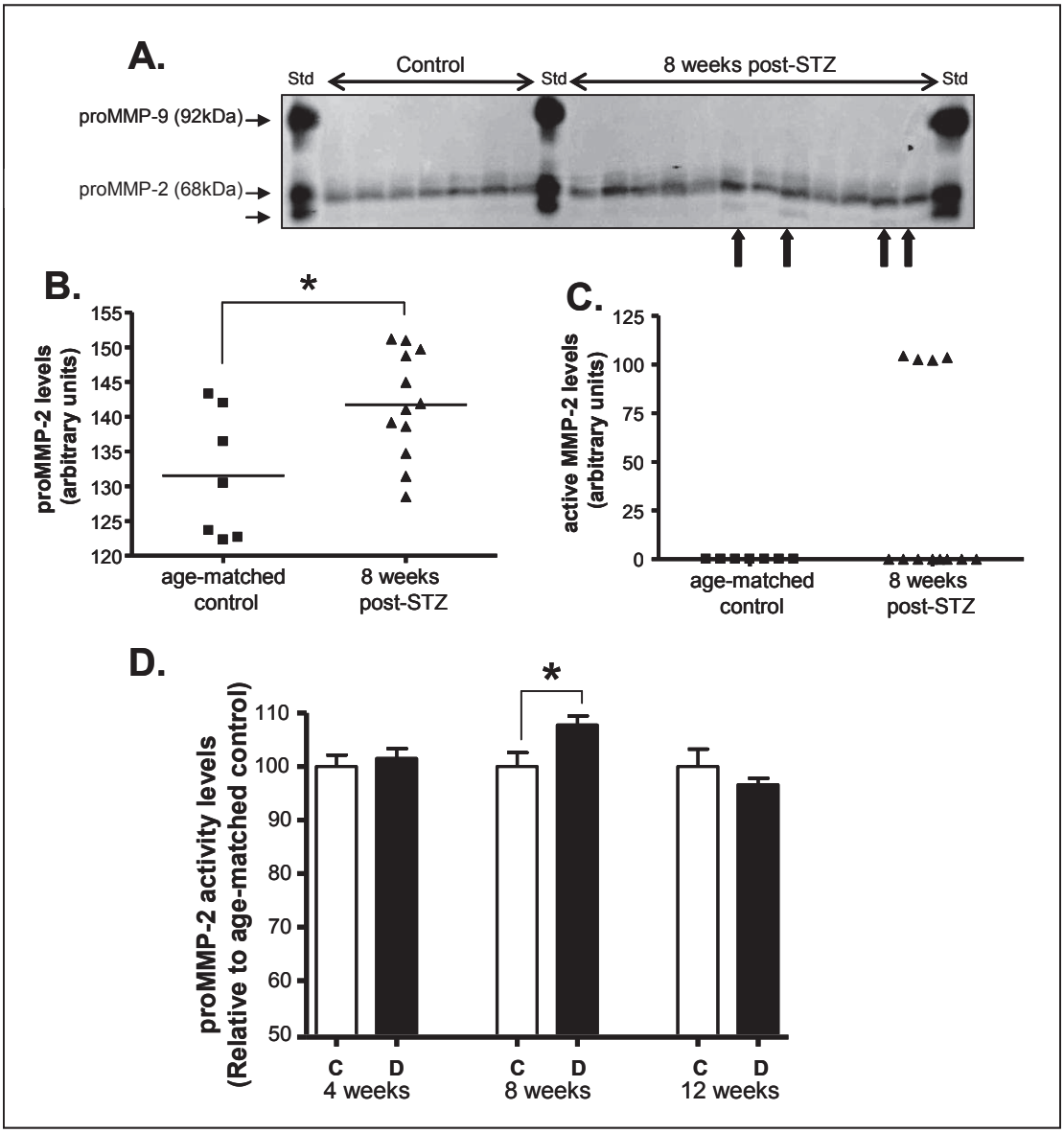


Figure 2.8: **proMMP-2 activity levels are significantly increased with conversion to active MMP-2 in the sciatic nerve of diabetic rats compared to age-matched controls.** A) Gelatin zymography gels showing proMMP-2 activity and conversion to active MMP-2 in the sciatic nerve of diabetic (8 weeks post-STZ) and age-matched control rats (Each band represents an individual animal; control (n=7) and 8 weeks diabetic adult rats (n=12); Std – MMP-2 and MMP-9 standard) with B) quantified using densitometry of proMMP-2 levels showing significant increase (* $p<0.05$) and C) active MMP-2. D) Graphical representation of proMMP-2.

It was not possible to discriminate which cell types in the sciatic nerve upregulated MMP-2 in diabetes. As in the control sciatic nerve, MMP-2-IR was localised to Schwann cells and axons in sciatic nerves from diabetic rats (8 weeks post-STZ; Figure 2.9).

MMP-9 expression in the sciatic nerve was also assayed by PCR (data not shown), Western blot (data not shown) and gelatin zymography (Figure 2.8A), with no changes in MMP-9 expression in sciatic nerve obtained from diabetic rats (4, 8 and 12 weeks-post-STZ).

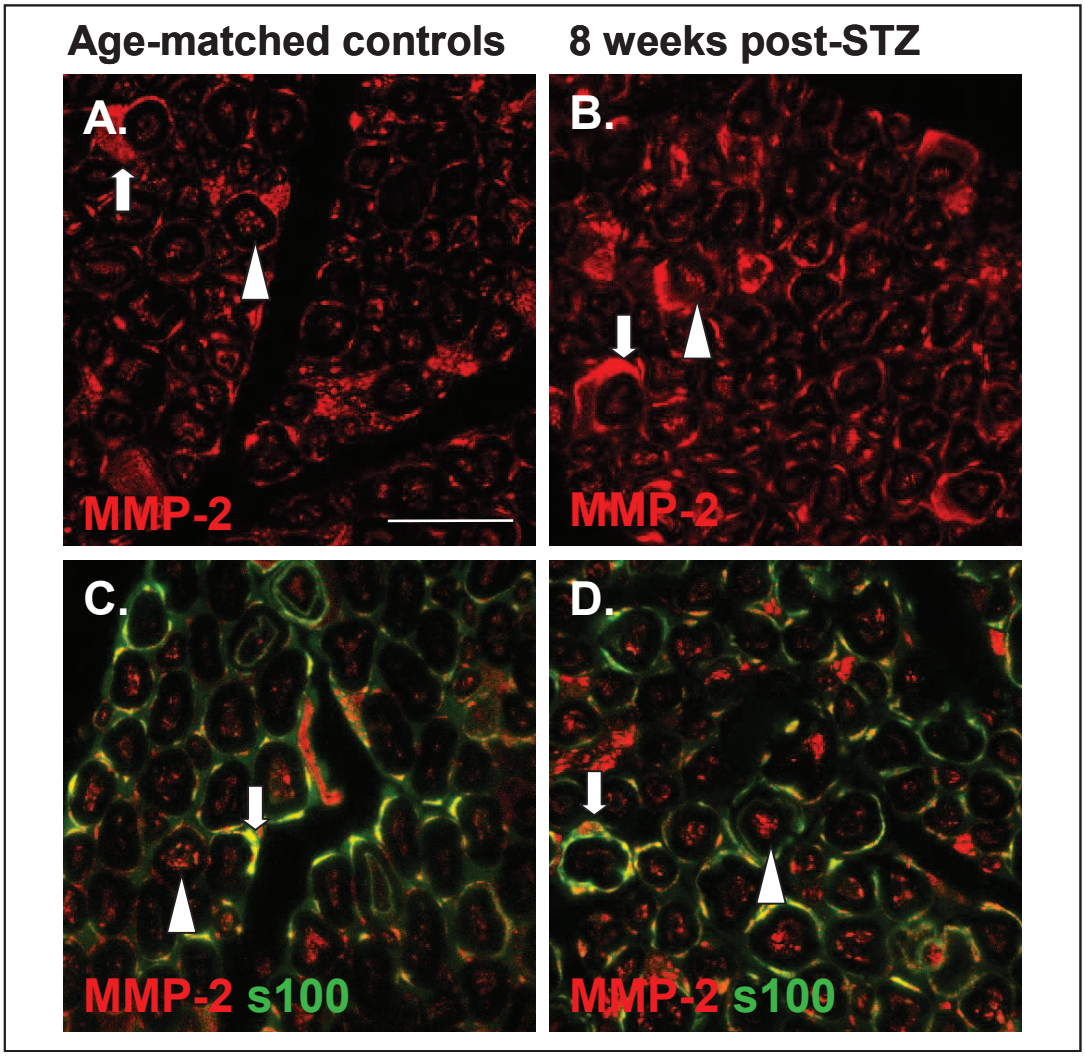


Figure 2.9: **Co-localisation of MMP-2 with axonal and Schwann cell markers in the sciatic nerve from age-matched control and 8 weeks post-STZ adult rats.** Sciatic nerve of age-matched controls and 8 weeks post-STZ were labeled for MMP-2 (A & B respectively; red stain) and dual labeled with s100 (C & D respectively**;** marker for Schwann cells; green stain). Arrow heads indicate areas of axonal immunostaining and full arrows indicate areas of Schwann cell staining. Scale bars = 50μm.

Unlike other MMPs, MMP-2 is resistant to activation by serine proteases, instead MMP-2 is activated at the cell surface through a unique multistep mechanism involving MT-MMP (MMP-14) and TIMP-2 (Strongin et al., 1995). Therefore, we investigated whether the expression of these endogenous regulators of MMP-2 was also altered in experimental diabetes using real-time PCR and Western blot analysis. At the mRNA level, there was no significant alteration in the expression of either membrane-type (MT)1-MMP or TIMP-2 (Supplementary figure 3). However, TIMP-2 protein was significantly upregulated in the sciatic nerve at 8 weeks post-STZ induction of diabetes (Control 14.1 ± 9.1 (*n=7*) vs Diabetic 76.4 ± 5.3 (*n=12)* arbitrary units, *** $p<0.001$, Supplementary figure 4), and may represent a potential mechanism by which MMP-2 is regulated.

2.5. Discussion

The results from this study show that control adult rat DRG and sciatic nerve constitutively express MMP-2 mRNA and protein. In the DRG, MMP-2 -IR was high in the small-medium diameter sensory neurons that co-express IB4 or CGRP but was low in large-diameter NF200-positive sensory neurons. Whilst in the sciatic nerve, MMP-2 expression co-localised with both Schwann cell (s100-IR) and axonal (β-III-tubulin-IR) markers, confirming previously published observations (Demestre et al., 1999;La et al., 1996;Muir, 1994). In contrast, MMP-9 mRNA and protein was undetectable in both the L4/5 DRG and sciatic nerve of control adult rats, again, these findings are consistent with work from other laboratories (Ferguson and Muir, 2000;Shubayev et al., 2006).

For the first time, we have shown that there is a small but significant increase in MMP-2 mRNA, protein and activity levels in the sciatic nerve of diabetic rats (8 weeks post-STZ). Diabetes-induced upregulation in gelatinase MMP expression has previously been reported in other tissue associated with other secondary complications including retinopathy (Giebel et al., 2005;Noda et al., 2003;Giebel et al., 2005;Noda et al., 2003;Navaratna et al., 2007;Yang et al., 2007), nephropathy (Thrailkill et al., 2009;van der Zijl et al., 2010), chronic non-healing diabetic ulcers (Lobmann et al., 2006) as well as coronary (Derosa et al., 2007) and peripheral arterial diseases (Signorelli et al., 2005).

2.5.1. MMP-2 expression in sensory neurons

In our study, MMP-2 was localised predominantly to the small-medium diameter sensory neurons in the DRG of control and diabetic rats. Whilst there was a trend towards an increase in MMP-2 expression at the mRNA and protein levels in the L4/5 DRG of diabetic rats, this was not significant at the time-points tested (4, 8 and 12 weeks post-STZ). The IB4 and CRGP positive sensory neurons are markers for the peptidergic and non-peptidergic neurons of the peripheral nervous system (Tucker et al., 2006;Kiernan, 2005). Unmyelinated axons from these neurons innervate the epidermis of the skin and are the first to be affected by the polyneuropathy ‘die-back’ effect associated with clinical (Lauria and Devigili, 2007;Beiswenger et al., 2008b) and experimental diabetic neuropathy (Wright et

al., 2004;Beiswenger et al., 2008a). In STZ-mice studies, damage to these small epidermal unmyelinated fibres has been shown to coincide with thermal hypoalgesia and mechanical allodynia in the early stages of diabetic neuropathy (Johnson et al., 2008;Christianson et al., 2003;Beiswenger et al., 2008a). However, altered pain thresholds in diabetic rats have shown conflicting results. Reports of mechanical allodynia and chemical hyperalgesia are fairly consistent in STZ rats (Courteix et al., 1993b;Calcutt et al., 1994;Calcutt et al., 1996), changes in thermal nociceptive thresholds have been highly variable, with thermal hyperalgesia, no change or hypoalgesia observed by different groups (Calcutt, 2004;Courteix et al., 1993a;Forman et al., 1986).

Studies investigating the role of MMPs in the regulation of chronic neuropathic pain following lesions in the peripheral (PNS) or central nervous systems (CNS) showed that MMP-9 plays a role in early phase pain hypersensitivity, whilst MMP-2 is involved in late phase of neuropathic pain (Kawasaki et al., 2008;Ji et al., 2009). After peripheral nerve injury, proinflammatory factors such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)α and MMP-2 and MMP-9 are upregulated in the nerve, DRG and by glial cells of the spinal cord (Kawasaki et al., 2008;Chattopadhyay et al., 2007;Ji et al., 2009). In the spinal cord, there is an initial early phase increase in MMP-9, which invokes microglial activation and a late stage increase in MMP-2 in astrocytes (Kawasaki et al., 2008). In DRG, there is a transient increase in MMP-9 expression in the neurons (Kawasaki et al., 2008), and a delayed and persistent expression of MMP-2 in the satellite cells (Ji et al., 2009). This injury-induced upregulation of MMP-9 in the DRG is induced by TNFα, since treatment with TNFα synthesis inhibitors following sciatic nerve ligation suppresses injury-induced MMP-9 expression (Kawasaki et al., 2008). The functional consequence of upregulation of MMP-2 and MMP-9 include cleavage of IL-1β in both PNS and CNS, whilst in the CNS, MMP-9 stimulates p38 activation in microglial (Kawasaki et al., 2008). Inhibition of IL-1β signalling and p38 activation, by neutralising antibodies or inhibitor respectively, blocked MMP-9 induced early stage allodynia (Kawasaki et al., 2008).

In support of the role played by MMP-9 in generation of pain, MMP-9 knock-out (KO) mice display a significant reduction in the development of tactile allodynia following either peripheral or central nerve lesions (Kawasaki et al., 2008;Chattopadhyay et al., 2007). An

intrathecal injection of either TIMP-1 (endogenous peptide inhibitor of MMP-9) or small interfering RNA (siRNA) against MMP-9 attenuates early stage neuropathic pain (Kawasaki et al., 2008). However, the MMP-9 deletion did not facilitate recovery from chronic neuropathic pain following either peripheral or central sensory nerve lesions (Kawasaki et al., 2008;Chattopadhyay et al., 2007). In the MMP-9 KO mice, a MMP-2 compensatory mechanism occurs; the treatment of these null mice with MMP-2 inhibitor produced a more profound anti-allodynia effect (Kawasaki et al., 2008). Similar approaches, the blocking of MMP-2 with TIMP-2 or MMP-2 siRNA, have shown attenuation of late-stage neuropathic pain with mechanical allodynia (Kawasaki et al., 2008). Other MMPs have also been implicated in the development of neuropathic pain. MT5-MMP deficient mice do not develop mechanical allodynia but their response to acute noxious stimuli was normal after sciatic nerve injury (Beiswenger et al., 2008a;Komori et al., 2004). This, therefore, has implications for diabetic neuropathy as the observation that increased MMP-2 in the sciatic nerve occurs in the absence of any alteration in MMP-9 could indicate that a similar compensatory mechanism occurs in the sciatic nerves of diabetic animals, as observed in MMP-9 KO mice following peripheral and central lesion injuries.

Work by Wright et al., (2004) has shown that as early as 3 weeks after diabetic induction, there is a significant upregulation of activating transcription factor (ATF)-3, a marker of neuronal damage, indicating the presence of injured neurons in the diabetic mice compared to age-matched controls. At 3 and 6 weeks of diabetes, it is predominately the small, unmyelinated neurons that are ATF-3-positive, suggesting the small unmyelinated neurons may be the first population affected by diabetes (Wright et al., 2004). However, at 6 weeks, this ATF-3 expression by the small diameter neurons coincides with a decrease in the number of unmyelinated neurons; thereby, suggesting that by 6 weeks the large, myelinated neurons are increasingly affected by the progression of diabetes (Wright et al., 2004). Since only 10% of sensory neurons in L4/5 DRG population are ATF-3 positive in diabetic mice compared to approximately 50% of sensory neurons following sciatic nerve injury (Wright et al., 2004), we would expect a much smaller upregulation in MMP-2 and MMP-9 in the DRG and spinal cord in response to diabetes then that observed following nerve injury. Therefore, this small percentage of neurons that are ATF-3 positive in

experimental diabetes could explain why we see a small, but not significant, increase in MMP-2 gene and protein expression in the DRG of diabetic rats.

These studies, taken as a whole, support the hypothesis that the small increase in MMP-2, seen in the small-to-medium sensory neurons of the DRG taken from diabetic rats, could be an indication of the development of peripheral sensitization of the DRG sensory neurons in response to C-fibre axonal damage. Further investigation would be required to confirm this. If this is an indication of diabetic-induced peripheral sensitisation we could expect increases in MMP-2 expression to be accompanied by an earlier increase of MMP-9 in the sensory neurons as well as upregulation in MMP-2 and MMP-9 in the spinal cord, which could be an indication of central sensitisation.

2.5.2. Upregulation of MMP-2 expression in the sciatic nerve

In this study, we demonstrated a significant increase in MMP-2 protein expression in the sciatic nerve of 8 week diabetic rats. Immunocytochemistry techniques were employed and allowed the localisation of MMP-2 expression to the axon and Schwann cell components of the sciatic nerve. However, due to the non-quantitative nature of immunocytochemistry, the cell type responsible for this increase in MMP-2 expression remains unknown. Therefore, we could be seeing either a diabetic-induced local synthesis of MMP-2 by the axons or an increase in MMP-2 expression by the Schwann cells, or indeed both.

Interestingly, proMMP-2 was cleaved and converted to its active form in the sciatic nerve of 4 of the 12 diabetic animals tested. MMP-2 activity is not typically regulated like other MMPs as MMP-2 is resistant to activation by serine protease (Strongin et al., 1995). Instead, MMP-2 is activated at the cell surface through a unique multistep mechanism involving MT1-MMP (MMP-14) and TIMP-2. Therefore, we also investigated the expression of these MMP-2 modulators and, as with active MMP-2, our results showed variability in the expression of TIMP-2, with higher protein expression seen in 5 of the 12 diabetic rats tested. Thus, we have demonstrated that the activation of MMP-2 is not homogenous in the sciatic nerve of diabetic animals; the reasons for this are unclear. Similar heterogeneous activation of MAPKs in L4/5 DRG has been described in

experimental diabetic neuropathy, phosphorylation of ERK1, ERK2, p38 and JNK1 correlated with the degree of mechanical hyperalgesia observed in diabetic rats (3 weeks post-STZ) (Daulhac et al., 2006).

2.5.2.1. A role for MMP-2 in ECM modulation?

Diabetes is associated with peripheral microvascular complications with the integrity of the blood nerve barrier (BNB), blood brain barrier (BBB) and blood retinal barrier (BRB) known to be altered in patients with diabetes (Beiswenger et al., 2008a;Poduslo et al., 1988;Ohi et al., 1985). In experimental diabetes, increased BBB and BRB permeability has been linked to increased circulating MMPs and loss of tight junction proteins such as occudulin and cadherin (Giebel et al., 2005;Hawkins et al., 2007;Navaratna et al., 2007). However, whether altered BNB integrity is due to altered transport through the endothelial-perineurial barrier or leakage of tight junctions is not clear.

In diabetic neuropathy, structural changes to the peripheral nerve ECM can be observed. One of the pathophysiological changes in diabetic neuropathy is the reduplication of the basement membrane as a result of the accumulation of collagen (COLL) type-IV and V in the perineurium and COLL type-I and III in the endoneurial ECM of the diabetic nerve, with no detectable changes seen in laminin (LM) and fibronectin (FN) expression (Bradley et al., 2000;Giannini and Dyck, 1995). The accumulation of inhibitory factors such as CSPG and heparin sulphate proteoglycans (HSPG) due to alteration in their expression, have been shown in the basement membrane of organs affected in other diabetic complications. In diabetic nephropathy, there is an increase in glomeruli CSPG staining, which is localised to the pericapillary basement membrane, with a concomitant decrease in HSPG levels (McCarthy et al., 1994). This alteration in the glomerular proteoglycans (PG) biosynthesis appears to affect the structural/functional dynamics of the glomerular basement membrane, which may directly contribute to the eventual renal dysfunction (McCarthy et al., 1994). Interestingly, this alteration in PG biosynthesis also occurs in the non-vascular basement membrane of the skin of type 1 diabetic patients with nephropathy (van der Pijl et al., 1998). However, there is no evidence to date of increased CSPG levels in the nerve in diabetes.

MMP-2 is involved in the degradation of COLL type-I, IV, V, VII and X, and inhibitory CSPGs (Butler and Overall, 2009;Coussens and Werb, 1996;Zuo et al., 1998a;Zuo et al., 1998b). Therefore, the increase in the expression and activity levels of MMP-2 in the sciatic nerve might reflect an attempt by the peripheral nerve to normalise diabetes-associated collagen deposition. The reduction in MMP-2 levels at 12 weeks post-STZ could be the result of neurons ceasing to respond to peripheral damage, collagen accumulation or suppression of MMP-2 expression as a consequence of the ongoing hyperglycaemia and oxidative stress. Multiple studies have shown that hyperglycaemia results in direct upregulation of MMP-2 and MMP-9 by endothelial cells from both bovine and human origin (Uemura et al., 2001;Death et al., 2003). An insulin intervention study would enable us to confirm whether hyperglycaemia is a contributing factor to this transient, heterogeneous upregulation of MMP-2 in the sciatic nerve of diabetic rats.

Evidence of MMP-2 remodelling of the basement membrane, as part of normal maintenance, is evident in the capillaries in the brain. MMP-2/MT1-MMP complex remodelling of the matrix has been shown to be essential in the maintenance of BBB via the prevention of excessive build-up of the ECM (Candelario-Jalil et al., 2009). In diabetic neuropathy, it is thought that the thickening of the perineurial and epineurial basement membrane as well as blood vessels in the peripheral nerve may impede the abilities of the BNB to function both as a diffusion barrier and as a nutrient delivery system, and thus, prevent the effective regulation of the endoneurial environment (Hill and Williams, 2004). This, in time, may lead to the axonal loss seen in diabetic neuropathy. Increased MMP-2 in the diabetic peripheral nerve could contribute to BNB leakage as MMP-2 is known to increase vascular permeability following nerve injury via the degradation of endothelial COLL type-IV (Rosenberg et al., 1992;Woessner, Jr., 1994). MMP-2 could also result in BNB leakage in diabetic neuropathy via degradation of tight junction proteins, a process that occurs in diabetic retinopathy (Giebel et al., 2005).

Another pathophysiological change that needs to be considered is the glycation of both the intracellular and extracellular proteins of the peripheral nerve in experimental diabetic neuropathy (Duran-Jimenez et al., 2009;Thornalley et al., 2003). Glycation of ECM occurs

as early as 3 weeks post-STZ (Duran-Jimenez et al., 2009). Glycation can alter cellular behaviour, modify protein function and cause structural distortion and activation of cellular pathways, as well as confer protein resistance to proteolysis (Lubec et al., 1982;Lubec and Pollak, 1980;Pollak et al., 1982). Therefore, an interesting concept to consider is whether the MMP-2 released in the sciatic nerve in the diabetic animals is able to degrade the glycated protein of the peripheral nerve. Hence, it is possible that the build-up of COLL type-IV and CSPG in the peripheral nerve may occur as a result of proteolytic resistance to MMP-2 enzymatic activity during diabetes.

2.5.2.2. MMP-2 in nerve regeneration

Following injury, successful peripheral nerve regeneration is important for the maintenance of a working peripheral nervous system. In diabetes, there is progressive distal nerve fibre loss due to the impaired ability of the diabetic nerve to regenerate in response to the degenerative process (Sima et al., 1988). Although there is evidence that the nerve attempts to regenerate in response to fibre loss, the number of regenerating fibres decreases during the progression of neuropathy (Kennedy and Zochodne, 2005). This results in an overall loss of nerve fibres as the level of regeneration does not compensate for the massive loss of fibres in diabetes (Kennedy and Zochodne, 2005).

Therefore, in our experimental model of diabetes, the increase in MMP-2 could be associated with degeneration or diabetic-induced neuronal damage. Even though the increase in MMP-2 in the sciatic nerve is much smaller (1.6 fold increase in diabetes compared to 7-fold increase following sciatic nerve crush injury) (Demestre et al., 2004;Ferguson and Muir, 2000), this increase is comparable when taking into account the estimate that 10% of the sensory neuron population shows evidence of neuronal damage compared to 50% observed following sciatic nerve crush and nerve transection (Wright et al., 2004).

Protein synthesis was originally thought not to occur in mature axons, however, recent studies have shown that an axonal synthesis mechanism, normally associated with neural development, can be “reactivated” by nerve injury (Wang et al., 2007). Many studies have

shown the synthesis of cytoskeleton protein and growth-associated proteins in the growing axons following injury (Wang et al., 2007). It is via this mechanism that enhanced injury-induced protein synthesis following pre-conditioning crush of the peripheral nerve is thought to contribute to enhanced regeneration capability (McQuarrie, 1978;McQuarrie and Grafstein, 1981;Gardiner et al., 2007). Therefore, it is possible that diabetes induced damage to the peripheral nerve may be stimulating the local axonal synthesis and upregulation of MMP-2; alternatively, axonal MMP may arise from increased anterograde / retrograde transport mechanisms.

Interestingly, unlike other nerve injury models, we saw no increase in MMP-9 expression in our experimental model of diabetes. Under normal injury response, MMP-2 and MMP-9 work in partnership towards successful nerve regeneration. MMP-9 is known to be involved in the breakdown and subsequent repair of the BNB, recruitment of macrophage to the site of injury and control of Schwann cell proliferation and migration during Wallerian degeneration (Chattopadhyay et al., 2007;Kobayashi et al., 2008;Leppert et al., 1995;Shubayev and Myers, 2002); all these processes are known to be affected in clinical and experimental diabetic neuropathy (King et al., 1989;Kennedy and Zochodne, 2005;King et al., 1989).

Minocycline is a semisynthetic second-generation tetracycline with broad-spectrum antimicrobial activity. It inhibits MMPs via binding to zinc or calcium in the MMP active site, and thereby, renders the proenzyme inactive (Keilhoff et al., 2007). Following nerve transection injury, daily intraperitoneal injections of minocycline resulted in downregulation of MMP-2 and MMP-9, reduced macrophage recruitment and activation, incomplete clearance of degenerated material and the migration and activation of phagocyte Schwann cells, which resulted in slowed Wallerian degeneration and subsequent nerve regeneration (Keilhoff et al., 2007).

However, other studies investigating the effects of pan-MMP inhibitor treatment following peripheral nerve injury showed contradictory results. Work using two different general MMP inhibitors (British Biotechnology BB11001; Chemicon Ilomastat) both indicated improved functional recovery with daily treatment of these inhibitors following sciatic

nerve crush (Demestre et al., 2004;Liu et al., 2010). Demestre et al,. (2004) showed improved muscle re-innervation and increased rate of recovery of muscle stimulation but this was not associated with an improvement in nerve regeneration rates. Whilst, Liu et al. (2010) showed enhanced axonal regeneration following inhibition of MMPs; the result of increased Schwann cell proliferation and promotion of dedifferentiation of myelin-forming Schwann cells. These conflicting results could be explained by the known roles of other MMPs in nerve maintenance and injury-induced repair, such as the constitutive expression of MMP-3 in control nerves, resulting in maintenance of Schwann cells in a non-proliferative state.

Following nerve injury, rapid and prolonged downregulation of MMP-3 results in Schwann cell proliferation. This downregulation coincides with rapid upregulation of MMP-9, a known activator of Schwann cell proliferation. Schwann cell interaction with the regenerating axon is essential for successful nerve regeneration. Therefore, any alteration in Schwann cell proliferation could have severe consequences on the success of peripheral nerve repair as axons rarely re-grow without association with activated Schwann cells (Ide, 1996).

Therefore, we hypothesise that the failure of nerve regeneration in diabetes could be the result of either a failure to sustain MMP-2 upregulation or a failure to upregulate MMP-9 which contributes to delayed Wallerian degeneration and regeneration failure via the lack of Schwann cell activation and macrophage infiltration. However, earlier time-points post-STZ should be assessed as MMP-9 expression may be rapid and transient. The altered expression of other MMPs in diabetes also needs to be considered due to their similar roles as MMP-9 in modification of Schwann cell behaviour and macrophage activation.

2.5.3. Other functional roles of MMPs

MMP functionality is not limited to the alteration of the composition of the ECM proteins (Butler and Overall, 2009;McCawley and Matrisian, 2001). There is an ever increasing number of studies that have revealed that MMPs also influence many non-matrix substrates including growth factors such as vascular endothelial growth factor (VEGF), fibroblast

growth factor, transforming growth factor-β and NGF (Bergers et al., 2000;Imai et al., 1997;Whitelock et al., 1996;Yu and Stamenkovic, 2000), cleavage and activation of cytokines such as TNFα and IL-1β (Haro et al., 2000;Schonbeck et al., 1998), integrins (Von et al., 1997;Von et al., 1997) and other cell-surface receptors including Fas-L and E-Cadherin (Lochter et al., 1997;Mitsiades et al., 2001;Noe et al., 2001;Powell et al., 1999).

The expression of many of these MMP substrates is known to be altered in the peripheral nervous system of diabetes; for example, VEGF (Pawson et al., 2010) and NGF (Fernyhough et al., 1995;Hanaoka et al., 1992), whilst in diabetic nephropathy, there is an increase in urinary E-cadherin levels. However, reasons for this increase are unclear (Jiang et al., 2009). Multiple studies have shown no alteration in the expression of proinflammatory cytokines in a number of organs associated with diabetic complications including TNFα and IL-1β in the plasma (Tuttle et al., 2004), TNFα in the serum and TNFα in the heart and aorta (Lu et al., 2010). There is, however, a significant elevation in urinary TNFα levels in diabetic nephropathy. This was shown to be due to an increase in intrarenal production but the reasons for this increase remain unclear (Navarro and Mora, 2006).

Therefore, the role of MMP-2 in the modification of the availability of growth factors, cytokines and many other proteins in diabetes needs to be considered, as any of the known non-matrix substrates of MMP-2 could modify axonal elongation and Schwann cell behaviour following nerve injury (Hughes et al., 2002).

2.5.4. Conclusion

Taken as a whole, this study shows that there is a diabetes associated upregulation in MMP-2 protein expression in the sciatic nerve, with conversion to its active form in a discrete population of diabetic rats. This increase in proteolytic activity could be involved in one of three processes including nerve regeneration, modulation of the peripheral nerve ECM and development of neuropathic pain. Further work is required to investigate the functional consequences of these changes.

2.6. *Supplementary data*

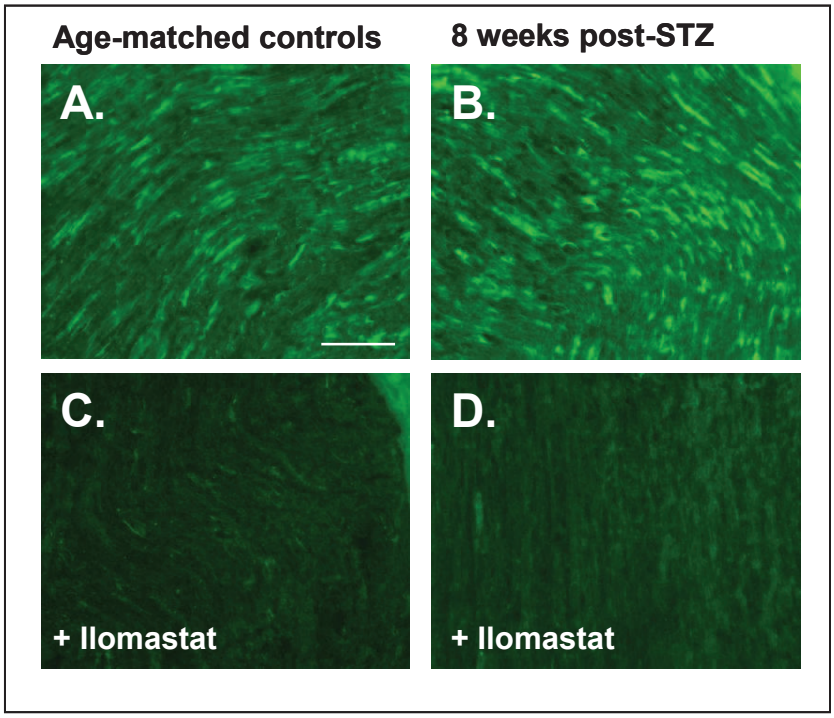
A.

	Number of animals	Start Weight (g)	End Weight (g)	Blood glucose (mmol/l)
4weeks				
Control	10	340 ± 9.4	471 ± 6.6	8.1 ± 1.0
Diabetic	12	339 ± 6.6	370 ± 13.5 ***	27.8 ± 0 ***
8weeks				
Control	10	335 ± 4.9	579 ± 18.0	9.1 ± 1.0
Diabetic	21	343 ± 3.3	383 ± 9.6 ***	27.8 ± 0 ***
12 weeks				
Control	10	334 ± 6.2	612 ± 14.2	9.9 ± 0.6
Diabetic	12	342 ± 4.9	396 ± 10.5 ***	27.8 ± 0 ***

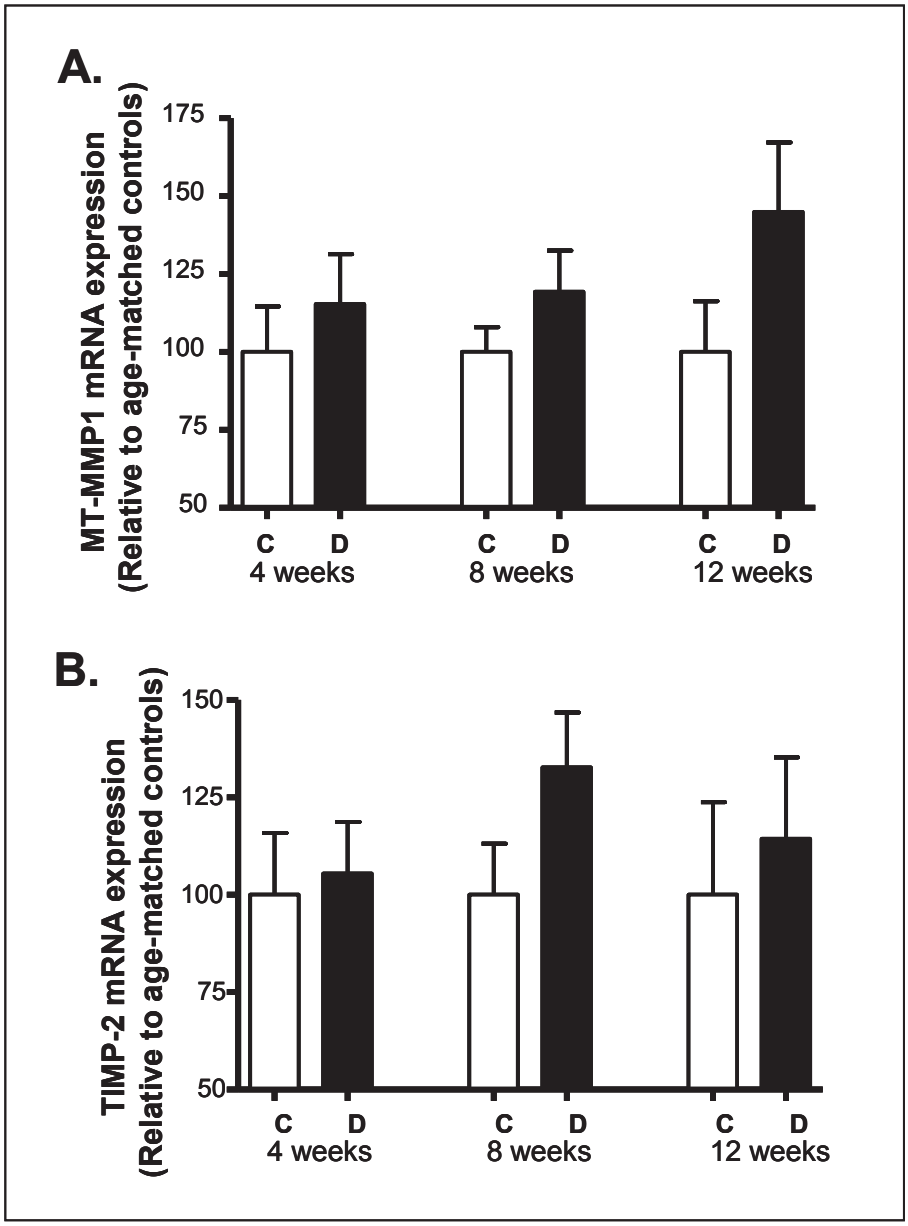
B.

	Number of animals	MNCV (m/sec)	SNCV (m/sec)
4weeks			
Control	3	61.4 ± 5.6	73 ± 10.2
Diabetic	11	54.1± 12.2	48.5 ± 8.0 ***
8weeks			
Control	6	58.1 ± 7.5	61.9 ± 6.5
Diabetic	7	46.5 ± 5.7 **	46.0 ± 8.4 **
12 weeks			
Control	8	81.0 ± 7.6	84.0 ± 7.1
Diabetic	8	37.0 ± 8.0 **	38.8 ± 9.5 **

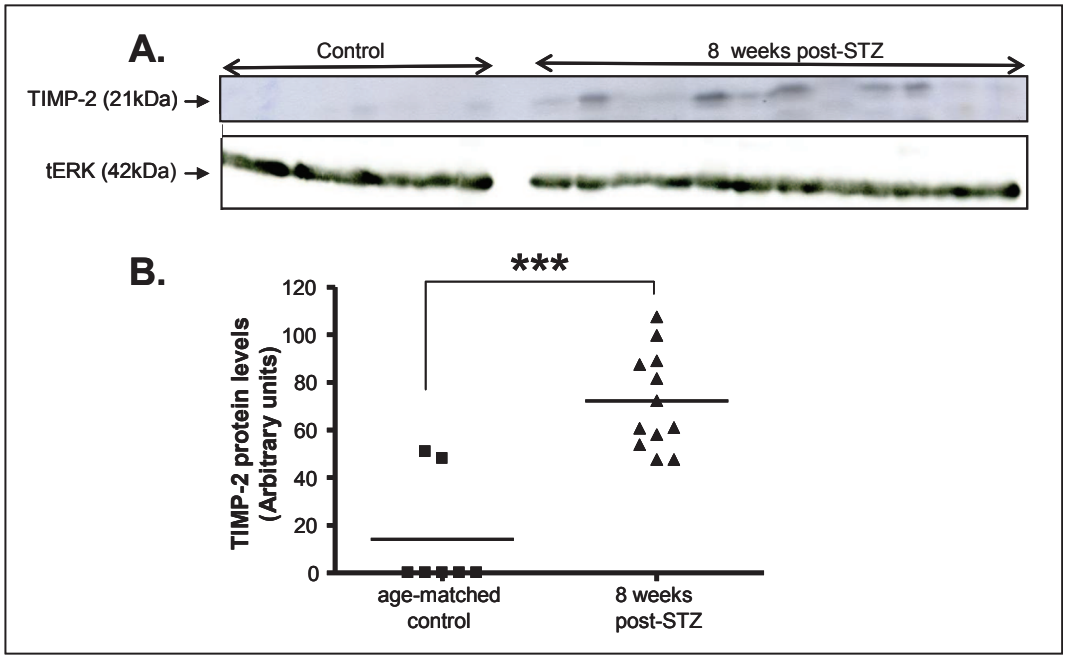
Supplementary Table 2.1: **Physiological deficits in diabetic rats.** A) Mean (±SD) body weight of all animals at the start and end of each study and mean blood glucose levels at the end of each study. B) Mean motor and sensory NCV measured at the end of each study compared to age-matched controls. Unpaired t-test compared to age-matched controls; *** $p<0.001$, ** $p<0.005$



Supplementary Figure 2.1: **In-situ zymography displays increased gelatinase MMP activity levels in sciatic nerve of 8 week post-STZ induced diabetic rats.** Fresh sciatic nerve of A) age-matched controls and B) 8 weeks post-STZ were incubated with fluorescein-labeled gelatin to visualise the areas of gelatinase MMP activity in fresh tissue. C & D) Inclusion of 1mM Ilomastat (pan MMP inhibitor) abolished immunofluorescence, indicating it to be due to MMP activity. Scale bars = 50μm.



Supplementary Figure 2.2: **Upregulation of mRNA expression of endogenous modulators of MMP-2 (MT1-MMP and TIMP-2) is not significant in the sciatic nerve at 8 weeks post-STZ.** Graphic representation of the relative expression of A) MT1-MMP (MMP-14) and B) TIMP-2 at 4, 8 and 12 weeks post-STZ compared to age-matched controls.



Supplementary Figure 2.3: **TIMP-2 protein levels are significantly increased in the sciatic nerve 8 weeks post-STZ.** A) Western blot displaying TIMP-2 protein expression comparing 8 weeks post-STZ sciatic nerve with age-matched control (Each band represents an individual animal; control (n=7) and 8 weeks diabetic adult rats (n=12). B) Graphic representation of TIMP-2 protein levels shows significant increase in TIMP-2 protein expression in the sciatic nerve of 8 weeks post-STZ rats (*** $p<0.001$).

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Modification of the extracellular matrix of sciatic nerve in diabetic rats contributes to the pathogenesis of diabetic neuropathy.

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3.1. Abstract

The extracellular matrix (ECM) plays an important role in mediating successful nerve regeneration in the peripheral nervous system. In order to assess whether modification of the ECM in diabetes affects the success of sensory axonal regeneration, we used an *in vitro* bioassay in which dissociated adult rat sensory neurons were plated onto sciatic nerve taken from streptozotocin (STZ)-induced diabetic or age-matched control adult rats. Adult sensory neurons plated onto sciatic nerve from diabetic rats had significantly shorter neurites compared to those plated on age-matched control nerves. This reduced neurite outgrowth was partially restored by pretreatment of the sciatic nerve sections with either active matrix metalloproteinase (MMP)-2 or chondroitinase ABC (ChABC).

To determine whether MMP-2 and -9 plays a role in direct modulation of adult sensory neuron outgrowth, we used two *in vitro* models of axonal regeneration where control or preconditioned dissociated sensory neurons were plated onto laminin. MMP-2 and MMP-9 were secreted in a time-dependent, but growth factor-independent, manner. However, the addition of either active MMP-2 (200ng/ml), MMP-9 (200ng/ml) or a general MMP inhibitor (Ilomastat; 100μM) did not alter the outgrowth of adult sensory neurons, indicating that MMPs do not directly modulate neurite outgrowth in these experimental paradigms.

The ability of MMP-2 and ChABC to remodel the ECM, by removal of inhibitory chondroitin sulphate proteoglycans (CSPGs) and enhance sensory neurite outgrowth on nerve sections obtained from STZ-diabetic rats suggests that modulation of CSPG levels in peripheral nerve in diabetes may be a potential therapeutic avenue to explore in the treatment of diabetic neuropathy.

Key words; Diabetes, peripheral nerve, neuronal regeneration, matrix metalloproteinases, chondroitin sulphate proteoglycans.

3.2. Introduction

The peripherally directed branches of sensory neurons, unlike the central branches or neurons within the central nervous system (CNS) are relatively successful at regenerating following nerve damage. The ability to successfully regenerate has been attributed to upregulation of growth-associated gene expression in sensory neurons (Chong et al., 1994;Costigan et al., 2002;Schreyer and Skene, 1993), the presence of neurotrophic factors (Zhou et al., 2006) and the conversion of the extracellular environment to one favourable to axonal regeneration (Ide, 1996;Venstrom and Reichardt, 1993).

In diabetes, however, peripheral nerve regeneration is impaired. Studies in both human diabetic subjects (Beiswenger et al., 2008;McArthur et al., 1998) and streptozotocin (STZ)-induced diabetes in rodents (Bisby and Chen, 1990;Ekstrom and Tomlinson, 1989;Kennedy and Zochodne, 2000) have shown delayed axonal degeneration during Wallerian degeneration and impaired axonal regeneration. This failure in nerve regeneration is thought to be a result of multiple factors; including delayed degeneration (Terada et al., 1998a;Terada et al., 1998b), delayed axonal sprouting from proximal nerve stump (Bisby and Chen, 1990;Ekstrom and Tomlinson, 1989;Kennedy and Zochodne, 2000) and failure in collateral sprouting (Theriault et al., 1998); all of which are thought to contribute to loss of skin innervation and the progressive 'die-back' of the distal axon terminals. Structural alterations of the extracellular matrix (ECM) of the peripheral nerve also contribute to nerve regeneration failure in diabetes. In diabetic human patients there is increased collagen (COLL) in the basal lamina of the perineurium and endoneurium (Bradley et al., 2000;Hill, 2009;Hill and Williams, 2002), reduplication of basement membrane of the endoneurial capillaries (Giannini and Dyck, 1995) and thickening of the basal lamina of epineurial cells (Bradley et al., 2000;King et al., 1989).

Matrix metalloproteinases (MMPs) are a family of calcium- and zinc-dependent proteolytic enzymes that are able to degrade the protein components of the ECM (Hijova, 2005). The expression of several MMPs (MMP-2, MMP-9, MMP-3, MMP-7 and MMP-12) is altered following nerve injury. All of these MMPs, with the exception of MMP-3, are significantly upregulated in the rat sciatic nerve immediately after nerve crush (Demestre et al., 2004;Ferguson and Muir, 2000;La et al., 1996;Shubayev et al., 2006;Gantus et al.,

2006;Hughes et al., 2002); MMP-3 expression is progressively down-regulated after nerve crush (Gantus et al., 2006;Hughes et al., 2002). MMP-2 and MMP-9 expression following injury are increased in Schwann cells (Demestre et al., 2004;La et al., 1996;Muir, 1994) (Chattopadhyay et al., 2007;Chattopadhyay and Shubayev, 2009), whilst MMP-3 expression following nerve injury is reduced in the Schwann cells (Gantus et al., 2006;Hughes et al., 2002). MMP-2 expression is also increased in the axons themselves (Demestre et al., 2004), MMP-9 is increased in resident and invading macrophages (Shubayev et al., 2006) of the peripheral nerve, whilst MMP-7 and MMP-12 are expressed by activated macrophages (Hughes et al., 2002). This coordinated expression of MMPs following sciatic nerve injury initiates the remodelling process required for successful nerve degeneration and regeneration.

In vitro studies have also suggested a pro-regenerative role for MMP-2 and MMP-9, in modulating axonal regeneration. Nerve growth factor (NGF) and fibroblast growth factor promote neurite outgrowth, and at the same time upregulate MMPs (including MMP-2 and MMP-9, in cultures of pheochromocytoma (PC12) cells and embryonic chick sensory neurons (Machida et al., 1989;Muir, 1994). Furthermore, exogenous MMP-9 enhances NGF-induced neurite elongation and inhibition of MMP-9 by either a MMP-9 neutralising antibody or a broad-spectrum MMP inhibitor blocks NGF-mediated axonal elongation and sprouting (Shubayev and Myers, 2004). MMP-2 plays a role in remodelling of the matrix during axonal elongation (Krekoski et al., 2002;Ferguson and Muir, 2000;Zuo et al., 1998a). Embryonic rat sensory neurons extended significantly longer neurites on adult rat peripheral nerve tissue sections after enzymatic degradation of the nerve ECM with MMP-2 compared to untreated nerve. Addition of a MMP inhibitor to the culture medium decreased neurite outgrowth to control levels (Zuo et al., 1998a). Pre-treatment of peripheral nerve sections with chondroitinase ABC (ChABC). which degrades and inactivates neurite growth-inhibiting chondroitin sulphate proteoglycan (CSPG), resulted in similar increased neurite elongation as that seen in response to MMP-2 pre-treatment (Ferguson and Muir, 2000;Zuo et al., 1998a;Krekoski et al., 2001). Furthermore, the addition of laminin (LM)-blocking antibodies to the culture media attenuated the enhanced neurite elongation following MMP-2 or ChABC pre-treatment (Zuo et al., 1998a). Therefore, MMP-2 appears to enhance ability of the peripheral nerve to support neurite outgrowth via the degradation and inactivation of inhibitory CSPGs (Krekoski et al.,

2002;Zuo et al., 1998a;Krekoski et al., 2001;Zuo et al., 1998a;Zuo et al., 1998b), which results in the exposure of neurite-promoting LM on the Schwann cell basal laminae (Zuo et al., 1998a).

In this study we show that, MMP-2, but not MMP-9, promotes neurite elongation in a very small number of dissociated adult rat sensory neurons. Unlike previous culture studies using PC12 cells or embryonic neurons, application of exogenous MMP-2, MMP-9 or a general MMP inhibitor failed to significantly modulate either NGF-dependent or preconditioned- (by sciatic nerve crush) induced neurite outgrowth from adult rat sensory neurons. We demonstrate that sensory neurons plated on sciatic nerve cryocultures prepared from STZ-diabetic rats extend significantly shorter neurites than those plated on nerve cryocultures prepared from age-matched control rats. Pretreatment of nerve sections from diabetic rats with either recombinant active MMP-2 protein or ChABC partially restored this deficit. These studies indicate that inhibitory CSPG may contribute to the regeneration failure seen in diabetic neuropathy and may represent a novel therapeutic strategy.

3.3. Materials and methods

3.3.1. Animals and surgeries

All experiments were conducted in accordance with the UK Animals Act (Scientific Procedures 1986) and institutional regulations. Adult male Wistar rats (Charles River, UK) with a starting weight of 250-300g were given a single intraperitonal injection of STZ (Sigma, UK, Cat No. S0130) at a dose of 55mg/kg in 0.9% NaCl, administered the morning after an overnight fast. Three days later, blood glucose levels were monitored using a strip-operated reflector photometer (MediSense Optimum Plus; MediSense, UK). Animals with blood glucose less than 15mmol/l were rejected from the study. All rats were provided with standard rodent chow (Beekay International) and tap water, and housed at a constant temperature of 21°C with 12:12 hour light/dark cycle. Animals were weighted and checked biweekly. Age-matched rats were used as non-diabetic controls. Animals were maintained for 8 or 12 weeks post-STZ.

Adult male Wistar rats (250-300g; Charles River, UK) were anaesthetised with isofluorane (2% in oxygen), and under sterile conditions, the left sciatic nerve was exposed at mid-thigh level. Sciatic nerve was crushed (2 x 15 seconds) with tips of watchmakers forceps the wound was closed and animals recovered under observation. 2, 7 or 14 days following nerve crush the animals were killed by exsanguination from the right ventricle. The sciatic nerve was removed and snap-frozen on dry-ice and stored at -80°C.

3.3.2. Primary dissociated neuron cultures

Adult male Wistar rats were killed by concussion followed by decapitation. The DRG were removed, cleaned of connective tissue and chemically dissociated by incubation in 0.125% collagenase (Worthington Biochemicals, UK, Cat No. LS004188) in Ham's F12 media (Gibco, UK, Cat No.21765) for 1 hour. DRG were then washed three times with F12 media and incubated in 0.125% collaganase as before. DRG were then transferred to 0.25% trypsin (Worthington Biochemicals, UK, Cat No. LS003703) and incubated for 30 minutes. The trypsin was inactivated using 30% foetal bovine serum (Gibco, UK, Cat No. 10437). DRG were washed three times in Ham's F12 media to remove serum and then

were mechanically dissociated via gentle titration with a glass pipette. Dissociated neurons were passed through a 70μm mesh (Cadisch, UK) to remove un-dissociated cells and myelin, and then centrifuged for 5 minutes at 300rpm. Cells were re-suspended in Ham's F12 media, and centrifuged through 15% bovine serum albumin (Sigma, Cat No. A9205) at 900rpm for 10 minutes to remove non-neuronal cells. Cells were re-suspended in Bottenstain and Sato's medium (BS medium containing 0.1mg/ml transferrin (Sigma, Cat No. T8158), 20nM progesterone (Sigma, Cat No. P8783), 100μM putrescine (Sigma, Cat No. P5780), 30nM sodium selenite (Sigma, Cat No. S5261), 1μg/ml BSA (Fluka, Cat No. 05473), 0.01mM cytosine arabinoside (Sigma, Cat No. C1768) and 10pM insulin (Sigma, Cat No. I3505)) in Ham's F12. All incubations took place in humidified temperature controlled chambers at 37°C in 5% CO_2 unless otherwise stated.

Neurons were either seeded onto 6-well plates for protein assays or onto Lab-Tek chamber slides for immunocytochemistry. The 6-well plates were pre-coated overnight at 4°C with 500μg/ml polyornithine (Sigma, UK, Cat No. P8638) followed by 2ug/ml laminin-1 (LM; Sigma, UK, Cat No.L2020) in Ham's F12 incubated for 2 hours. Cells were then incubated for 1 hour before treatment with either NGF (10ng/ml, Sigma, Cat No.N6009), NT3 (10ng/ml, Genentech, Gift), GDNF (50ng/ml, Promega, Cat No.G2781) or left un-treated for 18 or 48 hours. The Lab-Tek chamber slides (Nunc, VWR, UK, Cat No.154534) pre-coated with LM as described above. Neurons were treated with either general MMP inhibitor (25uM-100uM; Chemicon, UK, Cat No. cc1100); MMP-2, active, human (100-200ng/ml; Calbiochem, UK, Cat No. PF023) or MMP-9, active, human, recombinant (100-200ng/ml; Calbiochem, UK, Cat No. PF024) or left un-treated, in the presence or absence of 10ng/ml NGF for 18 hours.

3.3.3. Pre-conditioned dissociated primary neuron cultures

Seven days after nerve crush, animals were killed by concussion followed by decapitation. Ipsilateral and contralateral L4 and L5 DRG were removed and treated as described above. All cells were used for immunocytochemistry and neurite outgrowth analysis.

3.3.3.1. Immunocytochemistry assessment of neurite outgrowth

Cultured neurons were fixed with 2% paraformaldehyde (Sigma, UK) for 20 minutes at room temperature, and washed three times with PBS (Sigma, UK, Cat No. P4417). Fixed cells were visualised by incubating with an antibody against β (III) tubulin (1:500; Sigma, UK, Cat No. P7280) in PBS with 0.2% (v/v) Triton-X 100, (Sigma, UK; Cat No. MAB347), at 4°C overnight. Cells were then washed three times with PBS and incubated with Cy3-conjugated donkey anti-mouse antibody (1:500, Jackson ImmunoResearch, Cat No. 715-167-003) for 2 hours at room temperature. Cells were then washed three times with PBS and mounted in Vectorshield containing DAPI.

3.3.3.2. Immunocytochemistry image analysis

Immunofluorescence was visualised using Leica MPS60 DMR fluorescent microscope and images were acquired using HC imaging software (Hamamatsu, US). Neuron images were acquired from a minimum of 20 randomly selected fields of view per condition at 10x magnification. The mean number of neurite-bearing cells and the length of longest neurite (maximum length cell body were measured from the centre of the cell body) were calculated using SigmaScan Pro 5 software (SPSS, UK). Statistical analysis was conducted using GraphPad PRISM 4 (GraphPad Software Inc). Data were analysed using an one-way ANOVA with Bonferroni's Multiple Comparison test and shown as mean ± standard deviation.

3.3.4. Trypan blue neuronal survival assay

Trypan blue assay of cell viability is based on the principle that live cells possess intact cell membranes that exclude certain dyes, whereas dead cells do not. Treated neurons are grown overnight in Labtek chamber slides (as described above) and are incubated with 0.2% Trypan blue solution (v/v; Sigma, UK, Cat No.T8154) at room temperature for 15 minutes. The trypan blue solution was then removed and the neurons washed 3 times with F12 Hams media, or until all blue colour has been removed. Neurons viability was then calculated by dividing the total number of viable neurons (unstained) by the total number of neurons (stained and unstained) for each treatment condition.

3.3.5. Cryoculture

Cryoculture is a neurite outgrowth assay in which neurons are grown on a substratum of unfixed peripheral nerve. Fresh nerve segments (1cm) were repeatedly freeze-thawed (5 minutes at 37°C followed by 5 minutes in liquid nitrogen) to lyse membranes and expose the extracellular matrix (Ide et al., 1983;Gulati, 1988). The sciatic nerve sections were embedded in OCT before being cryosectioned (14μm) and thaw-mounted onto acid washed, sterile, poly-D-lysine (PDL; 1mg/ml: Sigma, Cat No. P7280)-coated coverslips. Sections were treated either with active MMP-2 (10μg/ml), chondroitinase ABC (ChABC: 0.1U/ml; Sigma, UK; Cat No.C3667), MMP vehicle (25mM Tris-HC;, pH 7.9 containing $CaCl_2$) or chondroitinase vehicle (50mM Tris-HCl, pH8.0 containing 50mM NaCl) for 3hours at 37°C in 5% CO_2. 100μM of broad-spectrum metalloproteinase inhibitor (Ilomastat; Chemicon; Cat No. CC1100) was used as a negative control. Sections were then washed twice in Ham's F-12 media before dissociated sensory neurons were seeded directly onto the sciatic nerve sections in the presence of 10ng/ml NGF, and incubated for 24 or 48 hours at 37°C in 5% CO_2.

3.3.5.1. Immunocytochemistry assessment of neurite outgrowth

Cryoculture sections were fixed with 2% paraformaldehyde (Sigma, UK) for 20 minutes at room temperature, washed 3 times with PBS (Sigma, Cat No. P4417) and then blocking buffer (10% normal donkey serum (NDS: Sigma, Cat No. D9663) in 0.2% Triton X-100 in PBS) for 2 hours at room temperature. Blocking solution was replaced with an antibody against growth associated protein (GAP)-43 (1: 2000; Millpore, UK) in blocking buffer for 48hours at 4°C. Coverslips were washed 3 times with PBS and incubated with Cy3-conjugated donkey anti-mouse antibody for 2 hours at room temperature. Cells were then washed three times with PBS and mounted in Vectorshield containing DAPI.

3.3.5.2. Immunocytochemistry image analysis

Immunofluorescence was visualised using Leica MPS60 DMR fluorescent microscope and images were acquired using HC imaging software (Hamamatsu, US). Images of all neurite elongation were taken at 20x magnification per condition. The length of longest neurite was calculated.

3.3.6. Immuohistochemistry

Following pre-treatment of either active MMP-2 or chondroitinase ABC, sciatic nerve sections were fixed with 2% paraformaldehyde (Sigma, UK) for 20 minutes at room temperature, washed 3 times in 0.2% Triton X-100 in PBS (Sigma, Cat No. P4417) and then placed in blocking buffer (10% normal horse serum (Vector, Cat No. S-2000)) in 0.2% Triton X-100 in PBS for 2 hours at room temperature. Blocking solution was replaced with an antibody against chondroitin sulfate (CS; 1:500; Sigma, Cat No. C-8035) in PBS with 0.2% (v/v) Triton-X 100 at 4°C. Coverslips were then washed 3 times with PBS and incubated with Fluorescein Horse Anti-Mouse IgG Antibody, rat adsorbed (1:200; Vector, Cat No. FI-2001) for 2 hours at room temperature. Coverslips were then washed three times in 0.2% Triton X-100 in PBS and mounted in Vectorshield containing DAPI (Vector Labs, Cat No. H-1200). Immunofluorescence was visualised using Leica MPS60 DMR fluorescent microscope and representative images acquired using Hamamatsu camera and HC Image Imaging Software (Hamamatsu, US).

3.3.7. Gel zymography

Cell media was removed and ice cold lysis buffer (0.5M Tris pH 7.6, 0.2M NaCl, 10mM $CaCl_2$ and 1% Triton X-100) and 5x sample buffer (400mM Tris pH 6.8, 10% SDS, 50% glycerol and 0.025% bromophenol blue) were added to the wells and left for 30 minutes in ice. Cells were collected by scraping. Protein levels of both cell lysis and media were quantified according to colorimetric Bramhall Assay with albumin used as a standard and F-12 media as blank.

10μg of proteins were separated on 0.2% gelatin / 8% polyacrylamide gel by SDS-PAGE in electrophoresis buffer. Gels were run at 60V until samples were in a concentrated line in the stacking gel and then increased to 100V. Gels were washed overnight at room temperature in wash buffer (50mM Tris pH 7.5, 5mM $CaCl_2$, 1μM $ZnCl_2$ and 2.5% Triton X-100) with constant agitation, to renature proteins by removing the SDS from the gel. The gels were then washed with distilled water twice for 10 minutes, then incubated in activity buffer (50mM Tris pH 7.5, 5mM $CaCl_2$ and 1μM $ZnCl_2$) for 24 hours at 37°C with constant agitation, to activate proteases. Followed by two washes in distilled water for 10 minutes. Proteins within the gel were visualised by incubation in development solution

(40% methanol, 10% acetic acid and 0.1% Coomassie Brilliant blue) for 1 hour at room temperature. Finally, the gel was placed in destain solution (10% acetic acid and 10% methanol) until bands appeared on a uniformly stained background. Areas of gelatinase activity (ie, MMP-2 and MMP-9) appeared as white bands on a blue background. The gels were dried using DryEase Mini-Gel Drying System (Invitrogen).

3.3.8. Western blots

Desheathed sciatic nerves were homogenised in a freshly prepared ice cold lysis buffer (25mM Tris HCl pH 7.4, 15mM NaCl, 10mM NaF, 10mM Na Pyrophospahte, 2mM EDTA, 0.2mM Na_4Ov_3, 1mM PMSF, Protease Inhibitor Cocktail (1:200, Sigma, Cat No. P8340) and 1% (w/v) Nonidet P40) for 30-60 seconds using a hand-held homogeniser (Omni International μH, Camlab). The tissue homogenates were centrifuged (9,000g, 30 minutes, 4°C) and the supernatant transferred to a clean Eppendorf tube. Proteins were denatured with a SDS sample buffer (0.25M Tris pH 6.8, 20% glycerol, 0.01% bromophenol blue, 2% SDS and 2% mercaptoethanol) by boiling for 5 minutes at 95°C. Protein levels were quantified according to the colorimetric Bramhall assay with albumin used as a standard. 20μg of total protein were resolved through 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in electrophoresis buffer (25mM Tris-base, 192mM Glycine and 0.1% (w/v) SDS). The gel was run at 100V until samples were in a concentrated line in the stacking gel and then increased to 120V until the samples had run to the required distance. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL, GE Healthcare) at 80V in transfer buffer (25mM Tris-base, 192mM Glycine and 20% (w/v) Methanol). The even loading and transfer of protein was confirmed by staining the membrane for 30 seconds with 10% Ponceau S (Sigma) followed by washing with blotting buffer (10mM Tris-base, 100mM NaCl and 0.1% (w/v) Tween 20) until Ponceau staining was removed.

Non-specific binding was blocked by incubating the membranes in 10% non-fat dry milk (Marvel, Premier International food Ltd) in blotting buffer either overnight at 4°C or for 2 hours at room temperature with constant agitation. The membrane was then incubated overnight at 4°C with either rabbit anti-Rat MMP-2 (1:2000; Millipore, Cat No. AB19015), rabbit anti-Rat MMP-9 (1:3000; Millipore, Cat No. AB19016) or rabbit anti- p44/42 MAP

kinase antibody (1:5000: Cell Signalling Technologies, Cat No. 9102 in 1% bovine serum albumin (BSA; Fluka, Cat No. 05473) in blotting buffer. The following day, the membranes were washed with blotting buffer and then incubated for 1 hour at room temperature in anti-rabbit horseradish peroxidase (HRP)-linked IgG antibody (1:5000; Cell Signalling Technology) diluted in 5% non-fat dry milk in blotting buffer. The membranes were washed three times for 15 minutes with blotting buffer. Proteins were visualised using LumiGLO^{TM} and peroxidase reagent (Cell Signalling Technology, Cat No.7003) according to the manufacturer's instruction. Membranes were exposed to Hyperfilm ECL (Amersham BioSciences, Cat No. 28-9068-37) for between 30 seconds and 1 hour depending on signal strength.

3.3.8.1. Analysis and statistics

Westerns films and zymograph gels were scanned using a flatbed scanner with back lighting (HP Scanjet 2400). The average intensity of each band was measured using SigmaScan Pro5 software (SPSS, UK). An average of three recordings per band was used to measure average intensities of total protein activity. Statistical analysis was conducted using GraphPad PRISM 4 (GraphPad Software Inc). Data were analysed using an unpaired t-test and shown as mean ± standard deviation.

3.4. Results

3.4.1. Peripheral nerve from diabetic rats is less supportive for neurite outgrowth

Previous work in our lab has shown that glycation of endoneurial ECM proteins occurs in STZ-diabetes and also that exogenous glycation of laminin and fibronectin is inhibitory to sensory neurite outgrowth *in vitro* (Duran-Jimenez et al., 2009). Therefore, to test the hypothesis that peripheral nerve ECM from diabetic rats is less supportive to axonal regeneration, we prepared sciatic nerve cryocultures from STZ- and age-matched control rats. Dissociated adult rat sensory neurons were seeded onto longitudinal sciatic nerve sections obtained from either STZ-induced diabetic rats (8 weeks or 12 weeks post-STZ) or age-matched controls in the presence of NGF (10ng/ml; 48 hours), in order to assess their ability to support neurite elongation. NGF-induced neurite outgrowth on sciatic nerve sections from diabetic rats was significantly less than neurite outgrowth from neurons plated on control sciatic nerve (Figure 3.1). Neurites were significantly shorter at both timepoints following induction of diabetes (8 weeks: age-matched control: 193.2 ± 11.6μm vs diabetic: 131.3 ± 6.5μm, (*n=5*), *** $p<0.0001$) and 12 weeks: age-matched control: 209.7 ± 11.6μm vs diabetic: 143.6 ± 6.0μm, (*n=5*), *** $p<0.0001$) (Figure 3.1). Neurons seeded onto control sciatic nerve extended long unbranched neurites which grow in close association with the basal lamina (Fig 3.1A, C). In contrast, a significant proportion of neurons seeded onto sciatic nerve obtained from STZ-diabetic rats produced less well ordered neurites which were shorter, more branched and appeared less associated with basal lamina (Figure 3.1B,D, 3.2B,E & 3.3B.E arrows). Unfortunately, due to the non-planar nature of this neurite outgrowth assay it was not possible to quantify these morphological differences. In addition, fewer neurons appeared to adhere to diabetic sections, again, however, this observation was not quantifiable due to seeding density differences.

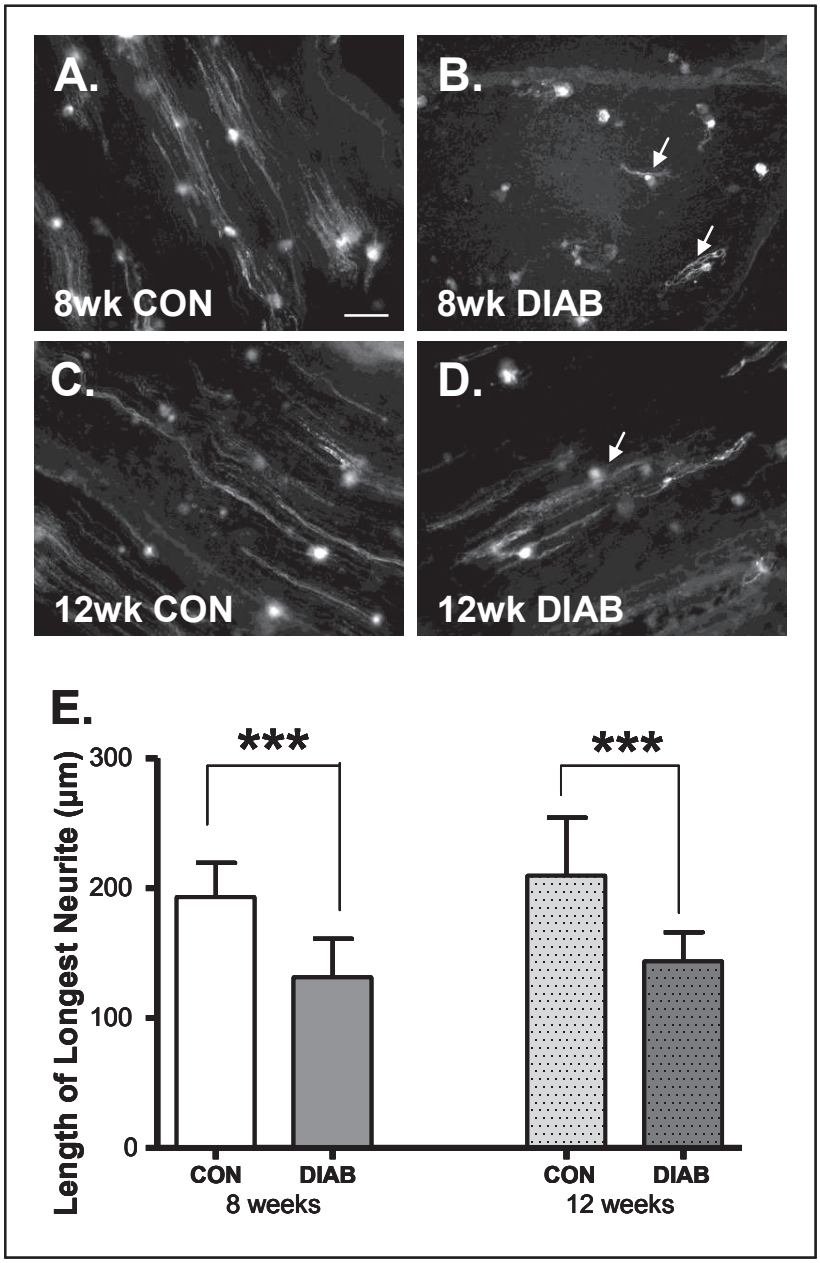


Figure 3.1 **Sciatic nerve cryocultures prepared from diabetic rats is significantly less supportive for sensory nerve regeneration than age-matched control nerve sections.** Representative micrographs of primary cultures of adult rat dissociated neurons plated on sciatic nerve cryoculture section from A) 8 week age-matched control, B) 8 weeks diabetic, C) 12 week age-matched control and D) 12 weeks diabetic sciatic nerve. Neurons were treated with NGF (10ng/ml, 48 hours). Arrows indicate the shorter, more branched neurites on diabetic sciatic nerve. Scale bar = 50µm. There is a significant reduction in the length of the longest neurite in neurons grown on diabetic sciatic nerve compared to age-matched control at both 8 and 12 week timepoints (E; n=5; all treatment groups; unpaired t-test; *** $p<0.001$).

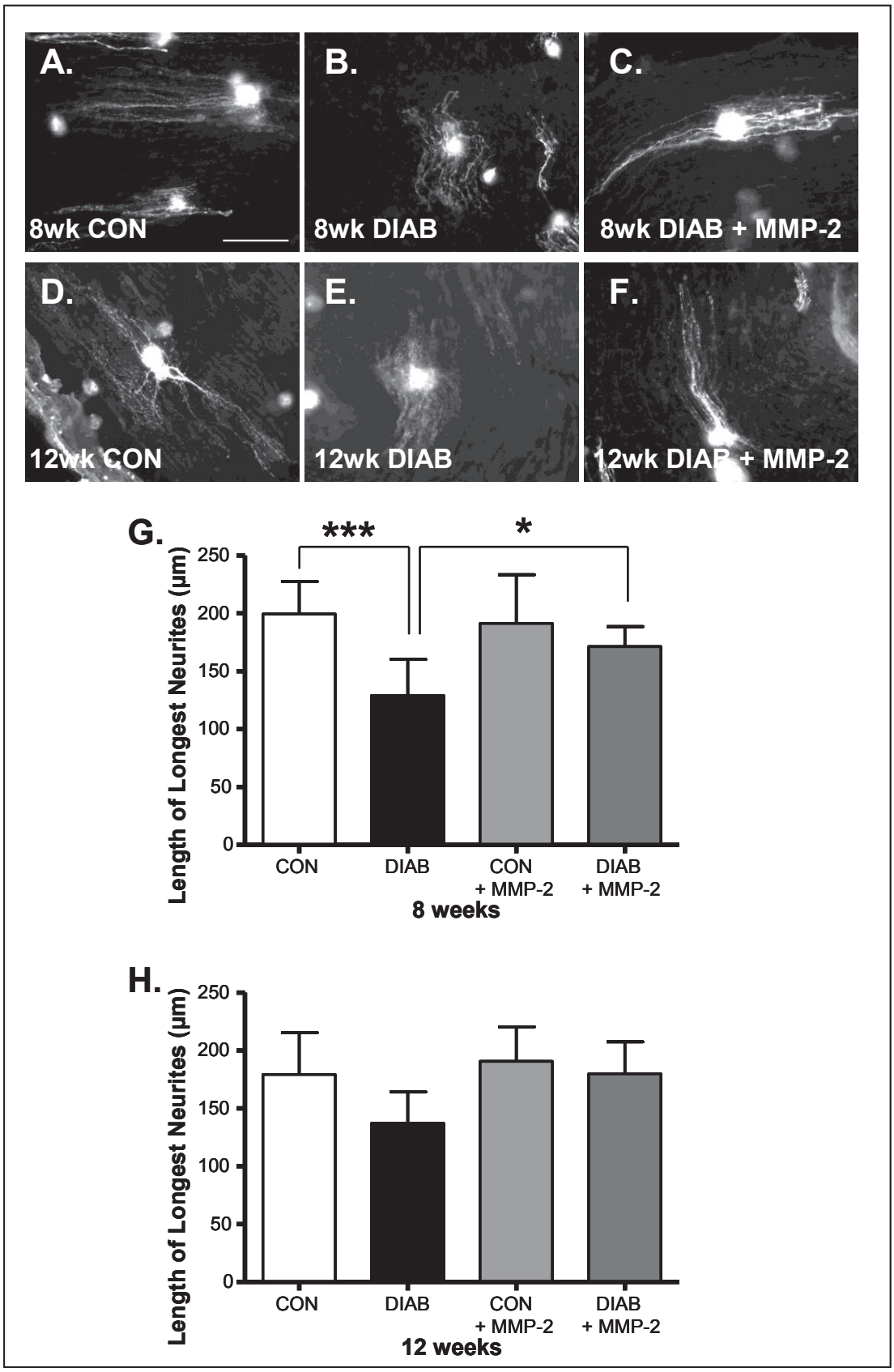
3.4.2. Enhanced nerve regeneration on cryoculture nerve section from diabetic rats pretreated with active MMP-2

MMP-2 pretreatment has previously been shown to enhance neurite elongation from both embryonic and adult rat sensory neurons grown on control rat sciatic nerve cryocultures (Ferguson and Muir, 2000;Zuo et al., 1998a;Zuo et al., 1998b). The aim of the next set of experiments was to determine whether MMP-2 is able to degrade the peripheral nerve ECM of diabetic rats thereby enhancing axonal regeneration.

Cryocultures of sciatic nerve sections from all treatment groups were pre-treated with either active MMP-2 (10μg/ml) or vehicle (V) prior to the seeding of the sensory neurons. MMP-2 pretreatment significantly enhanced neurite outgrowth on diabetic nerve sections at 8 weeks (Control + V: 199.5 ± 28.2μm vs diabetic + V: 129.0 ± 31.4μm vs Control + MMP-2: 191.3 ± 42.2μm vs diabetic + MMP-2: 172.7 ± 17.7μm (*n=5*), *** $p<0.001$, *$p<0.05$) but not at 12 weeks (Control + V: 179.3± 36.3μm vs diabetic + V: 137.3 ± 27.1μm vs Control + MMP-2: 190.7 ± 29.9μm vs diabetic + MMP-2: 179.9 ± 27.8μm, (*n=5*)) diabetic rats pretreated with active MMP-2 (Figure 3.2).

In contrast to previous studies (Ferguson and Muir, 2000;Zuo et al., 1998a;Zuo et al., 1998b), no improvement in nerve regeneration capabilities of age-matched sciatic nerve was achieved following active MMP-2 pre-treatment (Figure 3.2), which may reflect the different procedures used in preparing the cryocultures.

Figure 3.2: **Neurite elongation on diabetic nerve sections is partially rescued by pre-treatment with active MMP-2 protein.** Sciatic nerve sections were pre-treated with active MMP-2 (10μg/ml for 3 hours) or vehicle prior to plating with the primary dissociated sensory neurons in presence of NGF (10ng/ml for 48 hours). Gap43-IR allowed visualisation of cell bodies and neurite morphology (A-F). Representative micrographs of axonal elongation of neurons plated onto either age-matched control (A, D) or 8 or 12 week diabetic untreated or treated (B-F) sciatic nerve. The significant reduction in the length of longest neurite grown on diabetic sciatic nerve compared to age-matched control is rescued by pre-treatment of the diabetic sciatic nerve section with active MMP-2 protein (n=5; all treatment groups; one-way ANOVA with Bonferroni's Multiple Comparison test; *** $p<0.001$, * $p<0.05$) at 8 weeks (G). There is a trend towards similar rescue at 12 weeks however this was not significant (H). Scale bar = 50μm.

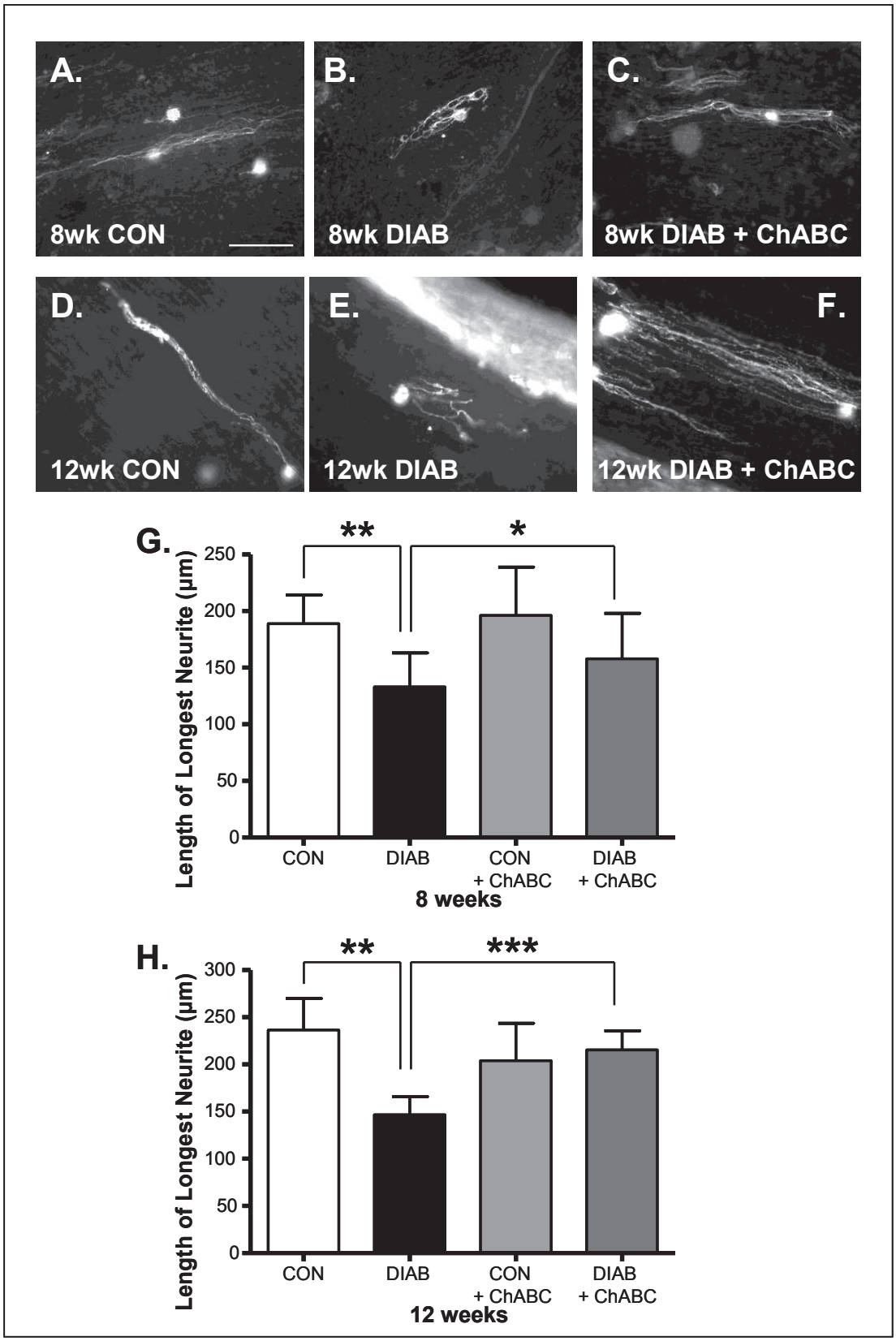

A.
8wk CON
B.
8wk DIAB
C.
8wk DIAB + MMP-2
D.
12wk CON
E.
12wk DIAB
F.
12wk DIAB + MMP-2
G.
***
*
Length of Longest Neurites (µm)
250
200
150
100
50
0
CON
DIAB
CON + MMP-2
DIAB + MMP-2
8 weeks
H.
Length of Longest Neurites (µm)
250
200
150
100
50
0
CON
DIAB
CON + MMP-2
DIAB + MMP-2
12 weeks

3.4.3. Degradation of chondroitin sulphate proteoglycans enhances nerve regeneration on cryoculture nerves section from diabetic rats

Since one of the actions of MMP-2 is the degradation of inhibitory CSPGs (Zuo et al., 1998a;Zuo et al., 1998b), we used ChABC, an enzyme that catalyses the removal of the inhibitory CS sidechains of CSPGs, to assess whether neurite elongation was similarly enhanced.

Identical cryoculture assays were performed following pre-treatment of nerve sections with ChABC (0.1Unit/ml) or vehicle. We observed similar levels of enhanced nerve regeneration on sciatic nerve from 8 week (Control + V: 188.9 ± 25.4μm vs diabetic + V: 133.1 ± 29.9μm vs Control + ChABC: 196.2 ± 42.6μm vs diabetic + ChABC: 157.7± 40.3μm ** $p<0.01$ ($n=5$)) and 12 week (Control + V: 236.4 ± 33.5μm vs diabetic + V: 146.7 ± 19.2μm vs Control + ChABC: 218.6± 36.4μm vs diabetic + ChABC: 215.4 ± 20.3μm ($n=5$), *** $p<0.001$, ** $p<0.01$) diabetic rats following pretreatment with ChABC as with active MMP-2 (Figure 3.3).

Figure 3.3: **Impaired neurite elongation on diabetic nerve cryocultures is partially rescued by pre-treatment with Chondroitinase ABC.** Prior to plating of the primary dissociated sensory neurons, sciatic nerve sections were pre-treated with Chondroitinase ABC (0.1 Unit/ml for 3 hours) or vehicle, and grown for 48 hours in the presence of NGF (10mg/ml). Representative micrographs of axonal elongation of primary dissociated neurons plated onto either age-matched controls (A, D) or 8 (B, C) or 12 week (E, F) untreated or pretreated diabetic sciatic nerve section. There is a significant reduction in the length of the longest neurite grown on diabetic sciatic nerve compared to age-matched control at 8 (G) and 12 weeks (H) duration of diabetes, which is rescued by pre-treatment of the sciatic nerve section with Chondroitinase ABC (n=5; all treatment groups); one-way ANOVA with Bonferroni's Multiple Comparison test; *** $p<0.001$, **$p<0.01$, *$p<0.05$). Scale bar = 50μm.


A.
8wk CON
B.
8wk DIAB
C.
8wk DIAB + ChABC
D.
12wk CON
E.
12wk DIAB
F.
12wk DIAB + ChABC
G.
**
*
Length of Longest Neurite (µm)
250
200
150
100
50
0
CON
DIAB
CON + ChABC
DIAB + ChABC
8 weeks
H.
**
***
Length of Longest Neurite (µm)
300
250
200
150
100
50
0
CON
DIAB
CON + ChABC
DIAB + ChABC
12 weeks

In order, to determine whether pre-treatment with active MMP-2 or ChABC effectively degraded CSPG in both control and diabetic nerve cryoculture sections, we conducted immunocytochemical analysis using an antibody raised against CS sidechains of CSPG. CS-immunoreactivity (-IR) was intense throughout the cryoculture nerve sections from both the 8 and 12 weeks diabetic rats and age-matched controls. CS-IR was associated with the Schwann cell basal lamina within the cryocultures sections (arrows, Figure 3.4A & B), as previously described (Zuo et al., 1998b). CS-IR was absent in cryocultures exposed to either ChABC or active MMP-2 (Figure 3.4C-F), indicating the effective permeation of enzymes throughout the nerve compartments and the thorough degradation of the CSPG sidechains in all treatment groups.

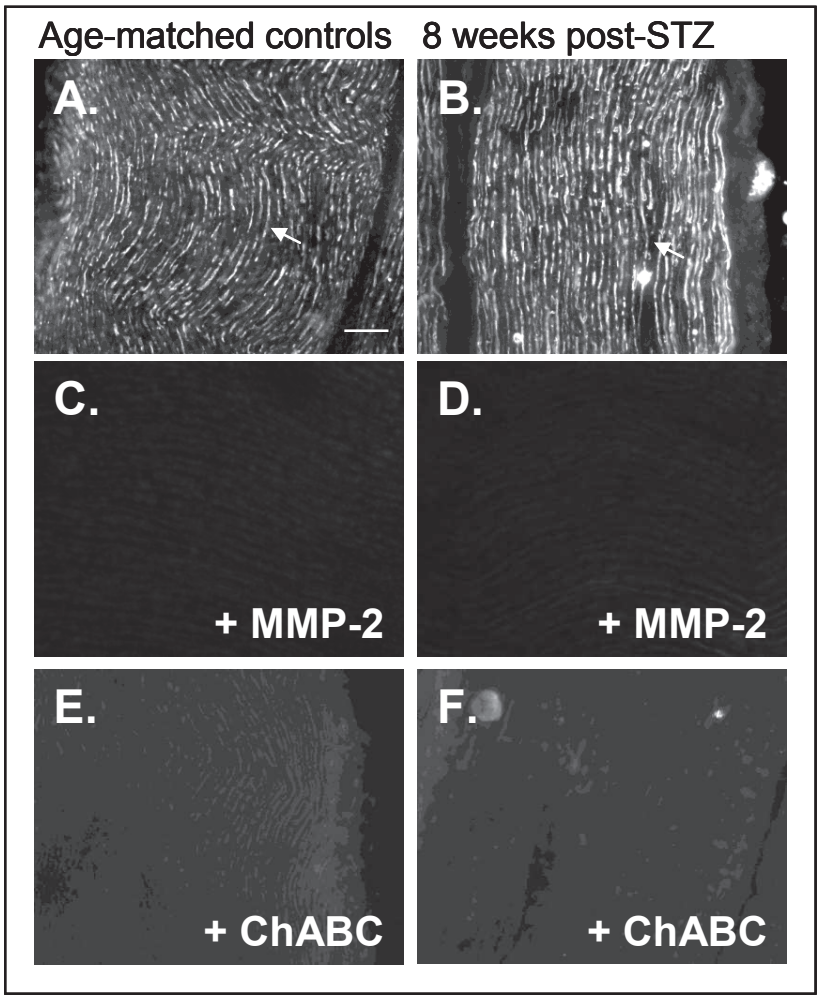


Figure 3.4: **MMP-2 or ChABC effectively degrade inhibitory CSPGs.** Respective micrographs of sciatic nerve cryocultures prepared from age-matched control (A, C & E) and 8 weeks diabetic rats (B, D & F) either pretreated with vehicle (A & B), active MMP-2 (C & D) and ChABC (E & F immunostained with an antibody against CS sidechains of CSPG in the sciatic nerve). Arrows indicate CS-IR association with the basal lamina. The absence of CS-IR indicates that both MMP-2 and ChABC effectively degrade CSPGs. Scale bar = 50μm.

3.4.4. MMP-2 and MMP-9 are secreted by dissociated adult rat sensory neurons

Sensory neurons are a heterogeneous population, which can be categorised according to their neurochemistry, morphology, neurotrophic requirements and sensory modalities into three cell groups (Tucker et al., 2006). The addition of neurotrophins (NGF, neurotrophin (NT)-3 or glial derived neurotrophic factor (GDNF)) to dissociated sensory neurons significantly enhances neurite outgrowth of different subpopulation of sensory neurons *in vitro* (Lewin and Barde, 1996).

Media collected from adult sensory neurons after 18 hour or 48 hour in cultures was assessed for the presence of latent and active forms of MMP-2 and MMP-9 by gelatin-zymography. The adult sensory neurons were cultured in the presence or absence of growth factors (NGF, NT-3 and GDNF), to assess whether MMP secretion is dependent on growth factor treatment/stimulation and whether the different subpopulations of sensory neurons expressed different MMPs in response to growth factor addition. Media collected from 18 hour sensory neurons cultures contained gelatinolytic activities corresponding with proMMP-9 (92kDa) and proMMP-2 (68kDa) (Figure 3.5A, B & D). Media collected from 48 hour sensory neurons cultures showed no alteration in proMMP-9 levels but did show conversion of pro- to active MMP-9 (Figure 3.5B & C), there was also a significant increase in proMMP-2 expression (18 hours 36.6 ± 0.4 vs 48 hours 83.7 ± 3.3 ($n=6$) arbitrary units; *** $p<0.001$; Figure 3.5D). Our data shows that MMP-2 and MMP-9 expression was increased in a time-dependent, growth factor independent manner in adult rat sensory neuron cultures.

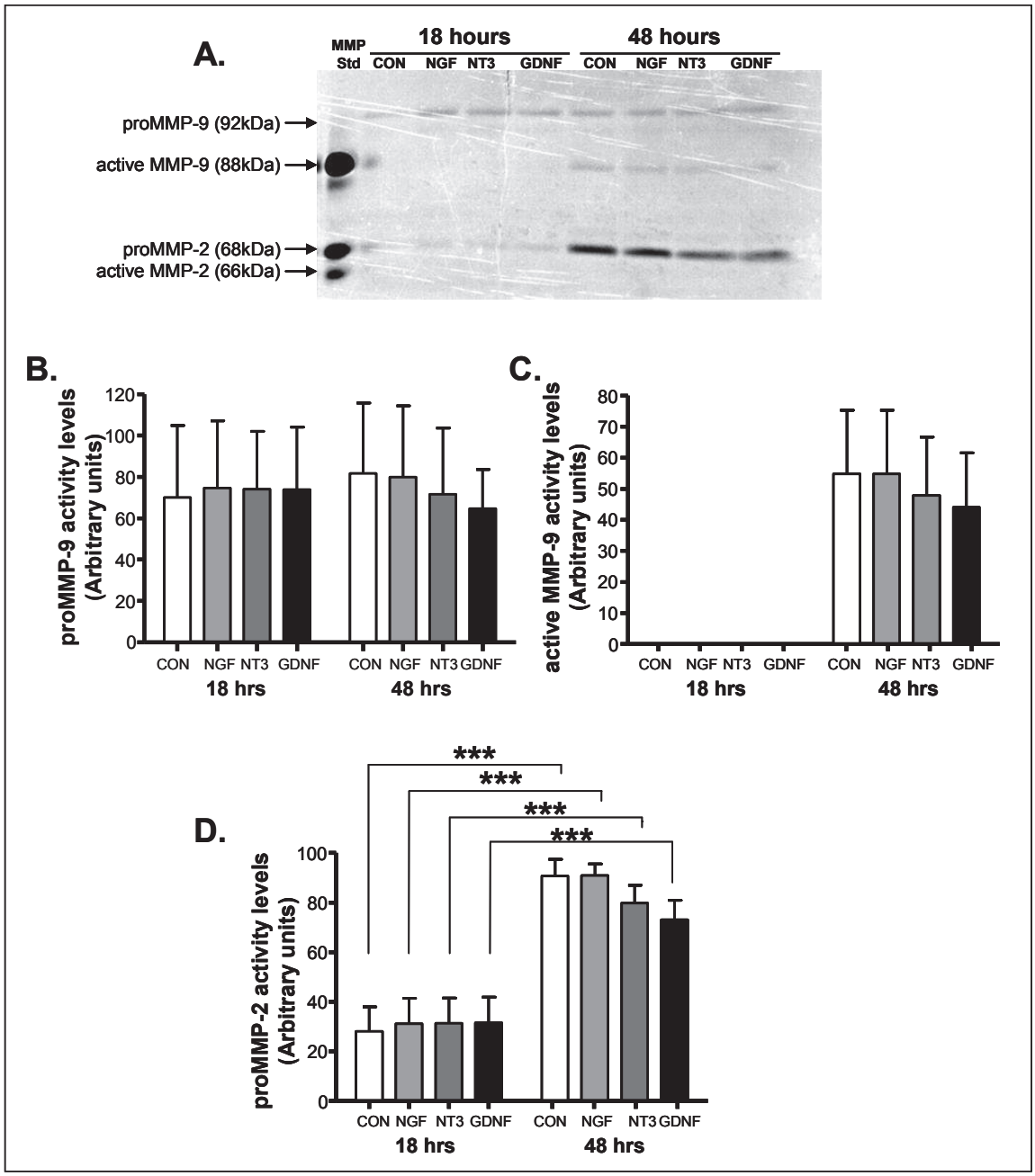


Figure 3.5: **Time-dependent increase in proMMP-2 and active MMP-9 secretion from dissociated sensory neuron cultures.** A) Gelatin zymography gel showing pro- and active MMP-2 and MMP-9 activity levels in the media collected from dissociated neuron culture after 18 or 48 hours incubation in the absence or presence of growth factors (NGF, NT3 and GDNF). B) Densitometric analysis of proMMP-9 activity levels showed an increase in proMMP-9 levels at 48 hours compared to 18 hours, in all treatment groups. C) Densitometric analysis of active levels showed conversion of proMMP-9 to its active form at 48 hours. D) ProMMP-2 activity levels shows a significant increase at 48 hours (n=6; two-way ANOVA with Bonferroni's Multiple Comparison test, *** $p<0.001$).

Immunocytochemical analysis was performed using an antibody which detected both MMP-2 and MMP-9 (Figure 3.6). Since, MMP-2/-9-IR was observed in neuronal cell body (clear arrowheads), neurites (white arrowheads) and in contaminating glial and fibroblasts (white arrows) of 18 hour adult sensory neurons cultures treated with NGF, all these cell types likely contribute to the increase in MMP-2/-9 secretion in culture (Figure 3.6A-C).

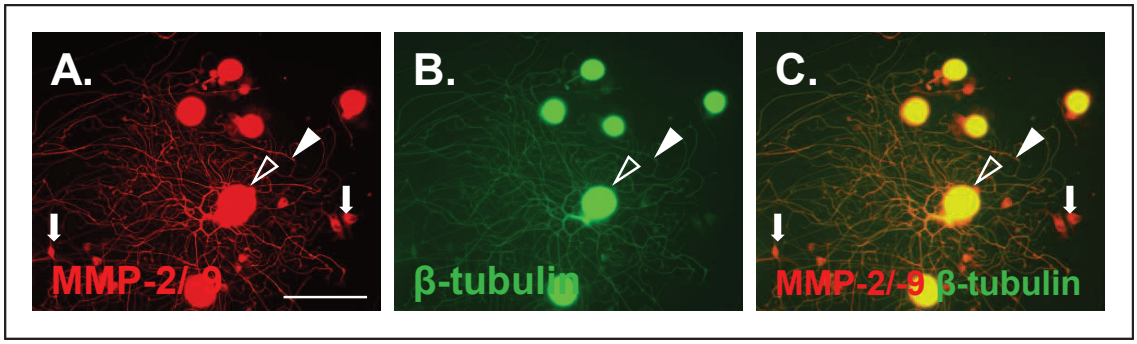


Figure 3.6: **MMP-2 and MMP-9 is expressed by sensory neuron cell body and neurite and contaminating glial cells *in vitro*.** Primary dissociated sensory neurons were plated on laminin and were treated with NGF and grown overnight for 18 hours. Neurite morphology was visualised with A) MMP-2/-9 (Red stain) and B) β-III-tubulin (Green stain) antibodies. C) MMP-2 and -9 staining co-localises with β-tubulin in the cell body and neurite of the primary dissociated neuron cultures. Clear arrowheads indicate neuronal cell bodies, white arrowheads indicate neurites and white arrows indicate contaminating glial and fibroblasts. Scale bar = 50μm.

3.4.5. MMP-2 and MMP-9 do not enhance neurite elongation and sprouting in adult dissociated sensory neurons

Previous work conducted using embryonic rat sensory neurons showed that the addition of general MMP inhibitor does not diminish neurite elongation when plated on planar ECM substrates (Ferguson and Muir, 2000), but does inhibit neurite elongation when plated on 3D matrix including cryoculture and matrigel (Krekoski et al., 2002;Ferguson and Muir, 2000;Zuo et al., 1998a). However, work using the PC12 cell line showed that MMP-9 enhanced NGF-induced neurite elongation when plated on planar PDL (Shubayev and Myers, 2004). In adult sensory neurons, the direct influence that MMP-2 and MMP-9 may have on neurite elongation has not been studied. We used two different dissociated sensory neuron culture systems (a model of neurotrophin-dependent collateral sprouting and a model of axonal regeneration) to assess the role MMP-2 and MMP-9 may play in the modulation of neuronal sprouting and neurite elongation.

The effect of MMP-2 and MMP-9 on adult sensory neuron sprouting and elongation when plated on LM was analysed by studying the effects of treatment with recombinant MMP-2, recombinant MMP-9 or a general MMP inhibitor. We showed a small proportion of sensory neurons extend neurites after 18 hours in the absence of neurotrophic factors, this was not affected by MMP-2, MMP-9 or Ilomostat ($p>0.05$; Figure 3.7A-D, F). The addition of MMP-2, MMP-9 or Ilomastat also had no effect on neurite survival ($p>0.05$; Figure 3.7A-D, E). The addition of recombinant MMP-2, but not MMP-9 resulted in a significant increase in neurite outgrowth (V: 82.3 ± 5.6μm vs MMP-2 (200ng/ml): 159.1 ± 44.9μm (*n=4*), * $p<0.05$, Fig 3.7A-C, G). Ilomostat did not affect basal control growth (Fig3.7A, D, G). The addition of NGF to dissociated sensory neurons induced neurite outgrowth in a discrete population of sensory neurons (Fig 3.8A-G). However, the addition of recombinant MMP-2, recombinant MMP-9 or general MMP inhibitor in combination with NGF did not alter neuronal survival (Fig 3.8E) neurite initiation (Figure 3.8F),or elongation (Figure 3.8G). The efficacy of Ilomostat in inhibiting MMP2 and 9 was confirmed by zymography (Supplementary Figure 3.1).

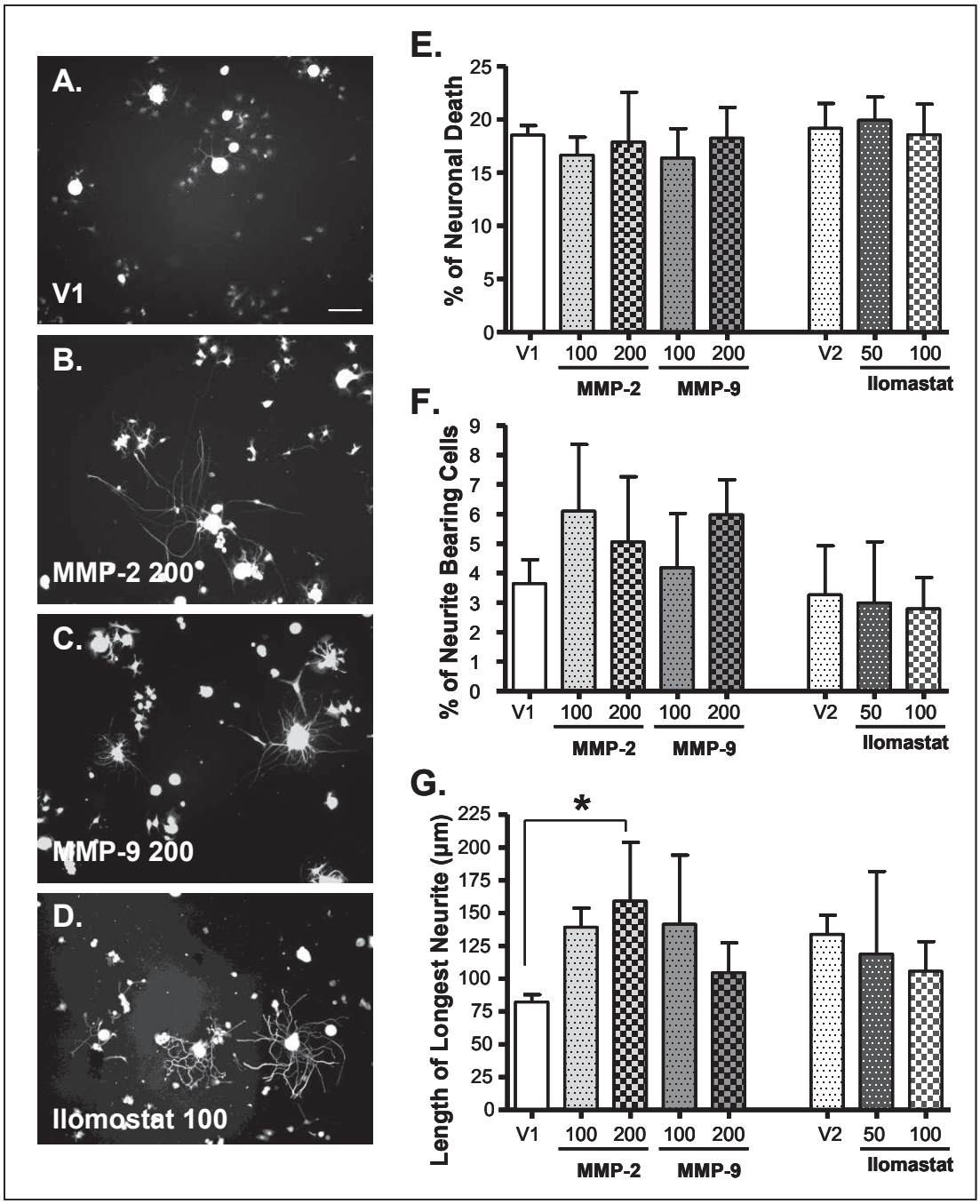


Figure 3.7: **Active MMP-2 treatment significantly increases neurite outgrowth in adult primary sensory neuron cultures.** Primary dissociated sensory neurons plated on LM were treated with active MMP-2 protein (100ng/ml or 200ng/ml), active MMP-9 human (100ng/ml or 200ng/ml) or general MMP inhibitor (Ilomastat; 50µM or 100µM) in the absence of NGF and grown overnight for 18 hours. Representative micrographs of axonal elongation of primary dissociated neurons plated onto LM in the presence of V1 (A), active MMP-2 (B), active MMP-9 (C) or Ilomastat (D). There was no alteration to the percentage of neurite death (E) and percentage of neurite bearing cells (F) following any of the MMP treatments. There was a significant increase in the length of the longest neurite grown on the presence of MMP-2 (200ng/ml), but not MMP-9 or Ilomastat (G) (n=8; one-way ANOVA with Bonferroni's Multiple Comparison test, * $p<0.05$). Scale bar = 50µm.

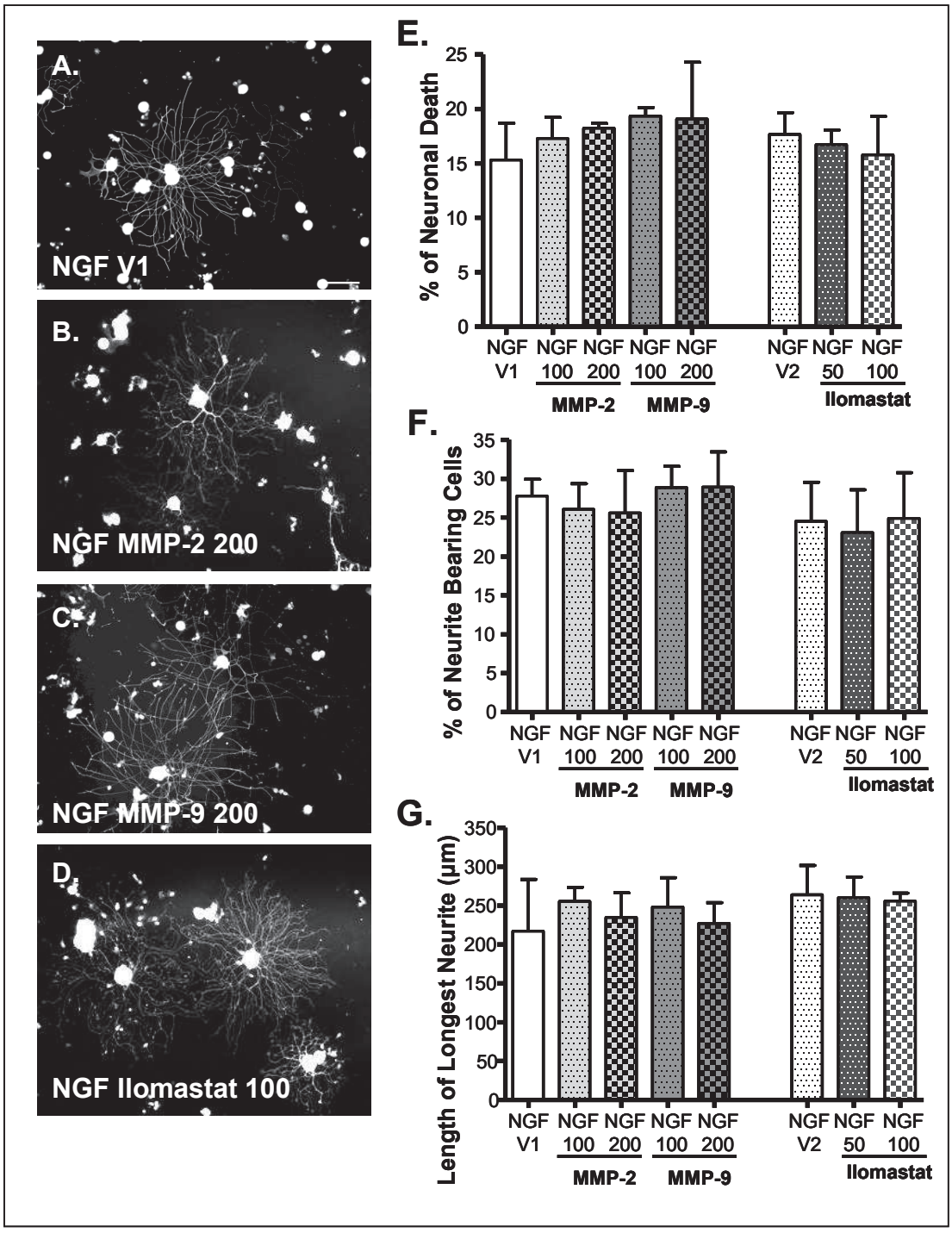


Figure 3.8: **Active MMP-2 and active MMP-9 treatment does not alter NGF-stimulated neurite outgrowth in adult primary neuron cultures.** Primary dissociated sensory neurons plated on LM were treated with active MMP-2 protein (100ng/ml or 200ng/ml), active MMP-9 human (100ng/ml or 200ng/ml) or general MMP inhibitor (Ilomastat; 50µM or 100µM) in the presence of NGF and grown overnight for 18 hours. Representative micrographs of axonal elongation of primary dissociated neurons plated onto LM in the presence of V1 (A), active MMP-2 (B), active MMP-9 (C) or Ilomastat (D), with NGF. There was no alteration to the percentage of neurite death (E) and percentage of neurite bearing cells (F) and length of longest neurite (G) in response to any of the MMP (n=8; one-way ANOVA with Bonferroni's Multiple Comparison test). Scale bar = 50µm.

We then used a culture system in which sensory neurons were preconditioned by an injury to the sciatic nerve *in vivo*, 7 days prior to the culture of sensory neurons from L4/5 DRG ipsilateral to the crush. Robust neurite outgrowth was observed in preconditioned sensory neurons in the absence of neurotrophin factors and this was enhanced further by addition of NGF ((Figure 3.9, 3.10). Similar to results obtained in control and NGF-treated cultures from control rats, addition of recombinant MMP-2 or MMP-9 or Ilomastat did not significantly alter pre-conditioned neurite elongation (Figure 3.9 & 3.10).

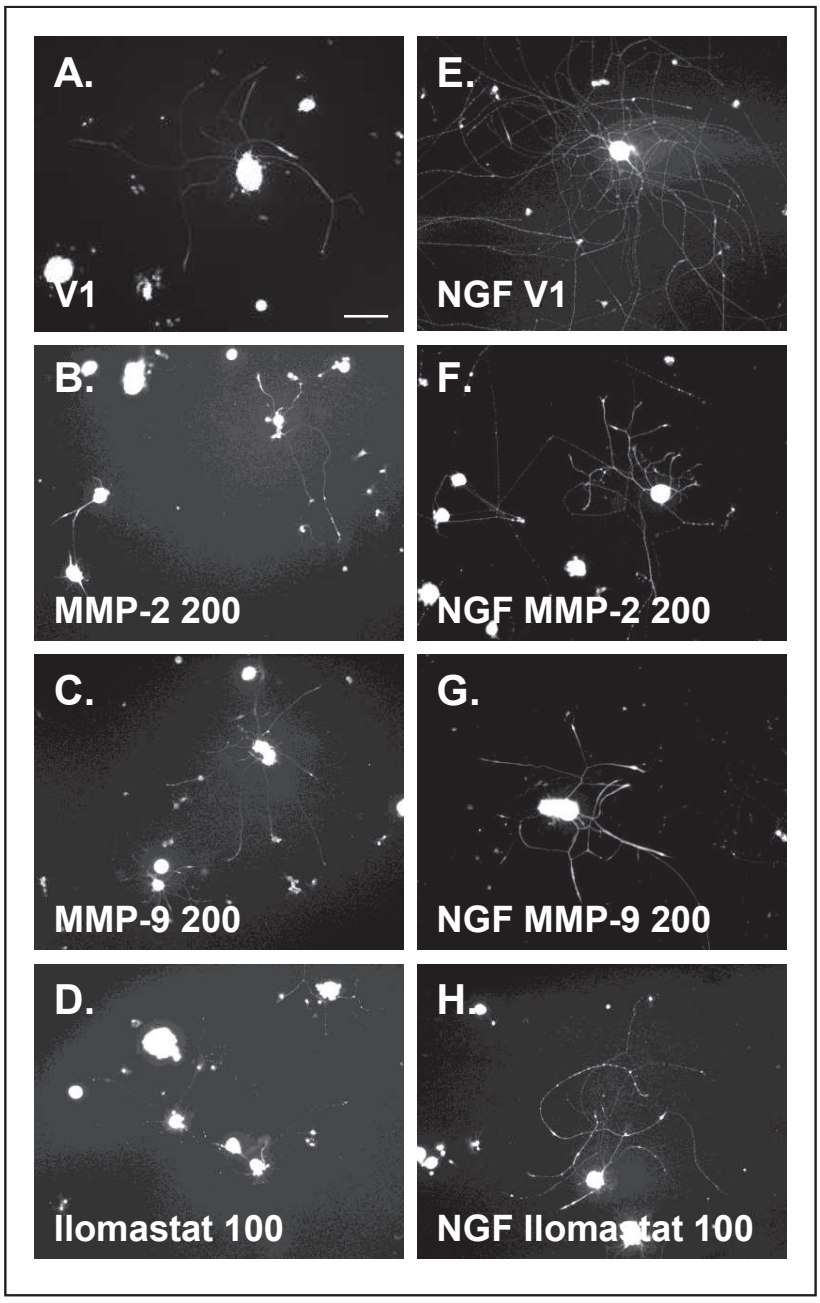


Figure 3.9: **Active MMP-2 and active MMP-9 treatment does not alter neurite outgrowth in pre-conditioned primary neuron cultures.** 7 days after sciatic nerve crush surgery primary dissociated sensory neurons of both the ipsilateral and contralateral (data not shown) were plated on LM, treated with either active MMP-2 (100ng/ml or 200ng/ml), active MMP-9 (100ng/ml or 200ng/ml) or general MMP inhibitor (100ng/ml or 200ng/ml), in the presence or absence of NGF and grown overnight for 18 hours. Representative micrographs of axonal elongation of primary dissociated neurons plated onto LM in the presence of V1 (A, E), active MMP-2 (B, F), active MMP-9 (C, G) or Ilomastat (D, H), in the absence or presence of NGF, respectively.

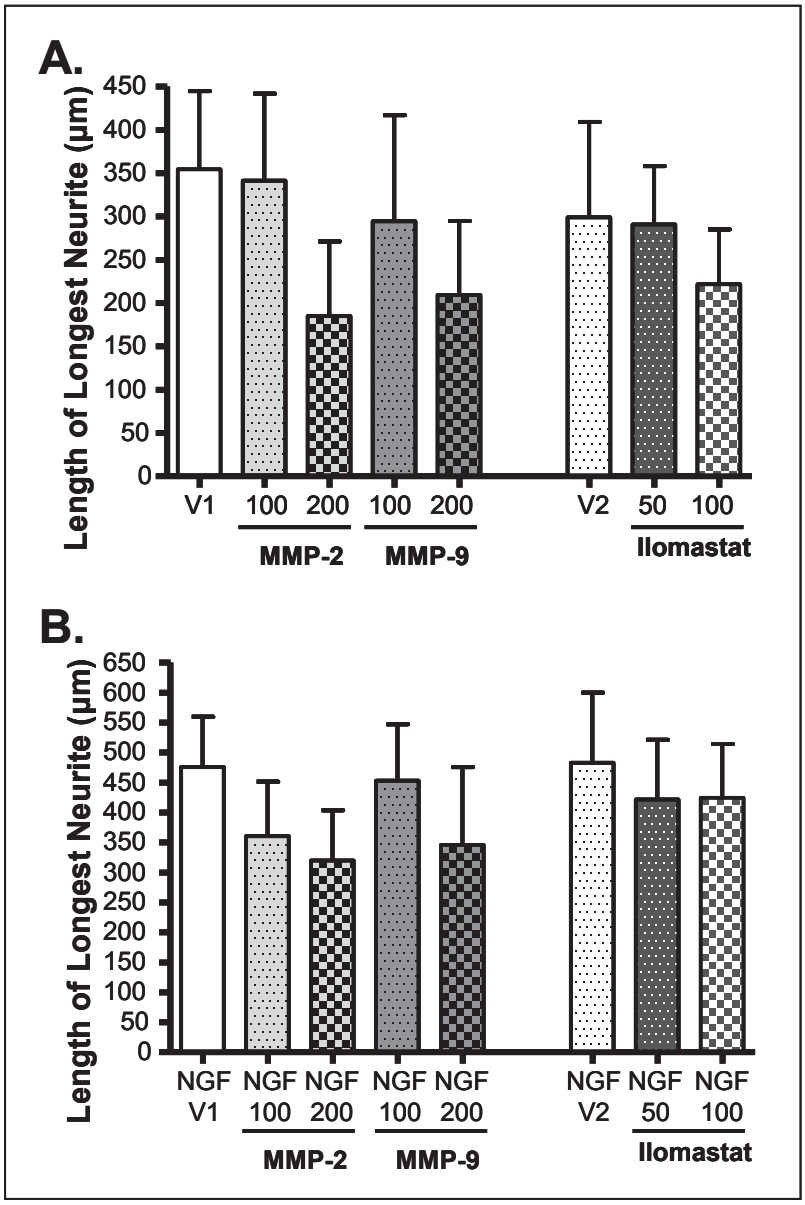


Figure 3.10: **Active MMP-2 and active MMP-9 treatment decreases, but not significantly, neurite outgrowth in pre-conditioned primary neuron cultures.** There was no alteration to the length of longest neurite in the absence of NGF (A) or presence of NGF (B) (n=6; one-way ANOVA with Bonferroni's Multiple Comparison test). Scale bar = 50µm.

3.5. Discussion

The results of the present study show that peripheral nerve ECM from diabetic rats is less supportive of sensory axon elongation compared to nerve from age-matched control rats. Dissociated adult rat sensory neurons which were plated onto sciatic nerve from diabetic rats (8 and 12 weeks post-STZ), extended neurites which were shorter than the neurons plated on age-matched control nerve sections. Both ChABC and active MMP-2 pre-treatment effectively degraded CSPGs, and resulted in partial rescue of diabetes-associated axonal elongation failure. This suggests that altered CSPG expression in the peripheral nerve ECM in diabetes may be a contributing factor to the pathogenesis of diabetic neuropathy. Whilst MMP-2 enhances ECM remodelling of the peripheral nerve in diabetes, it is unlikely to significantly modulate neurite outgrowth in sensory neurons.

Our previous work has shown increased expression of MMP-2 in the sciatic nerve of experimental diabetic rats. To further our understanding of the functional role this increased MMP-2 may be playing in the diabetic peripheral nerve, and whether this expression is beneficial or may contribute to the pathology of the diabetic nerve, we have investigated the role of MMP-2 using a number of *in vitro* culture systems. There is a large body of evidence that shows that MMP-2 remodels the peripheral nerve matrix of control or nerve injured rats, converting it from a non-permissive environment to one favourable to growth (Krekoski et al., 2002;Ferguson and Muir, 2000;Zuo et al., 1998a). Glycation of ECM proteins can render them resistant to proteolysis (Lubec and Pollak, 1980;Lubec et al., 1982;Pollak et al., 1982), and no data existed as to whether MMP-2 can effectively remodel the matrix in the diabetic peripheral nerve. We have now shown that MMP-2 can effectively promote regeneration on sciatic nerve cryocultures obtained from diabetic rats, via removal of inhibitory CSPGs.

We have also demonstrated that whilst MMP-2 and MMP-9 are secreted from primary sensory neuron cultures in a time dependent manner, secretion is not regulated by the addition of neurotrophic factors. These results are in contrast to previous studies in PC12 cells and embryonic chick sensory neurons where MMP-2 and MMP-9 are released in culture in an NGF-dependent manner (Muir, 1994;Shubayev and Myers, 2004). The secretion of other MMPs is also induced by NGF in PC12 cells (Machida et al., 1989).

MMP-2 and MMP-9 secretion could be the result of either the sensory neurons own increased production of MMPs or due to increasing proliferation of contaminating cell types such as microglia, Schwann cells and fibroblasts in the culture, since all these cell types can be seen co-localised with MMP-2/-9-IR in the culture system and indeed have previously been shown to produce MMP-2 and MMP-9 *in vitro* (Chattopadhyay et al., 2007;Chattopadhyay and Shubayev, 2009;Demestre et al., 2004;La et al., 1996;Muir, 1994). The presence of MMP-9 in the culture may reflect the axotomised state of the sensory neurons. We and others (Supplementary Figure 3.2 & 3.3; (Muir, 1994)) have demonstrated, a time-dependent upregulation of both MMP-2 and MMP-9 in ipsilateral L4/5 DRG. MMP expression following nerve crush injury is stimulated by injury-induced expression of cytokines such as tumor necrosis factor-α and interleukin-1β (Chattopadhyay et al., 2007;Shubayev and Myers, 2002;Shubayev et al., 2006), therefore the upregulation of these cytokines in the culture could increase MMP production and secretion.

Using two different in vitro models of sensory nerve regeneration, we determined that the addition of active MMP-2 or MMP-9 protein; or the addition of general MMP inhibitor (Ilomastat) to NGF-treated sensory neurons, does not influence the rate of neurite outgrowth of adult sensory neurons plated onto LM. Whilst, we did see a significant increase in the length of longest neurite of sensory neurons treated with active MMP-2. this response was seen in such a low population of sensory neurons the biological significance of this finding is uncertain. Similarly, we demonstrated that in preconditioned cultures, the presence of MMPs does not influence the rate of neurite outgrowth of adult sensory cultures plated onto LM. This is in contrast to previous work in PC12 cells where NGF-induced neurite elongation was enhanced by the addition of MMP-9, whilst MMP inhibition by treatment with either MMP-9 neutralising antibody or broad-spectrum MMP inhibitor reduced the degree of sprouting to the levels of non-NGF treated cells (Shubayev and Myers, 2004). However, our result is supported by work by the Muir laboratories, who found that the addition of spIMP (a synthetic peptides containing the conserved sequence corresponding to the prodomain of MMPs which inhibits MMP-2), to culture media has no significant effect on neurite outgrowth from embryonic chick sensory neuron and PC12 neurons plated on extracted extracellular matrix (EEM) (Muir, 1994).

Treatment of embryonic DRG neurons and PC12 cells with spIMP did attenuate NGF-induced neurite penetration into 3D-EEM (Muir, 1994). In this study, the matrix remodelling properties of the embryonic chick sensory neurons were demonstrated to be solely the result of increased MMP-2, as MMP-9 was absent in this culture system (Muir, 1994). This role of MMP-2 in remodelling of the native peripheral nerve was further investigated using embryonic mouse sensory neurons grown on adult rat sciatic nerve section, this was termed cryocultures. Pretreatment of the sciatic nerve with MMP-2 resulted in the NGF-treated embryonic sensory neurons extending significantly longer neurites compared to untreated nerve (Krekoski et al., 2002;Zuo et al., 1998a;Krekoski et al., 2001;Zuo et al., 1998a;Zuo et al., 1998b). This enhanced neurite outgrowth was linked to the degradation and inactivation of the neurite-inhibiting CSPG (Krekoski et al., 2002;Zuo et al., 1998a;Krekoski et al., 2001;Zuo et al., 1998a;Zuo et al., 1998b). Previous work has shown that CSPG associated with the basal lamina is a principle inhibitor of peripheral nerve regeneration (Ferguson and Muir, 2000;Bedi et al., 1992). The increase in neurite outgrowth following MMP-2 was blocked by anti-LM antibodies, showing that the removal of CSPG resulted in the exposure of neurite-promoting LM on the Schwann cell basal lamina (Zuo et al., 1998a).

In our study using a similar sciatic nerve cryoculture system as that described by the Muir laboratory we observed no differences in the neurite-promoting properties of control nerve pre-treated with either ChABC or MMP-2, compared to vehicle-treated nerve, despite the removal of inhibitory CSPGs. Pre-treatment of sciatic nerve from 8 weeks diabetic rats with either ChABC or active MMP-2 is able to promote neurite outgrowth, and significantly rescue this diabetes-induced regeneration failure. A similar trend towards partial rescue was observed following pre-treatment of sciatic nerve from 12 weeks diabetic rats. However, high variability in one set of experiments resulted in these data being insignificant.

In our current study, nerve cryocultures were prepared with additional freeze-thaw cycles to fracture/remove cells and expose ECM (Ide et al., 1983;Gulati, 1988) rather than on whole nerve sections as per the Muir studies(Chattopadhyay et al., 2007;Ferguson and Muir, 2000;Krekoski et al., 2001;Zuo et al., 1998a). We have shown a time-dependent increase in MMP-2 and MMP-9 secretion in our cultures which may be sufficient for local removal

of CSPGs in the vehicle-treated sections near the growing neurites over the 48 hour period of incubation, therefore by 48 hours no differences can be detected. In support of this hypothesis, we have preliminary evidence that after a shorter period in culture (24 hours; Supplementary Figure 3.4), neurons have longer neurites on the pretreated sections than the vehicle treated sections. However, this poses the question as to why we do not see similar time-dependent localised degradation of CSPG by the adult sensory neurons when plated onto cryoculture sections from diabetic rats. Zymography of media may indicate matrix-dependent differences in increased MMP expression and secretion depending on whether sensory neurons are plated on control or diabetic sciatic nerve sections. Indeed, recent microarray analysis of gene expression in sensory neurons plated on control or glycated laminin provide evidence that gene expression is significantly altered on glycated ECM (Ali et al., 2010). In addition, we suggest that presence of other growth-inhibitory factors such as COLL-IV and/or AGEs in the endoneurial ECM may contribute to reduced axonal regeneration.

In diabetes, the peripheral nerve undergoes a number of structural changes, including the reduplication of the basement membrane as a result of the accumulation of COLL type-IV and V in the perineurium and COLL type-I and III in the endoneurial ECM of the diabetic nerve (Bradley et al. 539-46;Giannini and Dyck 498-504). The increase in COLL-IV appears to be associated with basal laminae of Schwann cells in the endoneurium, however, the functional significant is uncertain (Bradley et al., 2000). However, work in our laboratory has shown that COLL type-IV is not supportive to neurite outgrowth (Gardiner., 2010; Personal communication). Another structural alteration to the basement membrane of organs associated with diabetic complications is the accumulation of inhibitory factors such as CSPG and heparin sulphate proteoglycans (HSPG). In diabetic nephropathy, there is an increase in glomeruli CSPG staining, localised to the pericapillaries basement membrane, with a decrease in HSPG levels (McCarthy et al., 1994). This alteration in the proteoglycans (PG) biosynthesis appears to affect the structural/functional dynamics of the glomeruli basement membrane that may directly contribute to the eventual renal dysfunction (McCarthy et al., 1994). This alteration in PG biosynthesis occurs in glomerular basement membrane and in the non-vascular basement membrane of the skin of type 1 diabetic patients with nephropathy (van der Pijl et al., 1998). CSPG is a potent inhibitor of axon regeneration within the peripheral nervous system and CNS (Snow et al.,

1990;Smith-Thomas et al., 1995). Schwann cells show a 7-fold increase in CSPG levels following sciatic nerve crush , which restrict neurite elongation (Zuo et al., 1998b;Zuo et al., 1998a). Inhibition of growth cone elongation is the result of CSPGs masking of LM (Zuo et al., 1998a). Increased levels of CSPGs are observed in the skin of diabetic patients (van der Pijl et al., 1998), which could be a contributing factor towards the failure of collateral sprouting and axonal regeneration seen in both clinical and experimental diabetic neuropathy, and will be investigated in future studies.

In vitro studies have shown that HSPG has a slight inhibitory effect on adult and embryonic sensory neurons (Tonge et al., 1997). Similar to CSPG, HSPG bind to LM thereby masking the growth-promoting effects of LM and inhibiting axonal regeneration (Tonge et al., 1997). Therefore in the sciatic nerve from diabetic rats, even after the unmasking of LM via the cleavage of inhibitory CSPGs by either active MMP-2 or ChABC, it is unknown if increased levels of other inhibitory ECM proteins, such as COLL-IV or HSPG, are high enough to inhibit neurite elongation.

Non-enzymatic glycosylation (glycation) of ECM proteins occurs as early as 3 weeks post-STZ (Duran-Jimenez et al., 2009). Previously in our laboratory, we have demonstrated reduced neurite outgrowth from adult rat sensory neurons plated on glycated LM or fibronectin (FN) 2D matrix compared to unmodified matrix (Duran-Jimenez et al., 2009). Whilst other studies have also shown reduced attachment and neuritogenesis in neonatal sensory neurons (Luo et al., 2002) and neurite extension from mouse DRG explants (Ozturk et al., 2006) plated onto glycated LM. These data suggest that AGE accumulation in the endoneurial ECM may contribute to the progressive failure of axonal regeneration in diabetic neuropathy via blocking axonal attachment to growth promoting ECM protein, LM and FN. Therefore, even if the sensory neurons plated onto cryoculture sections taken from diabetic rats are able to locally degrade inhibitory CSPG via MMP-2 secretion, it is not known if the elongating neurites attach to the unmasked LM due to possible presence of AGEs on LM. This could explain the partial rescue we observed following either active MMP-2 or ChABC pre-treatment.

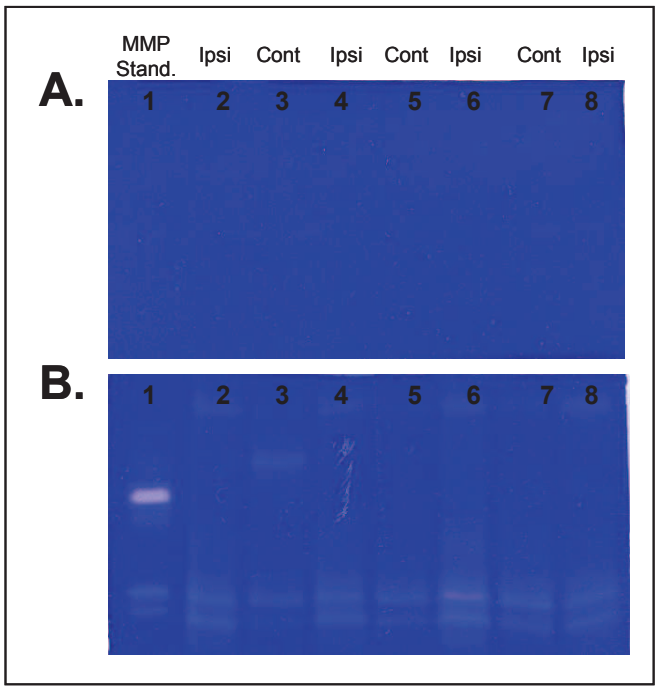
Other recent data from our laboratory have implicated altered gene expression as a contributing factor to glycation-induced reduction of sensory neuron outgrowth. Over 500

genes for which expression is significantly altered in NGF-treated adult sensory neurons plated on methylglyoxal (MG)-glycated LM compared to unmodified LM have been identified in a microarray study (Ali et al., 2010). LM is only one of many ECM proteins that could be glycated as a result of diabetes. This suggests that the expression of many more genes may be altered in clinical and experimental diabetes. Alteration of neuronal phenotype is thought to be the result of altered cell adhesion as previously demonstrated on glycated FN and COLL type-IV with MG and therefore compromised cell signalling. Previous studies showed that glycation of RGD domain resulting in the inhibition of αvβ3 integrin and endothelial cell adhesion when plated onto MG-glycated COLL type-IV (Dobler et al., 2006;Pedchenko et al., 2005). A similar mechanism has been suggested in the impaired adhesion of smooth muscle cells and endothelial cells plated onto MG-glycated FN (Bhatwadekar et al., 2008;McDonald et al., 2009) as α5β1 integrin is important in neurite outgrowth of preconditioned neurons (Gardiner et al., 2007).

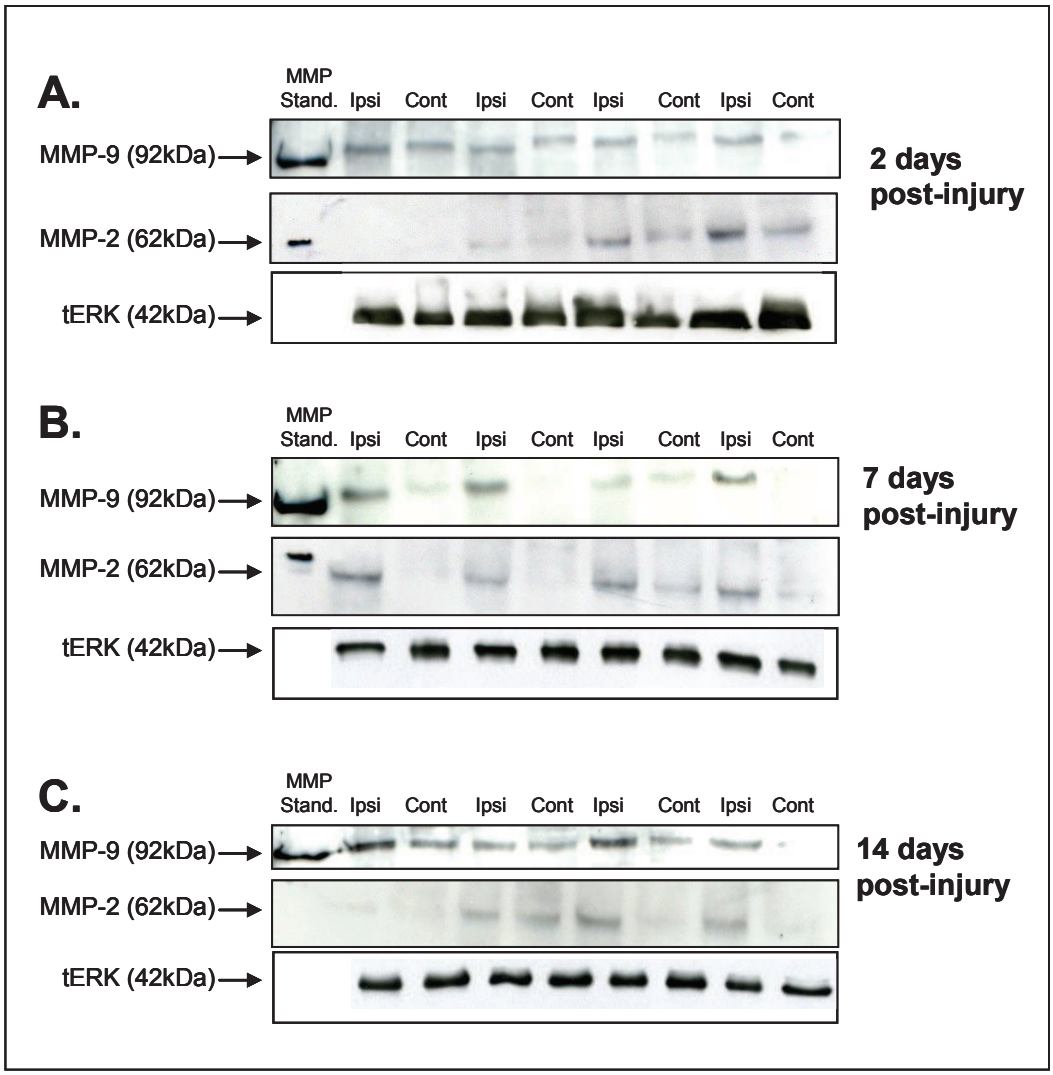
3.5.1. Conclusion

The main conclusion is that diabetic-induced alteration to the ECM of the peripheral nerve in diabetic neuropathy, results in axonal regeneration failure. We have shown that inhibitory CSPG plays a role in this diabetic-induced axonal regeneration failure. However, as only partial recovery was achieved, other factors must also be involved in this, rendering the peripheral nerve of experimentally diabetic rats less supporting to axonal elongation. Therefore, whilst this study identifies a potential therapeutic strategy in promoting axon regeneration in diabetic neuropathy, it also raises important mechanistic questions which need to be investigated further.

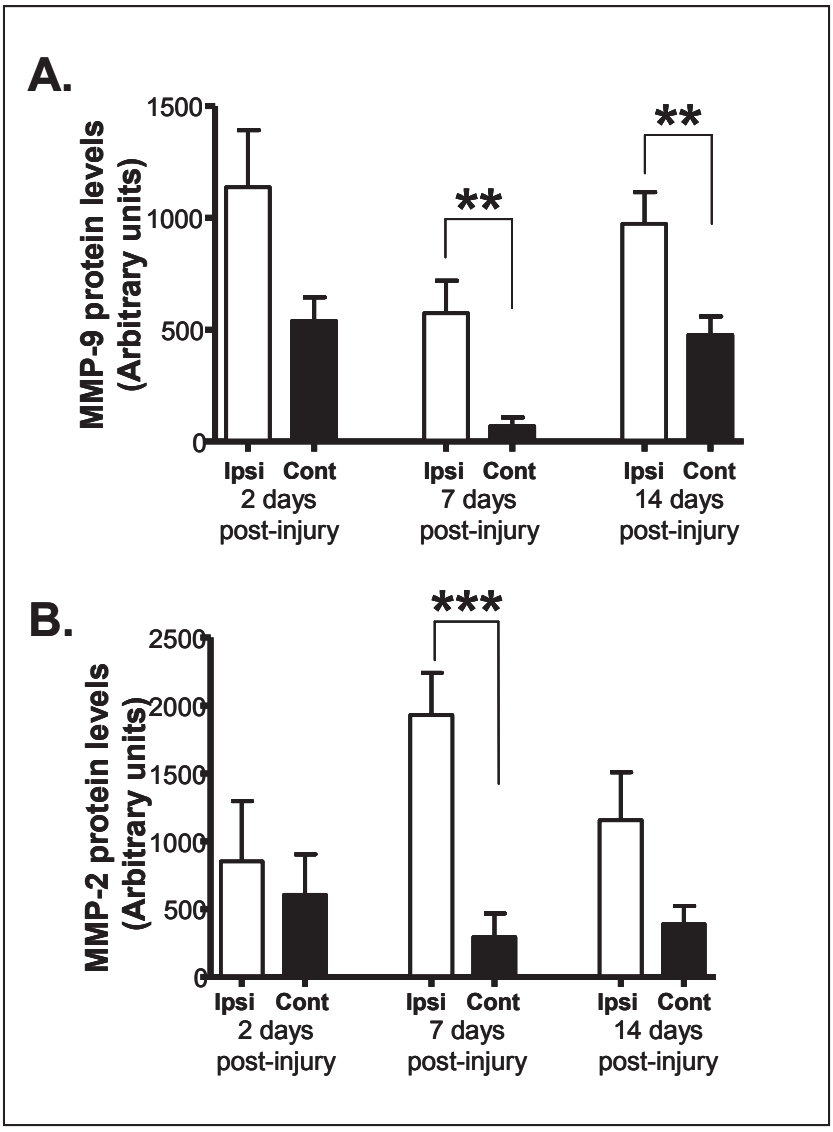
3.6. Supplementary data



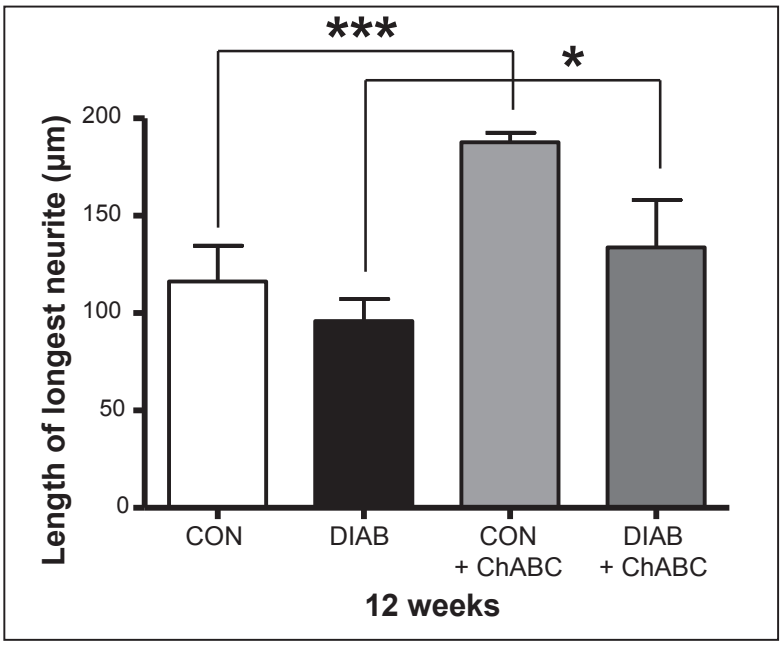
Supplementary Figure 3.1: **Ilomastat inhibits MMP-2 and MMP-9 when added to gelatin zymography gels.** Gelatin zymography showing the ability of general MMP inhibitor (1mM Ilomastat) to inhibit MMP-2 and MMP-9 expression in samples prepared from sciatic nerves ipsilateral or contralateral to nerve crush? Two identical gelatin zymography gels were incubated in identical incubation buffer either (A) in the presence of Ilomastat or (B) without Ilomastat.



Supplementary Figure 3.2: **Significant upregulation of MMP-2 and -9 protein levels in the sciatic nerve following nerve crush injury.** Western blots showing upregulation of MMP-2 and MMP-9 protein expression at 2 (A), 7 (B) and 14 days (C) following sciatic nerve crush injury (Ipsilateral (Ipsi)) compared to non-injured (contralateral (Cont)) side. Each ipsi and control pair is from an individual animal. Total ERK (tERK) is used a loading control.



Supplementary Figure 3.3: **MMP-2 and -9 protein expression is significantly upregulated in the sciatic nerve following nerve crush injury.** (A) Significant upregulation of MMP-9 in the ipsi sciatic nerve 7 and 14 days post-injury. (B) Significant upregulation of MMP-2 in the ipsi sciatic nerve 7 days post-injury. (n=4; unpaired t-test; ** $p<0.01$, *** $p<0.001$).



Supplementary Figure 3.4: **Enhanced neurite elongation on control sciatic nerve by pre-treatment with Chondroitinase ABC.** Prior to plating of the primary dissociated sensory neurons, sciatic nerve sections were pre-treated with Chondroitinase ABC (0.1U/ml for 3 hours) or vehicle, and grown for 24 hours in the presence of NGF (10mg/ml). There was no alteration in the length of the longest neurite grown on 12 week diabetic sciatic nerve compared to age-matched control. Neurite outgrowth is enhanced by pretreatment with ChABC of age-matched control and 12 week diabetic (n=4; all treatment groups); one-way ANOVA with Bonferroni's Multiple Comparison test; *** $p<0.001$, *$p<0.05$).

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Neuroprotective effects of the advanced glycation end-product inhibitors LR90 and pyridoxamine in experimental diabetes.

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4.1. Abstract

Aims/hypothesis: The aim of this study was to assess the neuroprotective effects of the advanced glycation end-products (AGE) inhibitors LR90 and pyridoxamine (PM) in experimental diabetic neuropathy.

Methods: Diabetes was induced in adult male Wistar rats using streptozotocin (STZ; 55mg/kg intraperitoneal). Diabetic and age-matched control rats were randomly allocated to untreated, or treated with LR90 or PM, groups and maintained for up to 12 weeks post-STZ. Tactile allodynia, sensory and motor nerve conduction velocity (NCV) and biochemical markers of oxidative stress, glycation and hyperlipidemia were assessed. Sciatic nerve cryocultures from age-matched control , untreated and treated STZ-rats were used to assess the ability of the peripheral nerve extracellular matrix (ECM) to support neurite outgrowth from dissociated adult rat sensory neurons.

Results: Untreated STZ-diabetic rats presented with hyperglycaemia, hyperlipidemia, slowed NCV, tactile allodynia, mechanical hyposensitivity and sciatic nerve cryocultures were less supportive for sensory neurite outgrowth. Sensory NCV, oxidative stress and regenerative deficits in diabetic rat sciatic nerve were prevented by treatment with either LR90 or PM. PM significantly reduced plasma hyperlipidemia in diabetic rats. Neither AGE inhibitor protected against the development of tactile allodynia or mechanical hyposensitivity in STZ-diabetic rats.

Conclusions/interpretation: Both AGE inhibitors are effective at preventing the development of some peripheral nerve dysfunctions in diabetes such as slowing of sensory NCV and the reduced supportive nature of peripheral nerve to regenerating axons, but at the doses tested were unable to prevent altered mechanical sensitivity. These agents have potential for the therapeutic treatment of diabetic neuropathy.

Keywords: Advanced glycation end-products (AGE), AGE inhibitors, advanced lipoxidation end-products (ALE), streptozotocin (STZ), diabetic neuropathy, lipid peroxidation, extracellular matrix (ECM), glycation.

4.2. Introduction

Peripheral neuropathy is a major complication of diabetes mellitus, affecting autonomic, sensory and motor nerve fibres in both human and animal models. Diabetic neuropathy is characterised by degenerative changes including axonopathy, segmental demyelination, and Schwann cell abnormities, and the progressive loss of sensation. The pathogenesis of diabetic neuropathy is complex and multifactorial; including increased reactive oxygen species (ROS) levels (1-3), increased flux through the polyol pathway (4;5), accumulation of advanced glycation end-products (AGEs) (6;7) and altered intracellular signalling in the peripheral nerve (8-10). This leads to development of functional deficits including reduced nerve conduction velocity (11;12), abnormalities of axonal transport (13-17), reduced capacity of nerve fibres to regenerate and the development of neuropathic pain (18-21).

Multiple experimental and clinical trials have been conducted on compounds designed to reduce or prevent these biochemical abnormalities including aldose reductase inhibitors (ARIs) which prevent polyol flux; antioxidants such as α-lipoic acid; transition metal chelating agents such as trientine, PKC-β inhibitors and p38 inhibitors (9;22-28). Although many of these compounds provided promising results in experimental models of diabetic neuropathy, there are very few treatments available clinically, with only Epalrestat, an ARI, licensed for clinical use, and only in Japan. The anti-oxidant, Fidarestat, is currently in phase III clinical trials. AGE inhibitors such as aminoguanidine (AG), pyridoxamine (PM), thiamine and LR90 have provided promising results in the treatment of diabetes (29;30-35).

Non-enzymatic glycation of proteins involves the covalent linkage of saccharides and saccharide derivatives to proteins. Glucose reacts with amino groups of lysine and N-terminal amino acid residues. Early stage reactions lead to the formation of fructosyl-lysine (FL) and related fructosamine residues, which degrade slowly to form stable AGEs (36;37). In addition, potent glycating agents such as glyoxal and methlyglyoxal (MG) react with proteins to form AGE residues directly and relatively rapidly. These highly reactive dicarbonyl compounds are glycating agents formed by the degradation of glycated protein, glycolytic intermediates and lipid peroxidation (38). Extracellular matrix (ECM) proteins are particularly long-lived and they are, therefore, potential targets for glycation. Glycation of ECM proteins result in morphological changes to the

basement membrane including structural distortion, altered cellular behaviour, receptor-mediated cell adhesion and confers resistance to proteolysis and subsequent ECM remodelling; via modification of protein-protein interactions, production of intermolecular cross-linking and activation of cellular pathways (3;39;40). We have previously shown that AGE residues accumulate in both intracellular and extracellular proteins of the peripheral nerve of STZ-induced diabetic rats as early as 3 weeks post-induction of diabetes (41;42). In addition, we and others, have shown that glycation of ECM components such as laminin (LM) and fibronectin (FN) dramatically impairs neuronal outgrowth and regeneration *in vitro* (41;43;44).

AG, the prototypic AGE inhibitor, has marked effects on peripheral neurovascular function including correction of sciatic nerve blood flow deficits, improved nerve conduction velocity and morphometry (34;45;45-47) thereby confirming AGE accumulation as a major factor in the pathogenesis of diabetic neuropathy. Other AGE inhibitors, such as PM, pyridoxine (48-51) and pyridoxal (intermediates of vitamin B_6 metabolism) (52), thiamine (vitamin B_1) and benfotioamine (a derivative of thiamine) (53;54); and LR90 an aromatic compound (31;55;56), are effective AGE/ advance lipoxidation end-product (ALE) inhibitors and are protective in experimental diabetic retinopathy (57;58) and nephropathy (59-61). Data presented in abstract form indicate that PM also has marked effects on peripheral neurovascular function including correction of sciatic nerve blood flow, nerve conduction velocity and thermal hyperalgesia (45). The protective efficacy of LR90 has not been tested, to date, in diabetic neuropathy.

In this study, we investigated the neuroprotective effects of PM and LR90 in the rat STZ-induced model of diabetic neuropathy by assessing behavioral, electrophysiological and biochemical endpoints. We also investigated whether neurite outgrowth from sensory neurons is impaired in peripheral nerve from STZ-diabetic rats, and if so, whether treatment with AGE inhibitors can prevent regenerative deficits. Treatment with PM or LR90 provided a degree of neuroprotection in experimental diabetic neuropathy.

4.3. Methodology

4.3.1. Animals and surgeries

All experiments were conducted in accordance with the UK Animals Act (Scientific Procedures 1986) and institutional regulations. Adult male Wistar rats (Charles River, UK) with a starting weight of 250-300g were given a single intraperitoneal injection of STZ (Sigma, UK, Cat No. S0130) at dose of 55mg/kg in 0.9% NaCl, administered the morning after an overnight fast. Three days later, blood glucose levels were monitored using a strip-operated reflector photometer (MediSense Optimum Plus; MediSense, UK). Animals with blood glucose less than 15mmol/l were rejected from the study. Age-matched rats were used as non-diabetic controls.

Age-matched control and diabetic rats were randomly allocated to treatment groups; untreated control (*n=15*), LR90 treated control (*n=6*; 100mg/L), PM treated control (*n=6*; 800mg/L), untreated diabetic (*n=9*), LR90 treated diabetic (*n=8*; 50mg/ml and PM treated diabetic (*n=7*; 400mg/L). LR90 and PM were administered in the drinking water and prepared fresh each day. The higher doses administered to the treated control groups were designed to compensate for the lower water intake of control rats compared to diabetic rats as per previous studies (31;55;56;62). All rats were provided with standard rodent chow (Beekay International), and housed at a constant temperature of 21°C with 12:12 hour light/dark cycle. Animals were weighed and checked biweekly, and maintained for 8 or 12 weeks post-STZ.

4.3.2. Behavioural assessment

The week before and 2, 4, 8 and 12 weeks post STZ-induction of diabetes behavioural testing was conducted. The animals were randomized for testing by using the animal identification number and a random number generator with no repeats (www.stattrek.com/Tables/Random.aspx) and the tester was blinded to the treatment groups. However, due to the obvious emaciation of the diabetic animals, it was not possible for the tester to be blinded to control and diabetic groups.

Von frey testing (Section 4.3.2.1) was conducted over 2 consecutive days. The following day, mechanical (Section 4.3.2.2) and thermal (Section 4.3.2.3) nociceptive

thresholds were measured and repeated the following day. Mechanical sensitivity was measured prior to thermal testing with at least 3 hours between the different tests.

4.3.2.1. Von frey

Von frey filaments (Stoelting, Wood Dale, IL, USA) were used to determine the 50% paw withdrawal threshold to a non-noxious mechanical stimulus, using the up-down method (63;64). Rats were placed in a plastic cage with a wire mesh bottom which allowed full access to the paws, and were left to acclimate for approximately 15 min, or until cage exploration and major grooming activities ceased. The hindpaw was touched with the first of a series of 8 Von frey hairs with logarithmic incremental stiffness (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g) starting with one that possessed a buckling weight of 2.00g. The filaments were applied in to the mid-plantar surface of the hindpaw with a pressure that caused the filament to buckle. The filament was held for approximately 6 seconds or until a positive response was recorded. A positive response was noted if the paw was sharply withdrawn. Flinching immediately upon removal of the hair was also considered a positive response, whilst moving about was considered a neutral response and the same filament reapplied. In the absence of a paw withdrawal response the next strongest stimulus was presented. In the event of paw withdrawal, the next weaker stimulus was chosen. This paradigm was continued until four measurements had been made after an initial change in the response or until five consecutive negative (given the score of 15 g) or four positive (score of 0.25 g) scores had occurred. Stimuli were presented at intervals of several seconds to allow animals to re-acclimate following previous stimuli. The cut-off of a 15.10 g hair was selected as the upper limit for testing, since stiffer hairs tended to raise the entire limb rather than to buckle. Data were recorded for each animal as median of all measurements recorded in both the left and right hindlimb over 2 days. In addition, groups means ± standard deviation (normalised to control group response) were also calculated.

4.3.2.2. Dynamic aesthesiometer latency withdrawal

Behavioural responses of rats to mechanical stimuli were also evaluated using a dynamic plantar aesthesiometer (37400 Ugo Basile, Milan, Italy). Animals were placed in an elevated wire mesh bottomed chamber allowing access to the underside of their paws, and allowed to acclimatise for approximately 15 minutes prior to measurements.

The aesthesiometer exerted an increasing mechanical force, by raising a single non-flexible filament (0.5mm diameter) at a force of 50g over a 15 second ramp on the plantar surface of the rat's hind paw, until either the paw was withdrawn or the cut-off force was reached. The nociceptive threshold is the latency prior to a withdrawal response by the animal. Paw withdrawal force was automatically recorded, and data was reported as means of the 3 consecutive measures in left and right hindpaws over 2 consecutive days. Groups means ± standard deviation (normalised to baseline response) were also calculated.

4.3.2.3. Hargreaves plantar test

The Hargreaves plantar test was used to measure the paw withdrawal latency to a noxious thermal stimulus. Animals were placed in an elevated transparent perspex box and the plantar surface of the hindpaw was exposed to a beam of radiant heat (set at 50 units, Ugo Basile Model 7370 Plantar Test). Paw withdrawal stopped the heat and the withdrawal latency was recorded to the nearest 0.1 s. An automated cut-off of 25s was applied in the absence of a response, to prevent tissue damage. Data were reported as means of the 3 consecutive measures in left and right hindpaws over 2 consecutive days. Groups means ± standard deviation (normalised to baseline response) were also calculated.

4.3.3. Nerve conduction velocity measurements

Motor NCV and sensory NCVs were measured at the end of the trials (8 and 12 weeks post-STZ). Rats were anesthetised with isofluorane (2% in oxygen). The sciatic nerve was stimulated via insertion of fine percutaneous needles electrodes firstly at the sciatic notch and then at the Achilles tendon. Stimulation, comprising 0.1ms pulses of varying amplitude was delivered using Neurolog stimulus isolator, a pulse generator and an AC/DC amplifier. Evoked electromyograms from the interosseous foot muscles were recorded using ABI Scope version 3.6.8 for Powerlab 4 software. Motor NCVs were measured from latencies of compound M waves, and sensory NCVs were measured from H reflex latencies from the same stimulation sites. H reflexes were considered genuine if they appeared at a lower stimulus voltage than M waves. Nerve length from sciatic notch to Achilles tendon was measured *ex vivo*. The latency difference between the two sets of H reflexes was calculated and related to the nerve length separating the

two stimulus points to calculate sensory NCV. M wave latency differences were used similarly to calculate motor NCV. Group means ± standard deviation were calculated

After NCVs were measured, rats were killed by exsanguination from the right ventricle and blood was collected directly from the heart into Labtek tubes containing Lithium Heparin (Teklab, UK, Cat No. H1137) and centrifuged at 4°C for 20 minutes at 2,500g. The collected plasma was stored at -40°C. The sciatic nerve and L4-L5 DRG were removed, snap-frozen on dry-ice and stored at -80°C.

4.3.4. Cryoculture

Cryoculture is a neurite outgrowth assay in which neurons are grown on a substratum of unfixed peripheral nerve section. Fresh nerve segments (1cm) from at least 5 separate animals per treatment group were repeatedly freeze-thawed to fracture cellular membrane and expose the ECM. The sciatic nerves were embedded in OCT before being cryosectioned (14µm longitudinal sections) and mounted onto acid washed, sterile, poly-D-lysine (PDL; 1mg/ml: Sigma, Cat No. P7280)-coated coverslips.

Adult male Wistar rats were killed by concussion followed by decapitation. The dorsal root ganglia (DRG) were removed, cleaned of connective tissue and chemically dissociated by incubation in 0.125% collagenase (Worthington Biochemicals, UK, Cat No. LS004188) in Ham's F12 media (Gibco, UK, Cat No.21765) for 1½ hours. DRG were then transferred to 0.25% trypsin (Worthington Biochemicals, UK, Cat No. LS003703) and incubated for 30 minutes. The trypsin was inactivated using 30% foetal bovine serum (Gibco, UK, Cat No. 10437). DRG were washed three times in Ham's F12 media to remove serum and then were mechanically dissociated via gentle titration with a glass pipette. Dissociated neurons were passed through a 70µm mesh (Cavendish and Sons, UK) to remove un-dissociated cells and myelin, and then centrifuged for 5 minutes at 300rpm. Cells were re-suspended in Ham's F12 media, and centrifuged through 15% bovine serum albumin (Sigma, Cat No. A9205) at 900rpm for 10 minutes to remove non-neuronal cells. Cells were re-suspended in Bottenstain and Sato's medium (BS medium containing 0.1mg/ml transferrin (Sigma, Cat No. T8158), 20nM progesterone (Sigma, Cat No. P8783), 100µM putrescine (Sigma, Cat No. P5780), 30nM sodium selenite (Sigma, Cat No. S5261), 1µg/ml BSA (Fluka, Cat No. 05473), 0.01mM cytosine arabinoside (Sigma, Cat No. C1768) and 10pM insulin (Sigma, Cat

No. I3505)) in Ham's F12. All incubations took place in humidified temperature controlled incubator at 37°C in 5% CO_2 unless otherwise stated.

Cryoculture sections were washed twice in Ham's F-12 media before dissociated neurons were seeded directly onto the sciatic nerve sections in the presence of 10ng/ml nerve growth factor (NGF). The cryocultures were fixed 48 hours later with 2% paraformaldehyde for 20 minutes at room temperature, washed 3 times with PBS (Sigma, Cat No. P4417) and then incubated in blocking buffer (10% normal donkey serum (NDS: Sigma, Cat No. D9663) for 2 hours at room temperature. Blocking buffer was replaced with an antibody against growth associated protein (GAP)-43 (1: 2000; Millpore, Cat No. MAB347) in blocking buffer (containing 10% normal donkey serum (NDS: Sigma, Cat No. D9663) 0.2% Triton-X-100 in PBS) for 2 hours at room temperature. Blocking buffer was replaced with an antibody against GAP-43 (1:2000 in blocking buffer; Millpore, Cat No. MAB347) and incubated for 48 hours at 4°C. Coverslips were washed 3 times with PBS and incubated with Cy3-conjugated donkey anti-mouse antibody (1:400; Jackson ImmunoResearch, Cat No. 711-166-150) for 1 hour at room temperature, washed three times with PBS and mounted in Vectorshield containing DAPI (Vector Labs, Cat No. H-1200).

4.3.4.1. Neurite outgrowth analysis

GAP-43 immunoreactivity was visualised using a Leica MPS60 DMR fluorescent microscope and images of all neurite-bearing cells on each individual nerve section were acquired using Wasabi imaging software (Hamamatsu, US). The length of longest neurite was calculated using Sigma Scan Pro 5 software (SPSS, UK). The mean length of longest neurite of neurons on each nerve section was calculated and data expressed as group mean ± standard deviation (*n=5-8* individual nerves per group).

4.3.5. ELISAs

Cholesterol (Cayman Chemical Company, Cat No 10007640) and triglyceride (Cayman Chemical Company, Cat No 10010303) levels were measured in plasma using commercial ELISA kits according to manufacturer's instructions. N^{ε}-(carboxymethyl) lysine (CML; Cell Biolabs Inc, Cat No STA-316) and 4-hydroxynonenal (HNE)-His adduct (Cell Biolabs Inc, Cat No STA-334) were measured in 20µg protein extracted from desheathed sciatic nerve homogenised in ice-cold lysis buffer (25mM Tris HCl pH 7.4,

15mM NaCl, 10mM NaF, 10mM Na Pyrophospahte, 2mM EDTA, 0.2mM Na_4Ov_3, 1mM PMSF, Protease Inhibitor Cocktail (1:200, Sigma, Cat No. P8340)).

4.3.6. Statistical analysis

All statistical analysis was conducted using GraphPad PRISM 4 (GraphPad Software Inc). For parametric data, data was analysed using one or two-way ANOVA and post-hoc tests as appropriate. For non-parametric data, data was analysed using Kruskal-Wallis followed by Dunn's multiple comparison test.

4.4. Results

4.4.1. LR90 and pyridoxamine have no effect on body weight or blood glucose levels

STZ-induced diabetes results in metabolic dysfunction, significant hyperglycaemia (**p<0.01 Control vs Diabetic; Table 4.1) and reduced weight gain compared to age-matched controls (Table 4.1). At both 8 weeks and 12 weeks, the mean body weight of diabetic animals was significantly lower than that of controls (Table 4.1).

	Number of animals	Start Weight (g)	End Weight (g)	Blood glucose (mmol/L)
8 weeks				
Control	6	316 ± 10	560 ± 68	7.7 ± 1.1
Diabetic	8	312 ± 1	341 ± 55 **	27.8 ± 0.0 **
Diabetic + LR90	8	312 ± 1	337 ± 48 **	27.8 ± 0.0 **
Diabetic + PM	8	312 ± 2	319 ± 55 **	27.8 ± 0.04 **
12 weeks				
Control	16	390 ± 21	562 ± 49	5.4 ± 0.9
Control + LR90	6	355 ± 25	508 ± 41	4.9 ± 0.8
Control + PM	6	365 ± 12	539 ± 15	4.5 ± 1.5
Diabetic	9	412 ± 18	398 ± 40 **	26.2 ± 2.8 **
Diabetic + LR90	9	404 ± 18	378 ± 20 **	27.1 ± 0.3 **
Diabetic + PM	6	386 ± 26	393 ± 23 **	27.2 ± 0.1 **

Table 4.1: **LR90- and PM-treatment did not affect hyperglycaemia or body weight in STZ-diabetic rats.** This table shows the body weight at start and end of trials (mean ± standard deviation (grams)) and final non-fasted blood glucose levels (mmol/l) . Statistical analysis was conducted using either Two-way ANOVA with Bonferonni's post-hoc test (12 week data) or One-way ANOVA with Dunnett's Multiple Comparison Test (8 week data), **p<0.01.

The development of these dysfunctions has long been used as an indicator for the presence of diabetes following STZ (65;66). Treatment of diabetic rats with LR90 or PM did not affect either body weight or blood glucose levels. Similarly, body weight and blood glucose levels in control animals treated with LR90 or PM were not

statistically different to untreated controls (Table 4.1). Therefore, neither LR90 or PM affected these parameters of diabetes.

4.4.2. Treatment with pyridoxamine partially corrects plasma lipid levels in STZ-diabetic rats

Diabetes is also associated with the development of hyperlipidemia (elevated level of any or all lipid and/or lipoproteins in the blood) and this has been suggested to contribute to the pathogenesis of diabetic neuropathy (67;68). LR90 and PM have both been shown to reduce hyperlipidemia in long-term (28-32 weeks duration) studies using STZ-induced diabetic rats (29-31) and in Zucker rats (56;69) (model of type 2 diabetes; 32-40 weeks duration).

In this current study, analysis of plasma lipid content (Figure 4.1) demonstrated a significant increase in untreated diabetic rats (12 weeks post-STZ) compared to age-matched controls. Plasma triglycerides were significantly increased in diabetic rats compared to age-matched controls (Control: 131.9 ± 63.8mg/dL vs Diabetic: 467.5 ± 114.2mg/dL; *n=9-10* per group; **p < 0.01; Figure 4.1A). Free cholesterol in plasma was also significantly increased compared to age-matched controls (Control: 26.9 ± 2.2 mg/dL vs Diabetic: 65.9 ± 35.1 mg/dL; *n=9-10* per group; **p < 0.01; Figure 4.1B).

Treatment of diabetic rats with PM significant reduced plasma triglycerides compared to non-treated diabetic animals (Diabetic: 467.4 ± 114.2mg/dL vs Diabetic + PM: 303.8 ±139.2mg/dL; *n=6-9* per group; *p<0.05). Treatment with LR90 had no significant effect on plasma triglyceride levels compared to untreated diabetic animals (Figure 4.1A).

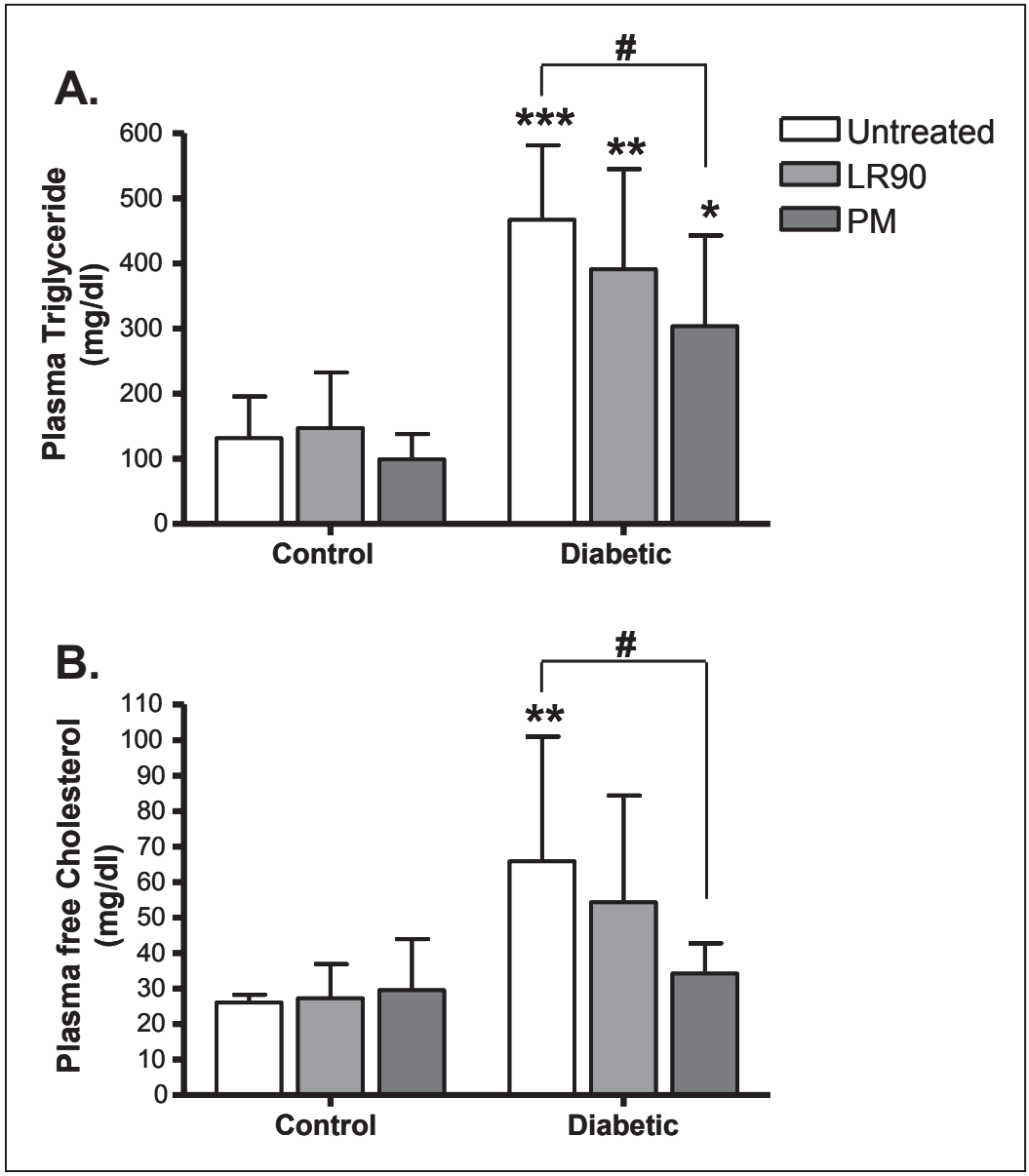

Figure 4.1: **PM significantly reduces hyperlipidemia in STZ-diabetes.** Diabetes was induced using STZ for 12 weeks and rats were untreated or treated with LR90 or PM in drinking water (50mg/dl and 400md/dl respectively) after 1 week post-STZ for the duration of the trial. Plasma triglyceride (A) and free cholesterol (B) levels were measured by ELISA. (A) Significant increase in plasma triglyceride levels occurred in all STZ-diabetic groups compared to appropriate control groups (***p<0.001, ** p<0.01, *p<0.05), PM-treatment significantly reduced triglyceride levels compared to untreated diabetic ($^{\#}$ p<0.05). (B) Significant increase in free cholesterol levels occurred in the plasma of untreated diabetic rats (** p<0.01), but not LR90 or PM treated diabetic rats, compared to appropriate control. PM-treatment significantly reduced free cholesterol levels in the blood compared to untreated diabetic animals ($^{\#}$ p<0.05). Data is represented as mean ± standard deviation (*n=6-11* per group). Statistical analysis was Two way ANOVA with Bonferonni's post-hoc test: ***p<0.001, ** p<0.01, *p<0.05 compared to appropriate control group; # p<0.05 compared to untreated diabetic group.

Treatment of diabetic rats with PM caused a significant reduction in free cholesterol levels in plasma compared to non-treated diabetic animals (Diabetic: 65.9 ± 35.1 mg/dL vs Diabetic + PM: 34.2 ± 8.6 mg/dL; *n=6-9* per group; *p<0.05). Treatment with LR90 partially reduced free cholesterol levels in plasma of diabetic animals, since cholesterol

levels of LR90-treated diabetic animals were not significantly different to either untreated diabetic or treated control rats (Figure 4.1B). Neither PM and LR90 altered plasma triglyceride or free cholesterol levels in age-matched control animals (figure 4.1A,B).

4.4.3. LR90 and pyridoxamine protects against development of sensory, but not motor, NCV deficits in experimental diabetic neuropathy

Slowed motor and sensory NCV is a hallmark of clinical and experimental diabetes (65;66). Untreated diabetic rats showed significantly reduced motor NCV and sensory NCV at both 8 (20% and 26% reduction) and 12 weeks post-STZ (23% and 27% reduction; **$p<0.01$, *$p<0.05$) compared to age-matched control rats (Figure 4.2). Treatment of diabetic rats with either LR90 or PM prevented sensory NCV deficits at 8 and 12 weeks post-STZ (LR90; 98% and 99% and PM; 92% and 93% of age-matched control values; *$p<0.05$; Figure 4.2A). Neither LR90 nor PM treatment prevented the development of diabetes-associated motor NCV deficits at 8 weeks. PM provided partial protection from motor NCV deficits at 12 weeks, as NCV measurements of PM-treated diabetic animals were not significantly different to untreated diabetic or treated control (Figure 4.2B). Neither LR90 nor PM treatment affected either motor or sensory NCV in control treated rats (data not shown).

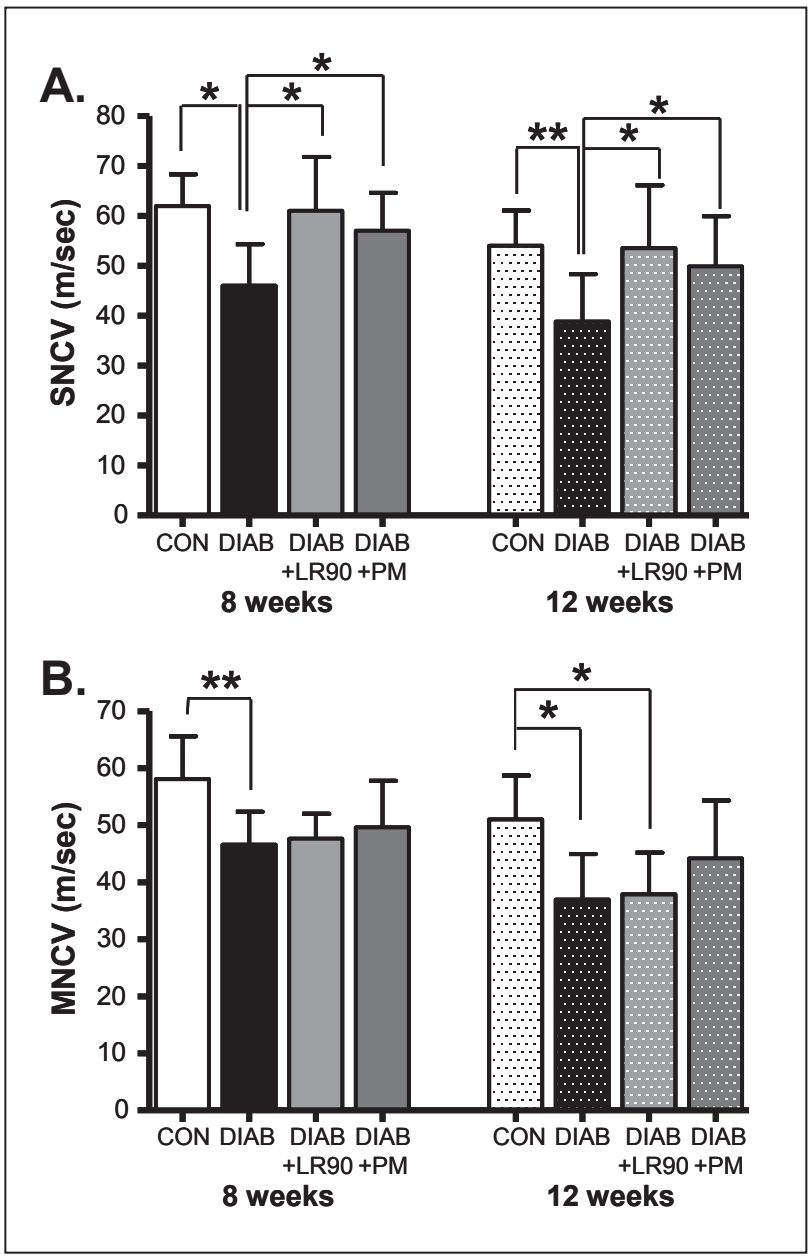

Figure 4.2: **LR90 and PM prevents the development of sensory NCV deficits in STZ-diabetic rats.** Sensory and motor NCVs were significantly lower in the both 8 (A) and 12 (B) week untreated diabetic rats than on age-matched controls. (A) Treatment with LR90 or PM prevents the development of sensory NCV deficits. (B) PM, but not LR90, provided partial protection against motor NCV deficits at 12 weeks. Data are represented as mean ± standard deviation (*n=6-12* per group). Statistical analysis was One way ANOVA with Newman-Keuls Multiple Comparison test, ** $p<0.01$, *$p<0.05$).

4.4.4. AGE inhibitors did not protect against development of tactile allodynia in experimental diabetic neuropathy

We observed the development of significant tactile allodynia by 4 weeks post-STZ as shown by decreased 50% paw withdrawal threshold to Von Frey stimulation compared to the untreated control group (Control: 100.0 ± 28.0% vs untreated Diabetic: 39.2 ± 11.4%; *n=9-10* per group*;* Figure 4.3A, B). This was normalised by 8 weeks post-STZ. Neither LR90 nor PM ameliorated this tactile allodynia. Using a dynamic plantar aesthesiometer, another method of assessing mechanical sensitivity, we were unable to detect the increased mechanical sensitivity at 4 weeks as observed with Von Frey testing. We did, however, observe the development of mechanical hyposensitivity at 12 weeks post-STZ in both untreated and treated STZ-diabetic rats (Control: 101.7 ± 24.9% vs untreated Diabetic: 142.2 ± 33%; *(n=9-10 per group;* Figure 4.4 A, B). Treatment with LR90 or PM did not attenuate the development of this reduced mechanical sensitivity at 12 weeks diabetes (Figure 4.4 A). No diabetes-associated changes in thermal threshold were observed in our diabetic rats over the 12 week timecourse, although we did observe a time-dependent increase in paw withdrawal latency in all 4 testing groups (Figure 4.5A).

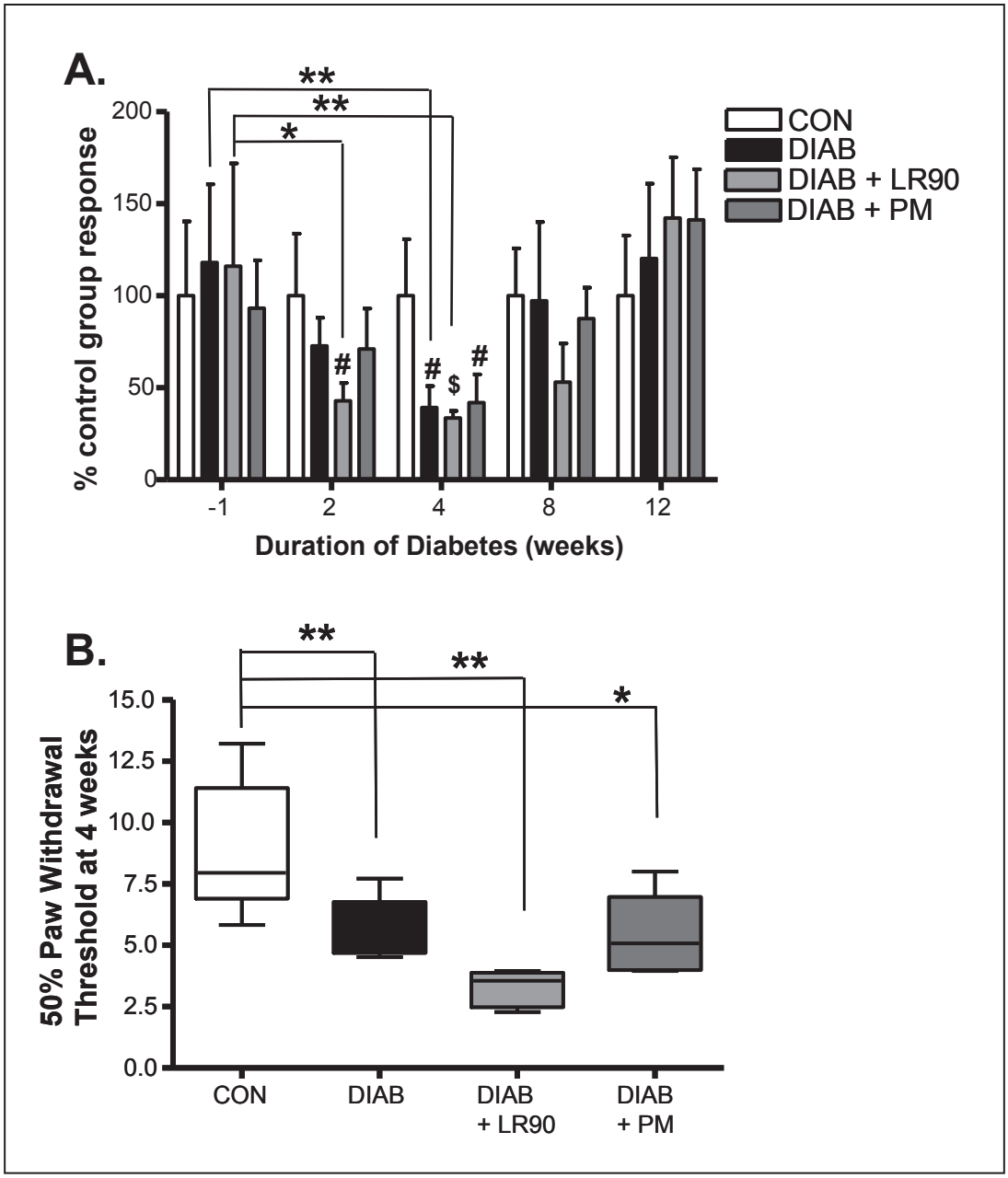

Figure 4.3: **Tactile allodynia was not prevented by LR90 or PM**. Diabetes was induced for 12 weeks and rats were treated with LR90 or PM after 1 week. Sensory behaviour testing was carried out prior to STZ (0 weeks), and then 2, 4, 8 and 12 weeks post-STZ. (A) Transient tactile allodynia developed in STZ-diabetic rats over the 12 week timecourse. 4 weeks post-STZ, a significant reduction in 50% withdrawal threshold was observed in untreated, LR90- and PM-treated diabetic. Data represent the 50% paw withdrawal threshold of animals expressed as % of the untreated age-matched control group mean ± standard deviation (*n=4-10* per group; Two-way ANOVA with Bonferroni posttest; **p<0.01, *p<0.05 compared to baseline value; $^{\$}$p<0.01, $^{\#}$p<0.05 compared to appropriate treated control group value). (B) Box and whiskers plot showing median and range of group data of 50% paw withdrawal threshold at 4 week spot-STZ (*n=4-10* per group; Kruskal-Wallis followed by Dunn's Multiple Comparison test, **p<0.01, *p<0.05).

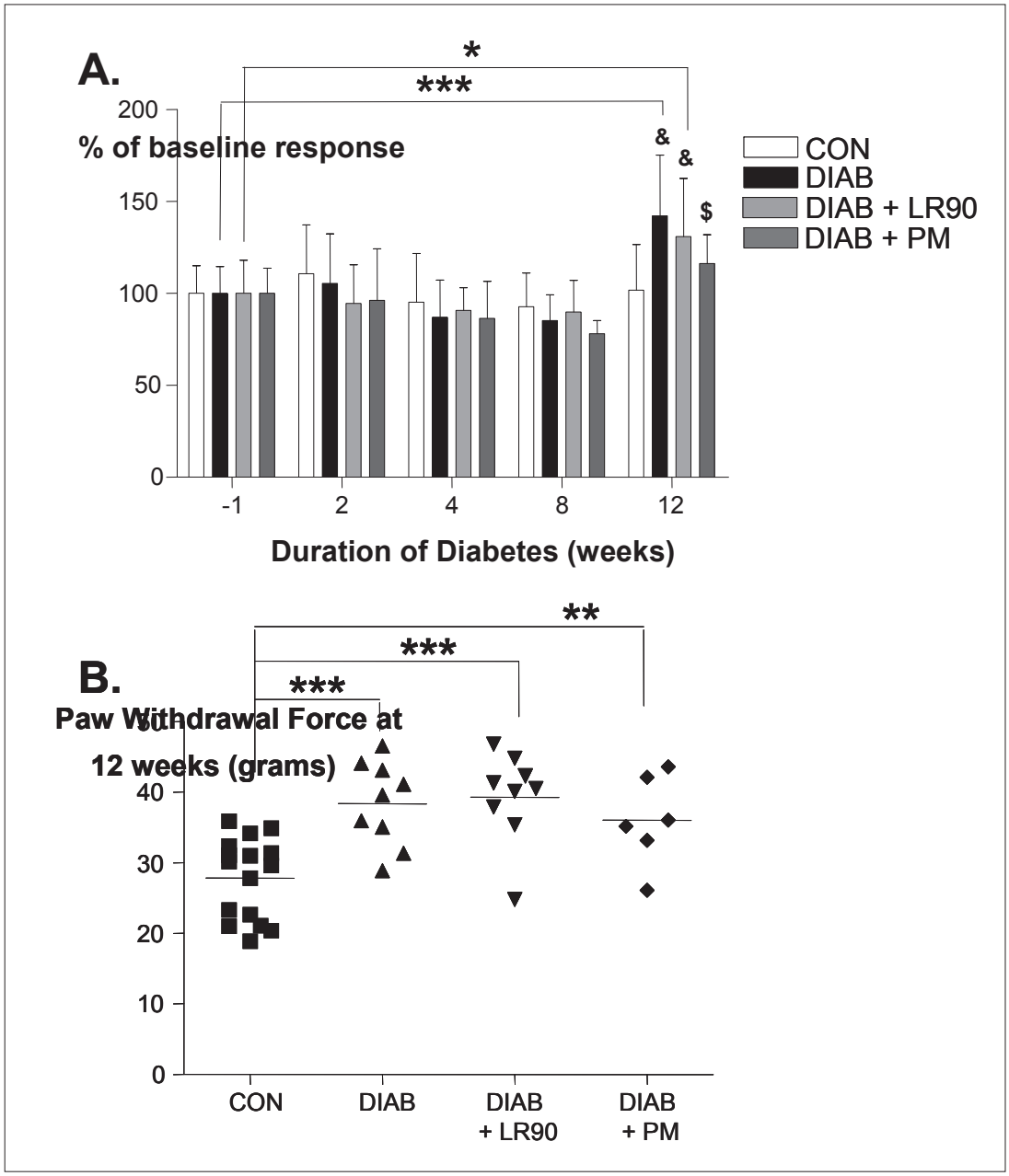

Figure 4.4: **Mechanical hyposensitivity was observed at 12 weeks in STZ-diabetic animals.** (A) Hyposensitivity developed in STZ-diabetic rats at 12 weeks post-STZ, a significant increase in withdrawal threshold was observed in untreated and LR90-treated diabetic. Data represent the paw withdrawal threshold of animals expressed as % of pre-diabetes baseline threshold of each group. Data is represented as mean ± standard deviation (*n=4-10* per group; Two-way ANOVA with Bonferroni posttest, ***p<0.001, *p<0.05 compared to baseline value; $^{\&}$p<0.001, $^{\$}$p<0.01 compared appropriate treated control group value). (B) Scatter graph showing mean of group data of paw withdrawal threshold at 12 week post-STZ (n=*4-10* per group; One-way ANOVA with Tukey's Multiple Comparison test, ***p<0.001, **p<0.01).

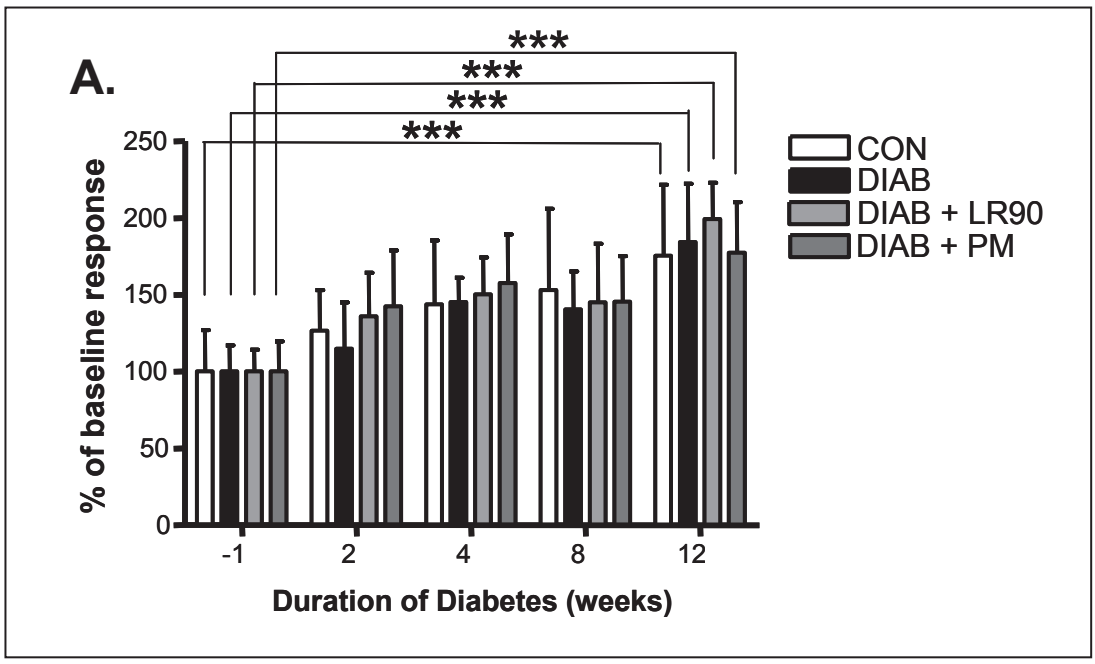

Figure 4.5: **Thermal hyperalgesia and hypoalgesia was not observed in STZ-diabetic rats.** (A) Thermal hyperalgesia and hypoalgesia was not observed over the 12 week diabetic timecourse. Data is represented as mean ± standard deviation (n=*4-10* per group; Two-way ANOVA with Bonferroni posttest, ***p<0.001 compared to baseline value).

4.4.5. LR90 and pyridoxamine reduce glycation and lipid peroxidation in the sciatic nerve of STZ-diabetic rats

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetic neuropathy (2;70). Oxidative stress causes lipid peroxidation, protein, DNA and RNA oxidation and therefore subsequent cellular dysfunction (71). Hyperglycaemia and hyperlipidemia gives rise to AGE residue formation and advanced lipoxidation endproducts (ALEs). Nε-carboxymethyl-lysine (CML) is a stable, nonenzymatic chemical modification of protein lysine residues resulting from both glycation and oxidation reaction (38). 4-hydroxynonenal (4-HNE) is a highly reactive α, β-unsaturated aldehyde and by-product of lipid peroxidation (72). Previously we, and others, have demonstrated the accumulation of AGEs on both the intracellular (42) and extracellular matrix proteins of the sciatic nerve of STZ-diabetic rats (41).

4-HNE levels were significantly increased in sciatic nerve 12 weeks post-STZ compared to age-matched controls (Control: 1.84 ± 0.4μg/ml vs Diabetic: 2.74 ± 0.8μg/ml; *n=9-10 per group*; ***p < 0.001, Figure 4.6A). Treatment of diabetic rats with LR90 or PM significantly reduced 4-HNE adducts in the sciatic nerve compared to

untreated diabetic animals (Diabetic + LR90: 1.40 ± 0.6µg/ml; Diabetic + PM: 1.96 ± 0.6µg/ml; *n=6-9 per group*; ***p<0.001, *p<0.05).

Using tandem mass spectrometry, we previously demonstrated a significant 3-fold increase in CML in ECM proteins from the sciatic nerve of STZ-diabetic rats (12 weeks post-STZ; Duran-Jimenez et al., 2009). In this study, we quantified the CML residue content of whole sciatic nerve lysates with a less sensitive method of detection (ELISA). We observed increased, but not significantly, levels of CML in sciatic nerve of diabetic rats compared to age-matched controls (Control: 2.41 ± 0.74ng/ml vs Diabetic: 3.95 ± 1.54ng/ml (*n=9*-10 per group; Figure 4.6B). Treatment with PM and LR90 reduced the CML adduct content in sciatic nerve proteins; however only LR90 treatment showed significance (Diabetic: 3.95 ± 1.4ng/ml vs Diabetic + LR90: 2.42 ±1.32 ng/ml, (*n=9* per group), *p<0.05).

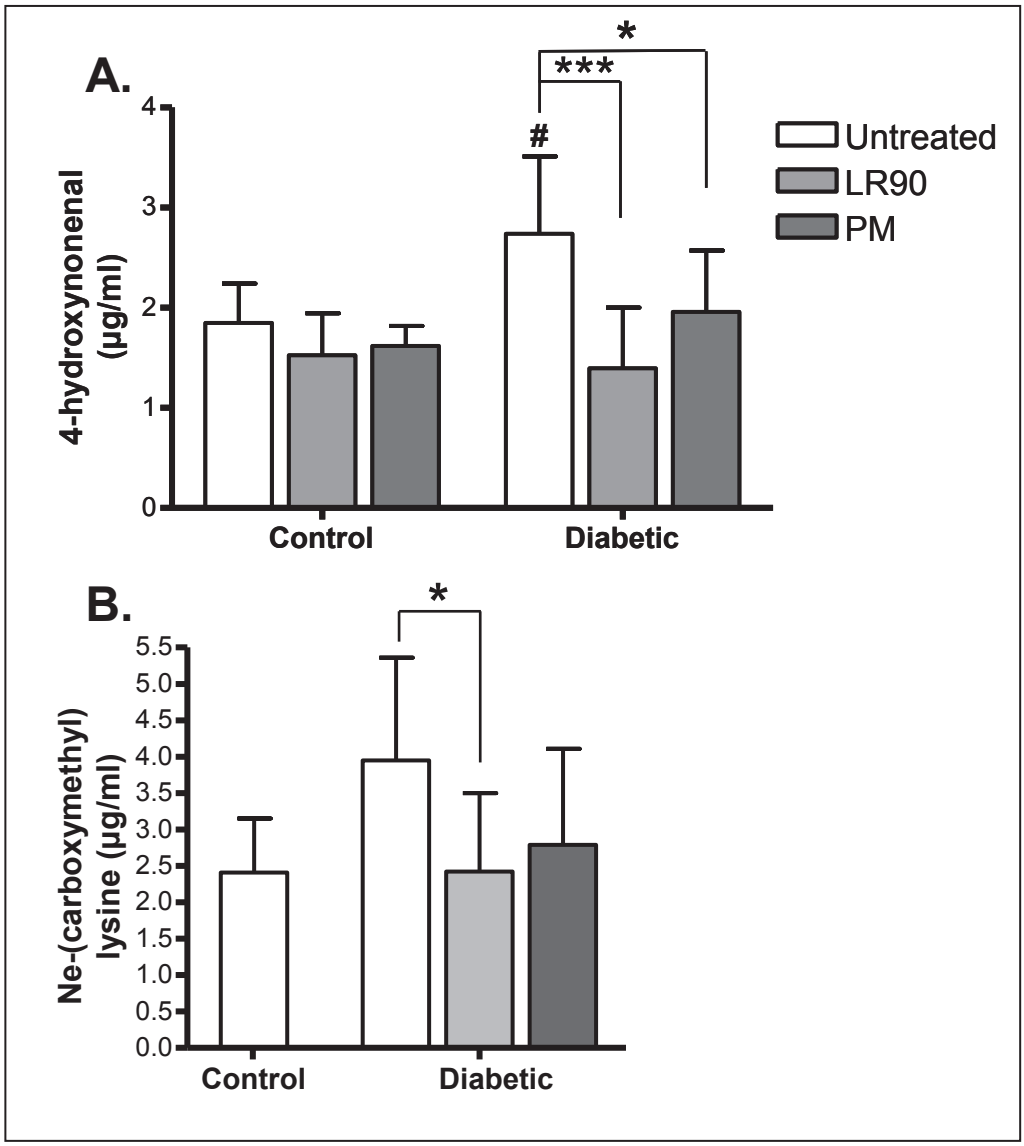

Figure 4.6: **LR90 and PM treatment prevent diabetes-induced oxidative stress.** CML and 4-HNE levels in the sciatic nerve were measured by ELISA. (A) Significant increases occured in 4-HNE levels in the sciatic nerve of untreated STZ-diabetic rats. LR90- and PM-treatment prevented diabetes-induced alteration in 4-HNE (*n=6-10* per group; Two way ANOVA with Bonferroni Comparison test; ***p<0.001, *p<0.05 compared to appropriate control group; # p<0.05 compared to untreated diabetic group). (B) Non-significant increases in CML levels were detected in the sciatic nerve of STZ-diabetic rats, LR90 treatment of STZ-diabetic rats reduced CML (*n=6-10* per group; One-way ANOVA with Dunnett's Multiple Comparison test; *p<0.05 compared to untreated diabetic group).

4.4.6. LR90 treatment prevents the development of diabetes-associated deficits in sensory nerve regeneration

Previously, we have shown that the peripheral nerve taken from diabetic rats is less supportive of axonal elongation compared to age-matched controls (73) and also that neurite outgrowth was significantly reduced on exogenously-glycated ECM (41). The aim of this experiment was to determine whether treatment of STZ-diabetic rats with the glycation inhibitors, LR90 or PM, would therefore prevent 'endogenous glycation' of peripheral nerve ECM and allow successful neurite outgrowth.

Adult rat sensory neurons were seeded onto longitudinal sections of sciatic nerve from either age-matched control, non-treated, LR90-treated or PM-treated 12 week STZ-diabetic rats to assess their ability to support neurite elongation. Neurons extended long unbranched neurites which grow in close association with the basal lamina on control sciatic nerve (Figure 4.7A). Neurons seeded onto sciatic nerve from STZ-diabetic rats produced less well ordered neurites which were shorter, less well-organised and more branched (Figure 4.7B). The mean neurite growth of sensory neurons plated on sciatic nerve taken from diabetic rats was significantly less than that plated on age-matched control nerve (Control: 236.4 ± 34μm vs Diabetic: 156.1± 37μm; 30% reduction (*n=5*), ** $p<0.01$; Figure 4.7E). However, this diabetes-associated deficit was almost totally prevented in nerves from the LR90 treated animals and partially prevented in nerves from the PM-treated animals (Diabetic + LR90: 98% of control growth; Diabetic + PM: 80% of control growth, *n=5;* ** $p<0.01$; Figure 4.7E). The neurons seeded onto sciatic nerve obtained from LR90- or PM-treated STZ-diabetic rats appeared morphologically similar to those seeded on control nerve, producing long, unbranched neurites which grow in close relation to basal lamina (Figure 4.7C, D, respectively).

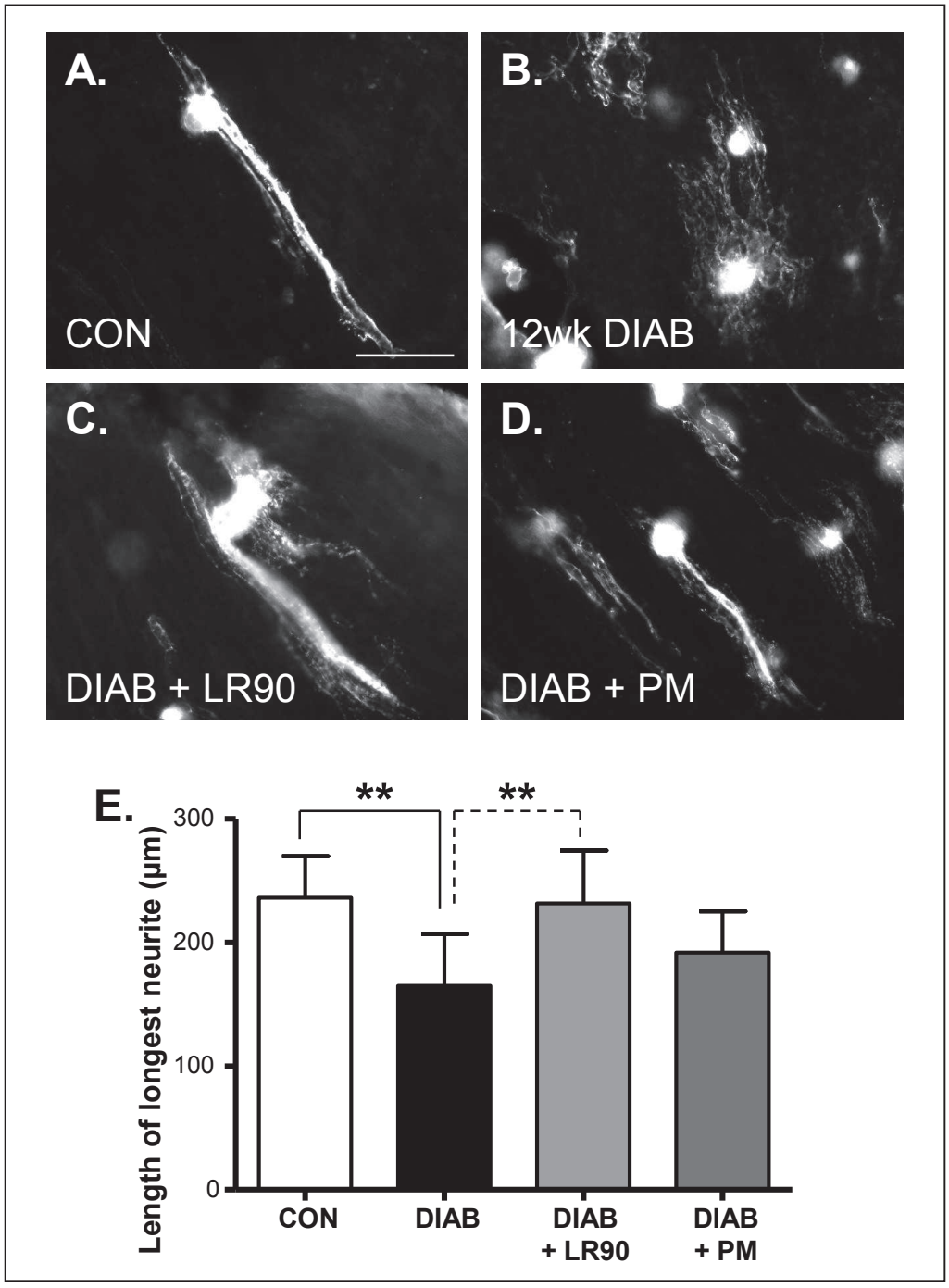

Figure 4.7: **Impaired neurite elongation on diabetic nerve cryocultures is rescued by LR90 and PM treatment.** Adult rat sensory neurons were plated onto sciatic nerve cryoculture sections and grown for 48 hours in the presence of NGF (10mg/ml). Representative micrographs of axonal elongation of primary dissociated neurons plated onto either age-matched controls (A) or 12 week untreated (B) or 12 week LR90- treated (C) or 12 week PM-treated (D) diabetic sciatic nerve section, scale bar = 50µm. (E) There is a significant reduction in the length of the longest neurite grown on 12 week diabetic sciatic nerve compared to age-matched. Treatment of diabetic rats with LR90 significant increase neurite outgrowth. Data expressed as group means ± standard deviation. (*n=5-8* nerve sections per treatment group; one-way ANOVA and Newman- Keuls Multiple Comparison test; **p<0.01).

4.5. Discussion

In this study we aimed to assess the therapeutic efficacy of LR90 and PM treatment in the STZ experimental model of diabetic neuropathy. We observed that PM treatment improved plasma hyperlipidemia and prevented lipid peroxidation in the sciatic nerve of STZ-diabetic rats. Whilst treatment with LR90 normalised lipid peroxidation and CML accumulation in the sciatic nerve of STZ-diabetic rats. LR90 and PM-treatment of STZ-diabetic rats normalised sensory NCV; but not motor NCV at 8 and 12 weeks duration of diabetes, or tactile allodynia or mechanical hyposensitivity deficits. Diabetes-associated regenerative deficits on sciatic nerve cryocultures were prevented in LR90 treated animals and partially prevented in nerves from the PM-treated animals.

PM was originally described as a post-Amadori inhibitor of AGEs (74;75) and recently has also been shown to inhibit the formation of ALEs. PM acts as a sacrificial nucleophile thereby trapping reactive intermediates of lipoxidation and glycoxidation reactions (50;51) such as glyoxal and glycolaldehyde, inhibiting AGE/ALE formation specifically CML and CEL (48;49). In addition, PM also inhibits chemical modification of proteins during metal-catalyzed oxidation reactions (49) and the production of superoxide radical (76),thereby further reducing lipid peroxidation and protein glycation. Mechanistic studies on PM show that PM are able to bind to and trap precursors of ALE and AGE formation (30). Derivatives of the PM-precursors interaction are readily detected in the urine of diabetic-treated rats thereby showing the PM is an effective AGE/ALE inhibitor (30).

LR90 is one of a series of LR compounds designed and screened by the Rahbar laboratory for their possible AGE inhibitory effects. These aromatic compounds were mostly derivatives of aryl (and heterocyclic) ureido and aryl (and heterocyclic) carboxamido phenoxy isobutyric acids. These LR compounds are potent inhibitors of AGE-protein cross-linking, post Amadori-derived AGE (31;55) and chelators of Cu^{2+} (55) providing comparable protection at a concentration 50 times less on a molar basis compared to AG and PM (55). The mechanism of action of LR90 is multifactorial. LR90 traps reactive carbonyls such as glyoxal and MG and thereby prevents the formation of protein crosslinks (55), and also has anti-oxidant properties and anti-inflammatory effects (55). LR90 prevents the expression of receptor for advanced

glycation end-products (RAGE) and other inflammatory molecules in human monocytes (55).

Multiple groups have shown that treatment of STZ-diabetic rats with AGE/ALE inhibitors significantly improves renal function (29-31), protects against retinopathy (30;32;33), and prevent neurovascular dysfunction in STZ-diabetic rats (35;45;77). This is the first description of the neuroprotective effects of LR90 in experimental diabetic neuropathy.

4.5.1. AGE inhibitors normalise diabetes-associated hyperlipidemia

Until recently, hyperglycaemia was thought to be the major driving force underlying the development of diabetic neuropathy (78-80). This was largely based on the results from The Diabetes Control and Complications Trail (DCCT), in which tight glycemic control was shown to reduce the incidence of diabetic neuropathy by 60% in Type-1 diabetic mellitus (T1DM) subjects (81). Over the last few decades, there has been an ever increasing body of evidence that further implicated AGE and AGE-proteins cross-linking as a major factor in the pathogenesis of diabetic complications. More recently, dyslipidemia has also been implicated as a significant and independent risk factor in diabetic complications (67;70;82;83). Lipid profiles are commonly abnormal in the early stages of type-2 diabetic mellitus (T2DM) in a temporal pattern (84;85), whilst in T1DM dyslipidemia develops in the later stage of the disease (83). In both T1DM and T2DM, abnormal lipid profiles coincide with the development of diabetic neuropathy (86;87).

Several reports have indicated that PM-treatment normalised diabetic-induced hyperlipidemia in STZ rats (29;88;89), and our current findings agree with these reports - treatment with PM normalised both plasma triglyceride and free cholesterol levels in STZ-diabetic rats compared to age-matched controls. However, we observed no significant protection from diabetic-induced hyperlipidemia in LR90-treated diabetic rats, in contrast to previous reports in STZ-induced diabetes (32 weeks diabetes; (55)) and Zucker rats (40 weeks diabetes; (56)). Whilst we do not see correction of dyslipidemia with LR90 at 12 weeks this may reflect the different duration of disease and LR90 treatment in these studies (31;55;56).

4.5.2. AGE/ALE accumulation may contribute to peripheral nerve dysfunction in STZ-diabetic rats

We observed protection of sensory NCV deficits in both LR90 and PM-treatment groups at 8 and 12 weeks duration of diabetes, and partial protection of motor NCV deficits by PM at 12 weeks. Previous work has shown other AGE inhibitors are able to inhibit the development of both motor NCV and sensory NCV deficits in STZ-diabetic rats (30;35;45;77;90). The neuroprotective effects of AG and PM were observed following 2 weeks of treatment that commenced after 4 weeks of untreated STZ-diabetes. Diabetic rats treated with either AG or PM showed recovery of sensory NCV and motor NCV compared with non-treated diabetic rats (45).

Reduced motor and sensory NCV develop in diabetic animals between 2 and 3 weeks after diabetes induction in rats (11). These deficits occur prior to diabetes-associated structural alteration to the peripheral nerve (12) and in the early stages of diabetes are with insulin treatment (12) or aldose reductase inhibitors (25;26). These finding strongly suggest that at this early stage of diabetes, NCV deficits are derived from hyperglycaemia-associated changes rather than structural alteration to the peripheral nerve. However, whether other diabetes-associated alteration to the peripheral nerve function may influence NCV deficits in longer tern diabetes is unclear. Evidence from numerous studies implicates impaired nerve and DRG blood flow as a major etiological factor in diabetic neuropathy (45). Reduced perfusion has been suggested as being entirely responsible for many early aspects of nerve dysfunction, such as diminished motor NCV and sensory NCV in experimental diabetic models (45). AG treatment prevented a decline in nerve blood flow in STZ-diabetic rats, which accompanied prevention / rapid correction of motor NCV and sensory NCV deficits (34;91). These observations taken as a whole suggest that inhibition of AGE/ALE accumulation by PM and LR90 treatment may result in correction of NCV deficits via normalisation of nerve blood flow, as seen following AG treatment. However, as nerve blood flow was not measured in this study, we can not confirm this hypothesis.

However, the reason why treatment with LR90 and PM of STZ-diabetic rats did not protect against the development of motor NCV deficits is unclear. It could simply be that a higher dose of the drug is required to normalise levels in motor NCV. Interestingly, a similar selective normalisation of only sensory NCV has also been previous studied using anti-oxidant therapies (α-lipoic acid) following STZ-induced

diabetes (22;23). Therefore the protective properties of LR90 and PM on NCV may be due to their anti-oxidant effects in the sciatic nerve.

We have previously shown CML residue content of the sciatic nerve is significantly increased as early as 3 weeks post-STZ using tandem mass spectrometry (41). In this current study, using ELISA, we observed a similar, but not significant, 1.6-fold increase in CML by 12 weeks post-STZ. The Cameron & Cotter group failed to detect significant increase in nerve CML, assayed by immunohistochemistry, 6 weeks post-STZ (45). This lack of significant CML accumulation may reflect these less sensitive detection methods (ELISA and immunocytochemistry) (92).

Oxidative stress, and lipid peroxidation in particular, have been implicated in the pathogenesis of diabetic neuropathy, with previous studies showing increased lipid peroxidation in the rat sciatic nerve after as little as 1 or 2 weeks duration of STZ-diabetes (93). Polyunsaturated fatty acids are particularly prone to lipid peroxidation; this reaction yields 4-HNE, which is used as a biochemical markers of lipid peroxidation. The accumulation of 4-HNE may contribute to neuronal degeneration in the PNS of diabetic rats in a number of ways. 4-HNE is known to cause microtubule dysfunction (94), mitochondrial dysfunction, and reduction in neurite outgrowth *in vitro* (95). All these consequences of 4-HNE accumulation suggest a role of lipid peroxidation in impaired nerve regeneration in diabetic neuropathy. We, also, confirmed reduction of CML and 4-HNE adducts in the sciatic nerve of STZ-diabetic rats by long-term LR90 and PM treatment. This reduction in AGE/ALE accumulation in the sciatic nerve coincided with normalisation of sensory NCV deficits thereby suggests that in long-term diabetes, both intracellular and extracellular accumulation of AGE/ALE may play a role in the maintenance of NCV deficits in long-term diabetes.

4.5.3. AGE inhibitor do not prevent altered peripheral sensitivity in STZ-diabetic rats

Neuropathic pain is multifactoral and development of diabetic neuropathy can be associated with abnormal sensation in patients extremities such as paresthesias, allodynia, hyperalgesia and spontaneous pain. The prevalence of pain varies between 10% - 20% in subjects with diabetes and 40%-50% in those with diabetic neuropathy (96). In STZ-mice studies damage to small epidermal unmyelinated fibres has been

shown to coincide with thermal hypoalgesia and mechanical allodynia in the early stages of diabetic neuropathy (97-99). However, altered pain thresholds in diabetic rats have shown conflicting results. Whilst reports of tactile allodynia and chemical hyperalgesia are fairly consistent in STZ rats (24;100;101), changes in thermal nociceptive thresholds have been highly variable, with reports of no change in thermal sensitivity, hypo- or hyperalgesia (102-104). The pathogenesis of diabetic painful neuropathy is poorly understood, although identification of impaired cutaneous endothelium-related vasodilatation and C-fiber mediated vasoconstriction as well as increased sural nerve epineurial blood flow in diabetic subject with painful, compared to those with painless neuropathy suggest the importance of hemodynamic factors (96). The development of tactile allodynia in clinical and experimental diabetes has been suggested to be the result of alteration in both peripheral and central nervous system, involving a lowering of the usually high receptor threshold of nociceptors, alteration to sensory neuron cell bodies, and therefore peripheral and spinal sensitisation (105).

In our study, we observed an early onset of tactile allodynia at 4 weeks post-STZ in untreated STZ-diabetic rats compared to age-matched controls, but did not see any protection by either the LR90 or PM treatment strategy. We also observed diabetic-induced mechanical hyposensitivity by 12 weeks post-STZ in untreated STZ-diabetic rats compared to age-matched control, again with no protection observed by either AGE inhibitor treatment strategies. Whilst this may suggest that AGE/ALE accumulation does not play a role in the development of tactile allodynia and mechanical hyposensitivity in experimental diabetes further experiment are required.

Questions regarding the limitations of this study need to be addressed. We used a dose of PM (400mg/L) which had previously been shown to reduce oxidative stress and AGE accumulation in plasma of STZ-diabetic rats (62), but this was lower than other studies (1g/L; (29;32;45;69)). PM plasma concentrations have been shown to reach 100µm in diabetic rats treated with 1g/L of PM (29). Therefore, assuming that proportionally lower steady-state levels of PM occurs, rats in our study should have plasma concentration of PM around 40µm. Plasma methylglyoxal (MG) levels are estimated at approximately 5µm (62), therefore 40µm should be sufficient to react with all the plasma MG. However, Degenhardt et al, 2002a also described high concentration of PM in the urine of treated rats suggests that most of PM is excreted unchanged in the urine (29). Studies using this higher dosage of PM did not see significantly different

results than we achieved with 400mg/ml. Both STZ-diabetic and Zucker rats treated with 1g/L, similarly, had normalised hyperlipidemia and reduced AGE/ALE accumulation (29;32;45;69). To date, due to the limited number of studies completed, LR90 has only been used at a single dose in all trials (50mg/ml) and little is known about the drug metabolism. Recent work from the Calcutt laboratory has shown that daily subcutaneous injection with a cocktail of vitamins B1, B6 and B12 is able to ameliorate tactile allodynia and formalin-evoked hyperalgesia as well as improve sensory NCV in a dose-dependent manner in 4 weeks STZ-diabetic rats (106). However, the relatively high dosage (180:180:1.8 mg/kg) required for these beneficial effects are equivalent to low and medium dose (20:20:0.2 mg/kg and 60:60:0.6 mg/kg, respectively) used in previous study (107), and could explain the lack of clear efficacy in clinical trials using B vitamins and also in this current study with LR90 and PM in experimental rats. Therefore the lack of neuroprotection from neuropathic pain following LR90 and PM treatment may be the result of insufficient dosage.

Another concept that needs to be considered is that the blood-brain/spinal cord-barrier (BBB) is a more effective barrier compared to the blood-nerve-barrier (BNB) therefore reduced diffusion of the AGE inhibitors into the CNS may limit the efficacy of the drugs, particularly in light of the failure to correct tactile allodynia which has a spinal component. AGE accumulation occurs in the CNS of diabetic patients; therefore, central administration of the AGE inhibitors by implanted intrathecal osmotic pumps or injections to the spinal cord may attenuate neuropathic pain in STZ-diabetic rats.

Tactile allodynia is difficult to prevent, for example the aldose reductase inhibitor (ARI; ICI 222155) did not prevent tactile allodynia (24), but did reverse other diabetes-induced functional deficits such as endoneurial blood flow, motor and sensory NCV (108-111) as well as lipid peroxidation and DNA oxidation (93). These studies concluded that early peripheral nerve dysfunction in diabetes such altered blood flow and NCV deficits are associated with oxidation damage to the nerve, which is ameliorated by ARI treatment. It was concluded that the mechanism behind tactile allodynia development was likely to be independent of these ARI effects. Similarly, we and others have observed reduced ROS production and lipid peroxidation by LR90 (55;56) and PM treatment (45;50;51;76), which coincided with normalisation of sensory NCV deficit.

4.5.4. AGE/ALE contributes to nerve regeneration failure in experimental diabetes

Previous work in our laboratory has demonstrated that AGE residues accumulate in both intracellular and extracellular proteins of the peripheral nerve of STZ-induced diabetic rats as early as 3 weeks post-induction of diabetes (41;42). In addition, we and other groups have shown that glycation of ECM components LM and FN, using MG or other glycating agents, dramatically impairs neuronal outgrowth and regeneration of sensory neurons *in vitro* (41;43;44). We have also recently demonstrated that diabetes-induced alteration to the ECM of the peripheral nerve in experimental diabetes results in reduced support for neurite outgrowth using an *in vitro* sciatic nerve cryoculture system (73).

In this current study, treatment of diabetic rats with LR90 and PM corrected the diabetes-associated regenerative deficit of sciatic nerve ECM, which suggests that the accumulation of AGE/ALE such as CML and 4-HNE in the peripheral nerve is a major contributing factor associated with nerve regeneration failure in diabetic neuropathy. Mechanisms by which AGE/ALE accumulation in the peripheral nerve ECM could contribute to reduced regeneration include altered Schwann cell phenotype, blocking of integrin activation, RAGE activation and failed MMP-mediated proteolysis of glycated ECM.

4.5.5. Conclusion

The main conclusion of this study is that AGE inhibitors are effective at preventing the development of peripheral nerve associated nerve dysfunction of diabetes such as slowing of sensory NCV and altered supportive nature of peripheral nerve to regenerating axons, but at doses tested were unable to prevent the development of alerted peripheral sensitivity. Therefore, whilst the study identifies a potential treatment strategy for promoting axon regeneration and correction of dyslipidemia and NCV deficits in diabetic neuropathy, further work is required in order to determine whether changes in mechanical sensitivity is independent of AGE/ALE accumulation in experimental diabetes.

4.6. References

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5. General Discussion

Diabetic neuropathy is the most common secondary complication of diabetes, development of which is typically associated with long-term diabetes and poor glycemic control (The Diabetes Control and Complications Trial Research Group, 1993). Diabetic neuropathy is characterised by the loss of protective sensation in patient's extremities as a result of distal "die-back" of the sensory nerve terminals. This can be accompanied by neuropathic pain, tactile allodynia, and thermal hyper/hypoalgesia and mechanical hyper/hypoalgesia. Diabetic complications are multifactoral in origin, however over the last decade increasing evidence has emerged indicating the biochemical process of AGE formation as one of the major pathways involved in the development and progression of both microvascular and macrovascular diabetic complications. AGEs accumulate within a multitude of organs that are damaged in diabetes including the kidney, retina, atherosclerotic plaques, skin and peripheral nerve (Sugimoto et al., 1997;Ryle and Donaghy, 1995) and experimental diabetes (Thornalley et al., 2003;Duran-Jimenez et al., 2009). The rate of AGE accumulation has been reported to be linked with glycaemic control, and the subsequent development of vascular complications (Goh and Cooper, 2008).

Our initial interest in examining whether MMP expression is altered in the peripheral nerve of diabetic rats derived from the observations that:

1. In diabetic neuropathy, structural changes are observed in the peripheral nerve including reduplication and thickening of the basement membrane as well as the build-up of ECM components, including COLL type-I,- III and -VI (Bradley et al., 2000;Giannini and Dyck, 1995). COLL is an substrate of MMPs, specifically MMP-2 and MMP-9 (Coussens and Werb, 1996;Butler and Overall, 2009).
2. We have previously shown accumulation of AGEs on intracellular and extracellular proteins of sciatic nerve endoneurium from STZ-induced diabetic rats, which occurs as early as 3 weeks post-STZ (Duran-Jimenez et al., 2009;Thornalley et al., 2003). In addition, we and others have demonstrated that neurite outgrowth from adult rat sensory neurons plated on glycated LM or glycated FN was significantly reduced compared to neurons plated onto unmodified ECM proteins (Duran-Jimenez et al., 2009;King, 2001;Luo et al., 2002;Ozturk et al., 2006).

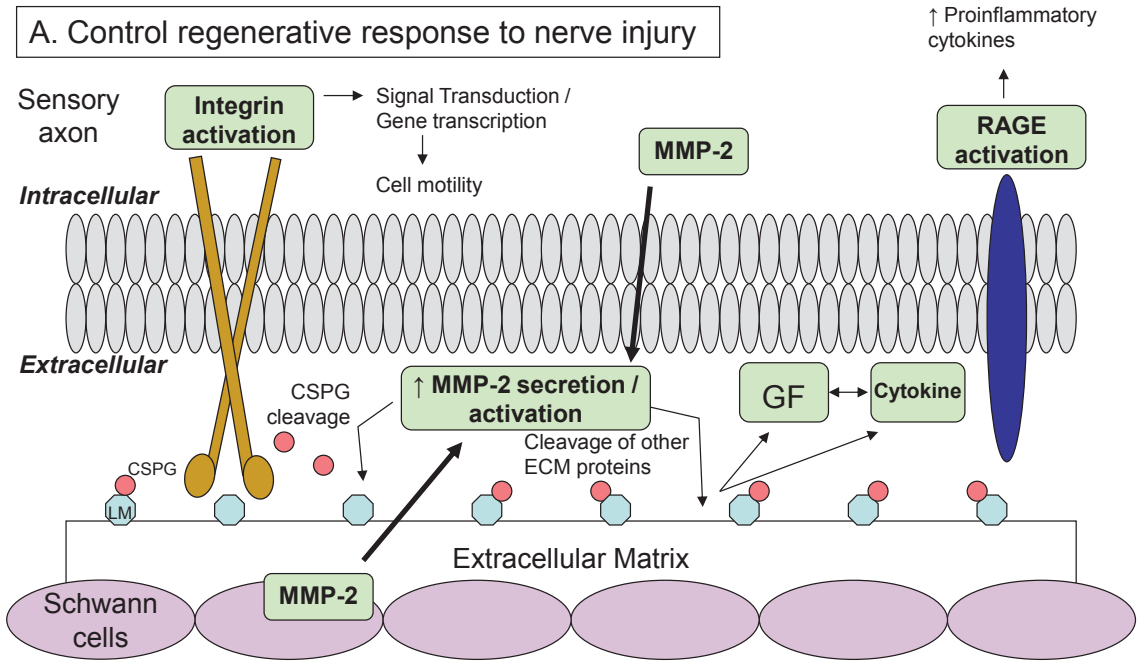
3. Impaired axonal regeneration is well characterised in clinical and experimental diabetes. In the STZ-model of diabetes the ability of nerves to regenerate is inversely proportional to duration of diabetes (Bisby, 1980;Ekstrom and Tomlinson, 1989;Kennedy and Zochodne, 2000), this may be linked with AGE accumulation.
4. Glycation of ECM protein confers resistance to proteolysis (Lubec et al., 1982;Lubec and Pollak, 1980;Pollak et al., 1982) and inhibits integrin binding (Dobler et al., 2006;Pedchenko et al., 2005).
5. MMP dysregulation occurs in diabetes with increased MMP-2, MMP-9 and TIMP-1 levels in serum and plasma samples from type 1 (Maxwell et al., 2001;Jacqueminet et al., 2006) and type 2 diabetic patients (Lee et al., 2005;Derosa et al., 2007) and also STZ-diabetic rats (Uemura et al., 2001).
6. Dysregulation of MMP activity has been implicated in the pathophysiology of other diabetic complications and dysfunctions including diabetic retinopathy (Giebel et al., 2005;Jacqueminet et al., 2006;Noda et al., 2003), chronic non-healing diabetic ulcers (Lobmann et al., 2006;Medina et al., 2005), coronary and peripheral arterial disease (Tayebjee et al., 2005a;Galis and Khatri, 2002;Tayebjee et al., 2005b) and, more recently, diabetic nephropathy (Thrailkill et al., 2007).

This current research project demonstrates the following key results:

- MMP-2 gene and protein is upregulated in the sciatic nerve of STZ-rats (8 weeks post-STZ), with conversion to the active form of MMP-2 in a discrete population of STZ diabetic rats.
- Peripheral nerve ECM of diabetic rats is less supportive of sensory axon regeneration compared to ECM from peripheral nerve of age-matched controls
- MMP-2 and -9 does not directly enhance neurite outgrowth from dissociated adult rat sensory neurons.
- Pre-treatment of peripheral nerve ECM of diabetic rats with either active MMP-2 or Chondroitinase ABC, removes inhibitory CSPGs and significantly increase neurite outgrowth on sciatic nerve from diabetic rats.
- Treatment with the AGE inhibitors, LR90 or PM, prevented development of sensory, but not motor, NCV deficits and regenerative deficits in diabetic rat ECM. However, neither AGE inhibitors protected against the development of tactile allodynia or mechanical hyposensitivity in diabetic rats.

This increased MMP-2 expression in the sciatic nerve of diabetic rats could be an attempt to remodel the ECM and potentiate axon regeneration in response to diabetes-induced structural alteration and/or axonal damage. Interestingly, this increase in MMP-2 expression is not maintained at 12 weeks post-STZ, which may coincide with regeneration failure. Indeed, in the early stages of diabetic neuropathy regenerating axons can be observed alongside degenerating axons in peripheral nerve biopsies (Sima et al., 1988), but over the timecourse of the disease the number of regenerating axons is decreased which leads to ultimate loss of distal nerve fibres (Ekstrom and Tomlinson, 1989). Therefore, could this failure to maintain MMP-2 production and activation signal the 'resignation' of the PNS in it's attempt to regenerate? We hypothesise that MMP-2 mediated proteolysis of inhibitory factors may also be compromised in the peripheral nerve of diabetic rats due to the build-up of AGEs and resistance to proteolysis. This failure to convert the peripheral nerve ECM to an environment favourable to growth would result in regeneration failure. Alternatively, MMP-2 may function normally; however, the presence of AGE-residues on unmasked ECM protein may result in altered cell-ECM and reduced axonal regeneration.

This final discussion chapter will focus on the potential mechanisms that could be altered in diabetes, leading to altered sensory neuron-ECM interaction and subsequent diabetes-induced failure of regeneration / reinnervation of target tissue. We will also discuss the potential therapeutic approaches of MMP-2 therapy and AGE inhibitors in the treatment of diabetic neuropathy.


A. Control regenerative response to nerve injury
↑ Proinflammatory cytokines
Sensory axon
Integrin activation
Signal Transduction / Gene transcription
Cell motility
MMP-2
RAGE activation
Intracellular
Extracellular
CSPG cleavage
↑ MMP-2 secretion / activation
GF
Cytokine
Cleavage of other ECM proteins
CSPG
LM
Extracellular Matrix
MMP-2
Schwann cells

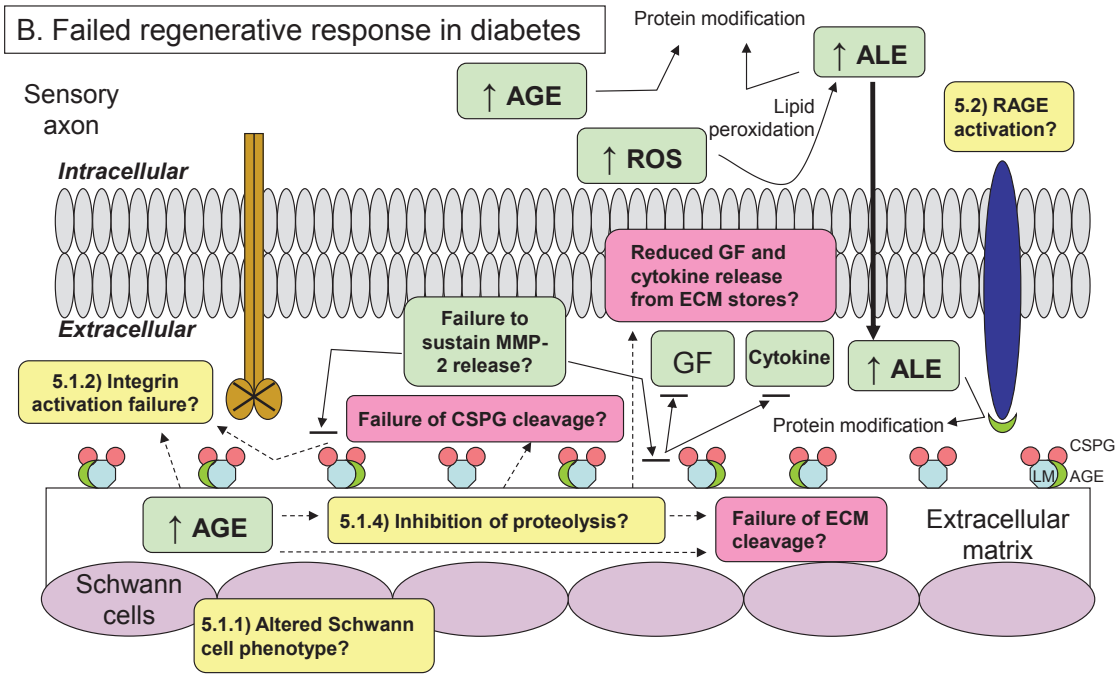

B. Failed regenerative response in diabetes
Protein modification
↑ ALE
↑ AGE
Lipid peroxidation
5.2) RAGE activation?
↑ ROS
Sensory axon
Intracellular
Extracellular
Reduced GF and cytokine release from ECM stores?
Failure to sustain MMP-2 release?
GF
Cytokine
↑ ALE
5.1.2) Integrin activation failure?
Failure of CSPG cleavage?
Protein modification
CSPG
LM
AGE
↑ AGE
5.1.4) Inhibition of proteolysis?
Failure of ECM cleavage?
Extracellular matrix
Schwann cells
5.1.1) Altered Schwann cell phenotype?

Figure 5.1: **A working hypothesis for the failed regenerative response in diabetes: focusing on the role of MMP-2 and AGE accumulation.** A) Following injury to a control peripheral nerve, MMP-2 is upregulated in sensory neurons and Schwann cells. This secreted/activated MMP-2 cleaves ECM proteins including CSPGs leading to the unmasking of LM and integrin binding sites. In addition, the cleavage of other ECM proteins leads to GF and cytokine release from ECM stores thus promoting axonal regeneration. B) In the peripheral nerve in diabetes, axon regeneration is reduced as the disease progresses. We hypothesise that the failure to sustain MMP-2 release over the timecourse of the disease and the concomitant accumulation of AGEs/ALEs on both intracellular and extracellular proteins may contribute to this failed nerve regeneration via: altered Schwann cell phenotype (Section 5.1.1.), failure of integrin activation (Section 5.1.2.), reduced proteolysis (Section 5.1.4) and altered RAGE activation (Sections 5.2.).

5.1. Functional consequences of glycation of the peripheral nerve

5.1.1. Changes in Schwann cell phenotype

Schwann cell interaction with both mature uninjured and regenerating axons is essential for successful nerve function. Following nerve injury, new outgrowth of axons beyond a transection of the peripheral nerve trunk involves an intimate partnership with extended Schwann cell processes (Chen et al., 2005). Following complete axonal transection, Schwann cell migration and proliferation precedes axon outgrowth. Proliferating Schwann cells migrate from the proximal stump forming a scaffold or "bridge" by secreting several basement membrane proteins, including LM and FN, onto which the regenerating axons can follow. In fact, few axons regenerate any significant distance without this partnership (Chen et al., 2005) and regenerating axons which enter the surrounding connective tissue instead of following the Schwann cell 'bridge' stop growing after a few millimetres (Ide, 1996). Following nerve crush, the Schwann cell basal lamina remains intact and this provides an essential scaffold for the successful elongation of the regenerating axons. Unlike nerve transection, new axon sprouts occur without any other cellular interaction and, interestingly, before full clearance of myelin and axon debris (Ide et al., 1983). These regenerating axons, without exception, associate with the inner surface of the Schwann cell basal lamina (Ide et al., 1983).

Bidirectional communication between the regenerating axons and Schwann cells occurs with each supporting the other. Axons signal to Schwann cells to proliferate and myelinate if they reach a certain calibre and maturity by producing mitogenic signals including

neuregulin (aka glial growth factor), PDGF-β, TGF-β, FGF, EGF and CGRP (Chen et al., 2005). These mitogenic signals overlap with autocrine Schwann cell molecules that further support Schwann cell survival, differentiation and proliferation (e.g. IGF-1, NT-3 and PDGF-BB). Schwann cells express LM, FN and other basement membrane constituents that are laid down into a basal lamina which provide an adhesive substrate for regenerating axons (Chen et al., 2005).

Multiple studies have used *in vitro* primary culture systems to investigate the direct effects on hyperglycaemia on sensory neuron and Schwann cell phenotypes (Gumy et al., 2007;Sango et al., 1995). Although culture systems cannot mimic the systemic changes that occur in diabetes, they do give a useful insight into the mechanism by which hyperglycaemia may acutely alter the autonomous functions of sensory neurons and Schwann cell (Vincent et al., 2005). The Tolkovsky group showed that the pro-regenerative functions of Schwann cells (i.e. proliferation and migration) are particularly sensitive to hyperglycaemia suggesting that in diabetes Schwann cell dysfunction could contribute to regeneration failure in diabetic neuropathy. Hyperglycaemia did not cause apoptosis of Schwann cell in culture (Gumy et al., 2007), this observation is consistent with *in vivo* observation where only a small population of Schwann cells show an apoptotic response in diabetes (Kalichman et al., 1998). Thereby confirming that reduced number of Schwann cells in the DRG explants was the result of a hyperglycaemia-mediated reduction in proliferation rate and not cell death (Gumy et al., 2007). Using a mitogenic inhibitor (AraC; cytosine arabinoside) a lower rate of neurite outgrowth was observed, which was more pronounced under hyperglycaemic conditions (Gumy et al., 2007). These results suggest that reduced axon regeneration in adult sensory neurons is partly dependent on interactions with proliferating Schwann cells, and also demonstrates that hyperglycaemia also affects neurons directly.

Other studies using an immortalised mouse Schwann cell line (IMS32) has shown that cells in high glucose (30mM) secrete significantly lower levels of NGF than those under the normal glucose condition (5.5mM) (Tosaki et al., 2008). Furthermore, dissociated adult mouse sensory neurons cultured in IMS32-(high glucose) conditioned media had significantly reduced neurite outgrowth compared to neurons cultured in IMS32- (normal glucose) conditioned media (Tosaki et al., 2008). This study demonstrates that the

hyperglycaemia results in altered expression and secretion of NGF, a pro-regenerative neurotrophin, by Schwann cells, which has a direct influence on axonal regeneration.

In addition to reduced neurotrophic support, there are altered expression of Schwann cell receptors, such as Erb B2 and $p75^{NTR}$, in diabetes. Erb B2 is a Schwann cell cell-surface receptor that interacts with neuregulin to control Schwann cell growth, survival and differentiation (Esper et al., 2006). Work by the Dobrowsky group observed that increased activation of neuregulin/Erb B2 in Schwann cells of sciatic nerve of STZ-diabetic rats correlated with decreased motor NCV and the development of mechanical and thermal hypoalgesia (McGuire et al., 2009). These diabetes-associated neuronal dysfunctions could be reversed by inhibition of Erb B2, and in addition Erb B2 activation alone was sufficient to mimic these accepts of peripheral neuropathy in the absence of diabetes (McGuire et al., 2009). These findings thereby indicate Erb B2 activation as a primary contributor to these particular indexes of diabetic neuropathy.

Increased $p75^{NTR}$ expression, a low affinity neurotrophin receptor, has been observed in Schwann cells of sciatic nerve in clinical and experimental diabetes (Conti et al., 2002;Scarpini et al., 1996). In adult PNS, $p75^{NTR}$ protects SCs against oxidative stress and apoptosis (Russell et al., 1999;Vincent et al., 2002). Therefore, it would be predicted that increase in $p75^{NTR}$ would be neuroprotective in diabetes. However, this remains unclear as Schwann cell may respond differentially to $p75^{NTR}$ signalling, depending upon their state of degeneration, regeneration and duration of hyperglycaemic exposure.

These studies raise the question as to whether axonal regeneration failure in diabetic neuropathy occurs in part as the result of altered hyperglycaemia-mediated changes in Schwann cell phenotype. Following nerve injury, Schwann cells upregulate MMP-9 mRNA and protein at the site of injury (Demestre et al., 2004;Ferguson and Muir, 2000;Hughes et al., 2002;Shubayev et al., 2006;Chattopadhyay and Shubayev, 2009). Following sciatic nerve crush injury, MMP-9 KO mice had significantly increased in resident, but not infiltrating, Schwann cells compared to wild-type mice, suggesting that MMP-9 suppresses Schwann cell proliferation *in vivo* (Chattopadhyay and Shubayev, 2009). MMP-9 controls myelin protein degeneration and macrophage migration into the injured sciatic nerve, with MMP-9 KO mice showing reduced Wallerian degeneration

(Chattopadhyay et al., 2007;Kobayashi et al., 2008;Shubayev et al., 2006). Multiple roles of MMP-9 have also been observed in adult mice treated with a general MMP inhibitor (Ilomastat) following nerve crush, in which MBP degeneration, macrophage influx and Schwann cell activation were attenuated (Kobayashi et al., 2008). Interestingly, MMP-9 also controls Schwann cell proliferation and phenotypic remodelling via IGF-1 and ErbB receptor-meditated activation of MEK/ERK 1/2 pathway (Chattopadhyay and Shubayev, 2009). Treatment of primary neonatal Schwann cells with recombinant MMP-9 inhibited Schwann cell mitosis, whilst both Erb B1 and Ilomastat reversed this anti-mitogenic action of MMP-9 (Chattopadhyay and Shubayev, 2009;Liu et al., 2010). MMP-9 also promotes Schwann cell migration via activation of LDL receptor-related protein (LRP-1) via ERK 1/2 and Akt activation (Mantuano et al., 2008). No information exists to date about the role of MMP-2 and Schwann cell phenotype.

We conducted preliminary experiments to assess the role of MMP-2, MMP-9 and Ilomastat on Schwann cell proliferation and migration in vitro (data not shown). Our results were largely negative, and with the release of the comprehensive papers above on the subject, these experiments were abandoned. Therefore, as we hypothesised that the upregulation of MMP-2 in the sciatic nerve observed at 8 weeks post-STZ is an attempt to regenerate, could this sustained release of MMP-2 and concomitant lack of MMP-9 upregulation by Schwann cells contribute to the reduced Schwann cell proliferation, MBP degeneration and macrophage influx and ultimately to regenerative failure in diabetes.

5.1.2. **Integrins-ECM interaction and axonal regeneration**

Successful elongation of regenerating axons depends on the expression of specific cell-surface receptors, such as immunoglobins, cadherin and integrins, and the presence of growth-promoting factors, such as LM, FN and COLL in the Schwann cell basal lamina. Signals to neurons from the ECM depend largely on integrins, a large family of α- β-heterodimeric transmembrane glycoproteins receptors (Humphries, 2000). Integrin binding with ECM ligands leads to conformational changes in the integrin heterodimer, clustering of activated integrin receptors and the formation of focal adhesion complexes. This adhesion complex provides the physical link between the intracellular actin cytoskeleton and the ECM, and also regulates the activation of numerous intracellular signalling

cascades and subsequent gene transcription, thereby influencing cell behaviour and phenotype (Taipale and Keski-Oja, 1997). Integrins have multiple ligands including various fibrous proteins such as COLL, FN and LM, as well as coagulation and fibrinolytic factors and CAMs (Humphries, 2000). In the PNS, integrins (predominately β1 associated integrins) mediate axonal guidance, adhesion and migration, Schwann cell migration and are therefore key molecules in neuronal and Schwann cell development and successful nerve regeneration (Milner et al., 1997;Previtali et al., 2001). A number of integrin subunits including α4, α5, α6, α7 and β1, are upregulated in sensory neurons during nerve regeneration, with their expression highest in the growth cones of developing and regenerating axons (Milner et al., 1997;Previtali et al., 2001). Multiple studies using adult sensory neurons have demonstrated the importance of interactions between neurotrophin and integrin-receptor signalling in neurite elongation (Tucker et al., 2005;Tucker et al., 2005;Gardiner et al., 2005;Tomaselli et al., 1993;Gardiner et al., 2007). ECM molecules, such as LM, COLL and FN, enhance neurite outgrowth from adult rat sensory neurons compared with an adhesive, but non-integrin activating, substrate poly-L-lysine (Tucker et al., 2005;Vogelezang et al., 2001). Integrin-dependent neurite outgrowth on matrigel and FN was attenuated by treatment with RGD (arginine-glycine-aspartate) peptides, and β1- and α5-integrin blocking antibodies (Tucker et al., 2005;Tucker et al., 2005;Vogelezang et al., 2001); whilst neurite outgrowth on LM is significantly reduced by β1- or α7-intergin blocking antibody (Tucker et al., 2005;Tucker et al., 2005;Gardiner et al., 2005;Tomaselli et al., 1993). These studies suggest that nerve regeneration is strongly correlated with, and perhaps dependent on, increased integrin expression. The lack of integrin upregulation in sensory neurons after central axotomy is thought to partly contribute to limited regeneration in the CNS (Wallquist et al., 2004). In addition, integrin-dependent neurite outgrowth can be further enhanced by the presence of neurotrophin such as NGF suggesting that integrin activation and neurotrophin signalling cooperate to enhance neurite outgrowth in adult sensory neurons (Tucker et al., 2005;Zhou et al., 2006). While neurotrophin and neurotrophin receptor levels are reduced in diabetes, there are no published data regarding integrin levels are diabetes. Although we have observed no alteration in integrin expression at mRNA levels (α5, α6, α7 and β1) in DRG from STZ-diabetic rats, post-translational modification may occur in diabetes (Gardiner, N.J., personal communication). In cell culture systems, glycation of COLL and FN has been shown to alter integrin activation.

Glycation of the RGD domains of COLL-IV inhibits renal and retinal endothelial cell adhesion and αvβ3 integrin binding (Dobler et al., 2006;Pedchenko et al., 2005). Similarly, glycation of FN results in reduced retinal endothelial cell attachment and spreading compared to on unmodified FN (Bhatwadekar et al., 2008b;McDonald et al., 2009). As our laboratory has previously shown that the RGD domain of FN is important for α5β1 integrin binding and subsequent neurite outgrowth, glycation of this domain may be responsible for impaired neurite outgrowth on glycated FN. Therefore, could we be observing hyperglycaemia / diabetes-induced reduction in integrin expression on the cell surface of sensory neurons, leading to reduced attachment to ECM and subsequent changes in intracellular signalling? Or could altered integrin activation result from glycation of integrin-binding sites on ECM? This may result in impaired protein-protein and protein-receptor interactions.

Interestingly, there is also evidence indicating the importance of MMP-integrin interaction in cell behaviour. MMP-2 directly binds to integrin αvβ3 on the cell surface on endothelial cells undergoing angiogenesis. It is thought that this anchoring of active MMP-2 to the cell surface enables collagen degradation and facilitates directed cellular invasion (Brooks et al., 1996). MMP-integrin interaction could also play a role in nerve degeneration and regeneration.

Little work has been done to investigate the direct effects of hyperglycaemia on cultured adult sensory neurons; however there is larger body of evidence of its effect on embryonic sensory neurons. Two studies completed by the Sango group, observed that DRG explants taken from STZ-diabetic mice have significantly greater number of regenerating neurites compared to control (that were longer then 400μm) when embedded into COLL-I solution in the presence of low or high glucose concentration (10mM or 30mM glucose) (Saito et al., 1999;Sango et al., 2002). These results suggests that experimental diabetes induces alteration in the production of growth enhancing genes independent of hyperglycaemia thereby enhancing the regenerative capacity of adult sensory nerve after axotomy in STZ-diabetic mice (Saito et al., 1999;Sango et al., 2002). However, this is in contrast to other studies, where no change in growth-associated genes in diabetes was observed (Burnand et al., 2004). Sensory ganglia taken from diabetic mice show considerable morphological

changes including swelling and migration of the nucleus, these changes are recognised to be chromatolysis, the cell body reaction after peripheral axon injury (Sango et al., 2002). This finding further suggests that this enhanced neurite sprouting could be the result of increased protein synthesis due to the initiation of regenerative response to injury resulting in the explanted DRG being pre-conditioned (Sango et al., 2002). However, as these studies used DRG explants and not dissociated sensory neurons the influence of other cell types on this enhanced neurite sprouting can not be excluded. Previous work by this group has studied the effects of diabetes on dissociated sensory neuron taken from adult mice (Sango et al., 1995). Sensory neurons taken from STZ-induced diabetic mice showed impaired attachment when plated on COLL, LM and FN, indicating that binding of sensory neurons to the ECM proteins is impaired by diabetes (Sango et al., 1995).

Interestingly, however, once attached the "diabetic" sensory neurons showed no difference in their survival and neurite elongation ability compared to control group (Sango et al., 1995;Akude et al., 2010). In fact, the Fernyhough group have only observed impaired adult sensory neurite outgrowth of "diabetic" neurons in the presence of oxidative stress (Zherebitskaya et al., 2009;Akude et al., 2010). 4-HNE is a highly reactive α, β-unsaturated aldehyde and a major product of lipid peroxidation, and is upregulated in the sciatic nerve of STZ-diabetic rats (Cunha et al., 2008;Brooke et al., 2010a). Following treatment with 4-HNE, sensory neurons from STZ-rats expressed morphological abnormalities and extended significantly shorter neurites compared to control (Akude et al., 2010). Using a sciatic nerve cryoculture assay, we observed a significant reduction (approximately 30%) in the ability of the peripheral nerve taken from diabetic rats to support axonal outgrowth of control sensory neurons compared to peripheral nerve from age-matched controls (Brooke et al., 2010b). Therefore, these results suggest that the production of ROS and AGEs/ALEs in the peripheral nerve ECM of diabetic rats may be contributing factors in nerve regeneration failure.

5.1.3. **Growth-inhibitory factors such as CSPGs / COLL IV**

We demonstrated a role for inhibitory CSPGs in regeneration failure in diabetic neuropathy using an *in vitro* sciatic nerve cryoculture system (Brooke et al., 2010b). Following nerve injury, the removal of CSPG by MMP-2 is a key event in successful regeneration.

Embryonic and adult sensory neurons plated onto control sciatic nerve sections pretreated with either active MMP-2 or ChABC extend significantly longer neurites compared to neurons plated onto non-treated sciatic nerve sections (Brooke et al., 2010b;Ferguson and Muir, 2000;Krekoski et al., 2001;Zuo et al., 1998). Removal of CSPGs (via either MMP-2 or ChABC treatment) improves axonal regeneration and functional recovery following CNS injury (Galtrey and Fawcett, 2007;Moon et al., 2001;Bradbury et al., 2002).

We have shown that exogenous active MMP-2 protein is able to cleave inhibitory CSPGs from the ECM of peripheral nerve from both diabetic and control rats, thereby significantly improving neurite outgrowth on sciatic nerve cryocultures from diabetic rats (Brooke et al., 2010b). However, this study has also raised some important mechanistic questions. We demonstrated that sensory neurons plated on LM release both MMP-2 and MMP-9 into the culture media over 48 hour incubation period (Brooke et al., 2010b). This increase in MMP-2 and MMP-9 secretion was sufficient to overcome the inhibitory nature of the peripheral nerve ECM when plated on sciatic nerve from control rats (Brooke et al., 2010b), presumably via local degradation of CSPG near the tip of the growing neurites. This hypothesis posed the question as to why we did not see similar degradation of CSPG by the sensory neurons plated onto cryoculture sections from diabetic rats. Assessment of the media for MMP-2 and MMP-9 activity should determine whether this increase occurs. Another important question to consider is does the endogenously produced MMP-2 cleave CSPGs in a similar fashion? Or are we in fact observing failed ECM proteolysis, resulting in compensatory MMP-2 upregulation? Furthermore, even if the endogenous MMP-2 is able to remodel the ECM via removal of inhibitory CSPGs and unmasking LM binding sites, this unmasked LM may be glycated, therefore inhibiting both integrin activation and axonal attachment. This work supported observations made in other diabetic complications, where accumulation of inhibitory factors such as CSPG and heparin sulphate proteoglycans (HSPG) in the basement membrane affected organ function (McCarthy et al., 1994;van der Pijl et al., 1998). In diabetic nephropathy, increased CSPGs and decreased HSPGs alter the structural/ functional dynamics of the glomeruli basement membrane directly contributing to renal dysfunction(McCarthy et al., 1994;van der Pijl et al., 1998). However, there is no evidence to date of increased CSPG levels in the peripheral nerve ECM in diabetic neuropathy. Although an increase has been described in the skin of Type 1 diabetic patients (van der Pijl et al., 1998), since this is the region where

degeneration and failure of nerve regeneration is first evident in diabetic neuropathy – this may be of crucial importance.

We therefore hypothesise that an increase in CSPGs may occur in the peripheral nerve and/or skin in diabetes, which contributes to the failure of axonal regeneration and loss of IENF, and remains an important avenue to explore further in future studies.

5.1.4. **Failure of MMP proteolysis**

The strongest evidence that failure of MMP proteolysis may occur during diabetic neuropathy is the accumulation of COLL type-I, -III and -VI in the peripheral nerve of diabetic patients (Bradley et al., 2000;Giannini and Dyck, 1995). This may be due to upregulation of ECM in diabetes (Price et al., 2006) or due to abnormal resistance of glycated ECM to digestion by MMPs (Lubec and Pollak, 1980;Lubec et al., 1982).

In addition to their matrix remodelling roles, MMPs also play a role in mediating bioavailability of growth factor and cytokines (Butler and Overall, 2009;McCawley and Matrisian, 2001). Proteolytic processing may release and activate, or disinhibit growth factors and cytokines anchored to cell surfaces associated with matrix proteins or sequestered by carrier proteins. This is a critical event in regulating active, "free" or dissociable growth factors or cytokines and facilitates their interactions with cell surface receptors (Fowlkes and Winkler, 2002). Numerous studies that revealed that MMPs influence many non-matrix substrates including growth factors (VEGF, FGF, TGF-β and NGF; (Bergers et al., 2000;Whitelock et al., 1996;Imai et al., 1997;Yu and Stamenkovic, 2000)), cleavage and activation of cytokines such as TNFα and IL-1β (Schonbeck et al., 1998;Haro et al., 2000), integrins (Von et al., 1997) and other cell-surface receptors including Fas-L and E-Cadherin, (Noe et al., 2001;Lochter et al., 1997;Powell et al., 1999;Mitsiades et al., 2001). Therefore could the diabetes-induced glycation of the peripheral nerve result in altered bioavailability of these growth factors and cytokines, thus contributing to failure of axonal regeneration in the diabetic peripheral nerve?

Pro-inflammatory cytokines including TNFα and IL-1β may stimulate "de novo" expression of MMPs, which in turn can also release other ECM-bound cytokines, which

can further upregulate MMP release in a positive feedback loop. The role of these cytokines in diabetic neuropathy is not well understood. In control nerve, IL-1β is upregulated by Schwann cells following injury and helps overcome the inhibitory nature of MAG and enhance nerve regeneration (Temporin et al., 2008b). In dissociated primary sensory neuron cultures, IL-1β-enhanced neurite outgrowth occurred via activation of the p38 MAPK pathway and deactivation of RhoA (Temporin et al., 2008a). Local administration of IL-1β to the site of nerve transection promoted sciatic nerve regeneration via increased Schwann cell proliferation, macrophage infiltration and upregulation of NGF synthesis from activated macrophages (Temporin et al., 2008b). In early stages of experimental diabetic neuropathy IL-1β co-localises with infiltrating macrophages and along nerve fibres. By 5 weeks post-STZ, macrophage clearance was completed and IL-1β became undetectable, and this study suggested that the decline in IL-1β by 5 weeks is a contributing factor to subsequent regeneration failure (Conti et al., 2002). This is a similar timecourse to the increase in MMP-2 seen in the sciatic nerve in early-stage of STZ-diabetes, with normalisation of MMP-2 by 12 weeks post-STZ.

TNF-α production has been shown to be increased in serum, plasma and urine in diabetic patients (Navarro and Mora, 2006;Gordin et al., 2008) and experimental diabetes (Tanaka et al., 1992). There is a significant increase in TNF-α levels in the spinal cord of diabetic mice (Comelli et al., 2010), but not in the peripheral nerve (Tanaka et al., 1992). Since IL-1β and TNF-α, are known to induce MMP-9 expression in the peripheral nerve, thereby promoting macrophages recruitment and subsequent MAG degradation following injury (Shubayev et al., 2006), it is surprising that we did not detect any MMP-9 upregulation in sciatic nerve in diabetes, an earlier timepoint should be assessed. It would be interesting to determine whether altered MMPs in diabetes leads to dysregulation of cytokine activity in the peripheral nerve and whether this plays a role in nerve dysfunction.

5.2. RAGE activation

Receptor of advanced glycation end-products (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules. RAGE is activated by one of its ligands; AGEs, HMGB1 (Amphoterin), S100b, and amyloid-β-protein. In both clinical and experimental diabetes, it is thought that the increased accumulation of AGE contributes to

RAGE upregulation and increased production of proinflammatory genes such as TNFα and IL-6 (Bierhaus et al., 2004;Toth et al., 2008).

The role played by RAGE in both peripheral nerve regeneration following injury and diabetes neuropathy has been extensively investigated by the Schmidt group (Rong et al., 2004;Bierhaus et al., 2004;Goova et al., 2001;Kislinger et al., 2001;Toth et al., 2008). Following sciatic nerve crush RAGE is upregulated in axons and infiltrating macrophages in the distal segment of the injured sciatic nerve and is involved in functional recovery. Adult control mice treated with either soluble RAGE or function blocking $F(ab')_2$ fragments of antibodies showed reduced macrophage infiltration, inhibition of recovery of NCV, reduced functional recovery assessed by walking track analysis and decreased numbers of myelinated fibers which suggests reduced muscle reinnervation (Rong et al., 2004). However, whether this suppression of regenerative mechanism is the result of diminished Wallerian degeneration or whether RAGE also plays a role in outgrowth of regenerating axons is unclear.

In experimental diabetic neuropathy, RAGE and its ligands, AGE and S100/calgranulin, is significantly elevated in DRG, sciatic nerve and epidermal fibres (Toth et al., 2008;Bierhaus et al., 2004). This increase in RAGE expression was shown to correlate with increases in RAGE-mediated signal transcription and the progression of pathological changes in diabetic nerve (Toth et al., 2008). One study by Beirhaus et al (2004) showed RAGE co-localises with accumulated AGE, and results in sustained activation of NF-κB and subsequently increased IL-6 expression, in the peripheral nerve of patients with diabetic neuropathy. In addition, exposure of sensory neurons to high glucose or CML caused the translation of NF-κB p65 to the nucleus in diabetic WT mice, whereas this response to the stimulus was largely prevented in diabetic RAGE KO mice. Finally, diabetic WT mice had activated NF-κB and increased IL-6 levels in sciatic nerve, which was attenuated in diabetic RAGE KO mice (Bierhaus et al., 2004). However, whether this activation of RAGE in the PNS of diabetic rats enhances nerve regeneration as seen in nerve injury model or contributes to nerve regeneration failure in diabetes is currently unknown. As this neurite-promoting role of RAGE of control sensory neurons stands in stark contrast to the beneficial role of RAGE inhibition observed in other diabetic complications. For example, treatment of STZ-diabetic mice with soluble RAGE restored

effective wound healing and attenuated vascular inflammation (Goova et al., 2001;Kislinger et al., 2001). In diabetic nephropathy and diabetic wound healing, blockage of RAGE suppressed levels of cytokines such as TNFα and IL-6, reduced the number of infiltrating inflammatory cells, decreased the components of ECM and suppressed matrix metalloproteinase -2, -3 and -9 expression (Goova et al., 2001;Kislinger et al., 2001). Therefore, RAGE activation in diabetic neuropathy could be a mechanism by which MMP-2 is increased in peripheral nerve.

The presence of RAGE in epidermal axons of skin footpads is of considerable interest, and coincides with reduction of epidermal nerve fibres, a physical alteration that is associated with loss of sensory function (Toth et al., 2008). Loss of pain perception was largely prevented in diabetic RAGE KO mice, but diabetic-induced loss of protein gene product(PGP)9.5- positive small diameter nerve fibres was still observed (Bierhaus et al., 2004). In fact, other structural and electrophysiological changes within the peripheral nerve were attenuated in the diabetic RAGE KO mice, including slowed NCV and altered epidermal fibre density (Toth et al., 2008). These finding suggested that the presence of RAGE in the small diameter nerve fibres is involved in loss of pain sensation, but not nerve fibre loss in diabetes. Therefore this data suggests that RAGE activation in the peripheral nerve in diabetes contributes to nerve dysfunction and development of neuropathic pain, but not nerve regeneration.

5.3. MMP-2 and -9 in neuropathic pain

Neuropathic pain is multifactoral and can arise from different levels including the terminals of the peripheral sensory nervous system in the skin (C and Aδ fibres), the involvement of Aβ and Aδ fibres to produce allodynia, reorganisation at spinal cord level and higher brain centres and dysregulation of descending inhibitory pathways (Vinik et al., 2004). The pathogenesis of diabetic painful neuropathy is poorly understood, although identification of impaired cutaneous endothelium-related vasodilatation and C-fiber mediated vasoconstriction as well as increased sural nerve epineurial blood flow in diabetic subject with painful, compared to those with painless neuropathy suggest the importance of hemodynamic factors (Obrosova, 2009). The development of tactile allodynia in clinical and experimental diabetes has been suggested to be the result of alteration in both

peripheral and central nervous system, involving a lowering of the usually high receptor threshold of nociceptors, alteration to sensory neuron cell bodies, and therefore heighted peripheral and spinal sensitisation (Woolf and Ma, 2007).

Studies investigating the role of MMPs in the regulation of chronic neuropathic pain following lesions in the PNS or CNS showed that MMP-9 plays a role in early phase pain hypersensitivity, whilst MMP-2 is involved in late phase of neuropathic pain (Kawasaki et al., 2008;Ji et al., 2009). After peripheral nerve injury, proinflammatory factors such as IL-1β, IL-6 and TNFα and MMP-2 and MMP-9 are upregulated in the nerve, DRG and by glial cells of the spinal cord (Kawasaki et al., 2008;Chattopadhyay et al., 2007;Ji et al., 2009). In the spinal cord, there is an initial early phase increase in MMP-9, which invokes microglial activation and a late stage increase in MMP-2 in astrocytes (Kawasaki et al., 2008). In DRG, there is a transient increase in MMP-9 expression in the neurons (Kawasaki et al., 2008), and a delayed and persistent expression of MMP-2 in the satellite cells (Ji et al., 2009). This injury-induced upregulation of MMP-9 in the DRG is induced by TNFα, since treatment with TNFα synthesis inhibitors following sciatic nerve ligation suppresses injury-induced MMP-9 expression (Kawasaki et al., 2008). The functional consequence of upregulation of MMP-2 and MMP-9 include cleavage of IL-1β in both PNS and CNS, whilst in the CNS, MMP-9 stimulates p38 activation in microglia (Kawasaki et al., 2008). Inhibition of IL-1β signalling and p38 activation, by neutralising antibodies or inhibitor respectively, blocked MMP-9 induced early stage allodynia (Kawasaki et al., 2008). Therefore, could the upregulation of MMP-2 in the DRG and sciatic nerve of STZ-diabetic rats be an indication of MMP-2 involvement in the development of diabetic neuropathic pain? The period of MMP-2 mRNA upregulation coincides with the development of tactile allodynia in diabetic rats 4 weeks post-STZ, MMP-2 may be transported to the spinal cord, or indeed upregulated within the spinal cord, thereby contributing to central sensitisation.

From the nerve-injury literature, we would expect the increase in MMP-2 to be accompanied by an earlier increase of MMP-9 in the DRG and sciatic nerve as well as upregulation in MMP-2 and MMP-9 in the spinal cord, therefore MMP expression at an earlier timepoint of diabetes should be examined. The role of MMP-9 in pain is well understood, MMP-9 KO mice display less tactile allodynia following either peripheral or

central nerve lesions (Kawasaki et al., 2008;Chattopadhyay et al., 2007). An intrathecal injection of either TIMP-1 (endogenous peptide inhibitor of MMP-9) or small interfering RNA (siRNA) against MMP-9 attenuates early stage neuropathic pain (Kawasaki et al., 2008). However, the MMP-9 deletion did not facilitate recovery from chronic neuropathic pain following either peripheral or central sensory nerve lesions (Kawasaki et al., 2008;Chattopadhyay et al., 2007). In the MMP-9 KO mice, a MMP-2 compensatory mechanism occurs; the treatment of these null mice with MMP-2 inhibitor produced a more profound anti-allodynia effect (Kawasaki et al., 2008). Similarly, blocking MMP-2 with TIMP-2 or MMP-2 siRNA attenuated late-stage neuropathic pain (Kawasaki et al., 2008).

Therefore, since MMP-9 is involved in early phase pain hypersensitivity, with its upregulation occurring prior to MMP-2 release following SNL(Kawasaki et al., 2008;Ji et al., 2009), perhaps MMP-9 is upregulated at an earlier timepoint in diabetes. Further studies to investigate spinal expression of MMP-2 and MMP-9 and their expression at earlier timepoints in the DRG and sciatic nerve of diabetic rats would provide a useful insight into their involvement in the development of diabetes-associated neuropathic pain. In addition, the regulation of other MMPs should also be considered. For example, MT5-MMP deficient mice develop mechanical hyperalgesia but not allodynia after sciatic nerve injury (Beiswenger et al., 2008;Komori et al., 2004).

5.4. MMP-2 as a potential therapeutic strategy in the treatment of diabetic neuropathy?

We hypothesise that upregulation of MMP-2 in the early-stage of diabetic neuropathy likely has beneficial pro-regenerative effects on the peripheral nerve, and that failure to sustain MMP-2 levels in the later-phase of diabetes coincide with the loss of regenerative ability. Therefore, modulation of MMP-2 levels, either pharmacologically or genetically, in the peripheral nerve at later stages of diabetes may boost regenerative ability and protect against loss of protective fibre and sensation.

A gene therapy approach either using adenovirus-mediated gene transfer or zinc finger protein-mediated endogenous gene activation (Pawson et al., 2010), could be utilised to sustain the increase in MMP-2 in diabetic neuropathy.

Overexpression of MMPs using adenoviruses has been described in arthritis, breast cancer and impaired wound healing; all diseases associated with altered ECM degradation (Joronen et al., 2004;Saghizadeh et al., 2010;Bendrik et al., 2008). For example, a single intra-articular injection of adenovirus coding for MMP-13 resulted in the recruitment of inflammatory cells and increased production of cytokines and chemokines in the knee joint of adult mice (Joronen et al., 2004). However, caution would be needed with such an approach, while upregulation in Schwann cells may be beneficial for nerve regeneration in the periphery, upregulation in the DRG and/or spinal cord may contribute to neuropathic pain. Therefore further studies are required using specific inhibitors of MMP-2 in diabetic rats to characterise its role in all aspects of nerve dysfunction (neuropathic pain, NCV deficits and regeneration failure) in diabetic neuropathy.

5.5. Use of LR90 or pyridoxamine as a treatment of diabetic neuropathy

Previously, we and others have shown AGE/ALE accumulation on both intracellular and extracellular matrix proteins in the sciatic nerve of diabetic rats (Cunha et al., 2008;Duran-Jimenez et al., 2009;Thornalley et al., 2003). We have also shown that exogenously glycated LM caused a significant reduction in neurite outgrowth from adult rat sensory neurons (Duran-Jimenez et al., 2009) and 'endogenously' glycated peripheral nerve taken from STZ-diabetic rats is less supportive of axonal elongation compared to nerve from age-matched controls (Brooke et al., 2010b).

Work described in Chapter 4 investigated whether two therapeutic AGE/ALE inhibitors (PM and LR90) were able to attenuate the development of functional deficits including slowed NCV, development of neuropathic pain and regeneration failure in STZ-diabetic rats. Previously, these AGE/ALE inhibitors have shown promising results in the treatment of both diabetic nephropathy (Degenhardt et al., 2002;Metz et al., 2003;Figarola et al., 2003) and retinopathy (Metz et al., 2003;Stitt et al., 2002;Bhatwadekar et al., 2008b). In this study, non-treated diabetic rats were hyperglycaemic, hyperlipidemic and showed functional deficits associated with diabetic neuropathy including NCV deficits and tactile allodynia. Treatment with LR90 or PM both prevented the development of sensory, but not

motor, NCV deficits at 8 and 12 weeks post-STZ. Diabetes-induced regenerative deficits observed when sensory neurons where plated onto sciatic nerve cryoculture sections taken from diabetic rats were significantly attenuated by LR90, but not PM, treatment. However, neither AGE/ALE inhibitor protected against diabetes-associated tactile allodynia and mechanical hyposensitivity.

Diabetes-associated hyperlipidemia was prevented by PM, and both LR90 and PM treatment prevented oxidative stress as measured by 4-HNE accumulation, but not AGE accumulation as measured by CML levels, in sciatic nerve of diabetic rats. Previously we have shown the accumulation of a number of AGE residues in the ECM of endoneurium from STZ-induced diabetic rats (Duran-Jimenez et al., 2009). This previous study used the highly sensitive method of liquid chromatography with tandem mass spectrometry to measure the accumulation of AGE residues over a 24 weeks timecourse of STZ-diabetes. In this current study, we used ELISA to quantify AGE accumulation in our treatment group, therefore the detection of no significant upregulation in CML in diabetes could reflect the lower sensitivity of this technique.

Several other groups have previously indicated that PM-treatment normalised diabetic-induced hyperlipidemia in STZ rats (Degenhardt et al., 2002;Degenhardt et al., 2002;Tanimoto et al., 2007;Hagiwara et al., 2009), and our current findings agree with these reports with PM treatment normalising both plasma triglyceride and free cholesterol levels in STZ-diabetic rats compared to age-matched controls. However, we observed no significant protection from diabetes-induced hyperlipidemia in LR90-treated diabetic rats, in contrast to previous reports in longer term STZ-induced diabetes (32 weeks diabetes; (Bhatwadekar et al., 2008a)) and Zucker rats (40 weeks diabetes; (Figarola et al., 2008)), which may reflect the different duration of disease (Figarola et al., 2003;Figarola et al., 2008;Bhatwadekar et al., 2008a).

In fact, the observation that LR-90 and PM did not normalise biochemical abnormalities in a similar fashion, points towards the different modes of actions of these drugs. PM inhibits AGE formation by binding to Amadori products thereby preventing their rearrangement into stable AGE residues, a process mediated by metal ions. Therefore could the metal chelation properties of PM contribute to this attenuation of diabetes-associated nerve

dysfunctions? Indeed, recent data presented in abstract form by Cameron and Cotter suggests that this is the case, PM which was modified to contain a metal-chelating imidazole group was over 2 orders of magnitude more effective in preventing diabetes-associated alteration to peripheral nerve function than unmodified PM (Cameron and Cotter, 2010). LR90 also has metal chelating properties; in addition LR90 is known to prevent the production of pro-inflammatory cytokines and the expression of RAGE and other inflammatory molecules in human monocytes (Bhatwadekar et al., 2008a). This additional anti-inflammatory property of LR90 could explain its ability to fully prevent axonal regeneration failure associated with diabetes-induced ECM alteration.

Neither LR90 nor PM protected against the development of diabetes-associated tactile allodynia and mechanical hyposensitivity. Neuropathic pain may therefore occur independently of AGE/ALE accumulation; or possibly the mode of treatment or drug dosage was ineffective. Neuropathic pain in diabetic neuropathy is notoriously difficult to treat or prevent. For example the aldose reductase inhibitor (ARI; ICI 222155) did not prevent tactile allodynia (Calcutt et al., 1996), but did effectively reverse other diabetes-induced functional deficits such as endoneurial blood flow, motor and sensory NCV (Matsumoto et al., 2008;Kawai et al., 2009;Matsumoto et al., 2008;Kawai et al., 2009;Greene et al., 1999;Matsuoka et al., 2007). Recent work from the Calcutt laboratory has shown that daily subcutaneous injection with a cocktail of vitamins B1, B6 and B12 is able to ameliorate tactile allodynia and formalin-evoked hyperalgesia as well as improve sensory NCV in a dose-dependent manner in 4 weeks STZ-diabetic rats (Jolivalt et al., 2009). However, the relatively high dosage (180:180:1.8 mg/kg) was required to achieve these beneficial effect, with low and medium dose (20:20:0.2 mg/kg and 60:60:0.6 mg/kg, respectively) used in previous studies (Ang et al., 2008). Therefore the lack of neuroprotection from neuropathic pain following LR90 and PM treatment may be the result of insufficient dosage.

To date, due to the limited number of studies completed, LR90 has only been used at a single dose in all trials (50mg/ml) and little is known about the drug metabolism. During, this current study, we used a lower dose of PM (400mg/L) than most published studies (For example 1g/L; (Alderson et al., 2003;Cameron et al., 2005;Degenhardt et al., 2002;Stitt et al., 2002)). However, this lower dose had previously been shown to reduce oxidative stress

and AGE accumulation in plasma of STZ-diabetic rats In previous reports, diabetic rats treated with 1g/L PM show plasma concentration up to 100µm PM (Degenhardt et al., 2002). If we assume that proportionally lower steady-state levels of PM occurs, rats in our study should have plasma concentration of PM around 40µm. Plasma methylglyoxal (MG) levels are estimated at approximately 5µm (Nagaraj et al., 2002), therefore 40µm should be sufficient to react with all the plasma MG. However, Degenhardt et al, (2002a) also described high concentration of PM in the urine of treated rats which suggests that most of PM is excreted unchanged in the urine (Degenhardt et al., 2002). Both doses of PM tested, reduced hyperlipidemia and AGE/ALE accumulation (Alderson et al., 2003;Cameron et al., 2005;Degenhardt et al., 2002;Stitt et al., 2002).

Another concept that needs to be considered is that the blood-brain/spinal cord-barrier (BBB) is a more effective barrier than the blood-nerve-barrier (BNB), therefore reduced diffusion of the AGE inhibitors into the CNS may limit the efficacy of the drugs. This may be particularly relevant in light of the failure to correct tactile allodynia which has a spinal component. Therefore, it would be useful to assess diabetes-associated tactile allodynia following central administration of the AGE inhibitors by implanted intrathecal osmotic pumps or injections. Of note, is the observation that AGE accumulation does occur in the CNS of diabetic patients.

5.6. Conclusion

In this study we describe upregulation of MMP-2 in the sciatic nerve in experimental diabetic neuropathy and suggest the potential for gene therapy in order to sustain MMP-2 levels, remodel the peripheral nerve ECM and facilitate nerve regeneration. Effective treatment of diabetic neuropathy will undoubtedly require a multiple pronged approach, including strategies that target biochemical abnormalities, NCV deficits, nerve regeneration failure and the development of neuropathic pain. We suggest that the AGE/ALE inhibitors PM and LR90 may be effective in such combination therapy in the treatment of diabetic neuropathy.

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