The role of Schwann cell c-Jun in restoring axon regeneration deficits in the peripheral nervous system

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I, Laura Jane Wagstaff confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Following peripheral nerve injury, myelin and Remak Schwann cells up-regulate a repair phenotype, controlled by c-Jun, to facilitate axon regeneration. Despite activation of this phenotype, nerve regeneration in humans is poor. An important factor is the deterioration of the distal nerve stump, a process involving a decrease in trophic factors initially up-regulated following injury and a decline in Schwann cell numbers, resulting in poor long-term regeneration. Regeneration deficits also develop with age - injured nerves in old mice regenerate slowly. I hypothesized that in old mice, and during chronic denervation, c-Jun levels are not maintained. This predicts that in young mice, c-Jun levels fall during chronic denervation, and that by maintaining Schwann cell c-Jun, the deterioration of the distal stump can be prevented. Similarly, by enhancing c-Jun levels in old mice, the regeneration deficit should be reversed.

In young mice, Schwann cell c-Jun significantly decreased during chronic denervation. Markers of the repair phenotype also declined. A mouse that over-expressed Schwann cell c-Jun (OE/+) was generated. Following chronic denervation, Schwann cells in denervated nerves maintained c-Jun levels. Regeneration was assessed by suturing a chronically denervated tibial nerve to a freshly cut common peroneal nerve, followed by neuronal backfilling. Improved motor and sensory neuron regeneration was observed in OE/+ mice following chronic denervation. In old OE/+ mice, the reduced c-Jun levels seen in old wild type mice were corrected. Regeneration was assessed by backfilling and old OE/+ nerves maintained the regenerative capacity of young animals. These results highlighted c-Jun as a pharmaceutical target. Sonic hedgehog is elevated after nerve injury and agonists significantly increased c-Jun and trophic factor expression in cultured Schwann cells.

This work implicates the c-Jun pathway in the regeneration deficit that occurs following chronic denervation and with advancing age, and highlights hedgehog signalling as an activator of c-Jun.

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List of abbreviations

ADS - Antibody Diluting Solution

ANOVA - Analysis of Variance

AP - Activator Protein

APS - Ammonium Persulphate

Ara-C - Cytosine Arabinoside

ATF - Activating Transcription Factor

BCA - Bicinchoninic Acid

BDMA - Benzyldimethylamine

BDNF - Brain-Derived Neurotrophic Factor

bLHL - Basic Helix-Loop-Helix

BrdU - Bromodeoxyuridine

BSA - Bovine Serum Albumin

BSU - Biological Services Unit

CAG - CMV Early Enhancer/Chicken β Actin

cAMP - Cyclic Adenosine Monophosphate

Cdc42 - Cell Division Control Protein 42

cDNA - Complementary DNA

cKO - Conditional Knock Out

CMT - Charcot-Marie-Tooth Disease

CNS - Central Nervous System

D - Days

DAPI - 4', 6-Diamidino-2-Phenylindole

DBD – DNA-Binding Domain

DDSA - Dodecenyl Succinic Anhydride

Dhh - Desert Hedgehog

DM - Defined Media

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide

DRG - Dorsal Root Ganglia

DS - Donkey Serum

E - Embryonic day

EDTA - Ethylenediaminetetraacetic Acid

ELISA - Enzyme-Linked Immunosorbent Assay

EM - Electron Microscopy

ER - Estrogen Receptor

ERK - Extracellular Signal-Regulated Kinase

FBS - Fetal Bovine Serum

Fl/fl - flox/flox

Fwd - Forward

GAP-43 - Growth Associated Protein 43

GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase

GDNF - Glial Cell-Derived Neurotrophic Factor

GFAP - Glial Fibrillary Acidic Protein

GFP - Green Fluorescent Protein

GFRα - GDNF-Family Receptor-α

Gli1 - Glioma-Associated Oncogene Homolog 1

HDAC - Histone deacetylases

Hh - Hedgehog

HRP - Horseradish Peroxidase

HS - Horse Serum

IFN - Interferon

IgG - Immunoglobulin G

IL - Interleukin

iNOS - Inducible Nitric Oxide Synthase

Jab1 - Jun Activation Domain-Binding Protein 1

JNK - c-Jun N-Terminal Kinases

LMCB - Laboratory for Molecular Cell Biology

LZ - Leucine Zipper Dimerization Domain

MAG - Myelin Associated Glycoprotein

MAPK - Mitogen-Activated Protein Kinase

MBP -Myelin Basic Protein

MCP - Monocyte Chemoattractant Protein

MNA - Methyl Nadic Anhydride

MRC - Medical Research Council

mRNA - Messenger Ribonucleic Acid

NgR - Nogo Receptor

NRG - Neuregulin

NTR - Neurotrophin Receptor

Oct-6 - Octamer-Binding Transcription Factor 6

OCT - Optimum Cutting Temperature

OE/+ - P_0 cre^+/c-$Jun^{OE/+}$ mice

OEC - Olfactory Ensheathing Cells

OE/OE - P_0 cre^+/c-$Jun^{OE/OE}$ mice

Olig1 - Oligodendrocyte Transcription Factor 1

P - Postnatal day

P_0 - Myelin protein zero

Pax - Paired Box

PBS - Phosphate-Buffered Saline

p-c-Jun - Phosphorylated c-Jun

PCR - Polymerase Chain Reaction

PFA - Paraformaldehyde

PLL - Poly-L-lysine

PLP - Proteolipid Protein

PMP22 - Peripheral Myelin Protein 22

PNS - Peripheral Nervous System

P/S - Penicillin/Streptomycin

qPCR - Quantitative Polymerase Chain Reaction

Rac1 - Ras-related C3 Botulinum Toxin Substrate 1

Rev - Reverse

RNA - Ribonucleic acid

RPM - Revolutions Per Minute

SAG - Smoothened Agonist

SCE - Schwann Cell Specific Enhancer

SDS - Sodium Dodecyl Sulphate

SEM - Standard Error of the Mean

Ser - Serine

Shh - Sonic Hedgehog

Smo - Smoothened

Sox - Sex Determining Region Y Box

Stat3 - Signal transducer and activator of transcription 3

T3 - L-Triiodothyronine

T4 - L-Thyroxine

TAE - Tris-Acetate-Ethylenediaminetetraacetic Acid

TBS - Tris-Buffered Saline

TBS-T - Tris-buffered Saline + Tween (1:1000)

TGF-β - Transforming Growth Factor Beta

Thr - Threonine

TMX - Tamoxifen

T-PER - Tissue Protein Extraction Reagent

TrkB - Tropomyosin Receptor Kinase B

UCL - University College London

UI - Uninjured

UT - Untreated

VEGF - Vascular Endothelial Growth Factor

Veh - Vehicle

W - Week/s

WT - Wild Type

1. Introduction

The peripheral nervous system has a remarkable capacity for regeneration compared to that of the central nervous system. This was thoroughly documented in the work of Henry Head over 100 years ago, who as well as describing the injuries of his patients, injured his own peripheral nerves and documented his recovery (Head & Sherren, 1905; Head et al., 1905). Ramón y Cajal observed that the neurons of the peripheral and central nervous systems were associated with different glia cells and that these glia were likely to be the reason for this contrast. Schwann cells had already been observed in the 19th century and are the myelin and non-myelin cells of the peripheral nervous system. They originate from the neural crest and go through various developmental stages that results in association with large or small calibre axons, the myelination of which is determined by size (Jessen & Mirsky, 2005; Monk et al., 2015; Feltri et al., 2016). In the adult nerve, Schwann cells maintain the myelin sheath and are neuroprotective for small calibre axons. When the nerve is injured a repair programme is activated to aid axon regeneration (Arthur-Farraj et al., 2012). Regeneration deficits are observed in the nerve with increasing injury time and age and few clinical advances have been made in the treatment of patients for over 50 years.

1.1 Schwann cell and peripheral nerve development

Schwann cell development is a multistage process that takes place from the initial generation of the nervous system and proceeds into early postnatal life. Schwann cells originate from neural crest cells which progress into Schwann cell precursors. Precursors differentiate into immature Schwann cells which become myelin or Remak Schwann cells depending on axon association. Both cell types then adopt a repair phenotype upon injury to aid peripheral nerve repair. Each of these stages is controlled by a variety of external and internal cues which will be described in the pages ahead (Jessen & Mirsky, 2005; Monk et al., 2015; Feltri et al., 2016). A summary of Schwann cell development is shown in Figure 1.1.

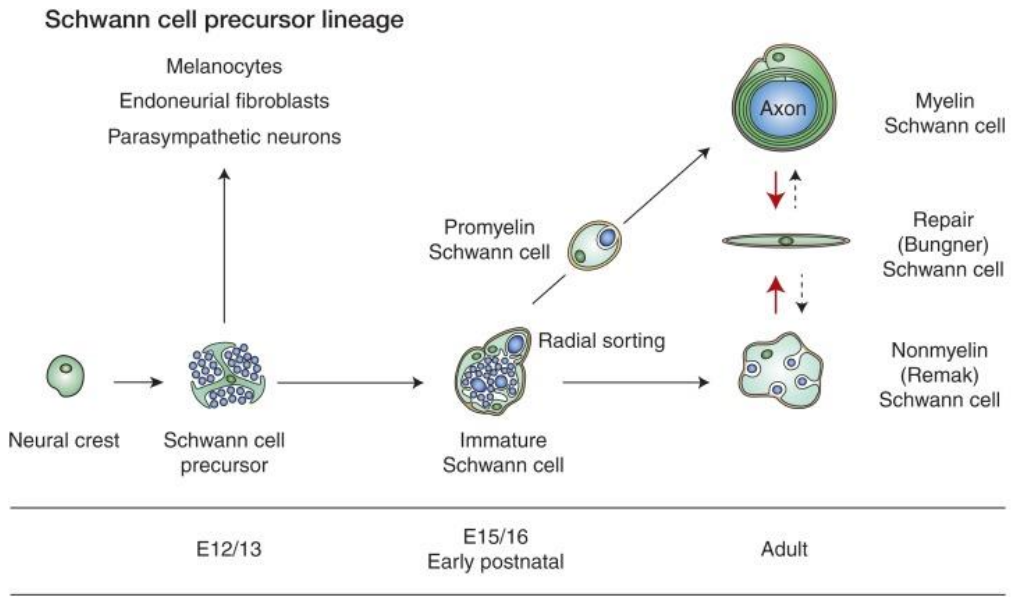


Figure 1.1 | A diagram of Schwann cell development

Neural crest cells initially express Sox 10. This is down-regulated by neuronal cells however remains elevated throughout the Schwann cell lineage. Schwann cell precursors differentiate to form immature Schwann cells. Immature Schwann cells become myelin or Remak Schwann cells after radial sorting pairs them with large or small calibre axons. Following injury, both cell types adopt a repair cell phenotype to promote axon regeneration. Taken from (Jessen et al., 2015)

1.1.1 The neural crest

Although Schwann cells are the primary glia of the PNS, other peripheral glia cell types include olfactory ensheathing cells - which associate with and protect olfactory axons, the terminal glia of neuromuscular junctions, satellite cells that associate with the cell body of sensory, sympathetic and parasympathetic ganglia, glia associated with sensory nerve endings and the enteric glia of the gut (Jessen & Mirsky, 2005). The peripheral nerves and glia originate from the neural crest (Harrison, 1924; Douarin, 1982; Le Douarin et al., 1991). Neural crest cells are highly mobile, multipotent cells that originate from the neuroepithelium. A single cell can give rise to multiple cells types (Bronner-Fraser & Fraser, 1988; Frank & Sanes, 1991; Baggiolini et al., 2015). Neural crest cells can migrate initially in a ventral direction and later in a dorsolateral direction. Peripheral neurons and glia are derived from the first waves of ventrally migrating neural crest cells (Weston, 1963; Le Douarin & Teillet, 1974).

Neural crest cells that express the transcription factor sex determining region Y box 10 (Sox 10) will generate cells of glia lineage and melanocytes. Mice that do not express Sox 10 do not develop peripheral glia despite normal neuron development, a feature also conserved in zebrafish (Britsch et al., 2001; Kelsh & Eisen, 2000). Neuregulin-1 (NRG1) promotes Schwann cell development and promotes the suppression of neurogenesis. *In vitro* neural crest cells differentiate to form neurons and glia, however in the presence in NRG1, only gliogenesis is observed. These experiments however examine a culture period of 2 weeks, allowing extensive precursor and Schwann cell proliferation, and use a culture medium that contains NRG. This finding therefore remains controversial (Shah et al., 1994). NRG1 is not required for the generation of all peripheral glia. Schwann cell precursors and Schwann cells are largely absent in the peripheral nerves of mice lacking the NRG1 co-receptor ErbB3, but satellite cells are still generated. Neurogenesis is not affected in these mutants, however excessive sensory and motor neuron death occurs during subsequent development due to the lack of neuroprotective Schwann cell precursors and Schwann cells (Riethmacher et al., 1997). Sox 10 regulates the expression of ErbB3 and together Sox 10 and NRG1 promote the survival of neural crest cells (Britsch et al., 2001; Paratore et al., 2001).

In turn, Sox 10 regulation is controlled by Pax 3. Pax 3 has been implicated in Schwann cell differentiation, proliferation and survival throughout the lineage (Kioussi et al., 1995; Blanchard et al., 1996; Doddrell et al., 2012). Pax 3 is activated by binding and activation of histone deacetylases 1 and 2 (HDAC1/2) to the Pax 3 promotor. Induction of myelin gene P_0 is also controlled by HDAC1/2 binding to the P_0 promoter. Satellite glia are reduced and no Schwann cell precursors are observed when HDAC1/2 is genetically deleted from neural crest cells. Pax 3 and Sox 10 expression are similarly reduced, demonstrating the importance of these transcriptional regulators in the specification of the Schwann cell lineage (Jacob et al., 2014).

With the activation of the appropriate transcription factors, neural crest cells specified towards gliogenesis mature into Schwann cell precursors.

1.1.2 Schwann cell precursors

Schwann cell precursors are observed in association with bundles of axons in the developing rat nerve at embryonic day 14 (E14) (Ziskind-Conhaim, 1988; Jessen et al., 1994). Although their appearance is correlated with the extension of nerves towards their targets, precursors are not required for initiation of this process but are required for the neuron survival and establishing connections with terminal targets (Reynolds et al., 1991; Grim et al., 1992; Riethmacher et al., 1997; Morris et al., 1999; Wolpowitz et al., 2000). Axon contact is similarly required for precursor survival, mediated by axonally derived NRG1. When precursors are removed from axonal contact *in vitro*, very few cells survive (Jessen et al., 1994). This effect however can be reversed in the presence of NRG1 (Dong et al., 1995). As previously described, a failure to respond to NRG1 signalling *in vivo* corresponds with an absence of Schwann cells in peripheral nerves (Riethmacher et al., 1997). This is not only due to a lack of precursor survival but an inhibition of their migration. ErbB2 mutants generate precursors within the DRG which proliferate normally but fail to migrate and associate with developing nerves. These however may be cells earlier in the lineage as Schwann cell precursors are always associated with axons (Morris et al., 1999). Schwann cell migration is similarly affected in zebrafish ErbB2 and 3 mutants (Lyons et al., 2005). NRG1 signalling is therefore required for Schwann cell precursor survival and migration and for the differentiation of precursors into immature Schwann cells (Dong et al., 1995; Brennan et al., 2000) .

1.1.3 Immature Schwann cells

The generation of immature Schwann cells from Schwann cell precursors is controlled by Notch dependent NRG1 signalling. Notch up-regulates ErbB2 receptors in precursors increasing NRG1 sensitivity. Notch also controls Schwann cell proliferation, with an increase observed following activation of the pathway (Woodhoo et al., 2009). Immature Schwann cells appear in the nerve at E15-17 in rats and E13-15 in mice (Jessen et al., 1994; Dong et al., 1995) where they control two important processes in the development of the nerve: (1) arteriogenesis and development of nerve structures and (2) the radial sorting of axons.

Arteries in the limb are aligned with the peripheral nerves. When Schwann cells or sensory nerves are absent, branching and arterial markers are disrupted. When nerves are present but their growth is disorganised, arterial alignment with misrouted axons is maintained (Mukouyama et al., 2002). Arteriogenesis is controlled by vascular endothelial growth factor (VEGF) derived from both nerves and Schwann cells (Mukouyama et al., 2005).

Immature Schwann cells also control the formation of normal nerve architecture of the epi, peri and endoneurium organisation through desert hedgehog signalling (Dhh) (Parmantier et al., 1999). Peripheral nerves are composed of 3 layer of connective tissue: an external epineurium, multiple fascicles surrounded by perineurium and axons surrounded by endoneurium. The perineurium is the barrier that controls cellular and molecular permeation of the nerve (Olsson, 1990) formed of compacted layers of epithelial-like cells with a basal lamina linked by gap and tight junctions. The epineurium is composed of collagen and fibroblasts (Thomas & Olsson, 1984). The epineurium of *Dhh* null mice however has reduced collagen and is less compacted. Lamination of the perineurium was reduced and layers that are present are decompressed. The cells of the perineurium have a disrupted basal lamina, blurring the boundary distinction between it and the epineurium. The blood nerve barrier is also compromised. Minifascicles are observed within the nerve, generated by perineural cells dividing groups of nerve fibres. The nerves of humans carrying *Dhh* mutations are also seen to contain these (Parmantier et al., 1999; Umehara et al., 2002; Sepideh N Bajestan et al., 2006; Sato et al., 2017). The expression of various

factors by immature Schwann cells are therefore required for normal peripheral nerve development.

Radial sorting is the process by which large and small calibre axons are sorted for myelination, a process which continues to postnatal day 10 in rodents (Figure 1.2). Immature Schwann cells mediate radial sorting by organising mixed calibre axons into bundles surrounded by basal lamina. This is performed by a unit or "family" of 3-8 Schwann cells. The larger axons are segregated to the periphery by the Schwann cells extension of cytoplasmic lamellipodia. As the Schwann cells proliferate they continually segregate single large axons from the bundle and form a 1:1 relationship with them. Smaller calibre axons remain unsegregated forming Remak bundles with one Remak cell associated with multiple small axons that remain unmyelinated (Webster et al., 1973; Jessen & Mirsky, 2005; Feltri et al., 2016) .

The molecular control of this process is not well characterised although many different molecules have been implicated in various stages of the process (Figure 1.2). Laminin is one of the components of the basal lamina and is required for the sorting of axons (Yang et al., 2005) and severe sorting defects have been reported as the consequence of laminin α2 gene mutation (Xu et al., 1994). A disruption in laminin receptors with the deletion of β1 integrin also results in disruptive sorting with immature Schwann cells failing to maintain and generate axon associated processes. This mutation is eventually lethal, emphasising the importance of radial sorting in development (Feltri et al., 2002; Pietri et al., 2004). *Cdc42* and *Rac1* Schwann cell mutant mice also show severe sorting defects, most likely by acting downstream of laminin signalling. Rac1 is required for the correct extension and stabilisation of Schwann cell processes whereas *Cdc42* is required for Schwann cell proliferation (Nodari et al., 2007; Benninger et al., 2007)

Similar deficits are observed in Jun activation domain–binding protein 1 (*Jab1*) null mice. The nerves of adult mice contain large bundles of unmyelinated axons of mixed calibre. Schwann cells surround these bundles and have extended lamellipodia but fail to proliferate. *Jab1* null Schwann cells presented with abnormal cell cycle progression caused by increased levels of p27, a negative regulator of the cell cycle. *Jab1* null mice also display lower levels of laminin suggesting *Jab1* regulates the laminin pathway (Porrello et al., 2014).

Radial sorting, in line with other stages of the Schwann cell lineage, is also regulated by NRG1. When signalling through ErbB is inhibited, Schwann cells are able to generate processes but are unable to extend them. Fewer Schwann cells are also observed in the nerve, suggesting that NRG signalling is required for Schwann cell proliferation and process extension during radial sorting (Raphael et al., 2011).

Sox 10, which is similarly required throughout the Schwann cell lineage is also required for radial sorting and the progression of Schwann cells from the immature stage. When Sox 10 is deleted specifically from Schwann cells at the precursor stage, the up-regulation of myelin and pro-myelination genes is not observed. Postnatally, myelination was absent resulting in alterations in nerve conduction and abnormal nerve architecture. The peripheral neuropathy that results from this mutation is lethal with poor survival past the seventh post-natal week (Finzsch et al., 2010).

Together these studies demonstrate the importance of both Schwann cell proliferation and process extension during radial sorting. Their work provides an insight into the molecular control of this process which is mediated by axons, Schwann cell receptors and nuclear activity.

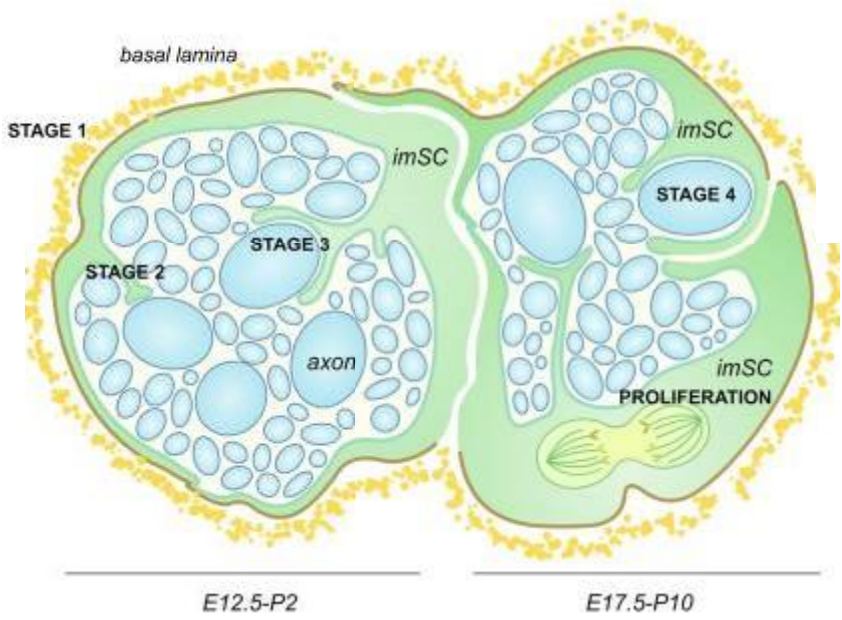


Figure 1.2 | A diagram of radial sorting

The diagram depicts the stages of radial sorting. Stage 1: Schwann cell axon units form and basal lamina is deposited. Stage 2: Schwann cells extend cytoplasmic lamellipodia. Stage 3 and 4: Larger calibre axons are recognised and segregated to the periphery. In the final stage (not shown) Schwann cells form a 1:1 relationship with the axon. Taken from Feltri et al., 2016.

1.2 Myelination

Radial sorting is the determining stage of Schwann cell fate. Schwann cells that remain associated with small calibre axons will become Remak (non-myelin) Schwann cells and these axons will remain unmyelinated. Schwann cells that have associated in a 1:1 relationship with axons greater than 1µm in diameter will become myelin Schwann cells and the axons will subsequently be myelinated (Matthews, 1968; Friede, 1972). During myelination, Schwann cells extend and wrap around axons in a spiralling motion. Schwann cells spread over the length of the axon then begin to deposit myelin as they rotate around the axon. The rate of movement around the axon is greatest during the early stages of myelination (Bunge et al., 1989). Myelination of the rat sciatic nerve results in axons with 100 layers of myelin with very few axons insulated by fewer than 10 (Friede & Samorajski, 1968). Myelin is essential for the rapid saltatory conduction of action potentials and conduction velocity increases with myelin thickness (Waxman, 1980). Failure of myelination or hypomyelination results in peripheral neuropathy and neuron death (Riethmacher et al., 1997; Lewis et al., 2003; Scherer & Wrabetz, 2008).

Myelination is controlled by the activation and repression of various factors including NRG1 (type III), Krox-20, Oct-6, and c-Jun.

1.2.1 Neuregulin

As previously described, NRG1, particularly type III, is essential for the progression of many stages of the Schwann cell lineage (Meyer et al., 1997). It is difficult to study the role of NRG1 in myelination as Schwann cell development is inhibited and the mutation is lethal. The relationship between NRG1 expression and myelin thickness was confirmed *in vivo* with mouse mutants expressing enhanced or reduced levels of neuronal NRG1 and ErbB receptors. Reduced NRG1 expression and signalling resulted in hypomyelination with very thin myelin sheaths observed on the axons. Enhancing NRG1 resulted in the increased wrapping of the axon with abnormally thick myelin sheaths present in adult mice (Michailov et al., 2004). *In vitro* studies have also been used to elucidate this mechanism. When NRG1 is removed from neurons, Schwann cells do not associate with or myelinate axons. This effect is reversed with the restoration of NRG1 through lentiviral infection. *In*

vitro appropriate NRG1 expression levels are not only required for the initiation of myelination but also determine the thickness of the myelin sheath formed. When neurons with varying levels of axonal myelination are compared, the axons with the thickest sheaths express higher levels of NRG1 (Taveggia et al., 2005).

1.2.2 Krox-20

The transcription factor Krox-20 is expressed by Schwann cells before the onset of myelination and is only observed in pro-myelinating Schwann cells (Murphy et al., 1996; Parkinson et al., 2004). Schwann cells of *Krox-20* null mice, although able to radially sort axons, form 1:1 relationships with axons and to perform one and a half turns around the axon, fail to form myelin sheaths (Topilko et al., 1994). Krox-20 controls Schwann cell myelination through the activation of myelin-differentiation genes (Parkinson et al., 2003) and inhibits further Schwann cell proliferation stimulated by NRG1 (Parkinson et al., 2004). Krox-$20^{-/-}$ Schwann cells do not exit the cell cycle and continue to proliferate resulting in increased postnatal apoptosis (Zorick et al., 1999).

1.2.3 Oct-6

Oct-6 is a POU domain transcription factor that is also referred to as SCIP or Tst-1. It is expressed in Schwann cells from E16. Expression of Oct-6 gradually decreases postnatally. Following nerve injury, it is briefly up-regulated following axotomy but is highly expressed in the regenerating nerve following a crush injury where it likely to aid remyelination (Scherer et al., 1994). Oct-6 expression is controlled during development and regeneration by the Schwann cell specific enhancer (SCE) located distally to the transcriptional start site (Mandemakers et al., 2000). Oct-$6^{-/-}$ Schwann cells myelinate however the process is delayed. Radial sorting is unaffected, with a 1:1 relationship observed and normal lamellipodia extension. Oct-$6^{-/-}$ Schwann cells are therefore able to initiate myelination but are unable to progress. Myelination is normally observed from P4 in mice yet is not observed in mutant mice. Myelin sheaths are first observed in mutants at P16 with only the largest calibre axons

myelinated. Of those that are myelinated, the majority present with normal nerve architecture (Jaegle et al., 2003).

Although myelination is delayed in Oct-$6^{-/-}$, it resumes at later stages in development. This suggests a compensatory mechanism is in place. Brn-2 is another POU transcription factor with almost identical binding to Oct-6, however its activation is independent of Oct-6. Over-expression of Brn-2 in Oct-$6^{-/-}$ mice reduces the delay in myelination however a double knock out of Brn-2 and Oct-6 further delays the myelination of nerves. Nerves are hypomyelinated and are consistent in appearance with those of early postal animals (Jaegle et al., 2003). It has been demonstrated that Oct-6 and Brn-2 directly bind to Krox-20 activating and enhancing myelination in cooperation with Sox 10 (Ghislain et al., 2002; Ghislain & Charnay, 2006), the dimeric binding of which with core element 1 to the SCE, regulates Oct-6 expression (Jagalur et al., 2011).

1.2.4 The role of c-Jun in myelination

So far, the activation of various transcription factors have been described to regulate myelination. However myelination also requires the down-regulation of many factors, such as Sox2 (Le et al., 2005) to commence. c-Jun is expressed in immature Schwann cells at E17. Krox-20 however down-regulates c-Jun before initiating myelination (Parkinson et al., 2004). If immature Schwann cells were to continue to express c-Jun, expression of Krox-20 regulated myelin genes such as P_0 and periaxin would be inhibited which actively prevents axon myelination. c-Jun is therefore a negative regulator of myelination (Parkinson et al., 2008). c-Jun is expressed at low levels in adult nerves and is almost undetectable. c-Jun is highly up-regulated again following injury (De Felipe & Hunt, 1994; Shy et al., 1996; Arthur-Farraj et al., 2012). The role of c-Jun in injury will be discussed later in this chapter.

1.2.5 Myelin sheath composition

The resulting myelin sheath that is generated is composed of protein and lipids. Other membranes have a high protein content whereas myelin is approximately 30% protein, 70% lipid (Morell & Quarles, 1999). Myelin protein zero (P_0) is the main component of the myelin sheath (Greenfield et al., 1973). P_0 is required for both the generation and compaction of the myelin sheath, as demonstrated by knock-out studies. Schwann cells in $P_0^{-/-}$ mice show wrapping defects around axons which become hypomyelinated. As the mice age, myelin sheaths decrease in thickness and onion bulbs form, indicative of active demyelination. Due to the lack of myelin sheaths, axon degeneration is later observed (Giese et al., 1992; Martini, Zielasek, et al., 1995; Martini, Mohajeri, et al., 1995). This demonstrates the role of P_0 not only in the generation of myelin and its importance in development but also its role in the maintenance of the myelin sheath.

PMP22 is a glycoprotein that comprises 2-5% of compact myelin (Snipes et al., 1992; Pareek et al., 1993). *PMP22* deficient mice display delayed myelination despite normal radial sorting. Some axons are myelinated, with excessive loops of myelin leading to hypermyelination. Due to the disorganised structure of the myelin, the sheath degrades and axons later degenerate (Adlkofer et al., 1995). *PMP22* over-expression results in hypermyelination of small calibre axons yet hypomyelination of large calibre axons (Verhamme et al., 2011; Hantke et al., 2014). Duplications in the *PMP22* gene are responsible for a majority of human Charcot-Marie-Tooth neuropathies (Shy et al., 2008). Appropriate expression of PMP22 is therefore required for the correct generation of the myelin sheath and regulates thickness and organisation in ways that are not properly understood.

Myelin basic protein (MBP) composes 15-20% of PNS myelin (Lees and Brostoff, 1984). It appears to play a greater role in CNS myelin as demonstrated by the *Shiverer* mouse which generates no MBP, fails to compact the myelin sheath and has a characteristic shivering gait. Although the CNS is hypomyelinated, peripheral nerve myelination is largely normal, suggesting MBP does not play an important role in the generation or maintenance of peripheral myelin (Readhead & Hood, 1990).

Myelin associated glycoprotein (MAG) exists as 2 isoforms, S-MAG and L-MAG (Lai et al., 1987). S-MAG is predominately expressed in the PNS throughout

development with only low levels of L-MAG observed (Tropak et al., 1988). MAG is confined to the periaxonal portion of the myelin sheath. It is also expressed in the uncompacted myelin of the Schmidt-Lanterman incisures (Sternberger et al., 1979).

As previously described, myelin is mainly composed of lipids. Cholesterol constitutes 26-28% of myelin lipid content (Norton & Cammer, 1984) and limits the rate of myelin production. Conditional knock outs in which cholesterol biosynthesis or lipid homeostasis is disrupted in Schwann cells generate hypomyelinated nerve fibres and peripheral neuropathy. *In vitro,* mutants are able to myelinate when in co-culture with DRGs, however this is largely dependent on the application of endogenous lipids (Saher et al., 2009; Verheijen et al., 2009). When tellurium, an inhibitor of cholesterol biosynthesis, is orally administered to newly weaned rats, progressive demyelination is observed (Lampert et al., 1970; Harry et al., 1989). This demonstrates that cholesterol is not only required for myelin synthesis, but also for maintenance of the myelin sheath.

Galactolipids comprised of galactosylceramides and sulfatides constitute 20-30% of the other myelin lipids and are the main components of the myelin sheath's outer surface (Eckhardt, 2008). Genetic mutations of sulfatides form regular myelin sheaths and display normal nerve conduction. Slight abnormalities are however present in myelin compaction and paranodal loop formation (Honke et al., 2002; Marcus et al., 2006). However if both galactosylceramides and sulfatides are mutated, nerve conduction velocity is impaired. The loss of these components is not compensated for by other lipids, therefore mutations result in severe neuropathy and eventual death (Bosio et al., 1996).

1.3 Remak Schwann cells

Schwann cells that do not form 1:1 relationships with axons and remain with bundles of small calibre axons do not generate myelin. These are referred to as Remak cells. They outnumber the myelin forming cells and are spaced closer together along axons compared to their myelin counterparts (Ochoa & Mair, 1969; Aguayo et al., 1973). Although they do not myelinate, NRG1 is still required for maintaining bundle size. Specific sensory neuron NRG1 ablation generates larger, closely packed Remak bundles, the axons of which are not separated by Schwann cell cytoplasm (Fricker et al., 2009). NRG1 signalling through ErbB is also essential for Remak cell survival in the adult nerve. When signalling is inhibited, Remak cell apoptosis and sensory function is impaired as a result of C-fibre degeneration (Chen et al., 2003).

Both Remak and myelin Schwann cells activate a repair cell phenotype following injury. This is controlled by c-Jun as will be discussed later in this chapter.

1.4 Types of injury

Peripheral nerve injuries are common, with approximately 100,000 peripheral nerve surgeries performed in the USA and Europe every year (Kelsey, 1997). Peripheral nerve injuries are common in the military. Combat induced injuries are largely localised to the extremities and nerve damage is caused from explosives and shrapnel (Korzinek, 1993; Chandler et al., 2017). In civilian life, the most common cause of peripheral nerve injury is motor vehicle collision (Noble et al., 1998).

The success and rate of axon regeneration can depend largely on the type of injury sustained by the nerve. Although the varying forms of these injuries have been classified (Seddon, 1942; Sunderland, 1951), axonal damage in nerve injury can be broadly attributed to a crush or cut injury. A crush injury is more favourable to regeneration. The basal lamina of both Schwann cells and axons remain intact allowing the axons to easily regenerate. After the nerve is cut however, the basal lamina and surrounding connective tissues are disrupted. If the proximal and distal nerve stumps are not re-joined, a tissue bridge, composed of matrix and inflammatory cells will form in the gap. The axons must overcome this distance to reinnervate targets. Schwann cells are sorted by fibroblasts and axons are guided through the bridge by Schwann cells, which move along blood vessels towards the distal nerve stump. (Morris et al., 1972; Jurecka et al., 1975; Friede & Bischhausen, 1980; Meller, 1987; Parrinello et al., 2010; Cattin et al., 2015).

1.5 Wallerian Degeneration

Following a peripheral nerve cut or crush injury, a cascade of events in the distal nerve stump occurs to allow for axon regeneration. This process was first described by Augustus Waller in 1850 and is aptly named Wallerian degeneration (Waller, 1850). Following insult, axons degrade and debris is cleared through macrophage infiltration. Schwann cells proliferate and aid axon regeneration through the formation of the bands of Büngner and the release of trophic factors. The careful orchestration of all these stages is required for successful axon regeneration and reinnervation of targets.

1.5.1 Axon survival and degradation

One of the earliest responses of the nerve to injury is the repair of plasmalemmal damage whereby the axons 'seal' the ends of the axons. This occurs within minutes of axonal damage and is mediated by Ca^{2+} influx resulting in the accumulation of Ca^{2+} dependent vesicles and other membrane-bound structures such as synaptotagmin and syntaxin. When vesicle trafficking and formation is inhibited, plasmalemmal sealing is impaired (Spaeth et al., 2010, 2012). Sealing is similarly impaired when Ca^{2+} binding to synaptotagmin or syntaxin is inhibited (Detrait et al., 2000), overall demonstrating the importance of Ca^{2+} dependent vesicle formation and fusion in plasmalemmal sealing. Plasmalemmal sealing impairment inhibits regeneration and compromises neuronal survival (Yoo et al., 2004; Nguyen et al., 2005).

Comparatively, axon degeneration is a much slower response. Axons appear normal for the first hours post injury with swelling and beading appearing 8 hours after injury (Zhai et al., 2003). The nerves are even able to conduct action potentials during this time (Moldovan et al., 2009). Degeneration is initiated after several days in humans (Chaudhry & Cornblath, 1992). In rodents, degeneration of thin fibres begins 22 hours after injury. The timing of fibre degeneration is determined by calibre, with the largest axons initiating degeneration last (after 32 hours). Fragmentation is initiated at the injury site and spreads distally following cut, but has been reported to proceed proximally following crush (Lubińska, 1977; Lunn et al., 1990; Beirowski et al., 2005).

The mechanism by which axon degeneration occurs is currently poorly understood. The Wld^s mouse undergoes slow axon degeneration following injury (Lunn et al., 1989). Studies have found that this response is intrinsic to the neuron (Glass et al., 1993) and is due to the over-expression of Ube4b/Nmnat (Wld) gene (Mack et al., 2001), implicating the deactivation of this pathway in axon degeneration following injury. The Schwann cell response to injury in these mice is also inhibited, demonstrated in the delayed activation of c-Jun (Jessen & Mirsky, 2008).

More recent experiments have shown that the nucleotide precursor of nicotinamide adenine dinucleotide (NAD), nicotinamide mononucleotide (NMN), accumulates in axons after normal nerve injury promoting axon degeneration (Di Stefano et al., 2015).

1.5.2 The macrophage response

In the uninjured nerves there is a small resident population of macrophages present that constitute 2-4% of the cell population (Oldfors, 1980; Perry et al., 1987). Following injury, macrophages are recruited within 1-3 days and remain present in the nerve for up to 3 weeks where they play a key role in the later stages of myelin breakdown. Macrophages largely gather around the injury site and diffuse in number distally (and proximally after crush) (Perry et al., 1987; Stoll et al., 1989; Raivich et al., 1991; Perry et al., 1995; Liu et al., 1995; Hirata et al., 1999; Hirata & Kawabuchi, 2002). Macrophage recruitment is also observed at a secondary site around the axotomized neuronal cell bodies where their presence is required for neurite outgrowth (Lu & Richardson, 1993; Schreiber et al., 1995; Niemi et al., 2013). Macrophage myelin phagocytosis is regulated through the complement receptor-3 and the scavenger receptor AI/II. Inhibition of these receptors results in reduced myelin clearance (Brück & Friede, 1991; Makranz et al., 2004). Macrophages leave the nerve with the re-emergence of axons through Nogo receptor (NgR) upregulation. In crushed nerves were NgR expression is inhibited, the efflux of macrophages is impaired (Fry et al., 2007).

When macrophage infiltration, migration and polarization in the injured nerve is altered, the rate of axon regeneration is reduced (Dailey et al., 1998; Barrette et al., 2008; Vargas et al., 2010; Chen et al., 2015). This is likely not due solely to the

presence of inhibitory debris which is normally cleared by macrophages because when macrophage infiltration is inhibited, myelin breakdown by Schwann cells is still observed particularly in the early days of injury (Perry et al., 1995). Macrophages also support axon regeneration through the secretion of trophic factors and cytokines (Brown et al., 1991; Reichert et al., 1996; Shamash et al., 2002) and promote vascularisation of the nerve bridge between proximal and distal nerve stumps through the secretion of vascular endothelial growth factor A (VEGF-A) (Cattin et al., 2015).

1.5.3 The Schwann cell response

Throughout the field, the Schwann cell response to injury has long been described as a process of de-differentiation in which the Remak and myelin Schwann cells revert to an immature Schwann cell to facilitate axon regeneration although other papers describe the response as activation (Chen et al., 2007; Jessen & Mirsky, 2008; Webber & Zochodne, 2010). Recent work however is arguing that the response is more complicated than this and that the cell is adopting a new phenotype which represents a combination of dedifferentiation and activation, a repair cell phenotype mediated by c-Jun activation. Although controversial, this is a logical theory. Schwann cells in the injured nerve exhibit a different genetic profile compared to immature Schwann cells. *Olig1, Shh* and *GDNF* are all up-regulated in Schwann cells following injury yet they are not expressed by immature Schwann cells (Arthur-Farraj et al., 2012). Novel behaviours are also exhibited by these cells. They break down myelin through the activation of autophagy (Gomez-Sanchez et al., 2015) and elongate to form the bands of Büngner, during which they elongate to 7-10x the size of immature Schwann cells (J A Gomez-Sanchez et al., 2017).

In response to injury, Schwann cells must undergo two phases of change. The first is the down-regulation of their myelin phenotype. Myelin genes such as MBP and P_0 are down-regulated (Chen et al., 2007; Jessen & Mirsky, 2008; Arthur-Farraj et al., 2012). As well as down-regulating myelin gene expression, Schwann cells initially clear myelin debris from the injured nerve in the days post injury, preceding the macrophage response (Perry et al., 1995). Myelin disruption originates at the Schmidt-Lanterman incisures 36 hours after injury, resulting in myelin ovoid

formation in an actin-dependant process (Ghabriel & Allt, 1979a, 1979b; Jung et al., 2011). The Schwann cell mediated removal of myelin has long been described as phagocytosis. Recent work however has demonstrated that it is however autophagy (Gomez-Sanchez et al., 2015), a process by which newly formed membranes (phagophores) engulf internal debris and transport it to the lysosome for degradation in a double-membraned autophagosome (Davidescu et al., 2012). The importance of autophagy in many mechanisms is now heavily emphasised and Yoshinori Ohsumi's work on determining the mechanism of autophagy lead to his award of the Nobel prize in 2016.

The second phase of transition Schwann cells undergo is the activation of the repair programme. This results in the up-regulation of trophic factors such as BDNF and GDNF (Meyer et al., 1992; Funakoshi et al., 1993; Naveilhan et al., 1997; Höke et al., 2000, 2002). Arthur-Farraj et al (2012) characterised the injury response in mice with c-Jun specifically knocked out in Schwann cells (c-Jun cKO mice). Although these mice develop normally, striking differences were found following nerve injury. First, c-Jun cKO Schwann cells did not down-regulate their myelin phenotype. Many myelin associated genes such as MBP and P_0 were still highly up-regulated following injury. Genes associated with axon regeneration such as GDNF, BDNF, artemin and GAP-43 where consequently not up-regulated. The formation of the bands of Büngner in these mice was distorted and Schwann cell numbers were reduced. Together, these factors result in poor axon regeneration and neuronal death, demonstrating the importance of Schwann cell c-Jun activation following nerve injury (Arthur-Farraj et al., 2012). These findings were further supported by the work of Fontana et al (2012). Also using the c-Jun cKO mouse, they observed reduced expression in GDNF and artemin following facial nerve injury. This finding was also accompanied by poor survival and regeneration of motor neurons (Fontana et al., 2012). Despite the importance of the c-Jun pathway, as highlighted by these authors, other members of the mitogen-activated protein kinase (MAPK) signalling pathway are also activated in Schwann cells following injury.

p38 MAPK is activated by Schwann cells almost immediately following injury (Myers et al., 2003; Zrouri et al., 2004). Activation of this pathway in myelin co-cultures results in the down-regulation of the myelin Schwann cell phenotype and active demyelination. Furthermore, inhibition of this pathway after injury prevents

the down-regulation of myelin genes. Activation of p38 is sufficient to induce c-Jun expression in Schwann cells, suggesting repair cell activation may be the consequence of down-stream p38 signalling (Yang et al., 2012). However recent work has demonstrated that axon regeneration is not affected when p38alpha is ablated in Schwann cells (Roberts et al., 2017).

The Ras/Raf/ERK signalling cascade controls the expression of many genes, including c-Jun (Minden et al., 1994). Following injury, phosphorylated ERK is up-regulated in Schwann cells. Activation of ERK signalling in myelin Schwann cells is sufficient to induce demyelination through the down-regulation myelin genes (Harrisingh et al., 2004). ERK is activated is both proximal and distal nerve stumps following injury and remains elevated in the nerve in the weeks that follow (Sheu et al., 2000). When ERK signalling is inhibited following nerve injury, expression of myelin genes continues, demonstrated that ERK is required for down-regulation of the myelin phenotype. Prolonged activation of the ERK pathway however prevents functional motor recovery, suggesting ERK signalling must be down-regulated for Schwann cells to initiate remyelination (Napoli et al., 2012). As well as down-regulating the Schwann cell myelin programme, ERK signalling also promotes macrophage recruitment. When ERK signalling is activated in uninjured nerves, inflammatory cells, such as macrophages, mast cells and neutrophils are recruited, in a timescale similar to that observed following injury. This is due to the activation of cytokines controlled by ERK signalling such a MCP-1 (Napoli et al., 2012). MCP-1 is up-regulated by Schwann cells and aides macrophage recruitment to the nerve with suppression resulting in decreased macrophage numbers (Fischer, Kleinschnitz, et al., 2008). When ERK signalling is inhibited, MCP-1 expression is reduced (Fischer, Weishaupt, et al., 2008). Together these studies highlight the importance of ERK signalling, not only in the control of the Schwann cell response to injury but in the recruitment of inflammatory cells also.

As highlighted in the c-Jun cKO mice, the bands of Büngner play an important role in guiding regenerating axons to their targets. The bands of Büngner are columns composed of overlapping Schwann cells inside the basal lamina. Recent work has demonstrated the extent of elongation and branching these cells undergo to form the bands of Büngner. Remak and myelin Schwann cells increase in length by 2-3 fold, adopting a branched, elongated profile (J A Gomez-Sanchez et al., 2017).

Following injury Schwann cells also proliferate, increasing in number from 19 hours after injury and peaking at 3 days, after which the rate of proliferation remains constant (Thomas, 1948; Bradley & Asbury, 1970). It has however been suggested that Schwann cell proliferation is not required for axon regeneration, at least initially. Schwann cells lacking cyclin D1, a G1 cell cycle protein, do not undergo proliferation following injury. Following a crush injury however, axons regenerate and remyelinate normally. (Kim et al., 2000; Atanasoski et al., 2001; Yang et al., 2008)

Once axons re-enter the nerve, Schwann cells undergo a second phase of proliferation and Schwann cell mediated remyelination begins 8 days after injury (Pellegrino & Spencer, 1985; Akassoglou et al., 2002). Any excess Schwann cells present in the nerve are removed by apoptosis (Yang et al., 2008). Recent work has confirmed the long standing assumption in the field that the repair cells become the myelin cells in the regenerated nerve. This was demonstrated by grafting a 4 week cut nerve segment from a mouse expressing Schwann cell GFP into a WT mouse. 6 weeks later, GFP positive myelin Schwann cells were observed in the WT nerve (J A Gomez-Sanchez et al., 2017).

1.6 The repair cell markers

The repair cell markers are defined as molecules, trophic factors, transcription factors and cell adhesion molecules that are up-regulated in Schwann cells following injury. They are either typically associated with aiding axon regeneration or their up-regulation is restricted to injury and they are not observed at other stages of the Schwann cell lineage. Their up-regulation is controlled by c-Jun, demonstrated by their dysregulation in the c-Jun cKO nerve. Although 172 genes are dysregulated in this mutant following injury, this thesis focuses on c-Jun, trophic factors GDNF, BDNF and the receptor p75 NTR, Shh and Olig1 (Arthur-Farraj et al., 2012).

1.6.1 c-Jun

The transcription factor c-Jun belongs to the JUN gene family, which also consists of JUNB and JUND. The activation of c-Jun controls many cellular pathways including apoptosis, proliferation and differentiation.

Phosphorylation of c-Jun by MAPKs, such JNK, in the N-terminal domain at serine (Ser) 63 and 73 and threonine (Thr) 91 and 93 is required for transcriptional activity (Smeal et al., 1991; Pulverer et al., 1991; Dérijard et al., 1994; Papavassiliou et al., 1995; Morton et al., 2003), whereas phosphorylation of C-terminal sites Thr 231, Ser 243 and 249 inhibits c-Jun DNA binding. Ser 243 is phosphorylated by glycogen synthase kinase 3 (GSK 3) whereas Thr 231 and Ser 249 are phosphorylated by casein kinase II (CKII). Activation of c-Jun can only be achieved through N-terminal phosphorylation coupled with C-terminal dephosphorylation (Boyle et al., 1991; Lin et al., 1992; Chou et al., 1992; Papavassiliou et al., 1995; Morton et al., 2003). A diagram of c-Jun protein structure, highlighting relivant binding and phosphorylation sites is displayed in Figure 1.3.

c-Jun forms and activates the transcription factors complex activator protein 1 (AP-1) either through homodimer formation with itself or heterodimer formation with c-Fos or activating transcription factor (ATF) proteins (Bohmann et al., 1987; Angel et al., 1988; Chiu et al., 1988; Rauscher et al., 1988; Franza et al., 1988; Halazonetis et al., 1988). The AP-1 complex activates TRE (12-O-Tetradecanoylphorbol-13-acetate responsive element) and CRE (cyclic AMP responsive element) through promotor

region binding (Lee et al., 1987; Sassone-Corsi et al., 1990). It is unknown what conformation of the AP-1 complex is formed in Schwann cells in response to injury, however recent work has suggested c-Jun forms heterodimers with Fosl2 and ATF3 due to their similar up-regulation patterns following peripheral nerve injury (Arthur-Farraj et al., 2017).

c-Jun is expressed in Schwann cell precursors and immature Schwann cells. It is down-regulated upon myelination with expression in adult nerves mainly observed in Remak Schwann cells although it has also been detected at low levels in adult myelin cells (Stewart, 1995; Shy et al., 1996; Klein et al., 2014; Hantke et al., 2014) c-Jun expression in pre-myelinating Schwann cells is likely to control cell numbers as, *in vitro* it has been shown that c-Jun participates in Schwann cell proliferation and apoptosis. Transforming growth factor β (TGFβ) induces apoptosis in high density cultured Schwann cells. It has been demonstrated that TGFβ apoptosis is mediated by c-Jun transcription. When Schwann cell cultures expressed v-Jun, a transcriptional active form of c-Jun (Nishimura & Vogt, 1988; Bohmann & Tjian, 1989), increased apoptosis was observed in serum free media. Similarly when c-Jun transcription was inhibited, cell survival increased (Parkinson et al., 2001). Conversely, c-Jun induces Schwann cell proliferation through activation by JNK. When JNK activity and thus c-Jun phosphorylation is inhibited, Schwann cell proliferation decreases in vitro as indicated by reduced BrdU incorporation. Pro-myelin gene Krox-20 inhibits c-Jun and Schwann cell proliferation through JNK signalling and drives the Schwann cell towards the myelin phenotype (Topilko et al., 1994; Parkinson et al., 2004).

Although c-Jun expression is low in adult neurons, it is rapidly up-regulated in both neurons and Schwann cells following peripheral nerve injury where it was thought to play a role in survival and regeneration (Leah et al., 1991; Jenkins & Hunt, 1991; De Felipe & Hunt, 1994; Stewart, 1995; Shy et al., 1996; Herdegen et al., 1997)

Raivich et al (2004) determined the role of c-Jun up-regulation in neurons of the central nervous system by creating a c-Jun neuronal knock out (c-jun$^{\Delta n}$). Following facial nerve injury, c-jun$^{\Delta n}$ neurons were atrophic and regenerated poorly compared to WT. It was therefore concluded that neuronal c-Jun up-regulation is required for sufficient axon regeneration (Raivich et al., 2004). This is an alternative function

compared to development where c-Jun activation has been demonstrated to induce apoptosis in sympathetic neurons *in vitro* in the absence of nerve growth factor. Depletion of c-Jun transcription was able to inhibit apoptosis and enhance cell survival (Ham et al., 1995).

Although c-Jun, Jun B and D are all expressed by Schwann cells *in vitro*, only c-Jun protein is up-regulated *in vivo* following injury (Stewart, 1995). In Schwann cells, c-Jun is the master regulator of the of the peripheral nerves response to injury, activating the repair cell phenotype in Schwann cells previously described in this Chapter.

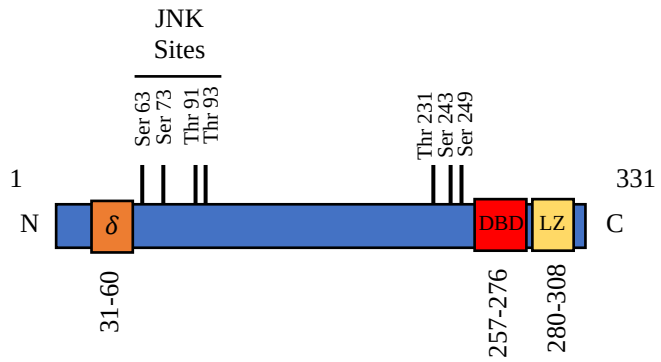


Figure 1.3 | A diagram of c-Jun protein structure

The c-Jun protein structure, highlighting relevant domains and binding sites. The N terminal phosphylation sites Ser 63, 73 and Thr 91 and 93 are located next to the delta (δ) domain. The delta domain regulates the phosphorylation of these N terminal sites (Adler et al., 1992, 1994). The C terminal phosphorylation sites Thr 231, Ser 243 and 249 are also annotated. The DNA-binding domain (DBD) and leucine zipper dimerization domain (LZ) are also located in the C terminus. Figure adapted from Barilá et al., 2000.

1.6.2 Olig1

Oligodendrocyte Transcription Factor 1 (Olig1) expression in Schwann cells is unique to the repair cell phenotype. The expression of Olig1 is more commonly observed, as its name suggests, in the glia of the CNS - oligodendrocytes. Olig1 is a basic helix–loop–helix (bHLH) transcription factor in the Olig gene family which also include Olig2 and Olig3. Olig1 expression, which is controlled by Shh, is observed in the ventral spinal cord at E9.5 in mice (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). *Olig1*$^{-/-}$ is a non-lethal mouse model that has been used to study the role of Olig1 in the CNS. Oligodendrocyte progenitor cell number is decreased in these mice and their differentiation impaired. The axons of these mice are hypomyelinated as a result of this. Although the initiation of myelination is not impaired, Olig1 is required for remyelination, through the differentiation of oligodendrocyte progenitor cells (Arnett et al., 2004; Dai et al., 2015).

Although we have previously reported that Olig1 is not observed during other phases of the Schwann cell lineage, a recent report suggests otherwise. Schmid et al (2014) have reported Olig1 expression in Remak bundles in P7 mouse nerves. qPCRs of nerves throughout postnatal development and adulthood, demonstrated increasing levels of Olig1 with Schwann cell maturation. Olig1 was expressed in the cytoplasm and nucleus of cultured cells but declined in the presence of cAMP, further enforcing the link between c-Jun and Olig1 (Schmid et al., 2014). The role of Olig1 in peripheral nerve regeneration has been examined. 4 days after crush, *Olig1* null mice demonstrate impaired regeneration compared to WT littermates. Fewer regenerating axons were observed at various distances in longitudinal sections of these nerves. It has however been observed that this regeneration deficit was not maintained. Olig1 null and WT mice demonstrated equal rates of regeneration at later time points (> 1 week). This suggests that Olig1 activation in Schwann cells plays a role in the initial stages of axon regeneration (S.Quintes, personal communication, unpublished data). As Shh is also up-regulated in Schwann cells following injury, it may activate Olig1 as it does during development.

1.6.3 Sonic Hedgehog

Sonic hedgehog (Shh), with Desert and Indian hedgehog, is a component of the hedgehog (Hh) signalling pathway. Hh was famously identified as a *Drosophila* larval patterning gene in the Nobel prize winning work of Christiane Nüsslein-Volhard and Eric F. Wieschaus (Nüsslein-Volhard & Wieschaus, 1980). Shh was named by Robert Riddle, a post-doc in the Tabin laboratory at Harvard University Massachusetts who first described the role of Shh in limb anteroposterior patterning (Riddle et al., 1993). Neural patterning and polarity in the developing CNS is controlled by Shh signalling. The differentiation of floor plate cells, motor neurons and ventral interneurons is mediated by Shh signalling from the notochord. Inhibition of signalling prevents the formation of the spinal column and differentiation of motor neurons (Martí et al., 1995; Roelink et al., 1995; Ericson et al., 1996; Chiang et al., 1996).

As previously mentioned, the Hh signalling pathway was initially determined in *Drosophila.* Here, a simplified version of pathway is described: Hh binds to Patched, a 12-pass transmembrane protein receptor. (Marigo et al., 1996; Stone et al., 1996). The binding of Hh removes Patched inhibition on Smoothened (Smo), a 7-pass transmembrane protein and G-protein coupled receptor (Chen & Struhl, 1996; Alcedo et al., 1996). Once the repression of Smo is alleviated, it translocates to the cell membrane (Zhu et al., 2003) where it activates the GLI transcription factors (Gli1, Gli2 and Gli3) (Hynes et al., 1997; Altaba, 1998). It is now known that Smo translocates to the cell membrane in the primary cilium. Cyclopamine antagonises the Hh pathway by binding directly to Smo and inhibiting Smo activity by preventing this translocation (Cooper et al., 1998; Incardona et al., 1998; Chen, Taipale, Cooper, et al., 2002; Huangfu et al., 2003; Corbit et al., 2005). Agonists of the Hh pathway such as purmorphamine and smoothen agonist (SAG) similarly activate signalling through direct binding with Smo (Chen, Taipale, Young, et al., 2002; Wu et al., 2004; Sinha & Chen, 2006). Figure 1.4 provides a basic summary of Hh signalling.

Shh is not expressed by myelin Schwann cells or during development but is rapidly up-regulated upon nerve injury in both neurons and Schwann cells (Arthur-Farraj et al., 2012; Lin et al., 2015; Martinez et al., 2015). Shh is neuroprotective and supports

axon growth. Martinez et al (2015) demonstrated reduced neurite out growth and branching was observed in dissociated DRG cultures that were transfected with *Shh* siRNA (Martinez et al., 2015). *In vivo* it has been demonstrated that inhibition of Hh signalling after injury through cyclopamine application increases motor neuron death whereas application of Shh to nerves following injury improves regeneration (Pepinsky et al., 2002; Akazawa et al., 2004; Hashimoto et al., 2008; Bond et al., 2013). The neuroprotective effects of Shh are attributed to the activation of BDNF. Hashimoto et al (2008) demonstrated this *in vivo* by continuously applying cyclopamine to the sciatic nerve after injury. This down-regulated Gli1 activity and subsequently decreased BDNF RNA and protein expression. Schwann cell cultures incubated with Shh significantly up-regulated Gli1 and BDNF expression whereas cyclopamine reduced BDNF expression (Hashimoto et al., 2008).

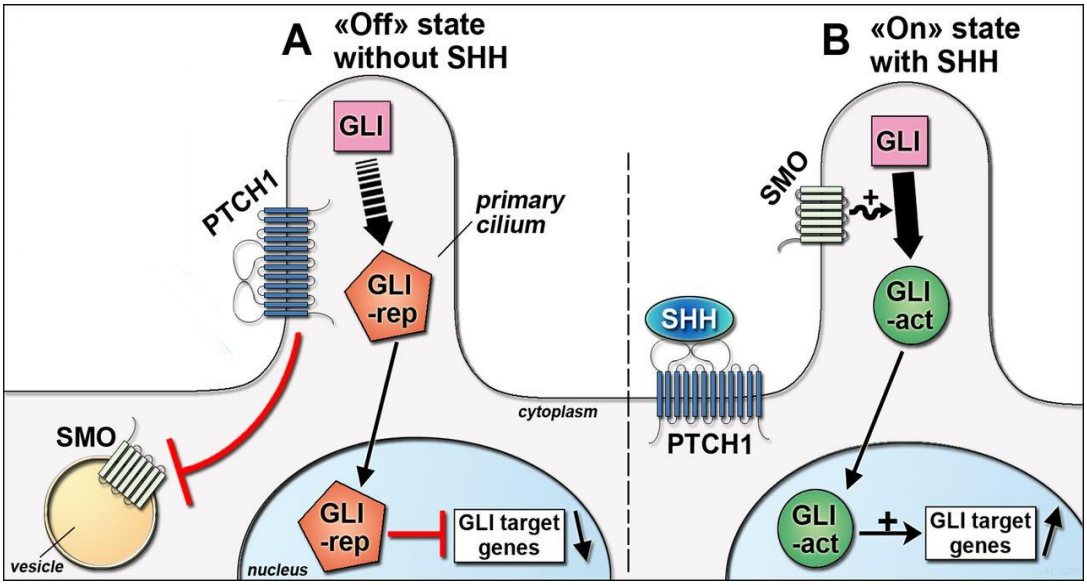


Figure 1.4 | A simplified diagram of Hh signalling

(A) In the absence of Hh signalling, Patched inhibits smoothened, preventing its translocation to the cilium. Gli transcriptional activation is repressed. **(B)** In the presence of Hh (Shh, Dhh or Ihh), Hh binds to Patched, removing the inhibition of smoothened which moves into the cilium. GLI is activated and promotes the transcription of GLI gene targets. Adapted from Mailleux et al., 2013.

1.6.4 Brain-derived neurotrophic factor

BDNF, like other members of the neurotrophin family to which it belongs, exists as a homodimer in its active form. BDNF was first isolated from the pig brain where its contribution towards neurite outgrowth and survival of sensory neurons *in vitro* was noted (Barde et al., 1982). It is expressed prominently in the CNS, however expression has been localised in internal organs including the stomach, liver, lungs, kidneys and colon (Leibrock et al., 1989; Lommatzsch et al., 1999).

Activation of the BDNF pathway contributes to a signalling network composed of 129 molecules (Sandhya et al., 2013). BDNF signals through its receptor trkB which is expressed throughout the central and peripheral nervous systems of both rodents and humans (Squinto et al., 1991; Klein et al., 1989; Allen et al., 1994; Klein et al., 1990). When the active signalling form of trkB is knocked out in mice the mice do not survive postnatally, due to their inability to feed due to motor and sensory abnormalities. A reduction in neural numbers was observed both centrally, in the facial motor nucleus and in the periphery. DRGs were reduced in size and contained fewer neurons. This demonstrates the importance of BDNF/trkB signalling in the developing nervous system (Klein et al., 1993). BDNF also binds with low affinity with the p75 NTR receptor, which will be described later.

Following peripheral nerve injury, BDNF is gradually up-regulated in the distal nerve stump. A significant elevation is only observed 7 days post transection and levels continue to increase up to 28 days post injury. This suggests the up-regulation observed in these studies is Schwann cell specific (Meyer et al., 1992; Funakoshi et al., 1993). BDNF up-regulation in motor neurons is far more rapid and short lived. Following axotomy of the facial nerve, *BDNF* mRNA is detected in motor neurons 3-8 hours following injury. mRNA levels peak after 1-2 days however protein remains elevated for 1 week (Kobayashi et al., 1996).

BDNF aids axon regeneration in the PNS. When endogenous BDNF signalling is inhibited with BDNF antibodies, axon regeneration is reduced by 24%. Remyelination is impaired likely due to the observed Schwann cell death and axon degeneration (Zhang et al., 2000). Similarly, when a nerve graft genetically lacking BDNF is used to repair an injured nerve, axon regeneration into the graft is poor (Wilhelm et al., 2012). Although exogenous application has been shown to enhance

peripheral nerve growth, the contribution of BDNF to axon regeneration is dose-dependent. BDNF at low concentrations promotes axon regeneration in the injured peripheral nervous system. When a low dose (0.5-2µg) of exogenous BDNF is applied to the sciatic nerve every day following chronic denervation, axons regenerate well. However higher levels of 12-20 µg inhibit regeneration through p75 NTR signalling (Boyd & Gordon, 2002a).

1.6.5 p75 neurotrophin receptor

Despite activation of p75 NTR through neurotrophin signalling, p75 NTR belongs to the family of tumour necrosis factor receptors. Signalling through this receptor is largely associated with apoptosis. Although p75 NTR binds with all members of the neurotrophin family, binding has also been observed with neurotoxic prion protein fragment PrP (26−106), a glycoprotein of the rabies virus envelope and the Aβ peptide of the amyloid precursor protein, inducing apoptosis (Kuner et al., 1998; Tuffereau et al., 1998; Della-Bianca et al., 2001). Apoptotic pathways are thought to be activated by the globular six-helix 'death' domain located in the juxtamembrane and carboxy-terminal domains. Activation of this domain has been demonstrated to activate apoptosis through calpain and caspase signalling (Liepinsh et al., 1997; Coulson et al., 2000). Activation of p75 NTR *in vitro* has induced apoptosis in embryonic motor and sensory neurons (Sedel et al., 1999; Barrett & Bartlett, 1994), neonatal sympathetic neurons (Bamji et al., 1998) and mature oligodendrocytes (Casaccia-Bonnefil et al., 1996).

In Schwann cells, p75 NTR is often used as a cell marker. It is expressed throughout Schwann cell development and in Remak cells. Signalling through the receptor has been shown to regulate myelination *in vitro.* Inhibition of the receptor with blocking antibodies reduces the expression of myelin proteins MAG and P_0 (Cosgaya et al., 2002) and *p75 NTR* null mice develop thin myelin sheaths, likely due to the reduced expression of these myelin proteins (Gjerstad et al., 2002; Song et al., 2006; Tomita et al., 2007). The control of myelination *in vivo* by p75 NTR is controlled by a series of downstream signalling cascades (Tep et al., 2012). Following peripheral nerve injury, it is gradually up-regulated in Schwann cells, peaking around 1 week post injury. Levels are seen to decline with the re-emergence of axons into the nerve

(Heumann et al., 1987; Taniuchi et al., 1988; You et al., 1997). The role of p75 NTR signalling in regeneration is complex. Many reports and observations have been made suggesting that p75 NTR activation is harmful to Schwann cells and regeneration. Inhibition of p75 NTR in neurons and glia reduces Schwann cell apoptosis (Firouzi et al., 2011), neurodegeneration induced by oxidative stress (Bradley R Kraemer et al., 2014) and enhances axon regeneration (Boyd & Gordon, 2001). Conversely, reports also demonstrate the importance of p75 NTR in regeneration. *p75 NTR* null mice display poor remyelination following sciatic nerve crush (likely due to the role of p75 NTR in the control of myelination) (Song et al., 2006), reduced apoptosis in P1 nerves following axotomy (Syroid et al., 2000) and impaired motor neuron regeneration and functional recovery (Tomita et al., 2007). The effect of p75 NTR signalling is dependent on the receptor partner binding interactions. Heterodimerisation with trk receptors promotes survival, differentiation and growth. Axon regeneration is inhibited through association with Lingo-1 and the Nogo receptor whereas apoptosis is induced by Sortilin association (Mi et al., 2004; Skeldal et al., 2012; Kraemer et al., 2014; Meabon et al., 2015).

1.6.6 Glial cell-derived neurotrophic factor

GDNF is a distant member of the transforming growth factor-β family. GDNF was originally purified from rat B49 cells, a glial cell line. It was characterisend as a glycosylated, disulfide-bonded homodimer that supported survival and differentiation of dopaminergic neurons *in vitro* (Lin et al., 1993). It was later demonstrated to be 75x more potent than members of the neurotrophin family in promoting motorneuron survival. GDNF however has no effect on embryonic sensory neuron survival (Henderson et al., 1994). Outside the nervous system, GDNF regulates the development of the kidney (Pichel et al., 1996; Sainio et al., 1997) enteric neurons (Sánchez et al., 1996) and spermatogonia differentiation (Meng et al., 2000). GDNF signalling is mediated by binding GDNF-family receptor-α (GFRα) receptors, with GDNF preferentially associating with GFRα1. (Jing et al., 1996; Wong & Too, 1998; Treanor et al., 1996). GDNF binding stimulates dimerization of GFRα with the RET receptor tyrosine kinase, promoting signalling through this pathway. However GDNF does not initiate RET dimerization

as when GDNF binding is inhibited, RET signalling is still activated (Eketjäll et al., 1999). RET activates various pathways, including phosphatidylinositol 3-kinase (PI3K)/AKT, JNK and p38 MAPK (Worby et al., 1996; Chiariello et al., 1998; Soler et al., 1999).

GDNF is expressed in Schwann cell precursors and the peripheral nerve at E14.5, however it is not observed in immature or mature Schwann cells (Henderson et al., 1994; Arthur-Farraj et al., 2012). Application of exogenous GDNF to intact adult nerves has resulted in myelination of previously unmyelinated small diameter fibres and enhanced Schwann cell proliferation (Höke et al., 2003). In Schwann cells, GDNF is a direct target of c-Jun activation. In c-Jun cKO nerves, the endogenous application of GDNF and artemin alleviates the regeneration defects associated with this knock out (Fontana et al., 2012). Following nerve injury, GDNF and GFRα are elevated in the distal nerve stump, with expression peaking at 1 week post injury (Naveilhan et al., 1997; Höke et al., 2000, 2002). Activation occurs in both myelin and Remak cells (Xu et al., 2013). In humans, GDNF is highly up-regulated in the peripheral nerve distal to the injury. Expression is observed in both Schwann cell and DRG neurons, whereas RET expression is confined exclusively to DRG neurons and axons (Bär et al., 1998). When the neuronal response to GDNF is inhibited through abolition of RET receptors, axon regeneration is impaired (Fontana et al., 2012). Many studies are now utilising GDNF as a method for enhancing nerve repair through exogenous application with microspheres, gels and pumps.

1.7 Factors affecting peripheral nerve regeneration

Despite the activation of the repair cell phenotype following injury and although the PNS does have an intrinsic capacity for regeneration, functional recovery in humans is poor. This is due to the fact that axons must travel long distances to reinnervate their targets. As axons regenerate at a rate of 1-2mm/day (Buchthal & Kühl, 1979), it can take months or even years for them to reach their targets. During this time, the initially growth permissive capabilities of both the axons and the Schwann cells decline, contributing to the poor regeneration observed. The effects of the declining regenerative capacity of the nerve are therefore present proximally and distally to the injury in the forms of chronic axotomy and denervation.

1.7.1 Chronic axotomy

For regeneration to occur, the axon must maintain its capacity to grow. It is debatable if chronic axotomy, in which axons proximal to the injury site are prevented from regenerating, affects repair. Holmes and Young (1942) transected the tibial nerve in rabbits and following 1 year of chronic axotomy, sutured the proximal stump to a freshly cut distal nerve stump of the common peroneal. They found no effect in the number of axons or the rate at which they regenerated between chronically axotomized and immediately repaired nerves (Holmes & Young, 1942). Conversely however, the work of Tessa Gordon claims that chronic axotomy does affect regeneration. Using the same paradigm as Holmes and Young, it was demonstrated that the number of regenerating motor neurons was significantly reduced following chronic axotomy. When the nerve was immediately repaired, 137 regenerating motor neurons made functional connections with the muscle. However, following chronic axotomy for any period over 6 months, only 50 motor neurons made functional connections. It was however noted that motor neuron branching and muscle fibre reinnervation were not affected (Fu & Gordon, 1995). This finding was later reinforced by examining motor neuron regeneration with retrograde labelling in the same model. The results demonstrated a significant decline in motor neuron regeneration following chronic axotomy (Boyd & Gordon, 2002b, 2003). Further studies have confirmed that this finding occurs even after only 2 months of axotomy (Furey et al., 2007). It could be argued however that these results are due to motor

neuron death. After injury, 20-30% of sensory neurons die in the adult DRG (Himes & Tessler, 1989). This effect is not observed in motor neurons however, the numbers of which are unaltered by axotomy (Carlson et al., 1979). This question was definitively answered by Xu et al (2010) who examined motor neuron numbers in uninjured, chronically axotomized and repaired chronically axotomized nerves. No difference in the number of motor neurons was observed between the first 2 groups, however after repair, only 50% of the neurons regenerated (Xu et al., 2010). It may be the Holmes and Young's observation is due to axonal sprouting, which is not representative of the number of neurons and could have over-estimated the number that are present. These experiments were also performed in rabbits so differences may be observed between species.

1.7.2 Chronic denervation

Following axotomy, the Schwann cells distal to the injury up-regulate the repair cell phenotype as previously described. One of the defining features of this is the up-regulation of trophic factors that support axonal growth. GDNF is rapidly up-regulated in the days following injury (Naveilhan et al., 1997; Höke et al., 2000, 2002) whereas BDNF's increase is gradual (Meyer et al., 1992; Funakoshi et al., 1993). This regenerative environment however is not maintained and declines with time in a process referred to as the deterioration of the distal nerve stump. This process is well defined and characterised by the decrease in trophic support and Schwann cell death. p75 NTR expression, although initially highly up-regulated 1 week after injury, begins to decline thereafter. Expression gradually declines for the first 6 months, after which the level is barely detectable. *In situ* hybridisation and dual labelling with S100 antibodies have demonstrated that this decline is Schwann cell specific (Heumann et al., 1987; Taniuchi et al., 1988; You et al., 1997).

The same is true of GDNF expression. GDNF is rapidly up-regulated following injury and is detectable within the first 48 hours. Expression peaks 1 week after injury, after which levels steadily decline over time. Levels are almost undetectable after 6 months of denervation. Interestingly, the expression of GDNF's receptor GFRα1 does not correlate with ligand expression. GFRα1, although up-regulated following injury, does not peak in expression until 3 months of denervation have

passed. Although reduced, levels of the receptor remain elevated even at 6 months. This is likely a compensatory mechanism, induced by autocrine signalling (Höke et al., 2000, 2002; Michalski et al., 2008; Eggers et al., 2010).

As previously mentioned, BDNF's up-regulation in Schwann cells is gradual with levels peaking 1 month following injury. Over the following month *BDNF* mRNA expression then decreases by half. Following 6 months of denervation *BDNF* mRNA levels are similar to those expressed 1 week after injury but protein expression is shown to be equal to that expressed in uninjured nerves after 4 months of denervation (Michalski et al., 2008; Eggers et al., 2010).

One of the causes of the decrease in trophic factor expression could be due to the reduced number of Schwann cells present in the nerve. Following injury, the number of Schwann cells in the distal nerve stump increases to approximately 3x of that present in uninjured nerves during the first 20 days. A slow decline in number is then observed. This is noted regardless of the length of the study and after 10 week denervation, Schwann cell numbers have declined to those observed in uninjured nerves (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; Benito et al., 2017). The disappearance of Schwann cells in the nerve results in the very gradual fading of the bands of Büngner. While it was initially hypothesised that the shrinkage and eventual disappearance of the bands could be caused by cell migration towards regenerating axons, due to the presence of necrotic cells observed in the nerve, apoptosis is likely the cause (Abercrombie & Johnson, 1946; Thomas, 1966; Weinberg & Spencer, 1978)

A secondary response to chronic denervation is the ability of the muscle to accept reinnervating nerves. Muscle maintenance requires both neurotrophic and electrical stimulation, both of which are lost during chronic denervation (Gibson et al., 1988; Chevrel et al., 2006). Without input, the muscle becomes atrophic and some studies report signs of necrosis. A decline in muscle mass is observed during the first week of denervation and mass continues to steadily decline with time. (Fu & Gordon, 1995; Cockman et al., 2001; Dow et al., 2004; Jonsson et al., 2013). The cross sectional area of fibres is reduced and although initially the fast type 2 fibres are more susceptible to atrophy, some studies find during chronic denervation all fibres are effected equally (Viguie et al., 1997; Bobinac et al., 2000; Ashley et al., 2007).

The tetanic tension and force of the muscle declines as a result of this (al-Amood et al., 1991; Fu & Gordon, 1995) and fibrous collagen and adipose tissue builds up in the muscle (Lu et al., 1997; Ashley et al., 2007).

Muscle satellite cells which are required to the generation of new tissue in adult muscle also decline in number during chronic denervation (Dedkov et al., 2001; Viguie et al., 1997). BDNF is required for the differentiation of muscle satellite cells (Clow & Jasmin, 2010). Although BDNF is expressed in muscle following nerve injury, its expression is largely Schwann cell specific (Griesbeck et al., 1995; Omura et al., 2005). The down-regulation of BDNF in chronically denervated Schwann cells therefore hinders the regeneration of muscle fibres. Together, these factors result in poor functional recovery and a reduced target innervation from the regenerating nerves.

The deterioration of the distal nerve stump implies that the repair cell phenotype is not maintained during chronic denervation. Stat3 was recently highlighted as a transcriptional regulator of the repair cell phenotype. Stat3 is phosphorylated in Schwann cells after injury (Sheu et al., 2000; Lee et al., 2009) and remains somewhat elevated during chronic denervation. When Stat3 is knocked out in Schwann cells, no difference is observed in the activation of the repair cell phenotype. However, during chronic denervation, c-Jun and repair cell markers such as GDNF, BDNF, Shh and Olig1 are significantly decreased in mutant nerves. Schwann cell survival is affected and fewer Schwann cells are observed in chronically denervated stumps. Irregular bands of Büngner form in shapes related to those observed 4 weeks after injury in the c-Jun cKO mouse. This demonstrated that Stat3, although not required for the activation of the repair cell phenotype, is required for maintenance during chronic denervation. The decrease in c-Jun expression and the repair cell phenotype suggests that Stat3 signals up-stream from c-Jun (Benito et al., 2017).

This thesis will however focus on the role of c-Jun in maintaining the Schwann cell repair phenotype and its contribution to the deterioration of the distal nerve stump.

1.8 Aging

The changes that occur with age are not simply confined to the greying of our hair and the wrinkling of our skin. Changes are observed in many systems and at every level. The telomeres of our chromosomes shorten, DNA becomes damaged, leading to the dysregulation of genes and proteins, cell to cell communication is impaired and regenerative systems fail. The nervous system is not exempt from the effects of aging and this is often observed in the form of motor and cognitive impairment. With advanced aging, the structure, function and regeneration of the PNS all undergo change.

1.8.1 Structural and functional changes in the peripheral nerve with age

The primary function of the PNS is to relay signals to and from the CNS via the propagation of action potentials. The ability of the PNS to perform this role is measured by the conduction velocity, the speed at which electrical impulses travel through nerves. Conduction velocity of peripheral nerves increases during youth and early adulthood and is associated with the maturation of the nervous system. This observation is conserved between rodents (Birren & Wall, 1956; Sato et al., 1985; Verdú et al., 1996) and humans (Rabben, 1995). These observations are refined to myelinated fibres, with unmyelinated fibres maintaining a constant conduction velocity (Sato et al., 1985). These observations are therefore likely due to the progressive myelination of the nerves, that continues through early adulthood and is the likely reason for plateaus observed in the data of young animals. Decreases in conduction velocity however are observed with advancing age. This is well documented in humans, with impairments observed in both motor and sensory nerves (Dorfman & Bosley, 1979; Bouche et al., 1993), a phenomenon also observed in rodents (Verdú et al., 1996).

As well as conveying electrical signals, peripheral axons must also physically relay information in the form of axonal transport. Axons in the PNS stretch across long distances between neuronal cells bodies and their targets. Information in the form of lipids, proteins and organelles is transferred between the axon and cell body by antero and retrograde transport. A decline in the rate of axoplasmic transport is observed with age. McQuarrie et al (1989) demonstrated a 40% decrease in axonal

transport in both the sciatic and optic nerves of 24 month old mice when compared to those aged 6 months (McQuarrie et al., 1989). Stromska and Ochs (1982) reported a similar, if not as steep a decline in axonal transport between 3 and 37-38 month old rats (Stromska & Ochs, 1982). More specifically, it has also been shown that the transport of choline acetyltransferase, an enzyme required for acetylcholine synthesis, is transported 5x faster in the sciatic nerves of young mice compared to old (Jablecki & Brimijoin, 1975)

The changes observed in nerve function with age are likely due to structural changes in nerve architecture. Although nerves maintain a level of relative morphological stability during adulthood, age-related changes become apparent with time. Ceballos et al (1999) studied these changes at length in the mouse, examining various ages and morphological parameters. Although no differences in the number of Schwann cells was reported, abnormalities in myelin were observed. Myelin loops formed irregular shapes around the axons and the number of myelinated and nonmyelinated fibres steadily declined with age, an observation commonly reported in the sciatic nerves of humans (Swallow, 1966; O'Sullivan & Swallow, 1968; Jacobs & Love, 1985; Kanda et al., 1991) and rats (Sharma et al., 1980). The number of resident macrophages present in the nerve was also seen to increase (Ceballos et al., 1999). The thickness of the myelin that remains decreases with age (Ugrenović et al., 2016) and its composition is altered. Decreases in mRNA expression of myelin proteins MBP, PMP22 are observed with age. A decline in P_0 expression is also observed at both the mRNA and protein levels (Melcangi, Magnaghi, Cavarretta, Martini, et al., 1998; Melcangi, Magnaghi, Cavarretta, Riva, et al., 1998; Melcangi et al., 2000).

1.8.2 Regeneration in the aging peripheral nervous system

Impaired healing and regeneration after injury is observed in many systems with advancing age. Liver (Bucher et al., 1964; Fry et al., 1984; Biondo-Simões et al., 2006), lung (Paxson et al., 2011) and skeletal muscle regeneration (McGeachie & Grounds, 1995), CNS remyelination (Shields et al., 1999), bone fracture (Meyer et al., 2001; Lu et al., 2005) and wound healing (Quirinia & Viidik, 1991; Ashcroft et al., 1997; Gosain & DiPietro, 2004) are all impaired with age. Regeneration in the peripheral nervous system is similarly affected, with functional impairment observed in both neurons and glia.

1.8.2.1 The axonal response

The age associated decline in peripheral nerve regeneration has been well documented. In rabbits, pinch tests (during with the nerve is re-exposed after an injury and pinched distally, a response that is equated to the distance regenerated) of crushed sciatic nerves demonstrate reduced response with age. This decline is very slight, however a greater response is observed in functional recovery. Toe spreading in older rabbits was found to be significantly lower after injury. This suggests a delay in target reinnervation (Gutmann et al., 1942). Black and Lasek (1979) demonstrated that the difference in the initial response to regeneration is not vastly impaired in old rats. 4 days after crush, although old rats displayed reduced motor neuron regeneration, the difference between young and old mice was not so different. However 8 days after crush, the effect of age was evident with an increased number of regenerating axons observed in younger rats (Black & Lasek, 1979). This response has been confirmed at 9 days after crush. Pestronk et al (1980) documented that 9 days after crush, the majority of regenerating axons in 2 month old rats had reached 24mm, axons in 10 month old rats had regenerated 12mm whereas axons in 28 month old rats had only reached 3mm from the crush site. They similarly reported no major difference in regeneration between age groups 4 days after crush (Pestronk et al., 1980).

Following injury, axon regeneration is initiated by sprouting whereby fine nerve processes extend from the intact axons of the proximal stump. In young mice, after crush injury, axon sprouts are observed 4 days after injury and increase in number

over a 2 week period. This initial response is similarly observed in older mice but is not maintained and a reduction is sprouting is observed (Vaughan, 1992). This reduction in axonal sprouting with age has been supported by other work (Pestronk et al., 1980). The reduced axonal transport described here previously, as well as the axons reduced capacity to respond to neurotrophins is also likely to contribute (Parhad et al., 1995). Axons that do regenerate are reported as being smaller in diameter with a thinner myelin sheath (Tanaka et al., 1992). However more recent work is proposing that the impaired regeneration seen in aged animals is occurring independently of the axon. Studies are now demonstrating that the regenerative capacity of the axon is maintained throughout life (Kang & Lichtman, 2013; Painter et al., 2014). It is instead the environment in which that regeneration is taking place that is impaired. This is mainly controlled by the Schwann cell and macrophage response to injury.

1.8.2.2 The Schwann cell and macrophage response

As previously described in this chapter, an essential aspect of regeneration is myelin clearance, mediated by both Schwann cells and macrophages. In young animals this readily occurs however with age, myelin debris persists (Tanaka & Webster, 1991; Vaughan, 1992). Kang and Lichtman (2013) performed a detailed time-lapse analysis of axon regeneration in young and aged mice. They demonstrated that axon growth in older regenerating nerves is continuously halted by axon and myelin debris. Once axons had passed the debris and reached the neuromuscular junction, synapse reinnervation readily occurred (Kang & Lichtman, 2013).

The persistence of myelin debris in the nerve could be due to a variety of factors. As mentioned previously, the myelin composition in aged nerves is different to young. This could impair the ability of macrophages and Schwann cells to break it down. However, parabiosis experiments, in which a young mouse is joined to an older mouse, demonstrate the ability of the young macrophages to clear myelin debris in the old CNS (Ruckh et al., 2012). Painter et al (2014) demonstrated that macrophage recruitment is impaired in old nerves. It has previously been demonstrated that macrophage suppression can diminish the regenerative response in young mice (Tanaka et al., 1992). However parabiosis studies performed by Painter et al (2014),

where young mice were joined to old, were unable to rescue the regenerative capacity of aged nerves. It is more likely that the impairment in myelin clearance lies in the intrinsic capacity of Schwann cells. Schwann cells from young and old nerves cultured in the presence of myelin display different rates of myelin clearance. A similar observation is conserved *in vivo* where Schwann cell internalization of myelin is delayed in aged nerves following injury. Due to nerve grafts from young mice enhancing axon regeneration in old nerves, it is therefore likely that the age associated decline in regeneration is Schwann cell specific (Painter et al., 2014) .

Painter et al (2014) go on to investigate the activation of the repair cell phenotype, the importance and induction of which was discussed previously in this chapter. Genetic screens of young and old nerve following injury revealed an impairment in the activation of several repair cell marker genes as well as an inhibition of myelin gene down regulation. c-Jun is highly up-regulated in young nerves 1 day after injury. This elevation is not observed in old mice, however levels of c-Jun gradually increase with time post injury at both ages. It is therefore suggested that the diminished regenerative capacity of peripheral nerves observed with advancing age is due to a delay in the activation of the repair cell phenotype (Painter et al., 2014). The field must now adjust its views on aging to accept that the deficits in regeneration observed are largely Schwann cell derived.

1.9 Current treatments in peripheral nerve repair

The greatest advances in peripheral nerve repair were made over half a century ago following World War II. During this time clinicians were exposed to large numbers of patients presenting with peripheral nerve injuries and were able to develop our understanding on how best to treat them. The majority of early treatments involved simply suturing the transected nerve back together (often described as end to end suture) (Platt & Bristow, 1924) (Seddon, 1947). It was believed that the stretching of the nerve aided its repair. However, it was later presented, as we now know, that this is not the case. Stretching the nerve over large gaps leads to extensive damage that prevents functional recovery (Highet & Sanders, 1943); Highet and Holmes, 1943). End to end suture is still a valuable technique for smaller nerves gaps. It has been developed further to enhance repair whereby fascicles are separated in both nerve stumps, matched and sutured together. This technique is however very delicate and increases risks of microbleeds and scarring (Lundborg, 1987).

A solution therefore had to be found to repair transected nerves where large gaps were present. Thus began the process of nerve grafting, using donor nerves to fill the gaps. Little success was initially observed with hetero and homogenous grafts. Heterogenous grafts were discredited as an idea very early. Homogenous grafts, in the form of nerves taken from other members of the same species, were found to become necrotic and become replaced by connective tissue (Seddon et al., 1942). Little regeneration is observed into these grafts even during long-term studies (Spurling et al., 1945). Autogenous grafts however proved highly successful. Autographs survived and featured Schwann cell proliferation and formation of the bands of Büngner. Myelin clearance and remyelination are also observed (Seddon et al., 1942; Titrud, 1947; Seddon, 1947; Aguayo et al., 1976). Grafts initially survive from the diffusion of molecules from the surrounding tissues before revascularisation begins a few days later (Almgren, 1975).

Autografts however give rise to various issues. Grafts are mainly obtained from the sural nerve due to its accessibility and thickness (Lundborg, 1987). The sural nerve is a sensory nerve and removing part of it reduces somatosensory function in patients. Furthermore, motor neurons preferentially reinnervate motor pathways and shown reduced regeneration into sensory branches (Brushart, 1988). Repair with a

sensory autograft has been shown to result in less successful regeneration with improved regeneration seen in the use of motor or mixed nerve grafts (Nichols et al., 2004). Patients with larger nerve gaps may also not be able to provide enough graft material. Many studies are now aiming to create artificial nerves *in vitro* for grafting.

There are many studies aiming to determine the best material and properties of tubes to emulate nerves and to enhance regeneration. The most common composition is collagen type 1 with aligned Schwann cells. Tubes with cells are shown to improve nerve regeneration compared to empty tubes (Phillips et al., 2005; Georgiou et al., 2013). However this procedure if yet again limited by the amount of neural tissue the patient can provide as Schwann cell cultures must be generated. Many studies are now generating Schwann-like cells from sources such as adipose-derived stem cells (Jiang et al., 2008), human dental pulp (Martens et al., 2014) and hair follicle stem cells (Amoh et al., 2005) which have been shown to improve nerve regeneration across gaps. However when much larger gaps are present (15mm in rat, 6mm in mice) regeneration is poor or not observed (Lundborg et al., 1982; Gómez et al., 1996). Indeed longer grafts have been associated with an increased rate of Schwann cell senescence (Saheb-Al-Zamani et al., 2013). In animal models, these grafts never achieve the rate of regeneration seen with autografts of peripheral nerves.

Electrical simulation has been shown to enhance nerve regeneration in models of both chronic axotomy and denervation (Al-Majed, Neumann, et al., 2000; Elzinga et al., 2015). Electrical stimulation increases the up-regulation of regeneration associated genes (Geremia et al., 2007) and activates trophic factors GDNF and BDNF (Al-Majed, Brushart, et al., 2000; Willand et al., 2016). New studies are aiming to pharmaceutically enhance the regenerative capacity of the axons and Schwann cells that are currently in the nerve. Many studies demonstrate enhanced regeneration following the application of trophic factors (Boyd & Gordon, 2002b, 2003; Lang et al., 2008; Wood et al., 2012, 2013). Studies investigating regeneration in crushed nerves have found Shh to enhance regeneration in both the sciatic (Pepinsky et al., 2002) and cavernous nerve (Bond et al., 2013; Angeloni et al., 2013).

Other work focuses on the effects of nerve regeneration from drugs that are already approved in humans by the US Food and Drug Administration. FK506 is an

immunosuppressant commonly administered to prevent organ rejection following transplant (Starzl et al., 1989). FK506 has been demonstrated *in vitro* to enhance neurite growth (Lyons et al., 1994) and *in vivo* enhances regeneration following nerve grafting or sciatic nerve crush (Gold et al., 1994, 1995; Doolabh & Mackinnon, 1999; Lee et al., 2000). During chronic injury however, FK506 is only able to improve motor neuron regeneration in models of chronic axotomy, not chronic denervation (Sulaiman et al., 2002) suggesting FK506 cannot restore the regenerative capacity of Schwann cells.

Ibuprofen is non-steroidal anti-inflammatory painkiller that has been suggested to improve nerve regeneration when administered directly to the injury site (Raisi & Mohammadi, 2015). Evidence for this however is poor. Although functional recovery is improved in rats receiving the drug, control littermates show no signs of recovery, an unusual finding for a rodent regeneration study. Furthermore, although functional improvements are present, no difference in the number of regenerating neurons is observed and only a slight improvement in remyelination is documented (Madura et al., 2011).

Although many scientific studies have aimed to enhance nerve regeneration, very few clinical advances have been made in this field. The gold standard for nerve repair remains the use of autografts. This thesis aims to aid our understanding of the effects of chronic denervation and advancing age and apply these findings to restore the regeneration deficits of the PNS.

1.10 Aims

The work in this thesis aims to contribute to our understanding to peripheral nerve regeneration deficits. Transgenic mouse lines will be generated and utilised to restore the regenerative capacity of peripheral nerves. Although each chapter will set out to achieve smaller, individual aims, the overall aims of this work are to:

1. Determine if c-Jun and thus the repair cell phenotype are maintained following chronic denervation (Chapter 3).
2. Determine if c-Jun and the repair cell phenotype can be maintained during chronic denervation (Chapter 4).
3. Determine if maintaining c-Jun restores the regenerative capacity of the nerve following chronic denervation (Chapter 5).
4. Confirm if c-Jun activation is impaired in aged nerves following injury (Chapter 6).
5. Determine if restoring c-Jun activation restores the regenerative capacity of aged nerves (Chapter 6).
6. Determine if c-Jun can be pharmacologically activated by Hh signalling (Chapter 7).

2. Materials and methods

2.1 List of recipes

Electron Microscopy

100ml of EM fix:

Component	Amount	Final Concentration	Company
8% PFA	25ml	2%	Fluka Chemicals Ltd
25% glutaraldehyde	10ml	2.50%	Agar Scientific
0.2 M cacodylate buffer (pH 7.3)	50ml	0.1M	N/A
ddH_20	15ml	-	N/A

EM resin:

Component	Amount	Company
Agar	12g	Agar Scientific
DDSA	8g	Agar Scientific
MNA	5g	Agar Scientific
BDMA	16 drops*	Agar Scientific

* BDMA added dropwise with 1.5ml pasteur pipette

Immunofluorescence

10x PBS, use at 1x diluted in ddH_20:

Component	Amount	Company
NaCl	80g	Fisher Scientific
Na_2HPO_4	11.5g	Sigma
KCl	2g	Sigma
KH_2PO_4	2g	Sigma
ddH_20	1L	N/A

ADS:

Component	Amount	Company
FBS	20ml	Perbio
Lysine	3.65g	Sigma
10% Sodium azide	400	Sigma
PBS	180ml	N/A

Tissue Culture

100ml defined medium:

Component	Amount	Final Concentration	Company
DMEM/F12 (1:1)	100ml	100%	Invitrogen
Glutamine	2ml	2mM	Sigma
BSA	1ml	0.04%	Sigma
Putrescine	1ml	16μg/ml	Sigma
Transferrin	1ml	100 mg/ml	Sigma
T4 (L-Thyroxine)	100μl	400 ng/ml	Sigma
Progesterone	100μl	60 ng/ml	Sigma
Insulin	100μl	10^{-6}M	Sigma
Dexamethasone	77μl	38 ng/ml	Sigma
T3 (L-Thyronine)	10μl	10.1 ng/ml	Sigma
Selenium	10μl	160 ng/ml	Sigma

Rat Schwann cell expansion media (in DMEM):

Component	Final Concentration	Company
FBS	3%	Perbio
NRG-1	10ng/ml in PBS	R+D Systems
Forskolin	2μM in ethanol	Calbiochem

Mouse Schwann cell expansion media (in DM):

Component	Final Concentration	Company
HS	0.5%	Thermo Scientific
NRG-1	10ng/ml	R+D Systems
Forskolin	2μM	Calbiochem

Cell lysis buffer:

Component	Amount	Company
T-PER	100μl per sample	Thermo Scientific
Halt protease & phosphatase inhibitor cocktail	1:100	Thermo Scientific

Western Blotting

RIPA buffer, for 20.2ml, use 75μl per sample:

Component	Amount	Company
1M Tris-HCl pH 8	5ml	Bio-rad Laboratories
5M NaCl	10ml	Fisher Scientific
20% Triton X-100	5ml	Sigma
5mM EDTA	200μl	Thermo Scientific

5x Laemmli buffer, use at 1x:

Component	Amount	Company
1M Tris pH 6.8	1.25ml	Sigma
20% SDS	5ml	BDH
100% Glycerol	2.5ml	Sigma
Bromophenol Blue	Some	Sigma
β-mercaptoethanol	1ml	Sigma
ddH_20	0.25ml	N/A

8% resolving gel, 1 gel:

Component	Amount	Company
ddH_2O	6.8ml	N/A
1.5M Tris pH 8.8	3.2ml	Bio-rad Laboratories
40% 19:1 Acr:Bis	2.5ml	Bio-rad Laboratories
10% SDS	125µl	BDH
10% APS	100µl	Sigma
TEMED	10µl	Sigma

5% stacking gel, 1 gel:

Component	Amount	Company
H_2O	3.1ml	N/A
0.5M Tris pH 6.8	1.25ml	Bio-rad Laboratories
40% 19:1 Acr:Bis	0.625ml	Bio-rad Laboratories
10% SDS	50µl	BDH
10% APS	37.5µl	Sigma
TEMED	7.5µl	Sigma

10x running buffer, pH to 8.3, use at 1x diluted in ddH_20:

Component	Amount	Company
Tris Base	30g	Sigma
Glycine	144G	Sigma
SDS	10g	BDH
ddH_20	Up to 1L	N/A

Transfer buffer:

Component	Amount	Company
48mM Tris	5.82g	Sigma
39mM Glycine	2.93g	Sigma
10% SDS	3.75ml	BDH
100% Methanol	200ml	BDH
ddH_20	Up to 1L	N/A

10x TBS, use at 1x diluted in ddH_20:

Component	Amount	Company
Tris HCL	78.6g	Sigma
NaCl	87.6g	Fisher Scientific

Genotyping

Alkaline lysis reagent pH 12 (Hotshot), use 75µl per sample:

Component	Amount	Company
25mM NaOH	50mg	Sigma
0.2mM disodium EDTA	3.72mg	Thermo Scientific
ddH_20	50ml	N/A

Neutralising reagent pH 5, use 75µl per sample:

Component	Amount	Company
40mM Tris-HCl	315.2mg	Sigma
ddH_20	50ml	N/A

2.2 List of antibodies

Primary antibodies – Immunofluorescence

Antibody	Host	Dilution	Diluent	Company
Arginase	Goat	1:100	ADS 0.2% Triton X-100	Santa Cruz
CD16/32	Rat	1:1000	1x PBS 0.1% BSA	BD Pharmingen
c-Jun	Rabbit	1:3200 (tissue) 1:800 (cells)	1x PBS 5% DS, 1% BSA, 0.1% Triton X-100	Cell Signaling Technology
DAPI	N/A	Various	1x PBS	Thermo Scientific
F4/80	Rat	1:200	ADS 0.2% Triton X-100	R + D Systems
Sox 10	Goat	1:100	1x PBS 5% DS, 1% BSA, 0.1% Triton X-100	R + D Systems

Secondary/biotinylated antibodies – Immunofluorescence

Antibody	Host	Dilution	Diluent	Company
Anti-goat (IgG) Alexafluor 488	Donkey	1:1000	1x PBS	Molecular Probes
Anti-rabbit (IgG) Cy3	Donkey	1:500	1x PBS	Jackson Immunoresearch
Anti-rat (IgG) biotinylated species	Goat	1:500	ADS 0.2% Triton X-100	Jackson Immunoresearch
Streptavidin Cy3	*Streptomyces avidinii*	1:500	ADS 0.2% Triton X-100	Jackson Immunoresearch

Primary antibodies – Western Blotting

Antibody	Host	Dilution	Dilutant	Company
c-Jun	Rabbit	1:1000	5% Milk TBS-T	Cell Signalling
p75 NTR	Rabbit	1:1000	5% Milk TBS-T	Millipore
Calnexin	Rabbit	1:1000	5% Milk TBS-T	Enzo Life Sciences
GAPDH	Rabbit	1:5000	5% Milk TBS-T	Sigma
p-c-Jun (Ser 63)	Rabbit	1:1000	5% Milk TBS-T	Cell Signalling

Secondary antibodies – Western Blotting

Antibody	Host	Dilution	Dilutant	Company
Anti-rabbit (IgG) HRP	Rabbit	1:1000	5% Milk TBS-T	Promega

2.3 List of primers

qPCR

House Keeping	Sequence	Primer conc (FWD/REV)
Calnexin Fwd	CAACAGGGGAGGTTTATTTTGCT	200/300
Calnexin Rev	TCCCACTTTCCATCATATTTGGC	
GAPDH Fwd	AGGTCGGTGTGAACGGATTTG	200/200
GAPDH Rev	TGTAGACCATGTAGTTGAGGTCA	
Yhaz Fwd	TAAATGGTCTGTCACCGTCT	200/300
Yhaz Rev	GGAAATACTCGGTAGGGTGT	

Repair Cell Markers	Sequence	Primer conc (FWD/REV)
BDNF Fwd	TCATACTTCGGTTGCATGAAGG	200/200
BDNF Rev	AGACCTCTCGAACCTGCCC	
c-Jun Fwd (Cells)	AATGGGCACATCACCACTACAC	200/300
c-Jun Rev (Cells)	TGCTCGTCGGTCACGTTCT	
c-Jun Fwd (Tissue)	CCTTCTACGACGATGCCCTC	200/300
c-Jun Rev (Tissue)	GGTTCAAGGTCATGCTCTGTTT	
GDNF Fwd	GATTCGGGCCACTTGGAGTT	200/300
GDNF Rev	GACAGCCACGACATCCCATA	
Olig1 Fwd	CCGCCCCAGATGTACTATGC	200/300
Olig1 Rev	AACCCACCAGCTCATACAGC	
Shh Fwd	AAAGCTGACCCCTTTAGCCTA	200/300
Shh Rev	TTCGGAGTTTCTTGTGATCTTCC	

Genotyping

Primer	Sequence (5'- 3')
c-Jun^{OE} Fwd	TGGCACAGCTTAAAGCAGAAA
c-Jun^{OE} Rev	GCAATATGGTGGAAAATAAC
P_0 cre Fwd	GCTGGCCCAAATGTTGCTGG
P_0 cre Rev	CCACCACCTCTCCATTGCAC
Rosa26 Fwd	GGAGTGTTGCAATACCTTTCTGGGAGTTC
Rosa26 Rev	TGTCCCTCCAATTTTACACCTGTTCAATTC
PLP Fwd	CACTCTGTGCTTGGTAACATGG
PLP Rev	TCGGATCCGCCGCATAACC

2.4 Genotyping: mastermix and PCR conditions

Mastermix recipe per sample giving final volume of 25µl:

Component	Volume (µl)	Company
Buffer 5x	5	Invitrogen
$MgCl_2$ (25mM)	1.5	Invitrogen
Fwd primer (10µM)	1.25	Invitrogen
Rev primer (10µM)	1.25	Invitrogen
dNTPs (10µM)	0.5	Invitrogen
Taq	0.2	Invitrogen
H_2O	13.05	-
DMSO	1.25	Sigma
DNA	1	-

P_0 cre PCR conditions:

Time	Temperature (°C)	
4 minutes	94	
30 seconds	94	x30 cycles
1 minute	50	
1 minute	72	
10 minutes	72	

Rosa26 PCR conditions:

Time	Temperature (°C)	
4 minutes	94	
15 seconds	94	x36 cycles
40 seconds	60	
40 seconds	72	
2 minutes	72	

c-Jun^{OE} PCR conditions:

Time	Temperature (°C)	
5 minutes	94	
30 seconds	94	x34 cycles
45 seconds	55	
1 minute	72	
7 minutes	72	

PLP PCR conditions:

Time	Temperature (°C)	
2 minutes	94	
30 seconds	94	x35 cycles
45 seconds	54	
45 seconds	72	
5 minutes	72	

2.5 qPCR conditions

20µl sample volume run:

Time	Temperature (°C)	
2 minutes	95	
15 seconds	95	x49 cycles
1 minute	55	
5 seconds	65	In 5°C increments
	95	

2.6 Animals

Animals were housed in the BSU at UCL in individually ventilated cages. All procedures were carried out in accordance with Home Office guidelines. All animals over P10 were culled via Schedule 1. Animals under P10 were culled by decapitation, a procedure for which we are licensed. All work was carried out with the 3Rs mind (reduce, replace, refine). The mice used in Chapters 3-5 are 6-10 weeks old. The young mice in Chapter 6 are also 6-10 weeks old and the old mice are 1 year old. All mouse lines are on a C57BL/6 background.

All rat pups were acquired from the UCL BSU where they are maintained and bred.

P_0 cre mice

P_0 cre mice were a gift from Dr L. Feltri, Buffalo, USA. A mP_0TOT(Cre) transgene is driven by the P_0 promoter (Feltri, D'Antonio, Previtali, et al., 1999).

c-Jun cKO mice

c-Jun cKO mice have c-Jun specifically knocked out in their Schwann cells under the control of the P_0 cre promoter. Mice were gifted from the Dr A. Behrens, London. Mice were generated by crossing c-Jun$^{fl/fl}$ mice (Behrens et al., 2002) with P_0 cre mice. Mice from this crossing were then crossed back with c-Jun$^{fl/fl}$ mice. c-Jun cKO mice are described at length by (Parkinson et al., 2008; Arthur-Farraj et al., 2012).

Homozygous mice were used for all experiments and were genotyped using P_0 cre primers (see 2.4). P_0 cre$^-$ littermates were used as experimental controls and are hereafter referred to as WT.

c-Jun $OE^{fl/fl}$ mice

Mice were gifted from the lab of Dr K. Rajewsky. These mice have the potential to over-express c-Jun in any cell in their body due to a c-Jun cDNA insert on the Rosa26 locus. The transgene expression is controlled by a lox-p flanked STOP cassette in front of a CMV early enhancer/chicken β actin (CAG) promotor (Figure 2.1). Homozygote mice were used in this thesis and homozygosity was confirmed by genotyping with both $c\text{-}Jun^{OE}$ and Rosa26 primers.

OE/+ and OE/OE mice

OE/+ and OE/OE mice over-express c-Jun specifically in Schwann cells. c-Jun $OE^{fl/fl}$ mice were crossed with P_0 cre mice generating P_0 $cre^{+}/c\text{-}Jun^{OE/+}$ mice, referred to hereafter as OE/+ mice. Homozygotes were generated by crossing OE/+ mice back with c-Jun $OE^{fl/fl}$ mice, hereafter referred to as OE/OE mice. P_0 cre^{-} littermates were used as experimental controls and are hereafter referred to as WT mice. Hetero or homozygosity was confirmed by genotyping with both Rosa26 and P_0 cre primers.

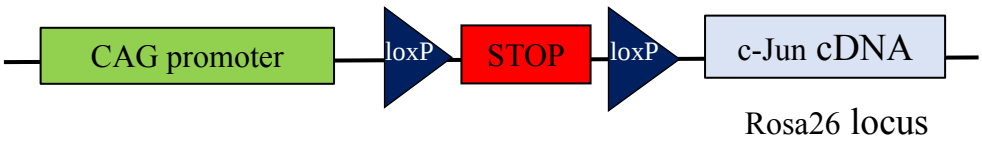


Figure 2.1 | Diagram of the c-Jun OE construct

A lox-P flanked STOP cassette is located in front of a CAG promoter. In the presence of cre, the lox-P sites will be excised, activating the transgene, leading to the over-expression of c-Jun.

TMX mice

To generate an inducible mouse line, PLP creERT2$^{+/-}$ mice (Leone et al., 2003), were crossed with c-Jun OE$^{fl/fl}$ mice. The resulting mice were then back crossed with c-Jun OE$^{fl/fl}$ mice generating a tamoxifen inducible c-Jun over-expressing mouse line. 170mg/kg tamoxifen dissolved in sunflower oil (Sigma) and 100% ethanol (10:1) was administered daily for 5 days. Mice were genotyped with PLP primers. Tamoxifen activates the ERT2 receptor which in turn excises the lox-P sites through cre-recombinase, leading to the over-expression of c-Jun in Schwann cells (Figure 2.2).

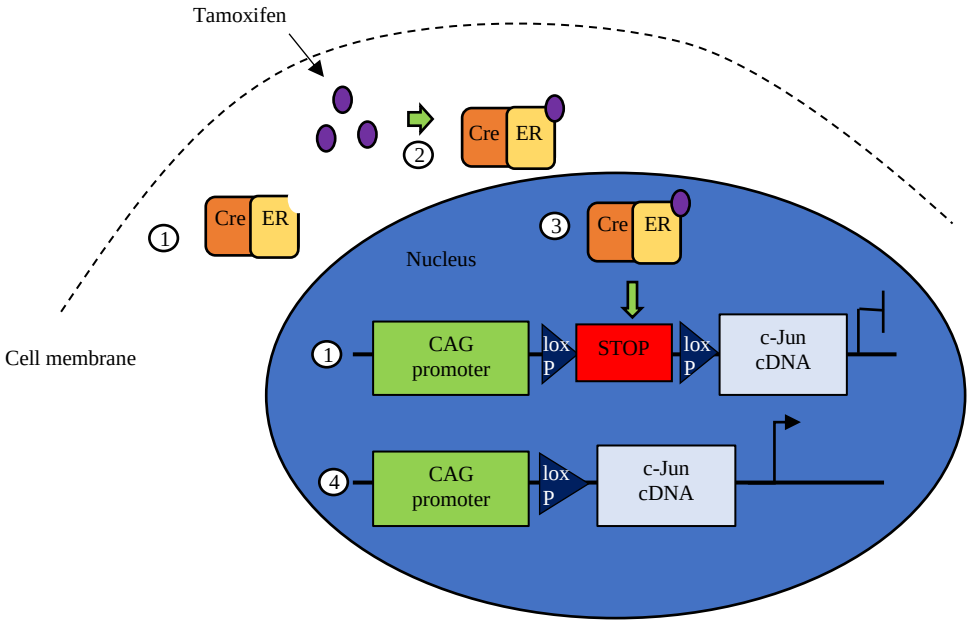


Figure 2.2 | A diagram of the tamoxifen inducible over-expression of c-Jun

Mice express a cre-recombinase linked estrogen receptor (ER) under the PLP promoter. The expression of this receptor is therefore Schwann cell specific. **1.** In the absence of tamoxifen, the receptor remains in the cytoplasm. In the nucleus, the loxP sites flank the stop codon preventing expression of the transgene. **2.** Injected tamoxifen crosses the cell membrane and binds to the ER receptor. **3.** The CreER receptor complex translocates to the nucleus where the cre excises the loxp sites releasing the stop codon. **4.** The cre-recombinase mediated removal of the stop codon induces gene expression resulting in the over-expression of c-Jun in Schwann cells.

2.7 Materials

General

15ml and 50ml Falcon tubes were bought from BD Biosciences. Stripettes (5ml, 10ml, 25ml) were bought from Costar. Tips were from Clear Line. Gloves were from Shield Scientific. Eppendorf tubes (0.5ml, 1.5ml and 2ml) were from Sarstedt. Pasteur pipettes (1.5ml and 3ml) were bought from Scientific Instruments Services. 100% ethanol and isopropanol were from BDH.

EM

Moulds were from Agar Scientific. Propylene oxide and osmium tetroxide were from Sigma.

Genotyping

Agarose was from Invitrogen. TAE 50x buffer was from VWR. 100bp ladder was from Fisher Scientific. Ethidium bromide was from Sigma. Agarose gels were viewed and imaged using a dual-intensity transilluminator from UVP.

Immunofluorescence

Tisse Tek OCT and moulds were from Agar Scientific. Super frost slides were purchased from Fisher Scientific. Southern Biotech provided Fluromount-G. 22 x 50 mm coverslips were from VWR.

qPCR

Primers from Invitrogen. PrecisionPLUS qPCR Mastermix with SYBR Green was from Primerdesign. White hard shell 96 well plates, microseal seals and the CFX96 Real-Time PCR Detection System were from Bio-rad. Tips were from Gilson.

Surgical

All surgical and dissection tools were provided by Fine Scientific Tools. Scalpel blades were purchased from Swann-Morton. Hydrex pink was from Ecolab. Sterile drapes, gowns, swabs, isoflurane, surgical gloves, 0.9% NaCl saline, bupivacaine, carprofen and vetergesic were provided by the UCL BSU. Silicon tubing was from Freudenberg Medical. Lacri-Lube was bought from Allergan. Flurogold was from Flurochrome. Clips were purchased from VET-TECH. Suture information is provided in the table below:

Suture use	**Needle size**	**Material**	**Absorbant**	**Company**
Wound closure	6	Coated vicryl	Yes	Ethicon
Tying back nerves	7	Prolene	No	Ethicon
Repairing nerves	8	Nylon	No	S&T

Tissue culture

All dishes, flasks and plates were bought from VWR. 13mm round cover slips were bought from BDH. Collagenase was purchased from Worthington. L15, DMEM media and 0.25% trypsin-EDTA were from Invitrogen. Penicillin streptomycin and trypsin were brought from PAA. Tips were from Star Labs. Purmorphamine was from Sigma. SAG was from Merk. Cyclopamine was from Insight Biotechnology.

Western blotting

Mini protean tank, semi-dry transfer system, extra thick blot paper and chemidoc were from Bio-rad. BCA protein assay kit and PageRuler prestained protein ladder were distributed by Thermo Fisher Scientific. Amersham provided nitrocellulose hybond membrane, ECL and ECL prime kits. Skirted 2ml tubes and easy grip screw caps were from Star Labs. Semi-skimmed milk powder was from Sigma.

2.8 Methods

2.8.1 Tissue culture

Rat Schwann cell cultures

P3/4 Sprague Dawley rats of either sex were obtained from the UCL BSU. 1 rat was needed per plate. Pups were culled by decapitation. Bodies were sprayed with 70% ethanol and the sciatic nerves and brachial plexuses were removed into L15 medium on ice. Nerves were cleaned and de-sheathed. In a hood, the L15 was removed and 300µl trypsin and 300µl collagenase was added. The nerves were incubated with the enzymes for 1 hour in the incubator (37°C, 5% CO_2). Nerves were triturated 10x with a 1ml tip then incubated for a further 10 minutes. Nerves were further triturated 10x will a 1ml tip and 10x with a 200µl tip. The digestion was halted with 1ml DMEM 10% FBS and the cell suspension was transferred to a 15ml Falcon tube. The 35mm dish was washed with a further 1ml to remove any remaining cells and the wash transferred to the Falcon tube. The cells were spun at 1000rpm for 10 minutes. The pellet was resuspended in 2ml DMEM 5% FBS + P/S and cells were plated on laminin and PLL coated dishes. 20µl of Ara C (1:100, final conc. 10^{-5}M) was added to eliminate fibroblasts (Brockes et al., 1979; Stevens et al., 1998). The cells were incubated at 37°C, 5% CO_2 for 3 days. The cells were then changed into rat expansion medium.

Mouse Schwann cell cultures

P8-10 mouse pups of either sex were culled by decapitation. 4 mice were needed per plate. Bodies were sprayed with 70% ethanol and the sciatic nerves were removed into L15 medium on ice. Nerves were cleaned and de-sheathed. In a hood, the L15 was removed and 300µl trypsin and 300µl collagenase was added. The nerves were incubated with the enzymes for 1 hour in the incubator (37°C, 5% CO_2). Nerves were triturated 10x with a 1ml tip then incubated for a further 10 minutes. Nerves were further triturated 10x with a 1ml tip and 10x with a 200µl tip. The digestion

was halted with 1ml DMEM 10% HS and the cell suspension was transferred to a 15ml falcon tube. The 35mm dish was washed with a further 1ml to remove any remaining cells and the wash transferred to the falcon tube. The cells were spun at 1000rpm for 10 minutes. The pellet was resuspended in 2ml DMEM 5% HS + P/S and cells were plated on laminin and PLL coated dishes. 20µl of Ara C (1:100, final conc. 10^{-5}M) was added to eliminate fibroblasts. The cells were incubated at 37°C, 5% CO_2 for 3 days. The cells were then changed into mouse expansion medium.

Schwann cell passaging

When cultures were 70-80% confluent, medium was removed and the cells were washed 2x in 1x PBS. 0.25% trypsin-EDTA (1x) was added to remove the cells from the dish. Cells were incubated in the enzyme for 5 minutes at 37°C, 5% CO_2. A half volume of 10% FBS DMEM was added to halt the reaction and the cell suspension was transferred to a 15ml falcon tube. The dish was washed with the remaining 10% FBS DMEM. The wash was added to the 15ml falcon tube. Cells were spun at 1000rpm for 10 minutes. The pellet was resuspended and the cells were plated in expansion medium.

Mouse Schwann cell virus infection

All virus infections were carried out in a dedicated containment level 2 virus lab in the UCL MRC laboratory for molecular cell biology (LMCB). Once cells were 70-80% confluent, cells were infected with 1:500 adenovirus in DM 0.5% HS and 2µM forskolin. The viruses had previously been titrated to determine the correct dilution for ~100% infection and only cultures with >95% Schwann cell purity were infected. The medium was changed after 24 hours to DM 0.5% HS. After 48 hours, cells underwent protein, RNA extraction or fixation.

Schwann cell culture experiments

During all experimental conditions, Schwann cells were incubated in DM and 0.5% of appropriate serum for 48 hours unless stated otherwise.

Purmorphamine, Smoothened agonist (SAG) and cyclopamine

Purmorphamine, SAG, and cyclopamine were dissolved in DMSO to generate a stock concentration of 10mM. Stock solutions were aliquoted and stored at -20°C until use. One aliquot was used per experiment to prevent multiple freeze thaw cycles.

Schwann cell immunofluorescence

For immunofluorescence experiments, 5000 Schwann cells were plated in a 35µl drop on a PLL laminin coated coverslip. Cells were given 24 hours to sit down, then were topped up with DM. At the experimental end point, cells were washed 2x with 1x PBS. Cells were fixed with 4% PFA for 10 minutes. Cells were then washed for 5 minutes in 1x PBS. Fresh PBS was added to the wells and the lid was parafilm sealed. Dishes were stored at 4°C until use.

Schwann cell ELISA

Cells were washed 2x with 1x PBS then incubated with 0.1M HCl for 10 minutes. A cell scraper was used to detach the cells from the plate and the suspension was kept on ice. Samples were spun at 1000rpm for 10 minutes at 4°C. The supernatant was stored at -80°C for no more than 1 week. A cAMP ELISA kit (Enzo) was used as per the manufacturer's instructions to determine the levels of cAMP present in the sample. This was then divided by the total protein of the sample which was calculated by BCA (described later in this chapter).

Schwann cell protein extraction

Cells were washed 2x with 1x PBS and incubated with 100µl cell lysis buffer. Cells were physically detached from dishes using a cell scraper and the cell lysate was kept on ice in a 1.5ml eppendorf tube. Lysate was spun for 2 minutes at 1000rpm to

pellet the debris. The supernatant was transferred to a fresh eppendorf tube and spun for a further 2 minutes at 1000rpm. The protein was stored at -80°C until use.

Schwann cell RNA extraction

RNA from cells was extracted using an RNeasy Micro Extraction Kit (Qiagen). 1,000,000 cells were used per sample. Extractions were carried out in a laminar flow hood. All surfaces and pipettes were sprayed with RNase Zap™ and gloves were changed regularly to prevent RNA degradation. Media were removed and cells were washed 2x with 1x PBS before the extraction. Extractions were carried out as per the manufacturer's instructions. An in-column DNase digest was included as part of the extraction protocol. RNA quality and concentration was determined after the extraction using a nanodrop 2000 machine (Thermo). RNA was stored at -80°C until use.

Schwann cell cDNA synthesis

1µg of RNA was converted to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. cDNA was stored at -20°C until use.

2.8.2 Nerve processing for EM

Mice were culled by Schedule 1 and the sciatic nerve was exposed. A few drops of EM fix was added to the nerve and left for 15 minutes. This was to ensure the nerve was fixed straight. The nerve was removed and left in EM fix overnight at 4°C. The nerve was washed in 0.1M cacodylate buffer 3x for 15 minutes. To osmicate the nerve, equal parts 2% osmium tetroxide in ddH_2O and 0.2M cacodylate buffer were mixed. The 1% osmium tetroxide solution generated was then added to the nerves for 2 hours at 4°C. The nerve was washed for 2x 15 minutes in ddH_2O. The samples were dehydrated by washing in progressively higher percentage ethanol solutions: 25% ethanol / 5 minutes, 50% ethanol / 5 minutes, 70% ethanol / 5 minutes, 90% ethanol / 10 minutes, 100% ethanol / 10 minutes (x4), propylene oxide / 10 minutes (x3).

The dehydrated nerve was incubated in a 25:75 resin:propylene oxide mixture for 45 minutes. The nerve was then incubated for 1 hour in a 75:25 resin:propylene oxide mixture. The nerve was then blocked in resin, shaking overnight. The nerve was re-blocked for 2 hours the next day in fresh resin. The nerve was then embedded in fresh resin in EM molds and baked for 24 hours at 60°C. Once ready, transverse ultrathin sections were cut 5mm from the injury site by Mark Turmaine (Electron Microscopy Unit). All the cells with a visible nucleus were counted in a whole tibial nerve profile at x8000 magnification on a Jeol 10p0 electron microscope equipped with a digital camera. Schwann cells were identified by the presence of a basal lamina and morphology, whereas fibroblasts, macrophages, mast and other cell types were confirmed by morphology. Counts were performed with the help of R. Mirsky and K.R Jessen.

2.8.3 Surgical procedures

All surgical procedures were performed under aseptic conditions in the surgical theatres of the BSU. All surgical procedures were carried out on the right sciatic nerve only. Mice were anesthetized with isoflurane. The surgical site was shaved and skin was sterilized with hydrex pink. Lacri-Lube was applied to the eyes to keep them hydrated during procedures. Mice were placed on a heat mat to maintain their body temperature. During every procedure, mice were given the minimum analgesia of 2 drops of bupivacaine diluted 1:10 in 0.9% saline administered at the incision site at the time of closure with number 5 forceps. After all surgical procedures, mice were placed in a recovery chamber at 27°C and returned to clean cages once fully ambulatory. Surgical clips were removed after 10 days and any sutures used to close were dissolvable.

Sciatic nerve crush

To ensure consistency, all sciatic nerve crushes were performed by Shaline Fazal. A vertical incision was made on the right hip using dissection spring scissors. If a second surgery was to be performed, a horizontal incision was made, this established an easier second incision and closure. The muscle was separated and the sciatic nerve exposed. The whole sciatic nerve was crushed at the sciatic notch with fine forceps for 3x 15 seconds at 3 rotation angles. The muscle was moved back into place and 2 drops of bupivacaine were administered to the site using number 5 forceps. The incision was closed using 2 autoclips.

Whole sciatic nerve cut (acute denervation < 1 week)

A vertical incision was made on the right hip using dissection spring scissors. The sciatic nerve was exposed and cut at the sciatic notch with fine scissors. The muscle was replaced and 2 drops of bupivacaine were administered to the site using number 5 forceps. The incision was closed using 2 autoclips**.**

Whole sciatic nerve cut (chronic denervation > 1 week)

If a cut nerve was to be examined at a time point of greater than 1 week, the proximal nerve stump had to be tied back into the muscle to prevent axon regeneration into the distal nerve stump. To perform this, a vertical incision was made on the right hip using dissection spring scissors and the sciatic nerve was exposed. A double knot was tied around the nerve at the sciatic notch using a prolene suture and the nerve was cut distal to this. The proximal stump was cleaned, allowing it be moved freely. A 'channel' was made from the nerve to the anterior of the biceps femoris by pushing number 5 forceps through the muscle. The suture was then passed through this channel and the proximal stump was pulled through to rest on the anterior of the biceps femoris. The proximal stump was anchored with a double knot. The distal nerve stump was left in place. 2 drops of bupivacaine were administered to the site using number 5 forceps and the incision was closed using 2 autoclips.

Tibial nerve cut and repair (immediate repair)

A 1.5cm horizontal incision was made using number 10 surgical blade from the hip towards the knee. The muscle was separated to expose the sciatic nerve from below the sciatic notch to the knee. The individual branches of the sciatic nerve were separated by inserting a pair of nerve 5 forceps between the visible branching at the knee. The forceps were gently opened and moved towards the notch, carefully separating the nerve branches. A knot was tied using a prolene suture where the sural nerve branched from the tibial nerve. The tibial nerve was cut distal to the suture leaving the other branches intact. The proximal stump of the tibial nerve was tied into the adjacent muscle to prevent axon regeneration. The common peroneal nerve was then cut and any visible distal stump was removed. This prevented the common peroneal nerve regenerating into its own distal stump as opposed to the tibial nerve distal stump. In order to repair the nerves and hold them together, a nylon suture and a silicon tube was used (Figure 2.3). The suture was inserted through the side of the tube and out of an opening. The common peroneal proximal stump was secured to the suture by the epineurium. The suture was then moved inside and through the tube. The tibial nerve distal stump was also secured to the suture by the epineurium.

Special care was taken not to push the suture through the whole nerve as this could impair axon regeneration. The suture was then passed back into the tube and pushed through the side of the tube where it had originally entered (Figure 2.3 A). Both ends of the suture were gently pulled bringing both nerves into the tube and connecting the stumps (Figure 2.3 B). A knot was then tied to hold the stumps in place (Figure 2.3 C). The muscle was closed with a horizontal mattress stich using a vicryl suture. 2 drops of bupivacaine were administered to the site using number 5 forceps and the incision was closed with a continuous subcuticular suture with a vicryl suture. Mice were given a subcutaneous injection of 0.1mg/kg vetergesic and carprofen was administered in their water for 5 days post-surgery (1 ml of 50mg/ml stock in 200ml drinking water).

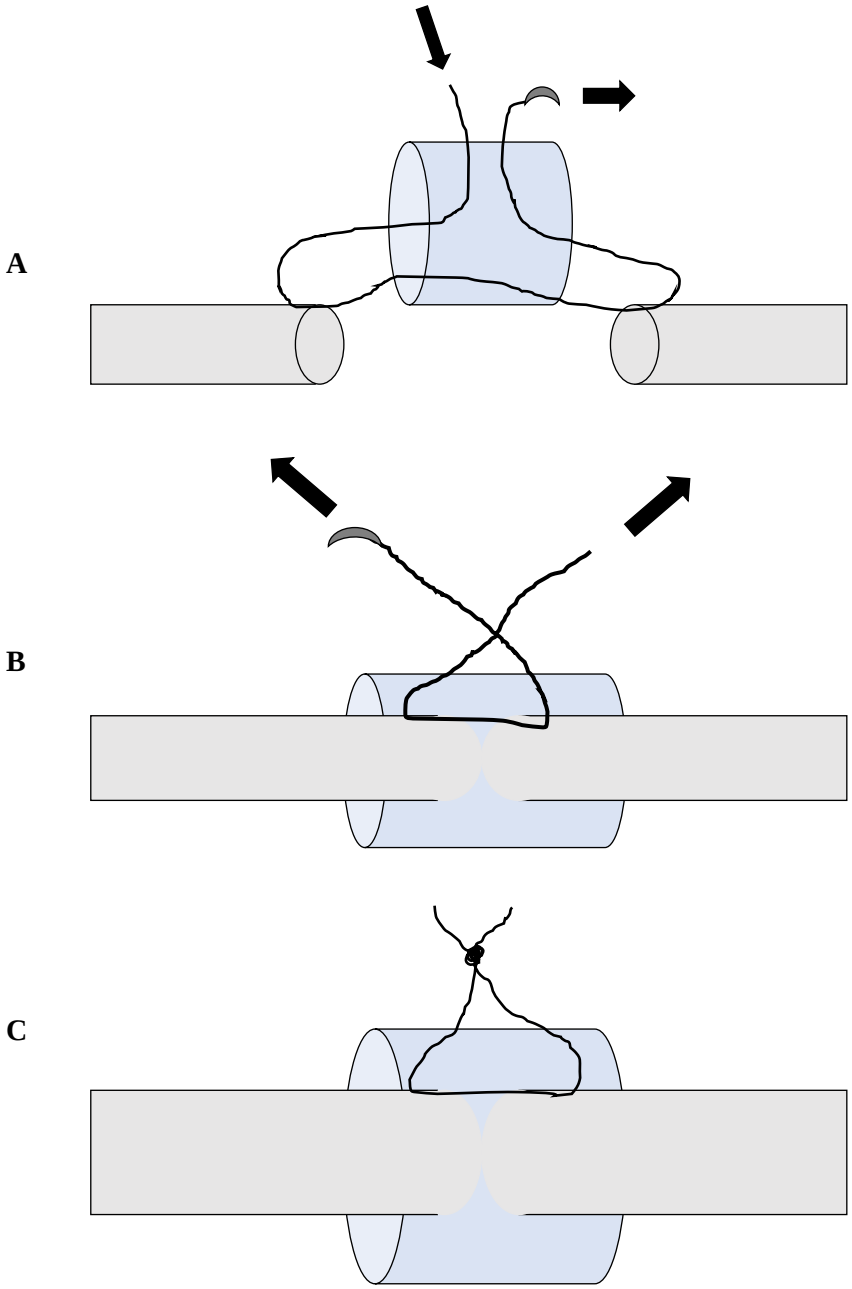


Figure 2.3 Diagram of surgical nerve repair

(A) The nylon suture is passed through the side of the silicon tube and out through the end opening. The suture secures the epineurium of the nerve and is passed through the tube. The suture goes through the epineurium of the adjoining nerve and is passed back inside the tube and out through the side it originally entered through. **(B)** Both ends of the suture are gently pulled to bring the nerves together inside the tube. **(C)** The suture is tied to secure the stumps.

Tibial nerve cut and repair (chronic denervation: 10 weeks)

Mice underwent the same early stages of the procedure described above for tibial nerve cut followed by immediate repair. However, once the tibial nerve was cut and the proximal stump was sutured into the muscle, the distal nerve stump was also tied back. A loop was made in a prolene suture and saline was added to the site. This allowed the distal nerve stump to be 'floated' through the loop, preventing damage, and a knot was tied to it. The distal nerve stump was then sutured into the adjacent muscle. This ensured the nerve could be easily located following chronic denervation, during which the nerve shrinks and due to myelin break down, becomes transparent. The site is then closed as described previously. 10 weeks later the site was re-opened and the nerve exposed (Figure 2.4 A). The tibial nerve distal stump was cut free from the muscle. The common peroneal nerve was cut and sutured to the tibial nerve distal stump as previously described in Figure 2.3 (Figure 2.4 B). The site was then closed and post-operative care was administered as previously described above.

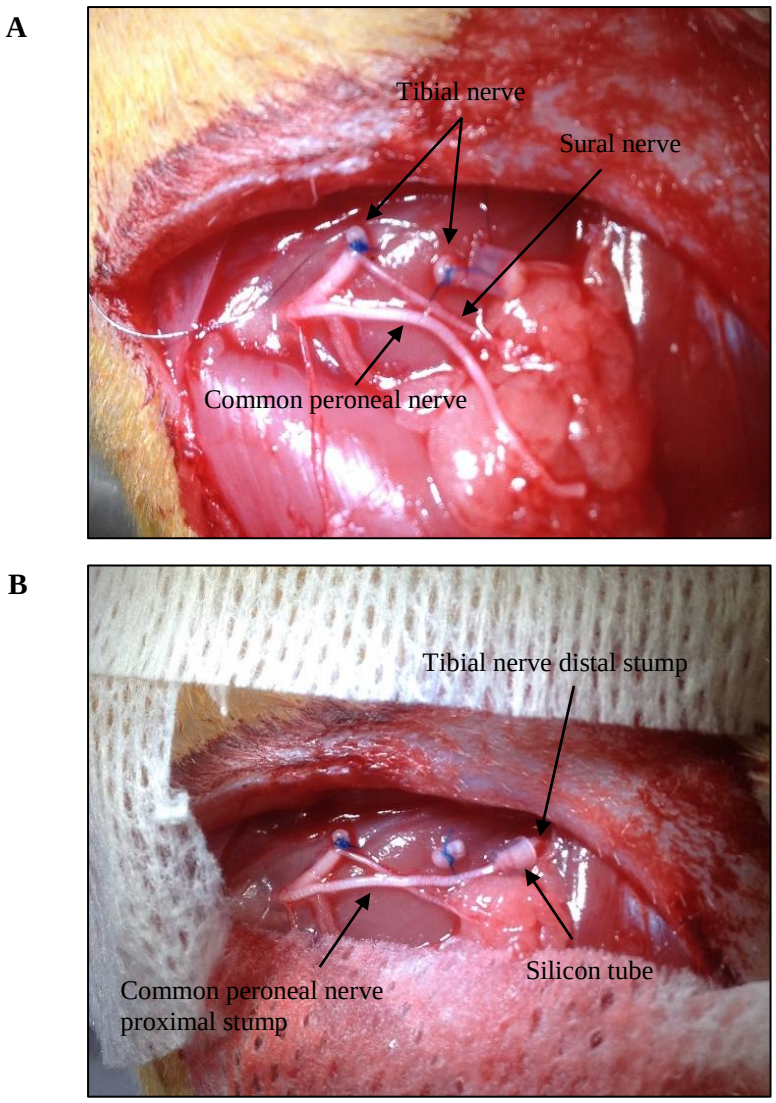


Figure 2.4 | Nerve cross suturing following chronic denervation

Representative images taken by the author. Here, a rat is used to demonstrate the procedure. The larger nerves provide more detailed images. The nerves in these images have not been chronically denervated to further improve clarity of the procedure. **(A)** Following 10 weeks of chronic denervation the injury site is reopened. The proximal and distal stumps of the tibial nerve were previously tied back into the muscle and are easy to locate due to the blue suture. The sural nerve remains intact. The common peroneal nerve is now cut with a long proximal stump allowing plenty of nerve to work with. **(B)** The distal stump of the tibial nerve is cut free from the muscle. This is sutured to the proximal stump of the common peroneal nerve (as previously decribed in Figure 2.3) and held in place with a silicon tube.

Retrograde labelling with Fluorogold

This technique was adapted from Catapano et al., 2016. Training in this technique was received directly from the authors of this work.

After sciatic nerve crush or tibial nerve repair, sufficient time was given to allow for axon regeneration. Retrograde labelling was then used to assess regeneration of motor and sensory neurons. In short-term crush injuries, the previous incision site was reopened. In nerve repair surgeries, a new incision was made parallel to the previous one using a number 10 blade. The muscle was opened and the sciatic nerve was exposed. In crush injuries, the desired distance was measured from the crush site at the sciatic notch. The nerve was then cut. With tibial nerve repairs, the tube used to hold the repair site together was carefully removed. 4mm was then measured from the site of repair down the tibial nerve and cut. The procedure uses a well technique to administer Fluorogold to the nerve (Figure 2.5). This method has been found to more accurate than the injection of tracers and is easier than the silicon cap technique, which yields similar results (Catapano et al., 2016). A 1cm x 0.5 cm rectangle of parafilm was cut. A hydrophobic barrier of petroleum jelly (Vaseline) was used to create a 0.5cm x 0.5cm well (Figure 2.5 A). The cut nerve was then placed into this well and the hydrophobic barrier reinforced (Figure 2.5 B). 4% Fluorogold in saline, which had been previously prepared and stored at -20°C, was thawed and added to the well (Figure 2.5 C). The nerve was left in the well for 1 hour. During this time, the open surgical site was covered with sterile gauze swabs soaked in sterile 0.9% saline to prevent infection and to keep the area hydrated. Mice were also injected with 0.9% saline (0.06ml/10g bodyweight) to remain hydrated. The isoflurane was reduced and breathing and heat mat temperature were closely monitored. After an hour, the nerve was removed from the well which was disposed of. If the incubation had been successful, the nerve tip that was exposed to the Fluorogold would be stained yellow (Figure 2.5 D). The site was washed 3x with 0.9% saline. The site was closed and post-operative care was administered as previously described.

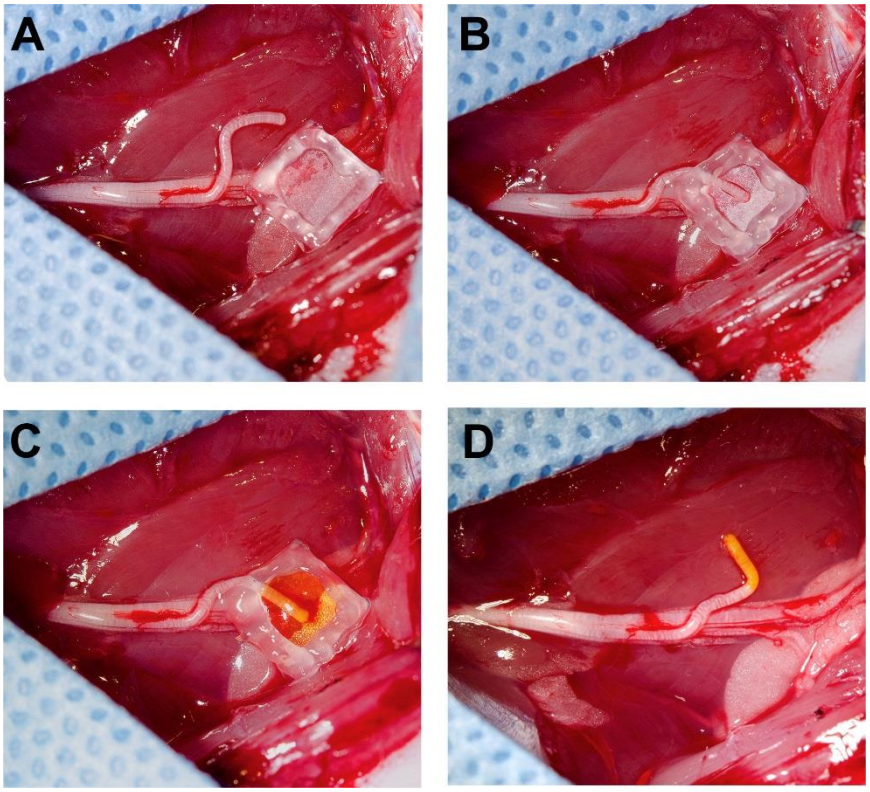


Figure 2.5 | Retrograde labelling with Fluorogold

For ease, a rat nerve is shown, which is larger in size than that of the mouse. **(A)** A hydrophobic barrier of petroleum jelly created a 0.5cm x 0.5cm well. **(B)** The cut nerve was placed into this well and the hydrophobic barrier reinforced. **(C)** 4% Fluorogold in saline is added to the well and the nerve was left for 1 hour. **(D)** The nerve is removed from the well and is stained yellow to indicate successful labelling. Figure taken from Catapano et al., 2016.

Harvesting tissue

1 week after retrograde labelling, mice were perfused. This allowed sufficient time for optimum labelling (Novikova et al., 1997). The spinal cord and L4 DRG were removed and fixed in 4% PFA/PBS for 24 hours at 4°C. Tissue was then transferred to 30% sucrose in 4% PFA for 72 hours at 4°C. Spinal cords were embedded in OCT in 24 x 24 x 5 mm moulds. DRGs were embedded in OCT in 15 x 15 x 5 moulds. Spinal cords were cut longitudinally at 50µm, DRGs were cut at 20µm. Sections were collected on super frost slides. All sections of the spinal cord were counted and the Abercrombie correction was applied to compensate for double counting (Abercrombie, 1946). This was calculated to be 0.625. To avoid double counting in the DRGs, every fifth section was counted. Neuronal cell bodies in the DRG have been observed to be 70-100µm in size in the mouse (Lawson, 1979)S. Fazal, unpublished observations). Each section was therefore counted 100µm apart. All counts were performed on a Nikon Labophot 2 fluorescence microscope.

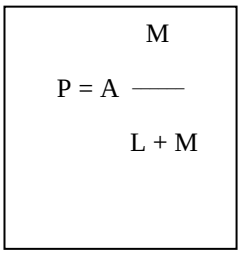


Figure 2.6 | The Abercrombie correction

Correction was applied to all counts from the spinal cord to prevent the double counting of cells. P is the average number of cells. A is the number of cells counted. Section thickness in µm is M. L is the average length of the cell in µm.

2.8.4 qPCR

Nerve RNA extraction

When experimental time points were reached, mice were culled by Schedule 1 procedure. The dissection area, cork board and tools were sprayed with RNase ZAP™ and tools were kept on an RNase free sheet of aluminium. Tools were re-sprayed between animals. Injured sciatic nerves were removed and snap frozen in liquid nitrogen in RNase free eppendorf tubes. The uninjured contralateral nerve was taken as a control. Extractions were performed under the conditions previously described. RNA was extracted using a RNeasy lipid tissue kit (Qiagen). Chronically denervated and uninjured nerves had to be pooled, 2 per n to generate a usable concentration of RNA. Acutely denervated nerves were not pooled using 1 nerve per n. An in column DNase step was performed and the RNA was suspended in 30μl RNase free water. RNA quality and concentration was determined after the extraction using a nanodrop 2000 machine (Thermo). RNA quality was further tested using an Experion™ RNA HighSens CHIP kit. Samples given a RNA quality indicator value of greater than 6.5 were converted to cDNA. RNA was stored at -80°C until amplification.

Tissue cDNA amplification

Due to low RNA concentration yields from nerves, it has previously been found that up to 8 nerves need to be pooled to generate quality cDNA to run an efficient qPCR reaction (Benito et al 2017). To reduce the number of animals needed for these experiments, RNA was amplified during the cDNA conversion using a QuantiTect whole transcriptome kit. 10ng of RNA was amplified as per the manufacturer's instructions using the standard amplification protocol (2 hour amplification) generating 10μg cDNA. cDNA was stored at -20°C until use.

qPCR

cDNA was diluted (Mouse Schwann cells 1:16, rat Schwann cells 1:32 and nerve 1:1024) in RNase free water and prepared with primers and PrecisionPLUS mastermix with SYBRgreen. GAPDH and calnexin were used as house keeping genes in cell experiments, GAPDH, calnexin and Yhaz were used for uninjured nerve experiments and denervated nerve experiments were compared with GAPDH.

2.8.5 Western Blotting

Tissue protein extraction

Nerves were dissected, placed in 1.5ml eppendorf tubes and snap frozen in liquid nitrogen. Samples were stored at -80°C until use. For protein extraction, nerves were placed in 2ml graduated skirted tubes with 9 10B lysing beads with 75ml RIPA buffer and homogenized using a Fastprep fp120 homogeniser. Samples were run twice at speed 6 for 45 seconds. Lysates were then centrifuged at 13000rmp for 2 minutes at 4°C to pellet the debris. The supernatant was transferred to a new 1.5ml eppendorf tube and centrifuged at 13000rmp for 2 minutes at 4°C. The supernatant was transferred to a new 1.5ml eppendorf tube and the protein extract was stored at -80°C until a BCA protein assay could be performed.

BCA protein assay

Protein concentrations from extracts were determined using a Pierce™ BCA protein assay kit as per the manufacturer's instructions. To ensure equal concentrations were added to Western blots, a standard test was used (range: 2000-25μg/ml). To determine the protein concentrations of samples extracted in 0.1M HCL for ELISA assays, an enhanced test was used (range 5-250μg/ml). The assay was performed on a 96 well plate with samples and standards run in duplicate. The plate was read at 510nm and sample protein concentrations were determined by creating a standard curve.

Western blots

Antibodies and dilutions can be found in 2.2.

Protein was diluted in the appropriate lysis buffer to ensure 10μg was loaded to each gel. 5x Laemmli buffer was added to samples at a working concentration of 1x. Samples were heated to 95°C for 5 minutes to denature the protein. 20μl of sample was loaded per well and run on an 8% gel at 60mV for 3 hours. Protein from the gel

was transferred to a nitrocellulose membrane. Extra thick blot paper, the nitrocellulose membrane and the 8% gel were soaked in transfer buffer for 5 minutes. A sandwich of paper, membrane, gel, paper was prepared. Between the addition of each layer, bubbles were rolled out using a glass Pasteur pipette. The transfer was performed on a semi-dry transfer system at 25mV for 45 minutes. Once the transfer was complete, membranes were briefly soaked in Ponceau S to determine that the transfer has been successful and that equal levels of protein had been loaded in the gel. Membranes were briefly washed in ddH_20 to remove excess Ponceau and blocked in 5% milk TBS-T for 1 hour shaking at room temperature. Membranes were then incubated with appropriate antibodies in heat sealable polyethylene bags to reduce the volume of antibody required. Membranes were attached to a rotatory wheel to ensure even antibody coverage and incubated overnight at 4°C. Membranes were washed 3x 10 minutes in 1x TBS-T then incubated with the appropriate secondary antibody in heat sealable polyethylene bags, rotating for 1 hour at room temperature. Membranes were washed 3x 10 minutes in 1x TBS-T before developing. For development of GAPDH, membranes were incubated with ECL for 1 minute and developed on a Bio-Rad Chemidoc machine. For the development of all other antibodies, membranes were incubated with ECL prime for 5 minutes then developed. Membranes were automatically exposed to prevent saturation. Blots were analyzed using Bio-Rad Imagelab. Protein levels were determined by dividing the protein of interest against the house keeping protein (GAPDH or calnexin). All blots were then normalized to one sample (e.g 1 week after injury, control cells) to account for any difference between each blot.

2.8.6 Immunofluorescence

Antibody dilutions and diluents can be found in 2.2.

Sox10 c-Jun dual immunolabelling

Distal nerve stumps were dissected and cut into 2mm segments. Segments were fresh frozen in OCT. Blocks were sectioned in transverse at 10μm on a cryostat. Sections were collected on super frost slides. Sections were stored at -80°C until use. Before use, sections were dried at room temperature for 10 minutes. Sections were fixed in 100% acetone for 10 minutes at -20°C. Sections were washed 6 x 5 minutes in 1x PBS and blocked for 1 hour with 5% DS, 1% BSA, 0.3% Triton X-100. Sections were washed 6 x 5 minutes in 1x PBS then incubated with Sox 10 antibody overnight at room temperature. Sections were washed 6 x 5 minutes in 1x PBS, 0.1% Tween-20 and incubated with Alexa 488 donkey anti-goat IgG for 1 hour at room temperature. Sections were washed 6 x 5 minutes in 1x PBS, 0.1% Tween-20, then incubated with c-Jun antibody over night at room temperature. Sections were washed 6x 5 minutes in 1x PBS then incubated with cy3 anti-rabbit IgG and DAPI 1:40000 for 1 hour at room temperature. Sections were finally washed 6x 5 minutes in 1x PBS, 0.1% Tween-20 and mounted in Citifluor mounting medium. When immunolabelling is performed on cell cultures, cells are fixed in 4% PFA and the acetone fixation is omitted. DAPI is incubated 1:1000.

Arginase/F4/80 dual labelling

Nerves were dissected and fixed in Bouin's solution for 4 hours. Nerves were washed in 1x PBS until it ran clear and incubated in 30% sucrose overnight at 4°C. Nerves were embedded in OCT and 10μm longitudinal sections were cut on a cryostat and collected on super frost slides. A hydrophobic barrier was drawn with a DAKO pen and sections were washed 6x 2 minutes in 1x PBS. Sections were blocked in 1% milk in 1x PBS for 1 hour at room temperature then incubated with arginase primary antibody overnight at 4°C. Sections were washed 6x 2 minutes in 1x PBS and

incubated with Alexa 488 donkey anti-goat IgG for 1 hour at room temperature. Sections were washed 6x 2 minutes in 1x PBS then incubated with F4/80 primary antibody overnight at 4°C. Sections were washed 6x 2 minutes in 1x PBS then incubated with anti-rat IgG biotin for 2 hours at room temperature. Sections were washed 6x 2 minutes in 1x PBS then incubated with streptavidin cy3 and DAPI 1:1000 for 15 minutes at room temperature. Sections were washed 6x 2 minutes in 1x PBS then mounted in Citifluor.

CD16/32 immunolabelling

Nerves were dissected and fixed in 4% PFA for 4 hours. Nerves were washed 3x 5 minutes in 1x PBS and transferred to 30% sucrose overnight at 4°C. Nerves were embedded in OCT and 10μm longitudinal sections were cut and collected on super frost slides. A hydrophobic barrier was drawn with a DAKO pen and sections were washed 6x 2 minutes in 1x PBS. Sections were blocked in 1% milk in 1x PBS for 30 minutes at room temperature then incubated with CD16/32 primary antibody overnight at 4°C. Sections were washed 6x 2 minutes in 1x PBS then incubated with anti-rat IgG biotin for 2 hours at room temperature. Sections were washed 6x 2 minutes in 1x PBS then incubated with streptavidin cy3 and DAPI 1:1000 for 15 minutes at room temperature. Sections were washed 6x 2 minutes in 1x PBS then mounted in Citifluor.

2.8.7 Genotyping

For initial genotyping, ear notches were performed at P15 and the tissue disks collected. For re-genotyping, tail tips were taken following Schedule 1 procedures. Tissue was incubated with 75µl hotshot buffer at 95°C for 1 hour. 75µl neutralizing reagent was then added to each sample (Truett et al., 2000). DNA was then added to the PCR mastermix and run under the appropriate conditions.

2.9 Statistical analysis

All surgeries and cell counts were performed blind. Cell counts of immunofluorescence were performed on ImageJ from images taken on a Nikon Labophot 2 fluorescence microscope. Data was analysed and graphs were prepared on Graphpad prism 6.

3.Repair cell maintenance during chronic denervation of peripheral nerves

Unlike the CNS, the PNS has a remarkable capacity for regeneration. Following peripheral nerve injury, the AP-1 transcription factor c-Jun is rapidly up-regulated in Schwann cells (De Felipe & Hunt, 1994; Shy et al., 1996). c-Jun is the master regulator of the repair cell phenotype. When c-Jun is specifically knocked out in Schwann cells and its activation is prevented after nerve injury, axon regeneration is compromised. This is contributed to by deficient up-regulation of trophic factors such as GDNF, BDNF and artemin. The regeneration tracks of the bands of Büngner do not form, inhibitory myelin debris is not cleared and Schwann cell survival is poor (Fontana et al., 2012; Arthur-Farraj et al., 2012). Despite the initial activation of the repair cell phenotype, peripheral nerve regeneration in humans is poor. Axons regenerate at a rate of 1-2mm per day (Buchthal & Kühl, 1979). In human injuries, axons travel vast distances to reinnervate their targets, which, due to their slow rate of regeneration can take months to years. It is known that the supportive, regenerative environment initially activated following injury, is not maintained. Growth supportive trophic factors activated upon injury gradually decline in the Schwann cells of the distal nerve stump (You et al., 1997; Höke et al., 2002; Michalski et al., 2008; Eggers et al., 2010). It has previously been shown, GDNF, BDNF and p75 NTR expression in Schwann cells is controlled by c-Jun (Arthur-Farraj et al., 2012). As these factors are decreasing the distal nerve stump Schwann cells, it is likely that c-Jun is also decreasing and thus the repair cell phenotype is not maintained during chronic denervation.

The aims of this chapter are therefore:

-To examine c-Jun expression in distal nerve stumps during periods of acute and chronic denervation

- To determine if any changes in c-Jun expression observed are Schwann cell specific

- To examine expression of other markers of the repair cell phenotype during acute and chronic denervation

3.1 c-Jun decreases in the distal nerve stump following chronic denervation

To examine c-Jun expression during acute and chronic denervation, WT mice underwent a sciatic nerve cut for periods ranging from 3 days to 10 weeks (Figure 3.1). Western blots of the distal nerve stumps demonstrated that c-Jun levels increased with injury and remained elevated during acute denervation (1-3 weeks) (Figure 3.1 A). However, from 6 weeks, c-Jun levels began to decrease. c-Jun levels then continued to decline consistently over time with levels even at lower at 8 and 10 weeks, the earliest time points classified as chronic denervation in rodent models (Sulaiman & Gordon, 2000; Boyd & Gordon, 2002a, 2003).

Western blots were quantified by dividing the c-Jun protein levels by those of the house keeping protein calnexin. c-Jun levels in blots were then normalised to 1 week post injury (Figure 3.1 B). Analysis of the data revealed that the increases in c-Jun levels after injury were significant, with highest expression observed 1 week after injury. It further demonstrated the gradual decline in c-Jun protein levels with time, with levels significantly lower (55.25%) 10 weeks after injury.

These results demonstrate that although c-Jun is activated after injury, levels decrease in the distal nerve stump during chronic denervation. It was thus decided in further experiments to compare c-Jun levels and repair cell markers after acute denervation 1 week post injury with levels after chronic denervation, classified as being 10 weeks after injury.

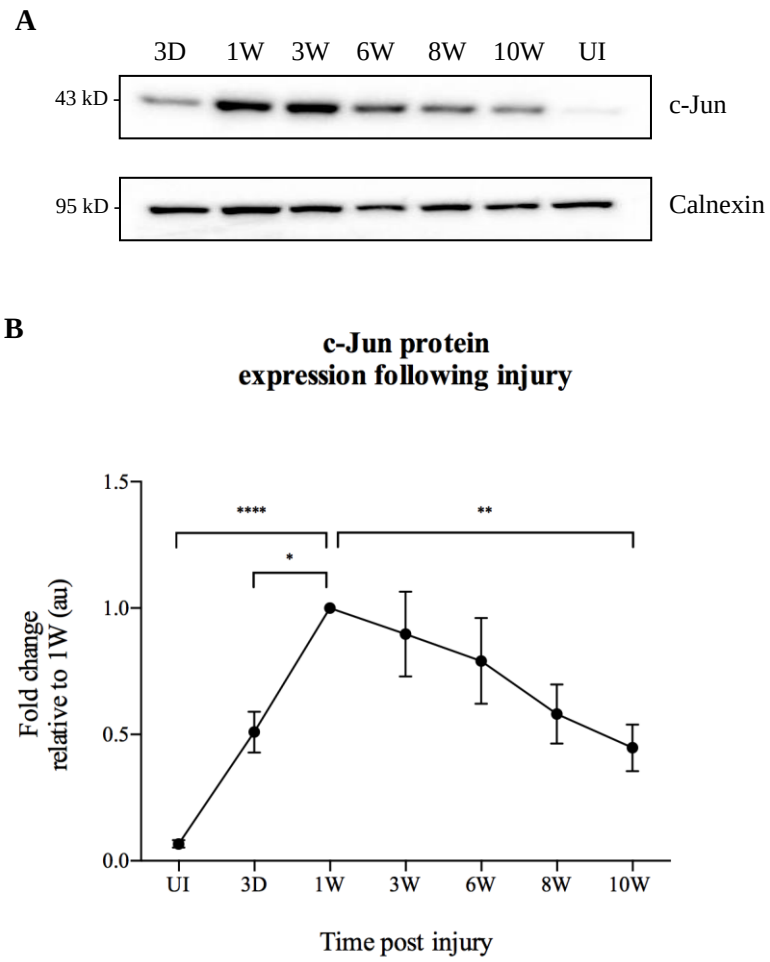


Figure 3.1 | c-Jun protein expression decreases in the distal nerve stump following injury

(A) Representative Western blot of c-Jun expression in distal nerve stumps following injury. Extracts from uninjured nerves (UI) and from distal nerve stumps taken 3 days (D) to 10 weeks (W) following injury were compared. c-Jun expression increased following injury then gradually declined over time. **(B)** Quantification of Western blots. c-Jun expression was normalised relative to calnexin and all time points were compared to 1 week after injury to determine the fold change in expression. c-Jun significantly increased following injury, with levels peaking 1 week after injury. Expression gradually declined with time and was significantly decreased by 10 weeks post injury. Graph of means ± SEM, n=5, one-way ANOVA and Dunnett's test, *=$p<0.05$, **=$p<0.005$, ****=$p<0.0001$.

3.2 Changes in c-Jun expression are not observed in other cells during acute or chronic denervation

Although Schwann cells make up the majority of the cell population of peripheral nerves, many other cell types are present, particularly after injury. Macrophages invade the injured nerve and remain present for up to 3 weeks, although a small population of resident macrophages is present in uninjured adult nerves (Perry et al., 1987; Stoll et al., 1989; Hirata et al., 1999). Macrophages have been observed to express c-Jun in injured nerves (S.Fazal, C. Benito, R. Mirsky, K.R Jessen, unpublished observations) and during macrophage differentiation in other systems (Gaynor et al., 1991; Hannemann et al., 2017). c-Jun is also expressed in fibroblasts, controlling proliferation and survival *in vitro* (Johnson et al., 1993; Behrens et al., 1999; Arthur-Farraj et al., 2012). The changes in c-Jun levels may therefore be due to the influx and gradual exodus or presence of other cell types into the nerve. To test this hypothesis, the c-Jun cKO mouse can be used. These mice, as previously described, have c-Jun conditionally knocked-out in Schwann cells. These mice develop normally, however, following peripheral nerve injury, do not up-regulate Schwann cell c-Jun, leading to disruptions in the activation of the repair Schwann cell phenotype, axon regeneration and Schwann cell survival (Parkinson et al., 2008; Arthur-Farraj et al., 2012; Fontana et al., 2012). As these mice do not up-regulate Schwann cell c-Jun, any c-Jun detected in the injured nerves of these mice is therefore expressed by other cell types.

To examine the c-Jun levels in these mice after injury, c-Jun cKO mice and WT littermates underwent a sciatic nerve cut for 1 or 10 weeks (Figure 3.2). Western blots of these nerve demonstrated that WT littermates up-regulate c-Jun after injury and, as previously observed, c-Jun protein levels decreased with time. The c-Jun cKO mice however did not up-regulate c-Jun following injury. c-Jun levels in these mice remained constant between uninjured and injured nerves with only a slight decrease observed after 10 weeks (Figure 3.2 A). Analysis of these blots revealed the WT mice significantly up-regulated c-Jun 1 week after injury and that these levels were significantly reduced by 30.1% at 10 weeks (Figure 3.2 B). No significant difference was observed in c-Jun up-regulation following injury in c-Jun cKO mice. Furthermore, although a slight decrease in c-Jun levels were observed between 1 week and 10 weeks this was not found to be significant. This suggests a baseline

expression of c-Jun is present in other cells in the nerve, however it is very low and is not affected by injury. Together, this data demonstrates that the changing levels of c-Jun expression following acute and chronic denervation are Schwann cell specific.

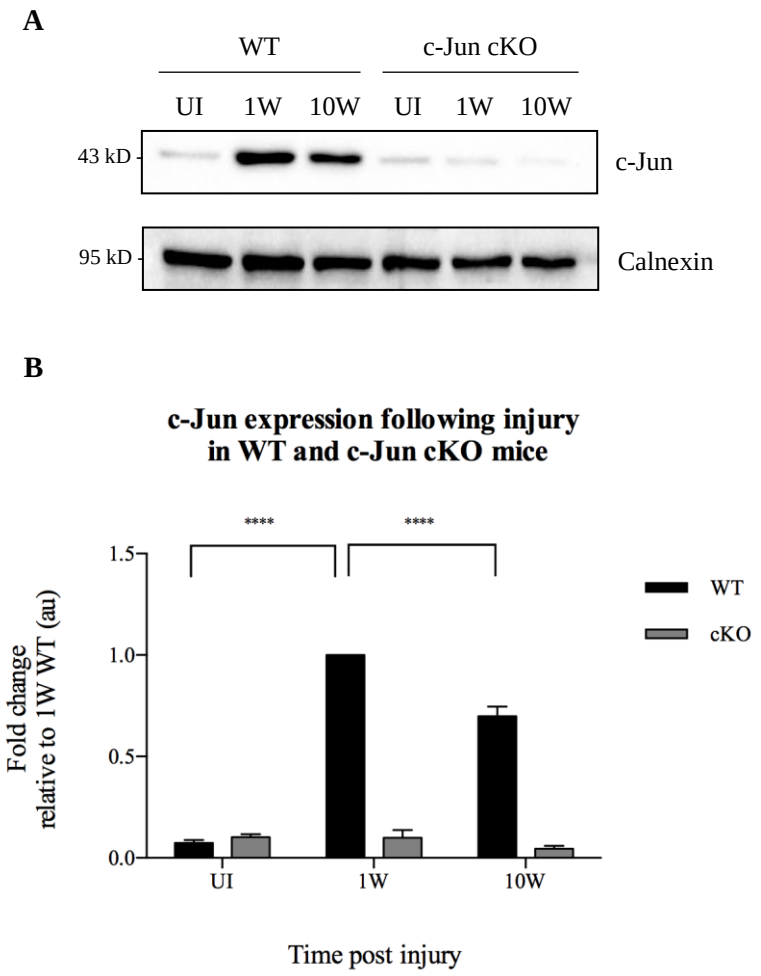


Figure 3.2 | c-Jun protein expression is consistant in the c-Jun cKO distal nerve stump following acute and chronic injury

(A) Representative Western blot of c-Jun expression in the distal nerve stumps of WT and c-Jun cKO mice following injury. Distal nerve stumps were examined 1 and 10 weeks post injury. c-Jun levels increased post injury in WT mice but had decreased by 10 weeks. In the c-Jun cKO mice, no difference in c-Jun levels were observed. **(B)** Quantification of Western blots. c-Jun expression was normalised relative to calnexin and all time points were compared to WT 1 week after injury to determine the fold change in expression. In WT mice, c-Jun levels significantly increased following injury but were significantly lower 10 weeks post injury. No significant difference in c-Jun levels were observed between any of the time points examined in the c-Jun cKO nerves. Graph of means ± SEM, n=5, two-way ANOVA and Dunnet's test, ****=p<0.0001.

3.3 c-Jun decreases in the Schwann cells of the distal nerve stump following chronic denervation

The results so far have established that the decrease in c-Jun observed during chronic denervation is Schwann cell specific. However, Schwann cell numbers change during acute and chronic denervation. Schwann cells proliferate following injury, however numbers decline with time post injury (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; Hall, 1999; Benito et al., 2017). Therefore, to further confirm that the decrease in c-Jun observed is the result of Schwann cell down-regulation as opposed to a decline in cell numbers, immunofluorescent dual staining of c-Jun with Schwann cell marker Sox10 can be performed. Sox10 is a marker of Schwann cells. Unlike other cell markers, it is the only marker that is present through all stages of development as well as being present in the adult cells (Kuhlbrodt et al., 1998). It is essential for Schwann cell development, with Sox10 knock out mice failing to develop myelin or Remak Schwann cells, leading to nerve abnormalities and axon death. Knock out mice do not survive past postnatal week seven (Britsch et al., 2001; Finzsch et al., 2010)

As with the previous experiments, mice underwent a sciatic nerve cut, and were examined after 1 or 10 weeks. Nerves were removed, cut into 2mm segments and fresh frozen in OCT. Transverse 10μm frozen sections were cut and immunolabelled with antibodies for Sox10 and c-Jun (Figure 3.3). From the images, it can be seen that 1 week after injury, almost all the Sox10 positive cells are also c-Jun positive (Figure 3.3 a and b). Some cells however were c-Jun positive, Sox10 negative (Figure a' and b', arrows). This demonstrates the importance of determining the specific cells in which the c-Jun decrease is occurring. Figure 3.3 a and b does however demonstrate that c-Jun is largely expressed in Schwann cells. The images also demonstrate that after 10 weeks of chronic denervation, large populations of Schwann cells have down-regulated c-Jun (Figure 3.3 c and d), with many Sox10 positive, c-Jun negative cells being observed (Figure c' and d', arrows). The number of Sox10 c-Jun positive cells in WT mice is quantified in comparable experiments described in a later chapter (Chapter 4, Figure 4.5).

Overall, this experiment demonstrates that following injury and during acute denervation, c-Jun is up-regulated predominately in Schwann cells. It subsequently

shows that during chronic denervation, Schwann cells down-regulate c-Jun. Together, this demonstrates that the changes in c-Jun expression observed are occurring in Schwann cells, concluding that during chronic denervation, Schwann cell c-Jun is not maintained.

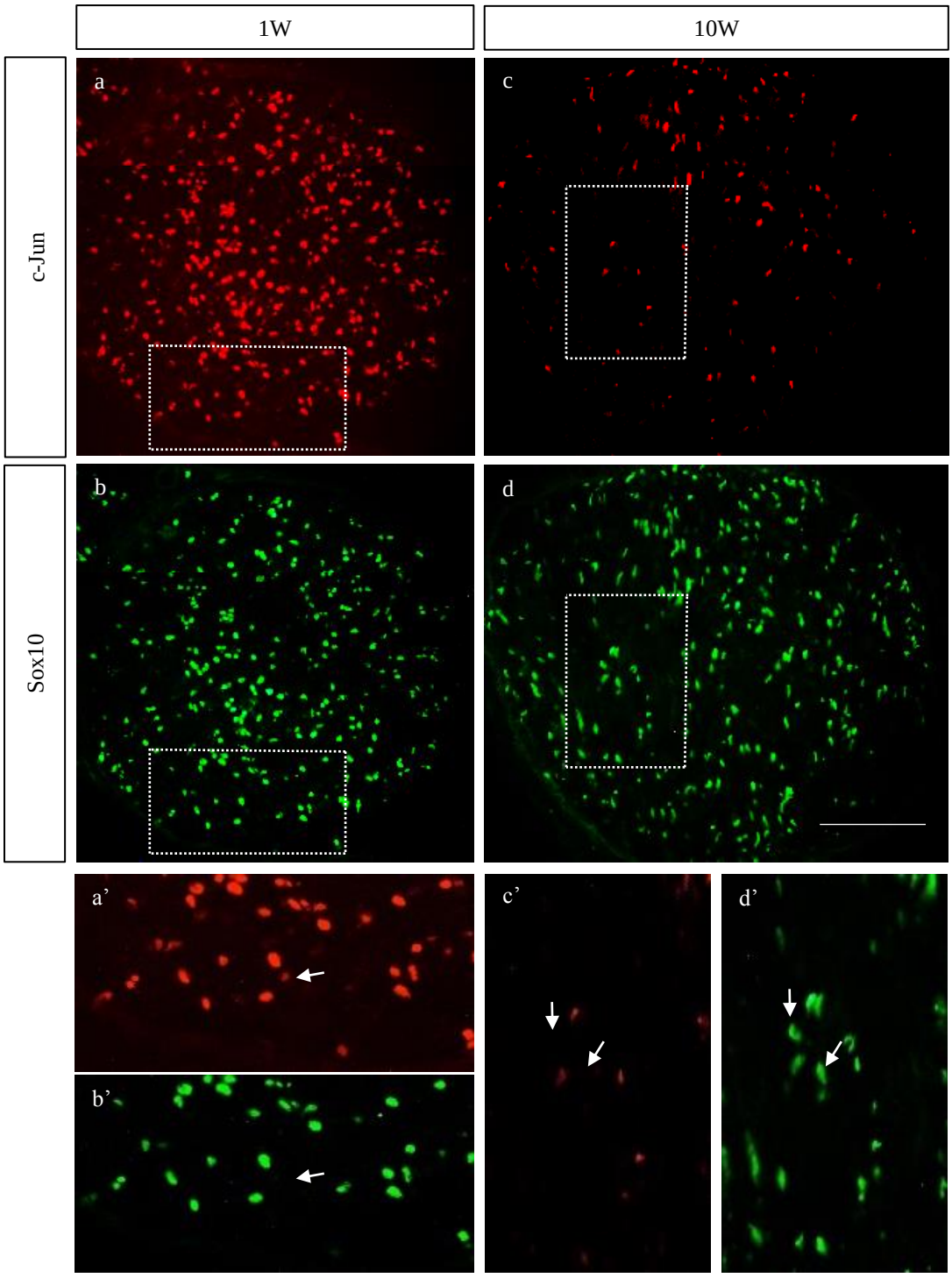


Figure 3.3 | c-Jun expression decreases in the Schwann cells of the distal nerve stump during chronic denervation

c-Jun was elevated in almost every Schwann cell 1 week after injury (a and b). However, cells that were not Schwann cells, characterised as Sox 10 negative cells, were also seen to express c-Jun (a' and b', arrows). 10 weeks after injury, many Schwann cells, although still expressing Sox 10, were no longer c-Jun positive (c, d, c' and d' arrows). Scale bar: 100μm.

3.4 Repair cell marker p75 NTR declines in the distal nerve stump during chronic denervation

Now that I have shown that c-Jun decreases in Schwann cells during chronic injury (Figures 3.1-3.3), it was hypothesized that other markers of the repair cell phenotype might also not be maintained. Markers of the repair cell phenotype are defined as cell surface proteins, trophic factors and transcription factors controlled by c-Jun that contribute to peripheral nerve regeneration (Arthur-Farraj et al., 2012). p75 NTR is a low affinity nerve growth factor receptor. It is up-regulated in the distal nerve stump during the first days post injury, peaks 1 week after sciatic nerve cut and levels remain elevated during the first month after injury (Taniuchi et al., 1986, 1988; Heumann et al., 1987; Roberson et al., 1995; You et al., 1997).

c-Jun regulates p75 NTR protein expression but does not up-regulate mRNA levels - c-Jun cKO mice have significantly lower levels of p75 protein yet normal mRNA levels (Arthur-Farraj et al., 2012). p75 NTR expression has been previously found to decrease in Schwann cells following chronic denervation (You et al., 1997). It was therefore hypothesised that p75 NTR protein expression might decline in the distal nerve stump following injury in line with c-Jun levels. To examine this, WT mice underwent a sciatic nerve cut as described previously. Following acute (3 days - 3 weeks) or chronic denervation (6-10 weeks), Western blots of nerves were run (Figure 3.4 A). It was found that p75 NTR was highly up-regulated 1 week post injury. Protein levels were then found to gradually decrease with time in a similar trend to that of c-Jun levels previously observed (Figure 3.1). Quantification of blots showed that the increase in p75 NTR after injury is significant (Figure 3.4 B). Interestingly, no significant difference was observed between levels in UI nerves and at 3 days post injury, demonstrating that p75 NTR is not immediately activated following injury but that it gradually increases. After peaking at 1 week, p75 NTR levels were found to decrease over time with a significant decrease in levels (58%) observed 10 weeks after injury. The p75 NTR levels were almost equal to those present in the early stages of injury (3 days) and in UI
nerves. These results confirm that a down-stream target of c-Jun that contributes to the repair cell phenotype is down regulated during chronic denervation.

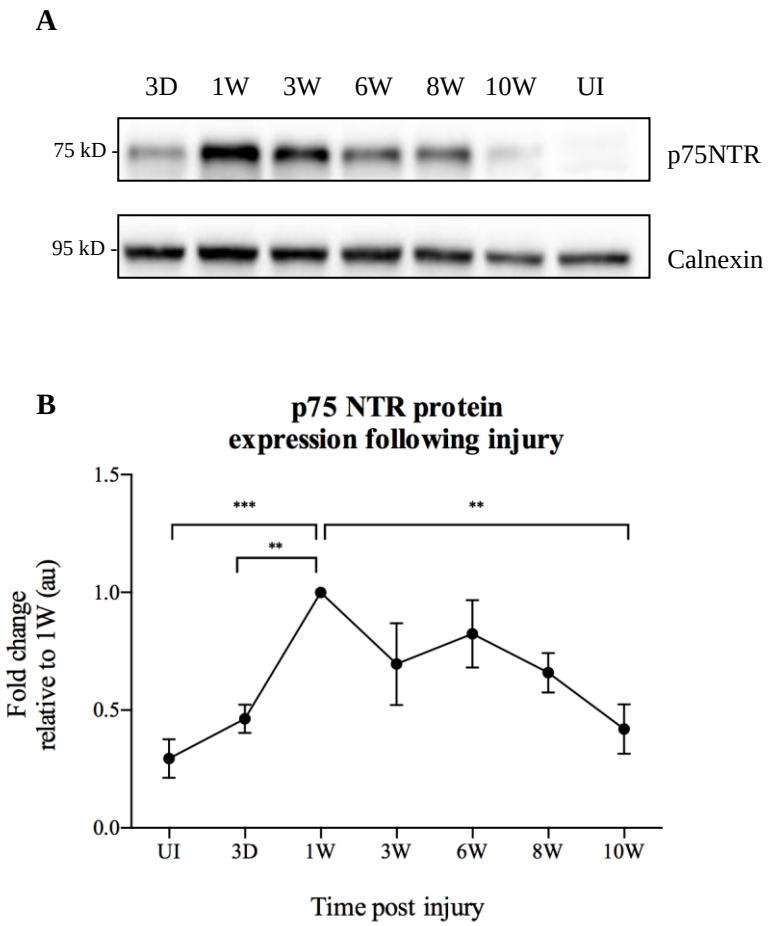


Figure 3.4 | p75 NTR protein expression decreases in the distal nerve stump following injury

(A) Representative Western blot of p75 NTR expression in distal nerve stumps following injury. Distal nerve stumps were examined 3 days to 10 weeks following injury. p75 NTR levels increased post injury and levels then declined over the 10 week period. **(B)** Quantification of Western blots. p75 NTR expression was normalised relative to calnexin and all time points were compared to 1 week after injury to determine the fold change in expression. p75 NTR significantly increased after injury with levels peaking after 1 week. Expression declined with time and levels were significantly lower 10 weeks post injury. Graph of means ± SEM, n=4, one-way ANOVA and Dennett's test, **=$p<0.005$, ***=$p<0.001$.

3.5 Discussion

c-Jun is a master regulator of the Schwann cell repair cell phenotype that facilitates axon regeneration in the peripheral nervous system (Arthur-Farraj et al., 2012). Despite the activation of this repair phenotype, peripheral nerve regeneration in humans is poor. This is in part due to the deterioration of the distal nerve stump which is characterised by the decline in trophic support for the axons and Schwann cell death (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; You et al., 1997; Hall, 1999; Höke et al., 2002; Michalski et al., 2008; Eggers et al., 2010; Benito et al., 2017)

As the expression of many of these factors (GDNF, BDNF and p75 NTR) have been shown to be controlled by c-Jun, this chapter aimed to examine if the deterioration of the distal nerve stump during chronic denervation was caused by the Schwann cells inability to maintain the expression of c-Jun and therefore aspects of the repair cell phenotype. This chapter therefore examined the expression of c-Jun and began to assess markers of the repair cell phenotype during acute and chronic denervation.

Western blots of distal nerve stumps of the sciatic nerve following transection demonstrated that c-Jun is significantly elevated following injury. These levels are not maintained however, with c-Jun gradually decreasing with prolonged denervation (Figure 3.1). As these are transected nerves in which the proximal stump has been tied back to prevent regeneration, it can be concluded that this down-regulation is not occurring due to axonal contact (Stewart, 1995).

However, changes in c-Jun expression could still be due to a number of factors. The rate of Schwann cell proliferation increases during the first week post injury while during chronic denervation, Schwann cells numbers decline (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; Hall, 1999; Benito et al., 2017). Changes in c-Jun levels observed here could be due simply to changes in Schwann cell numbers. Other cells are also present in the nerve following injury. Although there is a small resident population of macrophages present in the nerve (Oldfors, 1980; Perry et al., 1987), numbers increase very significantly after injury. Macrophages invade the nerve as early as 2 days after injury. Their numbers increase in the first 2 weeks and they remain in the nerve up to 21 days after cut and crush injuries (Perry et al., 1987; Stoll et al., 1989; Hirata et al., 1999). Macrophages

are even observed in the distal nerve stumps of chronically denervated tibial nerves, however in reduced numbers compared to those observed following acute injury (Benito et al., 2017). The changes in c-Jun expression could therefore have been due to the influx and exodus of macrophages in the injured nerve.

It was therefore important to determine that the changes in c-Jun expression were not due to changes in macrophage or Schwann cell numbers.

To determine that the changes in c-Jun expression were not due to macrophage or fibroblast population shifts, Western blots of acute and chronically denervated sciatic nerves from the c-Jun cKO mouse were examined. No significant difference in c-Jun expression was observed in cKO mice at any time point examined (Figure 3.2). As c-Jun expression in fibroblasts and macrophages in these nerves is normal (Arthur-Farraj et al., 2012) and no difference in c-Jun was seen during denervation, it can be concluded that the changes in c-Jun expression observed in WT nerves is due to Schwann cell expression.

Surprisingly, the decrease in c-Jun expression in WT mice in Figure 3.2 was different to that of WT mice in Figure 3.1. Figure 3.1 found WT nerves express 55.25% less c-Jun at 10 weeks compared to 1 week nerves. Figure 3.2 found only a 30.1% decrease at 10 weeks. Both results however were significant. It is likely that the difference observed in the percentage decrease between WT mice in Figure 3.2 and the WTs previously examined is due to the samples in Figure 3.2 being compared with samples with very low levels of c-Jun during the development of the blots. However this difference is more likely to be observed due to strain differences. The WT mice in Figure 3.1 are pure C57/Bl6 mice, however the WT control compared in Figure 3.2 is a WT littermate - a transgenic mouse that expresses the c-$Jun^{fl/fl}$ gene however does not express P_0 cre. The differences observed in c-Jun expression are therefore likely to be due to the different genetic backgrounds of these animals.

Although quantifying c-Jun expression in c-Jun cKO nerves demonstrates that the decline in c-Jun is Schwann cell specific, it does not account for the number of Schwann cells present in the nerve. Changes in c-Jun expression could still be due to Schwann proliferation and death in the nerve. To ultimately determine that changes are c-Jun expression are Schwann cell specific, c-Jun was dual immunolabelled with

antibodies for Schwann cell marker Sox 10. Sox 10 is one of the most reliable markers of Schwann cells as it is expressed throughout the Schwann cell lineage (Kuhlbrodt et al., 1998). Other markers of Schwann cells such as glial fibrillary acidic protein (GFAP) and S100 are less reliable. GFAP is only expressed from E18 in immature Schwann cells and is supressed by myelin Schwan cells. It is however activated in the absence of axons by Schwann cells *in vitro* (Jessen et al., 1990). S100 is expressed by Schwann cells (Stefansson et al., 1982) however expression declines during chronic denervation (Cicero et al., 1970; Stefansson et al., 1982; You et al., 1997). Protein and RNA screens of cultured Schwann cells have found delays in S100 activation in culture compared to Sox 10 (Liu et al., 2015). p75 NTR is another marker of Schwann cells, however its expression is regulated by injury and is variable in chronic denervation (Heumann et al., 1987; You et al., 1997). Sox 10 expression however, when studied by microarray, has found to be unchanged following nerve injury and in the weeks that follow (Nagarajan et al., 2002; Arthur-Farraj et al., 2012). It was therefore concluded that Sox 10 was the most reliable marker for examining Schwann cells in acute and chronically denervated nerves.

1 week after cut, almost all cells that were Sox 10 positive were c-Jun positive, however not all c-Jun positive cells were Schwann cells, although the majority were. It was observed that 10 weeks after injury, many cells in the nerves were Sox 10 positive however they were also c-Jun negative.

The final experiment of this chapter aimed to examine if the down-regulation of c-Jun resulted in the fading of the repair cell phenotype. To examine this, p75 NTR, a marker of the repair cell phenotype was examined during acute and chronic injury. Activation of p75 NTR was gradual, with no difference observed between injured and 3 day cut nerves. Previous work however has found only a small increase in p75 NTR mRNA at 3 days relative to 1 week where levels are highest (Taniuchi et al., 1986; Heumann et al., 1987). However, after 1 week, p75 NTR expression gradually decreased with time. This is consistent with previous findings that p75 NTR protein and mRNA levels decrease specifically in Schwann cells during chronic denervation. mRNA levels have been shown to decline between 7 and 20 days, a similar trend observed with these protein levels. (Heumann et al., 1987; You et al., 1997).

To further examine repair cell marker expression during chronic denervation, qPCR experiments are required. These will be performed in the next chapter where the expression of *GDNF*, *BDNF*, *Shh* and *c-Jun* will all be quantified.

4. Maintaining the repair cell phenotype during chronic denervation of peripheral nerves

The results described in Chapter 3 of this thesis demonstrated that c-Jun and some markers of the repair phenotype are not maintained during chronic denervation of peripheral nerves. If the repair cell phenotype were maintained during chronic denervation, the deterioration of the distal nerve stump might be prevented. The characteristic features of the distal nerve stump deterioration, such as the previously described down-regulation of trophic support and cell adhesion molecules, and Schwann cell death. Many of the trophic factors and cell adhesion molecules down-regulated have been previously found to be controlled by c-Jun and are disrupted in the c-Jun cKO mouse (Arthur-Farraj et al., 2012; Fontana et al., 2012).

Previous work has demonstrated that over expression of c-Jun in cultured Schwann cells leads to increased expression and secretion of GDNF and BDNF and increased proliferation (Huang et al., 2015). Furthermore, exposure of chronically denervated Schwann cells to trophic factors has previously been shown to enhance their ability to aid axon regeneration (Boyd & Gordon, 2003; Wood et al., 2013) It was therefore hypothesized that maintenance of c-Jun during chronic denervation might maintain the repair cell phenotype and prevent the deterioration of the distal nerve stump.

To examine this, we were gifted the $OE^{fl/fl}$ mouse. This mouse has the ability to over-express c-Jun in every cell as the transgene is expressed under the control of the Rosa26 promoter. In the presence of *cre*, the loxp sites flanking the transgene will be excised, activating the transgene and leading to the over-expression of c-Jun. Schwann cell cultures from these nerves can initially be used to examine the effect of c-Jun over-expression in Schwann cells *in vitro*. This mouse can then be crossed with a mouse expressing *cre* under the control of the P_0 promoter to generate a mouse that over-expresses c-Jun specifically in Schwann cells (Jose A Gomez-Sanchez et al., 2017; Fazal et al., 2017). This mouse can then be used to examine the effect of c-Jun over-expression and maintenance in acute and chronic injury.

The aims of this chapter are therefore:

- To determine if c-Jun and markers of the repair cell phenotype are elevated in $OE^{fl/fl}$ Schwann cell cultures following *cre* recombinant adenovirus infection
- To determine if c-Jun and markers of the repair cell phenotype are maintained in OE/+ Schwann cells following chronic denervation
- To characterize differences in WT and OE/+ nerves following acute or chronic injury.

4.1 Infection with *cre* recombinant adenovirus elevates c-Jun in $OE^{fl/fl}$ Schwann cell cultures

To initially examine the effect of over-expressing c-Jun in Schwann cells, cultures from $OE^{fl/fl}$ mice can be generated and infected with a *cre* recombinant adenovirus. To first confirm that the transgene system is working in this mouse, Schwann cells were cultured from P8-10 $OE^{fl/fl}$ mice. Schwann cell cultures were infected with a *cre* recombinant adenovirus or a GFP adenovirus as a control. Naïve cells which underwent no viral infection were also examined as a further control (referred to as control cells). c-Jun protein levels in these cultures were then examined by Western blot (Figure 4.1).

The results showed no difference in c-Jun levels between the control cells and the GFP virus infected cultures, demonstrating that viral infection of Schwann cells has no effect on c-Jun expression. Infection with the *cre* virus however led to a big increase in c-Jun expression (Figure 4.1 A). Quantification of this increase revealed it to be a significant 3-fold increase relative to both control cells and GFP virus infected cells. No significant difference was observed between control and GFP virus infected cells (Figure 4.1 B). To visualise the increase in c-Jun in $OE^{fl/fl}$ *cre* infected cultures, GFP and *cre* infected cultures were fixed and immunolabelling of c-Jun performed (Figure 4.1 C). Greater c-Jun fluorescence was observed in the *cre* virus infected cultures compared to GFP virus infected ones.

Together, Figure 4.1 confirms that the transgene insert in $OE^{fl/fl}$ mice can be activated by cre. c-Jun was successfully over expressed in Schwann cells cultured from this mouse infected with an *cre* adenovirus. The double control used in this study of naïve cells and an infection control in the form of GFP virus, which showed no elevation in c-Jun enforces the findings made.

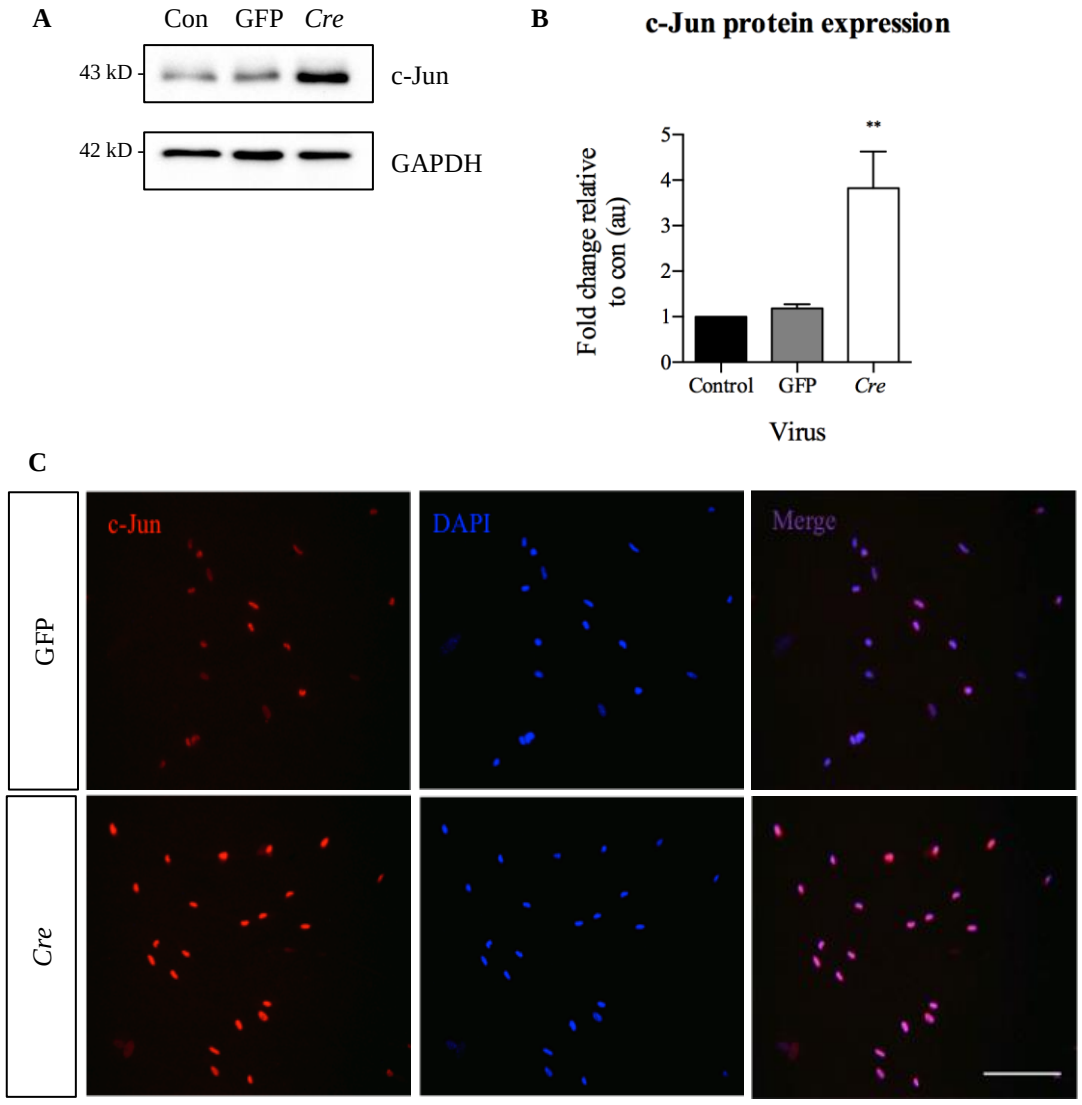


Figure 4.1 | c-Jun protein expression increases in *Cre* infected OE/OE$^{fl/fl}$ mouse Schwann cell cultures

(A) Representative Western blot of c-Jun expression in OE/OE$^{fl/fl}$ mouse Schwann cell cultures. Schwann cell cultures were infected with GFP virus, *cre* virus or no virus (con) for 48 hours. Infection with GFP virus had no effect on c-Jun levels however *cre* viral infection resulted in an increase in c-Jun levels. **(B)** Quantification of Western blots. c-Jun expression was normalised relative to GAPDH and samples were compared to the non-viral infection (control) to determine the fold change in expression. Infection with *cre* virus significantly increased c-Jun levels almost 4 fold compared to GFP virus and control infections. No significant differences in c-Jun levels were observed between control and GFP infected Schwann cells. **(C)** OE/OE$^{fl/fl}$ mouse Schwann cell cultures were immunolabelled for c-Jun and DAPI following viral infection. Cultures infected with *cre* virus expressed higher levels of c-Jun compared to those infected with GFP virus, confirming Western blot results. Graph of means ± SEM, n=4, one-way ANOVA and Tukey's test, **=p<0.005. Scale bar: 100µm.

4.2 *c-Jun* and *Shh* mRNA is elevated in *cre* recombinant adenovirus infected $OE^{fl/fl}$ Schwann cell cultures

The previous section has confirmed that c-Jun can be successfully over-expressed in the Schwann cells of the $OE^{fl/fl}$ mouse. This model can be used to examine the effect of c-Jun over-expression in Schwann cells *in vitro*. Huang et al (2015) over expressed c-Jun in Schwann cell cultures using a lentivirus with a *c-Jun* vector. This study found that over expression of c-Jun in Schwann cells by this method led to increased levels of trophic factor expression and secretion by the cells. GDNF, BDNF and artemin were all examined. This increase in trophic factor production was credited for the increased neurite outgrowth observed when DRG explants were co-cultured with the over-expressing cells (Huang et al., 2015). Based on these findings, it was hypothesized that over-expression of c-Jun in $OE^{fl/fl}$ *cre* infected cultures could lead to increased trophic factor expression. GDNF and BDNF are both contributors to the repair cell phenotype (Arthur-Farraj et al., 2012). If over-expression c-Jun leads to increased trophic factors expression, other markers of the repair cell phenotype might also be affected.

To examine effect of c-Jun over-expression on levels of repair cell markers *in vitro*, qPCRs for *GDNF, BDNF, Olig1, c-Jun* and *Shh* were performed on GFP virus and *cre* virus infected $OE^{fl/fl}$ Schwann cell cultures (Figure 4.2). Results from this experiment further confirmed the activation of the c-Jun transgene in these mice with *c-Jun* expression increasing 3-fold in *cre* virus infected cells relative to those infected with GFP virus ($p=0.0017$) (Figure 4.2 A). Controversially, no significant increase in *GDNF* or *BDNF* expression was observed following *cre* infection (Figure 4.2 B and C). Although a slight increase in *BDNF* was observed, this result was found not to be significant ($p=0.413$). *GDNF* expression was observed to be decreased in the *cre* infected cells although this was not a significant trend ($p=0.0857$). *Olig1* expression was similarly lower in the *cre* infected cells but this data also proved to not be significant ($p=0.2405$) (Figure 4.2 D). *Shh* expression however was found to increase in the *cre* infected cells ($p=0.0496$) (Figure 4.2 E). The relationship between *Shh* and *c-Jun* will be discussed at length in Chapter 5.

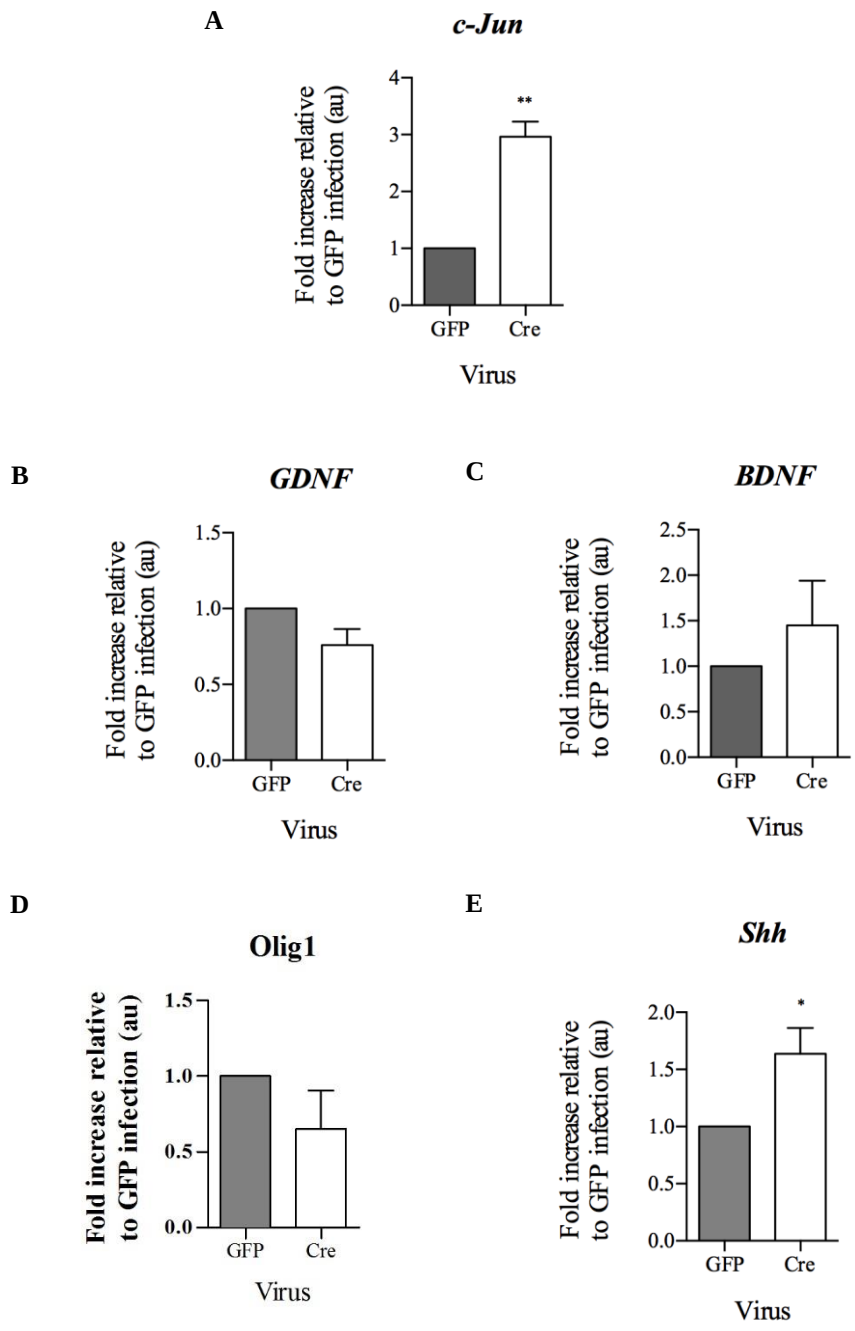


Figure 4.2 | *c-Jun* and *Shh* mRNA expression increases in *Cre* infected OE/OE$^{fl/fl}$ mouse Schwann cell cultures

(A) Viral infection of OE/OE$^{fl/fl}$ mouse Schwann cell cultures with *cre* virus for 48 hours significantly increased *c-Jun* RNA expression (p= 0.0017). No significant difference in RNA expression was observed in **(B)** *GDNF*, **(C)** *BDNF* or **(D)** *Olig1*. **(E)** *Shh* RNA expression also significantly increased following *cre* virus infection relative to the GFP virus control (p=0.0496). Graph of means ± SEM, n=3, unpaired Student's T-test.

4.3 p75 NTR protein expression is not affected by over-expression of Schwann cell c-Jun *in vitro*

As previously described, p75 NTR is regulated by c-Jun at the protein level and is a marker of the repair cell phenotype (Arthur-Farraj et al 2012). Although over expression of c-Jun *in vitro* only led to a significant increase in Shh, it may lead to alterations in p75 NTR expression. To examine this, Western blots were performed on $OE^{fl/fl}$ mouse Schwann cell cultures infected with *cre* or GFP adenovirus. Naïve cells with no viral infection were also examined (referred to as control). The results showed that the infection did not lead to an increase in p75 NTR with control and GFP cultures expressing similar levels (Figure 4.3 A). The same effect was observed in c-Jun Western blots previously (Figure 4.1). No increase in p75 NTR was observed once the cells were infected with *cre* virus either however. Overall it was found that there was no significant difference in p75 NTR levels between any of the experimental conditions (Figure 4.3 B).

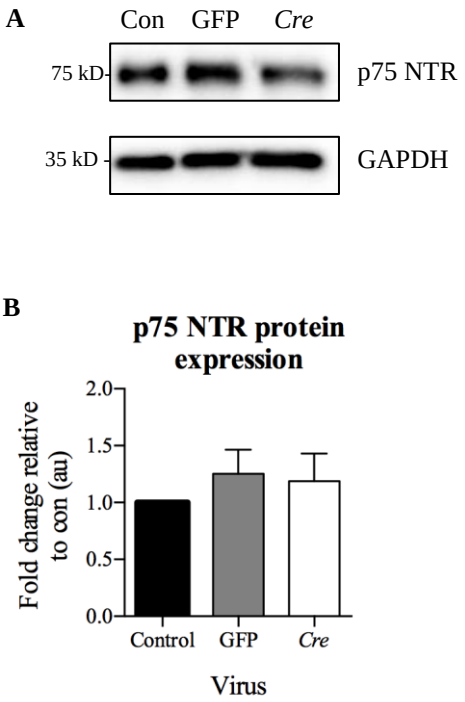


Figure 4.3 | p75 NTR protein expression is not altered in *Cre* infected OE/OE$^{fl/fl}$ mouse Schwann cell cultures

(A) Representative Western blot of p75 NTR expression in OE/OE$^{fl/fl}$ mouse Schwann cell cultures following viral infection. p75 NTR expression was similar under all conditions. **(B)** Quantification of Western blots found no significant difference between con, GFP virus or *cre* virus infected cultures. Graph of means ± SEM, n=3, one-way ANOVA with Dunnett's test.

Overal the results from these experiments to examine the effect of c-Jun over-expression *in vitro* on the expression of repair cell markers only produced one significant finding. The expression of trophic factors GDNF and BDNF were not seen to increase, as they were expected to, based on the previous literature (Huang et al., 2015). It was further surprising that no increase in p75 NTR was observed despite the significant increase in c-Jun up-regulation, considering the relationship previously described between them (Arthur-Farraj et al., 2012). These results could be the consequence of various factors that will be discussed at the end of this chapter. However, most importantly, this work does demonstrate that the transgenic mice over-express c-Jun in the presence of *cre* recombinase.

4.4 Uninjured OE/+ Schwann cells do not express repair cell characteristics

The work in the previous section of this chapter described examining the ability to increase c-Jun levels in Schwann cells *in vitro* by infecting cells from the $OE^{fl/fl}$ mouse with a *cre* adenovirus. By crossing the $OE^{fl/fl}$ mouse with a P_0 cre mouse, a mouse mutant that specifically over-expresses c-Jun in Schwann cells can be generated.

The cre-mediated recombination in Schwann cells occurs between embryonic day (E) 13.5 and 14.5 and the transgene remains active throughout life (Feltri, D'Antonio, Previtali, et al., 1999; Feltri, D'Antonio, Quattrini, et al., 1999)

OE/+ and OE/OE mouse development has been extensively characterized (Fazal et al., 2017). c-Jun is a negative regulator of myelination (Parkinson et al., 2008) so it is important to determine not only the level of c-Jun activation in these mice but also the normality of their Schwann cells and nerve architecture. Uninjured OE/+ nerves express 6 times more c-Jun protein compared to WT nerves at postnatal day 7 whereas OE/OE nerves express a 19-times more. Despite the elevation of c-Jun, the OE/+ mouse development is relatively normal with only a slightly thinner myelin sheath forming compared to WT littermates with a slightly delayed onset of myelination. The OE/OE nerves however have no myelin sheaths around larger axons or alternatively thin myelin sheaths, a larger nerve area and characteristics of peripheral neuropathies (Fazal et al., 2017).

As Fazal *et al* (2017) have demonstrated, c-Jun is over-expressed in the uninjured nerves of OE/+ and OE/OE mice. As we know that c-Jun is the global regulator of the Schwann cell repair phenotype, it is important to determine if repair cell characteristics can be seen in the uninjured nerves of these mice. To examine this, qPCRs of repair cell markers in uninjured nerves from OE/+, OE/OE and WT littermates were compared (Figure 4.4). Trophic factors GDNF and BDNF were examined. Shh was also examined as work earlier in this chapter found that over-expression of Schwann cell c-Jun led to a significant up-regulation of Shh (Figure 4.2). c-Jun was also examined to determine the level of mRNA in the uninjured nerves. The results also demonstrate the increase of c-Jun required to elevate repair cell markers. As uninjured WT nerves were found to have very low expression of

repair cell markers, qPCR data was analysed as fold increase relative to the uninjured OE/+ mouse.

Interestingly, the data from these qPCRs found no significant difference in *c-Jun* levels expressed between WTs and OE/+ mice, despite the differences in c-Jun protein levels previously reported (Figure 4.4 A). OE/OE mice however had a significant 3-fold increase in *c-Jun* mRNA compared to both the WT and OE/+ nerves. In line with *in vitro* work described earlier in this chapter, *Shh* increased when c-Jun was also elevated (Figure 4.4 B). A significant 6-fold increase in *Shh* occurred in OE/OE nerves relative to OE/+ and WT, in which *Shh* levels were found to be equal.

No significant difference in trophic factor expression was found either between WT and OE/+ nerves (Figure 4.4 C and D). *GDNF* and *BDNF* levels were consistent between these samples. A large increase in *GDNF* levels was however observed in OE/OE nerves (Figure 4.4 C). These uninjured nerves showed an almost 90-fold increase in expression. This up-regulation was found to be significant compared to low levels observed in WT and OE/+ nerves. Despite the over-expression of c-Jun in OE/OE nerves, no change was observed in *BDNF* expression (Figure 4.4 D). Levels were even observed to be lower in this mutant compared to the OE/+ nerves.

Together, these experiments provide further evidence that the nerves of OE/OE mice are abnormal. Schwann cells in these nerves have several markers of the repair cell phenotype activated. This suggests that a 3-fold increase in *c-Jun* mRNA levels *in vivo* is sufficient to activate markers of the repair cell phenotype. It is however surprising that no change is observed in *c-Jun* expression between WT and OE/+ nerves, despite the increase in protein. However, no increase in repair cell markers were observed in these nerves either. This finding is positive as it demonstrates that the uninjured nerves of these mice are relatively normal. The insertion of the transgene is not sufficient to elevate c-Jun RNA levels even if increased protein levels are seen. The lack of repair cell markers expressed in these nerves demonstrates the Schwann cells present in the nerve are currently adult myelin and Remak cells and are not actively expressing markers characteristic of repair cells.

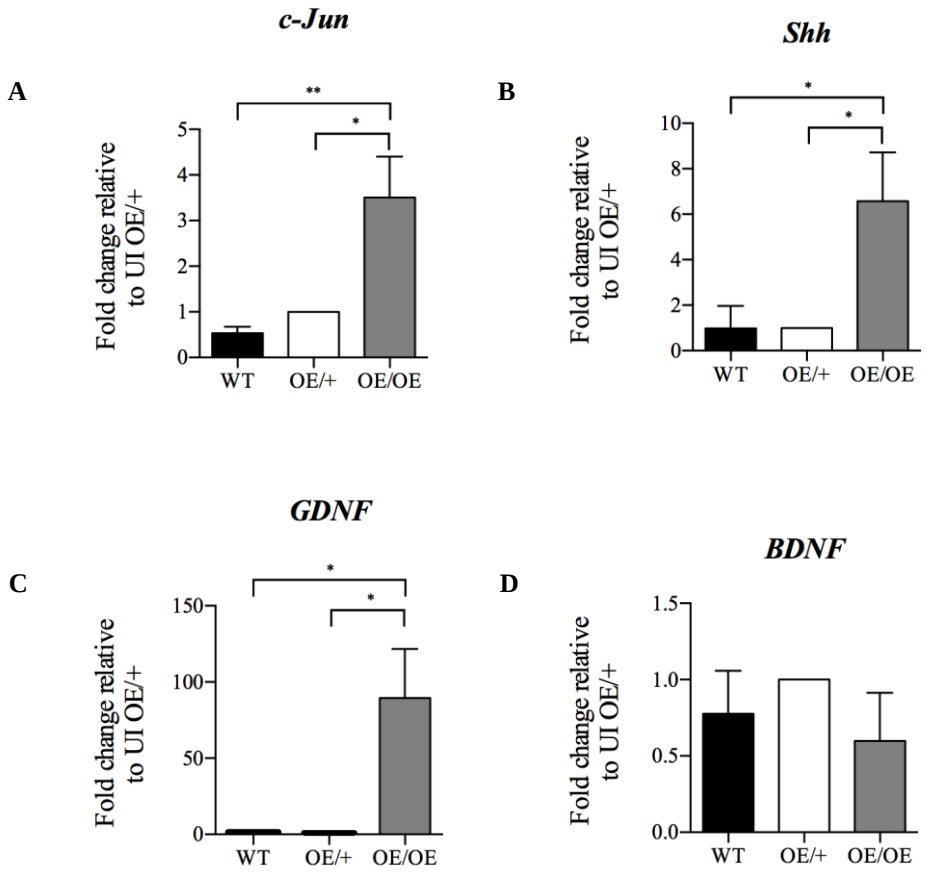


Figure 4.4 | Uninjured OE/OE nerves express increased levels of *c-Jun, Shh* and *GDNF* mRNA

(A) qPCR of uninjured nerves showed that no significant difference was observed in *c-Jun* expression between WT and OE/+ nerves. OE/OE nerves however expressed significantly higher levels of *c-Jun* relative to WT ($p=<0.005$) and OE/+ nerves ($p=<0.05$). **(B)** *Shh* was highly expressed in OE/OE nerves ($p=<0.05$) whereas no change was observed between WT and OE/+. **(C)** *GDNF* was significantly up-regulated in OE/OE nerves ($p=<0.05$) compared to WT and OE/+ nerves. **(D)** *BDNF* up-regulation however was not observed across any of the genotypes. Graph of means ± SEM, n= 4, one-way ANOVA and Tukey's test.

4.5 OE/+ nerves maintain c-Jun during chronic denervation

Now it has been confirmed that uninjured OE/+ nerves are largely normal, the c-Jun expression in these mice can be examined after injury. OE/+ mice and WT littermates underwent sciatic nerve cut followed by 1, 3 or 10 weeks denervation (Figure 4.5). Subsequent Western blots found that there was no difference in the levels of c-Jun 1 week after injury (Figure 4.5 A). WT samples were found to down-regulate c-Jun following the period of chronic denervation, as was previously observed in Chapter 3. Samples from the OE/+ mice however showed consistent levels of c-Jun expression throughout the period examined. OE/+ uninjured nerves were found to have increased levels of c-Jun protein relative to WT, although this was not found to be significant (Figure 4.5 B). No significant difference was observed between the WT and OE/+ c-Jun levels during acute denervation (1-3 weeks). However, after chronic denervation, the difference in c-Jun observed between WT and OE/+ was found to be significant ($p=<0.05$).

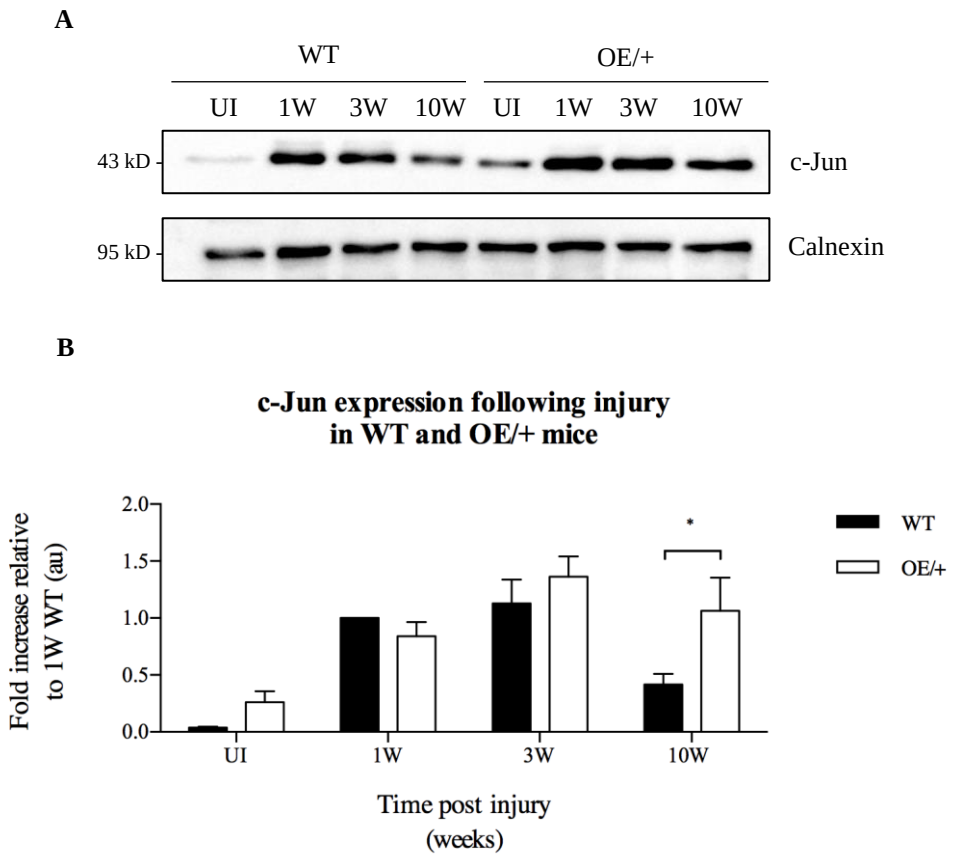


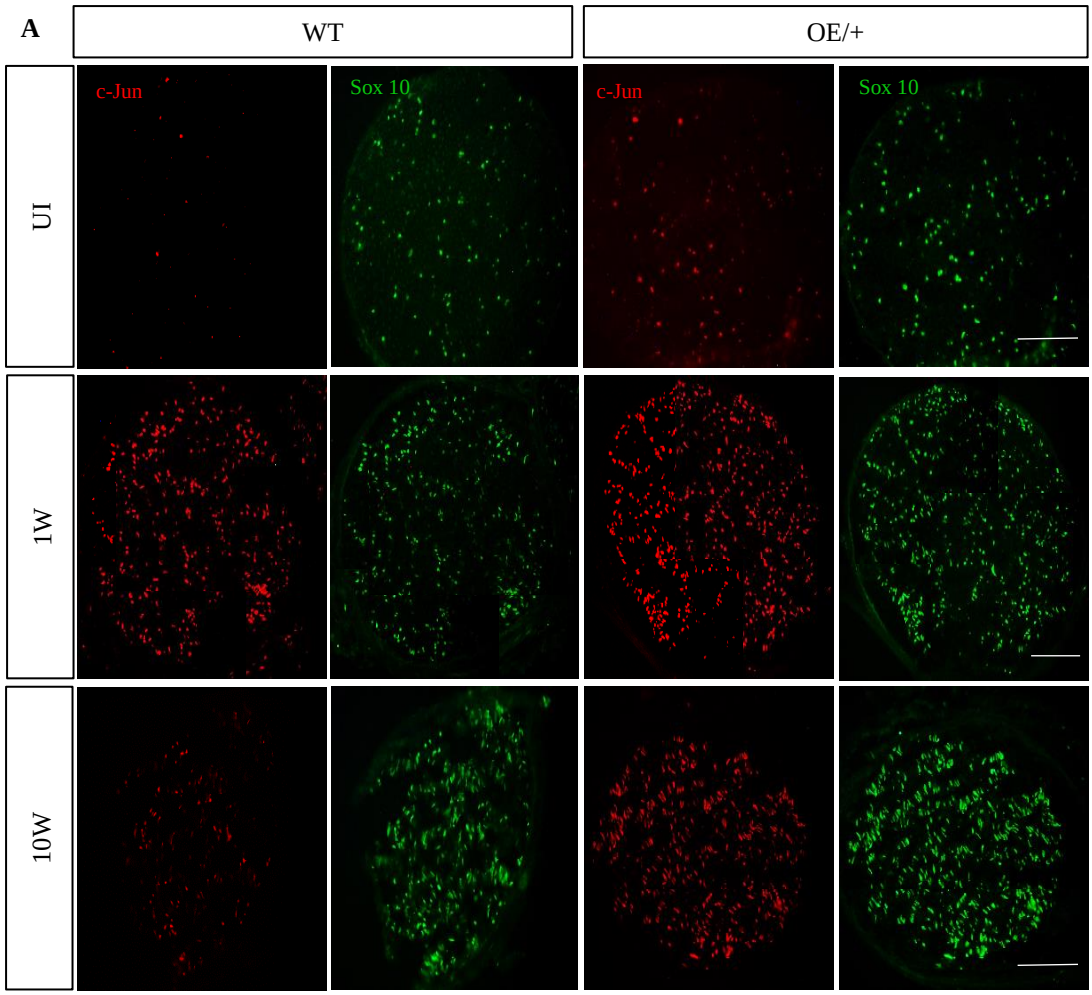
Figure 4.5 | OE/+ distal nerve stumps maintain c-Jun protein expression following chronic denervation

(A) Western blots showed that WT nerves followed previously observed patterns of c-Jun up-regulation and decline during injury. In contrast, OE/+ nerves maintained consistent levels of c-Jun throughout chronic denervation. **(B)** Analysis of the blots showed that WT nerves down-regulated c-Jun following chronic denervation. OE/+ nerves however maintained c-Jun expression from acute injury through chronic denervation. OE/+ nerves expressed significantly higher levels of c-Jun following chronic denervation compared to WT littermates ($p=<0.05$). Graph of means ± SEM, n= 5, two-way ANOVA with Sidak's test.

4.6 c-Jun maintenance in OE/+ nerves in chronic denervation is Schwann cell specific

OE/+ mice maintain c-Jun in the distal nerve stump during chronic denervation, however, as previously discussed, the peripheral nerve is composed of several cell types after injury. As was shown in Chapter 3, cells other than Schwann cells express c-Jun (Figure 3.3 a' and b'). It is therefore necessary to confirm that the maintenance of c-Jun observed in these mice is Schwann cell specific. Sox 10 c-Jun dual immunolabelling described previously can be utilized to examine this. OE/+ mice and WT littermates underwent a sciatic nerve cut for 1 or 10 weeks. Transverse sections of nerves were sectioned and immunolabelled with antibodies for c-Jun and Sox10 (Figure 4.6). Very few c-Jun positive cells were observed in the uninjured nerves of WT mice. Almost all cells however were c-Jun positive and Sox 10 positive 1 week after injury. After 10 weeks however, many cells were Sox10 positive c-Jun negative, while OE/+ uninjured nerves had many c-Jun positive Sox 10 positive cells. Similarly to the WT nerves, at 1 week, almost all cells were c-Jun positive Sox 10 positive, however unlike in WT nerves, 10 weeks after injury many cells were still c-Jun positive, Sox 10 positive. The number of Sox10 positive cells were counted as was the number of these cells that also expressed c-Jun. The percentage of c-Jun Sox 10 positive cells after injury was then calculated (Figure 4.6 B). 1 week after sciatic nerve cut, 94% of WT Schwann cells had up-regulated c-Jun. Similarly, 98% of OE/+ Schwann cells expressed c-Jun. However, following chronic denervation, 30% of Schwann cells present in WT nerves had down-regulated c-Jun while in OE/+ nerves, 94% of Schwann cells still express c-Jun.

Together, these data demonstrate that during chronic denervation, OE/+ Schwann cells maintain c-Jun levels. If the repair cell phenotype is therefore similarly maintained, the deterioration of the distal nerve stump might be prevented and thus sustain the supportive environment required for axon regeneration.


A
WT
OE/+
UI
1W
10W
c-Jun
Sox 10
c-Jun
Sox 10

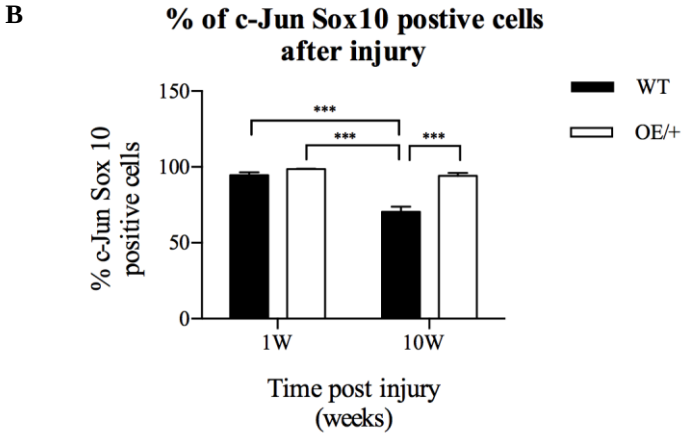

B
% of c-Jun Sox10 postive cells after injury
% c-Jun Sox 10 positive cells
150
100
50
0
***
***
***
1W
10W
Time post injury (weeks)
WT
OE/+

Figure 4.6 | OE/+ Schwann cells in the distal nerve stump maintain c-Jun expression during chronic denervation

(A) Sox 10 c-Jun dual immunolabelling of transverse sections from WT and OE/+ nerves **(B)** The percentage of c-Jun positive Sox 10 positive cells. WT Schwann cells had significantly down-regulated c-Jun following chronic denervation ($p=<0.001$) OE/+ Schwann cells did not down-regulate c-Jun and after 10 weeks denervation expressed significantly higher levels compared to WT littermates at 10 weeks after denervation ($p=<0.001$). Graph of means ± SEM, n= 3, two-way ANOVA with Tukey's test. Scale bars: 100µm.

4.7 p75 NTR is not maintained in WT or OE/+ nerves during chronic denervation

Having demonstrated that OE/+ Schwann cells maintain c-Jun levels during chronic denervation, it is now possible to assess whether the repair cell phenotype is also maintained. To start examining this, p75NTR expression following acute and chronic denervation can be measured in OE/+ nerves compared to WT. As previously shown, p75 NTR is down-regulated in WT nerves during chronic denervation (Chapter 3, You et al; 1997). OE/+ Schwann cell may maintain p75 NTR during chronic denervation in response to the maintenance of c-Jun. As c-Jun only regulates p75 NTR protein and not mRNA expression, Western blots of WT and OE/+ nerves were performed following acute or chronic denervation (Figure 4.7 A). As in Figure 3.4, p75 NTR expression was highly up-regulated following injury in WT nerves and steadily declined with time. Surprisingly however, p75 NTR was not initially as highly up-regulated in OE/+ nerves and these lower levels were maintained with time. Quantification of this data demonstrated that there were no significant differences in p75 NTR expression between WT and OE/+ nerves during acute or chronic denervation (Figure 4.7 B). Uninjured nerves had equal levels of expression which is consistent with the findings in this chapter that OE/+ uninjured nerves c-Jun elevation is not elevated enough to activate the repair cell phenotype. During acute denervation of 1-3 weeks injury, no difference is observed in c-Jun levels and no significant difference in p75 NTR expression is observed between the two genotypes, although the OE/+ expression is slightly but not significantly higher at 3weeks. However, as shown earlier following chronic denervation, c-Jun OE/+ mice maintain c-Jun in the distal nerve stump at 10 weeks after denervation (Figure 4.5-6), yet despite the previous links between c-Jun and p75 NTR protein levels, OE/+ mice only show slightly higher levels of p75 NTR expression from WT nerves at 10 weeks with no significant difference observed between them.

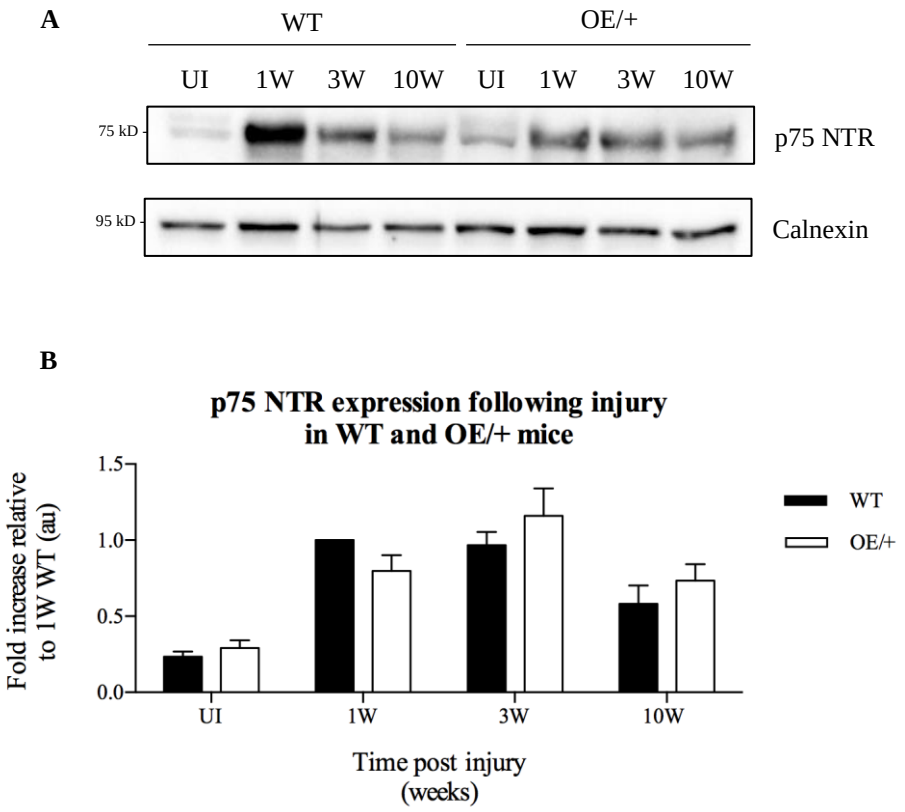


Figure 4.7 | p75 NTR protein expression is not maintained in WT or OE/+ nerves during chronic denervation

(A) WT nerve expression of p75 NTR was similar to that observed previously. Protein was highly elevated at 1-3 weeks after injury with levels declining by 10 weeks after injury. OE/+ nerves however initially expressed slightly lower levels of p75 NTR following injury compared with WT nerves, although the difference was not significant **(B)** Quantification of blots showed that WT and OE/+ nerves displayed similar expression patterns of p75 NTR following injury with no significant differences in expression observed at any time point. Graph of means ± SEM, n=5, two-way ANOVA with Sidak's test.

4.8 Trophic factors are not maintained during chronic denervation by WT or OE/+ nerves

As it was found that p75 NTR expression was not maintained in the distal nerve stump during chronic denervation, other markers of the repair cell phenotype were examined. Nerves from WT mice 1 and 10 weeks following injury and nerves from OE/+ mice 10 weeks after injury were examined for *c-Jun, Shh, GDNF* and *BDNF* mRNA expression by qPCR (Figure 4.8). It was found that *c-Jun* was significantly down-regulated in WT nerves following chronic denervation with *c-Jun* mRNA expression decreasing 70% (Figure 4.8 A). OE/+ nerves did down-regulate c-Jun with time after denervation, but this finding was not significant and levels were higher compared to those in chronically denervated WT nerves. *Shh* was similarly found to significantly decrease during chronic denervation in both genotypes (Figure 4.8 B). *Shh* expression decreased by 89% in WT nerves and 59% in OE/+ nerves. Nevertheless, OE/+ nerves retained significantly higher *Shh* expression compared to WT nerves at 10 weeks. Trophic factors *GDNF* and *BDNF* decrease in the distal nerve stump following chronic denervation (Höke et al., 2002; Michalski et al., 2008). This finding was confirmed here. *GDNF* was significantly down-regulated in WT nerves following chronic denervation. Interestingly, *GDNF* expression was not elevated in OE/+ nerves and levels were even lower than those observed in WT nerves (Figure 4.8 C). *BDNF* expression was decreased in WT nerves following chronic denervation however this was not a significant finding (Figure 4.8 D). *BDNF* expression is gradually up-regulated following injury, therefore WT nerves express similar levels during acute and chronic denervation (Michalski et al., 2008). OE/+ nerves however did significantly down-regulate BDNF by 86%. These results demonstrate that some elements of the repair cell phenotype, such as expression of *c-Jun* and *Shh,* are higher in OE/+ nerves compared to WT following chronic denervation. However trophic factors *GDNF* and *BDNF* are not elevated in these nerves and are observed to be even lower. Possible reasons for these findings will be discussed at the end of this chapter.

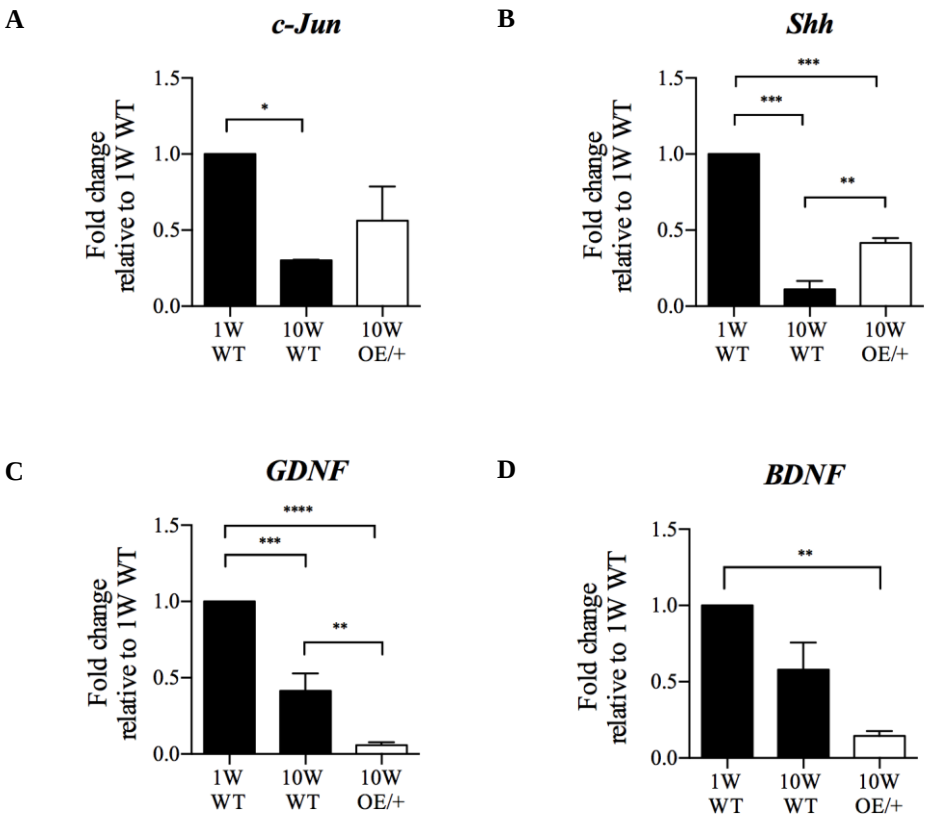


Figure 4.8 | Repair cell marker mRNA is not maintained in WT or OE/+ distal nerve stumps following chronic denervation

qPCR of nerve stumps of WT and OE/+ nerves following chronic denervation compared with acutely denervated WT nerves. **(A)** *c-Jun* significantly decreased in WT nerves following chronic denervation. Levels were elevated in OE/+ nerves and were not significantly lower than levels expressed during acute injury. **(B)** *Shh* was significantly decreased in both genotypes however OE/+ expressed significantly more than chronically denervated WT nerves. **(C)** *GDNF* was also significantly decreased in both genotypes with OE/+ expressing even lower levels than WT nerves. **(D)** *BDNF* expression was decreased in both genotypes, however this was only significant in OE/+ nerves. Graphs are means ± SEM, n=3, two-way ANOVA with Tukey's test. *=p=<0.05, **=p=<0.005, ***=p=<0.001, ****p=<0.00001.

4.9 OE/+ nerves have significantly more Schwann cells and macrophages than WTs following chronic denervation

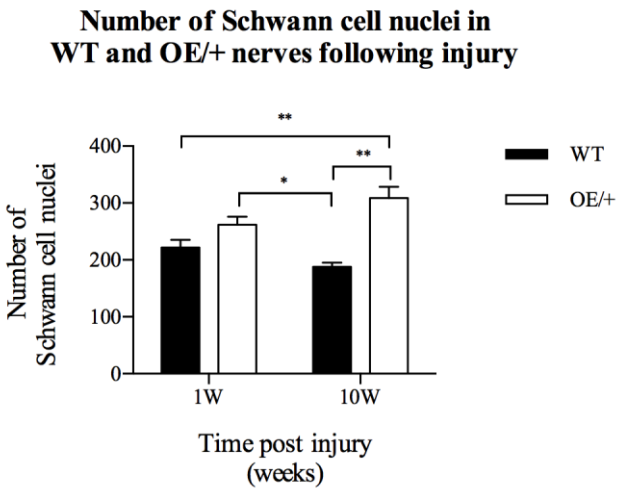
The deterioration of the distal nerve stump, as well as being defined by the nerve down-regulating trophic factors that promote axon regeneration, is further defined by the death of Schwann cells (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; Hall, 1999; Benito et al., 2017). c-Jun has previously been shown to control Schwann cell proliferation through activation via JNK signalling although c-Jun cKO nerves only have reduced proliferation under certain conditions (Parkinson et al., 2004; Arthur-Farraj et al., 2012). Uninjured adult OE/+ Schwann cells also demonstrate an increased rate of proliferation compared to WT (Fazal et al., 2017).

It was therefore hypothesized that maintaining c-Jun expression in the distal nerve stump, might prevent Schwann cell death. To investigate Schwann cell numbers in acute chronically denervated nerves, nerves were processed and counted by EM (Figure 4.9 D). In contrast to some previous findings, no significant Schwann cell death was observed in chronically denervated WT nerves at the time period examined (Figure 4.9 A). Although no significant difference in Schwann cell number was observed between genotypes in acute denervation, 1 week injured OE/+ mice had significantly more Schwann cells than WT 10 weeks after injury. OE/+ Schwann cell numbers were maintained during chronic denervation, with numbers slightly increasing. These numbers were significantly higher compared to WT nerves at any stage examined.

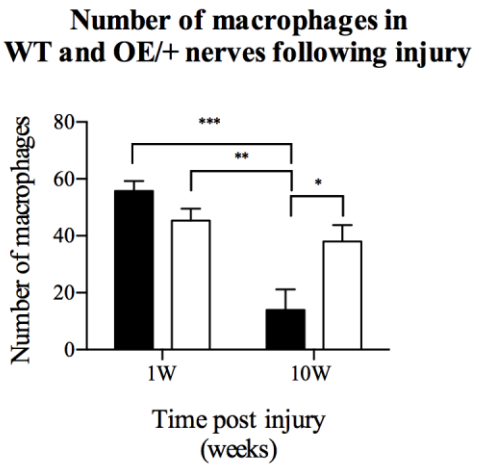
Other cell populations can be examined by EM such as macrophages and fibroblasts. Macrophages were counted in acute and chronically denervated nerves. Cells with typical macrophage morphology and a visible nucleus were counted (Figure 4.9 D). Counts showed that WT and OE/+ nerves had equal numbers of macrophages present in the nerve following injury (Figure 4.9 B). Macrophage numbers then significantly decreased in WT nerves during chronic denervation. Macrophage numbers in OE/+ nerves however did not decline. OE/+ nerves had significantly more macrophages remaining in the nerve compared to WT nerves. Fibroblasts were also counted. Cells that displayed typical fibroblast morphology with a visible

nucleus were counted (Figure 4.9 C and D). No difference in fibroblast numbers were observed between genotypes or time points.

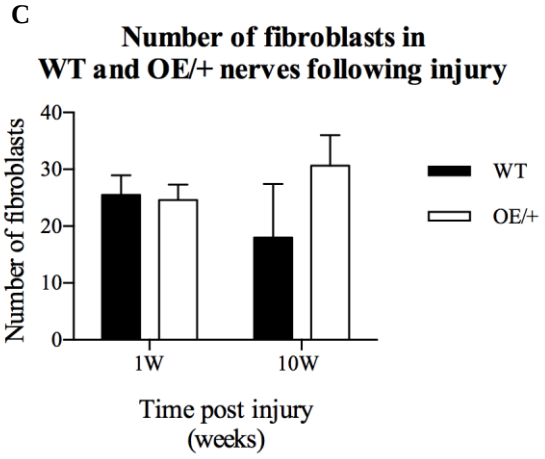
A


Number of Schwann cell nuclei in WT and OE/+ nerves following injury
Number of Schwann cell nuclei
400
300
200
100
0
1W
10W
Time post injury (weeks)
WT
OE/+
**
*
**

B


Number of macrophages in WT and OE/+ nerves following injury
Number of macrophages
80
60
40
20
0
1W
10W
Time post injury (weeks)
***
**
*

C


Number of fibroblasts in WT and OE/+ nerves following injury
Number of fibroblasts
40
30
20
10
0
1W
10W
Time post injury (weeks)
WT
OE/+

D

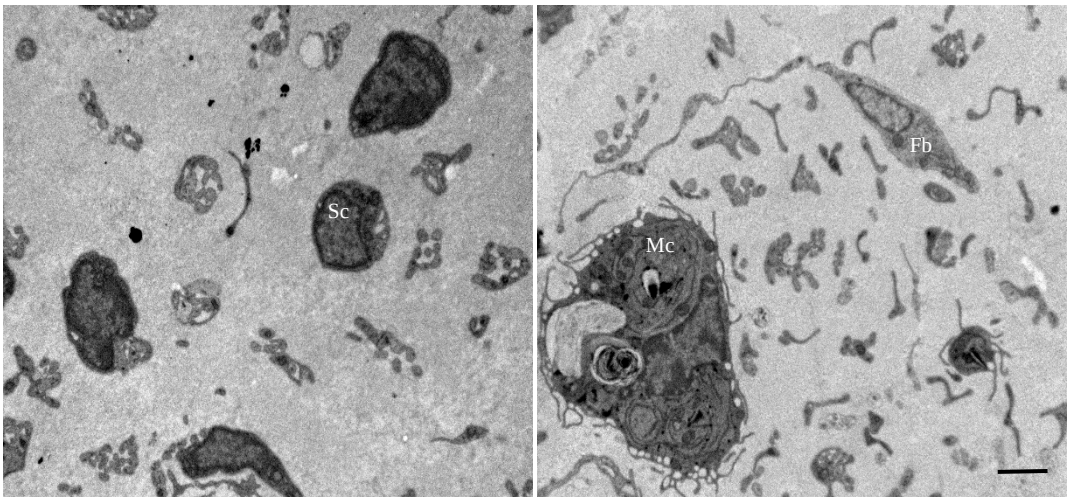
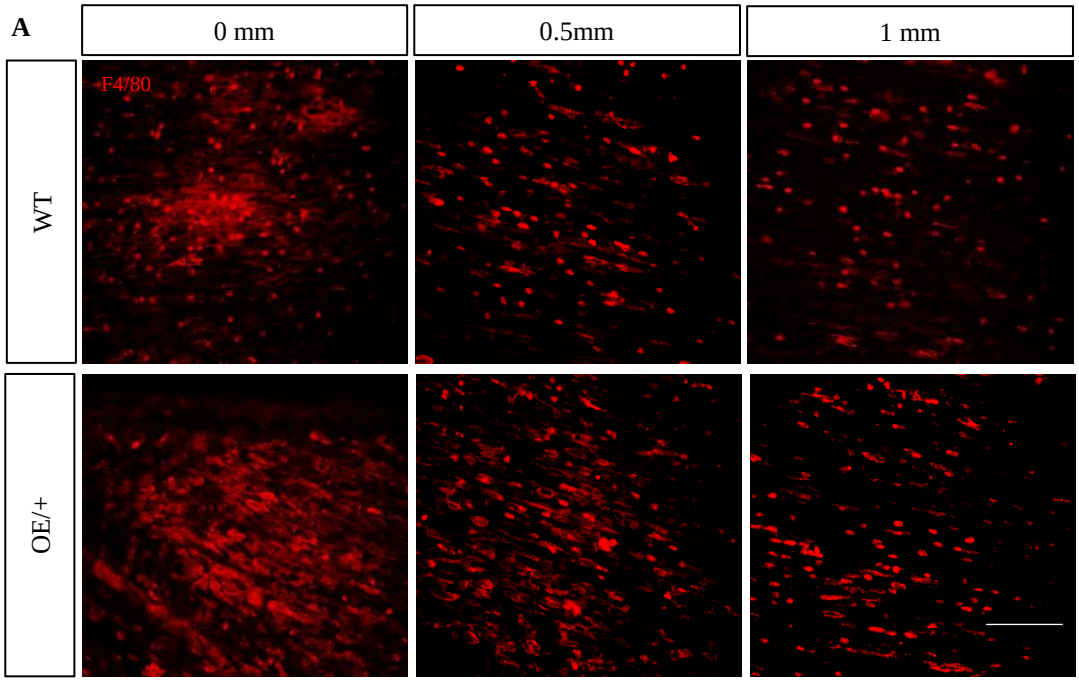

Sc
Mc
Fb

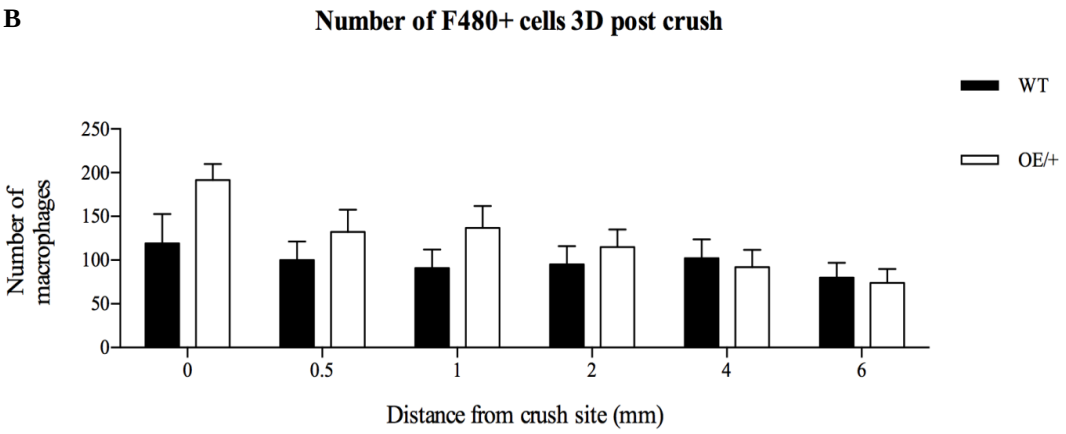
Figure 4.9 | OE/+ nerves have more Schwann cells and macrophages than WT following chronic denervation

(A) Schwann cells were counted by EM in WT and OE/+ nerves following acute and chronic denervation. Equal numbers of cells were observed during acute injury, with OE/+ nerves containing an increased number of Schwann cells following chronic denervation relative to WT. **(B)** Macrophage numbers following acute and chronic denervation in both genotypes. Numbers significantly decreased in WT nerves following chronic denervation. Macrophages however remained present following chronic denervation of OE/+ nerves. **(C)** Fibroblast numbers remained consistent in both genotypes following both injury paradigms. **(D)** Representative images of each cell type counted. EM images taken 10 weeks after injury. Four Schwann cells (Sc) are depicted in the left panel. A fibroblast (Fb) and macrophage (Mc) are shown in the right. The fibroblast is characterised by its bipolar morphology whereas the macrophage extends multiple processes. Graphs of means ± SEM, 1W WT n= 4, 1W OE/+ n=5, 10W WT and OE/+ n=3, two-way ANOVA with Tukey's test, *=p=<0.05, **=p=<0.005, ***=p=<0.001. Scale bar 2µm.

4.10 OE/+ nerves recruit a greater number of macrophages to the injury site

OE/+ nerves have a greater number of macrophages present following chronic denervation. Although OE/+ nerves did not have higher macrophage numbers following acute injury than WT nerves when examined by EM, there may still be higher numbers present at earlier time points. c-Jun cKO mice have a reduced number of macrophages 1mm from the cut site in the nerve 3 days after cut. This difference is only observed close to the injury site and was attributed to the c-Jun cKO Schwann cells reduced capacity to recruit macrophages to this site (Arthur-Farraj et al., 2012). It could therefore be argued that OE/+ Schwann cells may recruit more macrophages to the injury site compared to WT in the early days of injury. The EM data discussed previously (Figure 4.9) is taken 5mm from the injury site. The injury site and the distal nerve stump should therefore both be examined. To examine macrophage numbers in OE/+ and WT mice, nerves were crushed for 3 days and transverse sections of the whole nerve taken. Sections were labelled for the universal macrophage marker F4/80 (Austyn & Gordon, 1981; Hirsch et al., 1981) (Figure 4.10 A). F4/80 positive cells with a visible nucleus were counted within the field of view grid at various distances distal to the crush site (Figure 4.10 B). No overall differences were observed throughout the nerve in macrophage numbers, however a trend towards higher numbers at the crush site was observed in OE/+ nerves. When the data from the crush site was individually examined (Figure 4.10 C), it was found that OE/+ nerves had greater numbers of macrophages present at the injury site ($p=0.0054$). Individual examination of other distances found no significant differences. This increase in macrophage numbers to the injury site may help improve axon regeneration.


A
0 mm
0.5mm
1 mm
WT
OE/+
F4/80


B
Number of F480+ cells 3D post crush
WT
OE/+
Number of macrophages
250
200
150
100
50
0
0
0.5
1
2
4
6
Distance from crush site (mm)

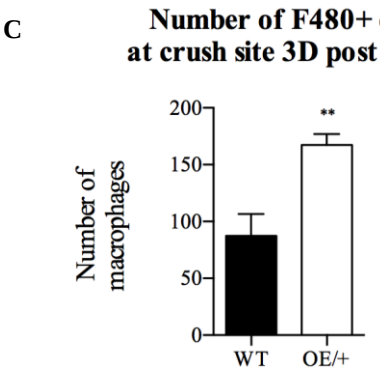

C
Number of F480+ cells
at crush site 3D post crush
Number of macrophages
200
150
100
50
0
**
WT
OE/+

Figure 4.10 | OE/+ nerves attract more macrophages to the crush site

(A) F4/80 immunolabelling in transverse nerve sections. **(B)** Counts of macrophage numbers throughout the nerve. OE/+ nerves were observed to attract more macrophages to the injury site. No difference was found in the number of macrophages present in the nerve overall, two-way ANOVA with Sidak's test. **(C)** The number of macrophages at the crush site. OE/+ nerves attracted significantly more macrophages to the injury site compared to WT ($p=<0.005$), unpaired Student's T-test. Graphs of means ± SEM, WT n=5, OE/+ n=5. Scale bar: 100µm.

4.11 OE/+ nerves recruit higher numbers of M2 macrophages to the injury site

Current work in immunology is emphasizing the importance not only the presence of macrophages but the polarization of subpopulations. Macrophages can be classified broadly as M1 or M2. M2 subtypes M2a, M2b and M2c are present and are normally grouped as M2 (Mills et al., 2000; Stein et al., 1992) (for review - (Mosser, 2003; Mantovani et al., 2004). Generally, M1 macrophages are classified as inhibitory and pro-inflammatory whereas M2 are involved with regeneration and tissue remodelling (Kigerl et al., 2009; Ruffell et al., 2009; Zhang et al., 2010; Sindrilaru et al., 2011; Mokarram et al., 2012)

To examine the different subpopulations of macrophages after injury, transverse sections of 3 day crushed nerves were stained with arginase, a marker of M2 macrophages (Hesse et al., 2001; Edwards et al., 2006; Kigerl et al., 2009) and F4/80 to confirm macrophage specification (Figure 4.11 A). Counts of these sections found a very significant increase in M2 macrophages present at the injury site in OE/+ nerves, with almost double the number of M2 macrophages present (Figure 4.11 B). No difference in M2 numbers was observed at any other distance distal to the injury site, with cell numbers steeply declining from 2mm onwards. As M2 macrophages have been shown to improve regeneration in peripheral nerves (Mokarram et al., 2012), OE/+ nerves may have improved axon regeneration initially during short term injury due to the increased number of this macrophage subpopulation.

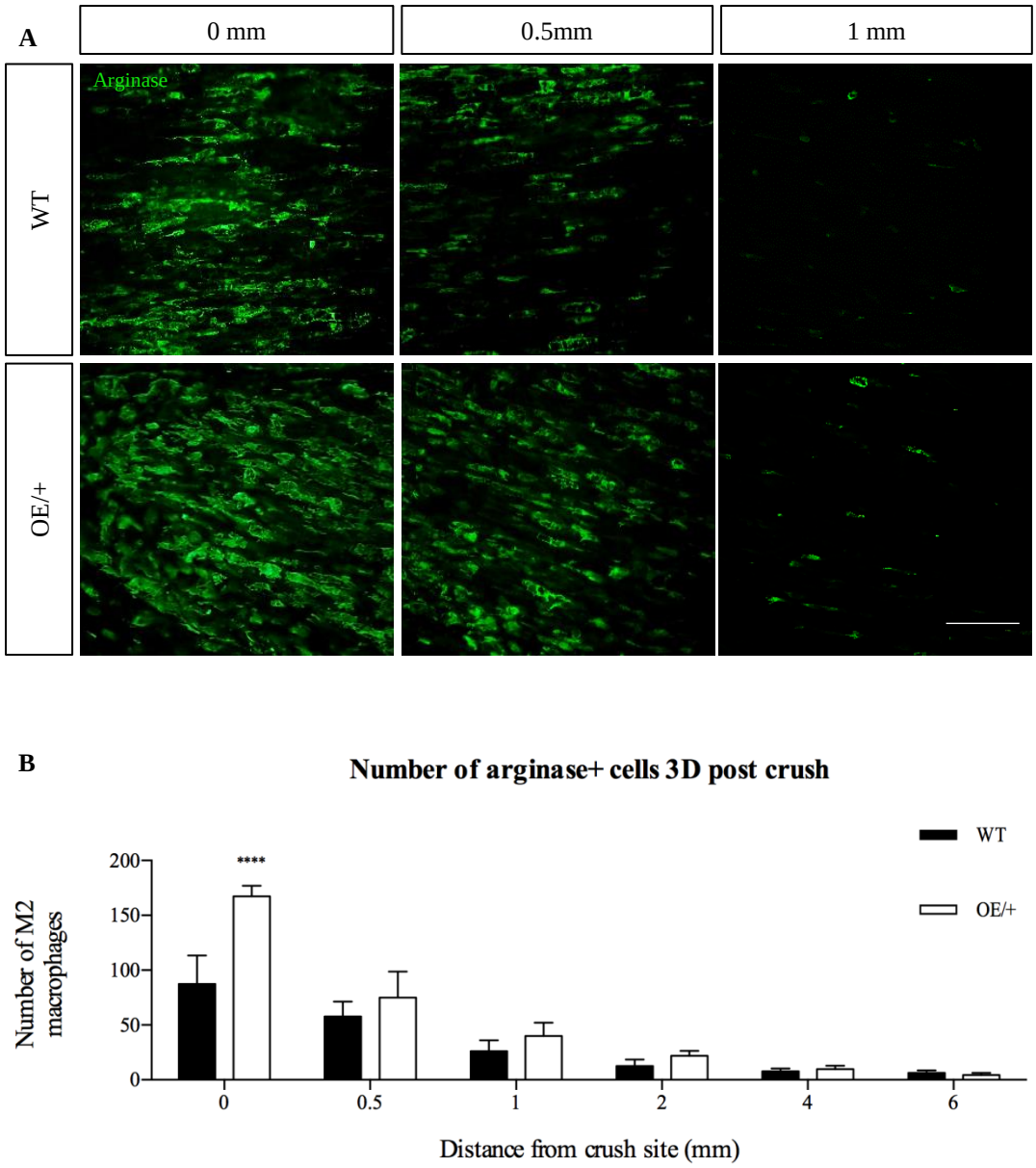


Figure 4.11 | OE/+ nerves attract more M2 macrophages to the crush site

(A) Transverse sections of 3 days crushed nerves were immunolabelled with the M2 macrophage marker arginase. **(B)** Counts of M2 macrophages as various distances distal to the crush site found the OE/+ nerves had significantly more cells present at the crush site compared to WT (p=<0.0001). No difference was observed at other distances distal to the crush. Graph of means ± SEM, WT n=4, OE/+ n=5, two-way ANOVA with Sidak's test. Scale bar: 100µm

4.12 M1 macrophages are present in the peripheral nervous system

Ydens et al (2012) have suggested that the regenerative capacity of the PNS is due to the presence of M2 macrophages. They suggested that inhibitory, inflammation inducing M1 macrophages are not present in the PNS and that only M2 macrophages are activated following nerve injury (Ydens et al., 2012). It was hypothesized that this may be incorrect and that due to the significantly higher numbers of M2 macrophages present at OE/+ injury sites, OE/+ may have a reduced number of M1 macrophages. Nerves were initially labelled with antibodies for the M1 macrophage marker iNOS but no positive labelling was observed (data not shown) (Kigerl et al., 2009; Ydens et al., 2012) . It was therefore concluded that M1 macrophages in the PNS may not express iNOS and that other markers should be examined. Pilot studies of labelling with CD16/32 antibodies demonstrated labelled cells in the peripheral nerve 3 days after crush (Figure 4.12 A). A trend towards OE/+ nerves potentially recruiting more M1 macrophages was observed (Figure 4.12 B). As with previous work, the greatest difference between the two genotypes was observed at the injury site (4.12 C). However, due to the low sample size examined, a large spread is observed in the data. Further experiments are required to determine if a true difference is present. This work however suggests that M1 macrophages are present in the peripheral nerve after injury.

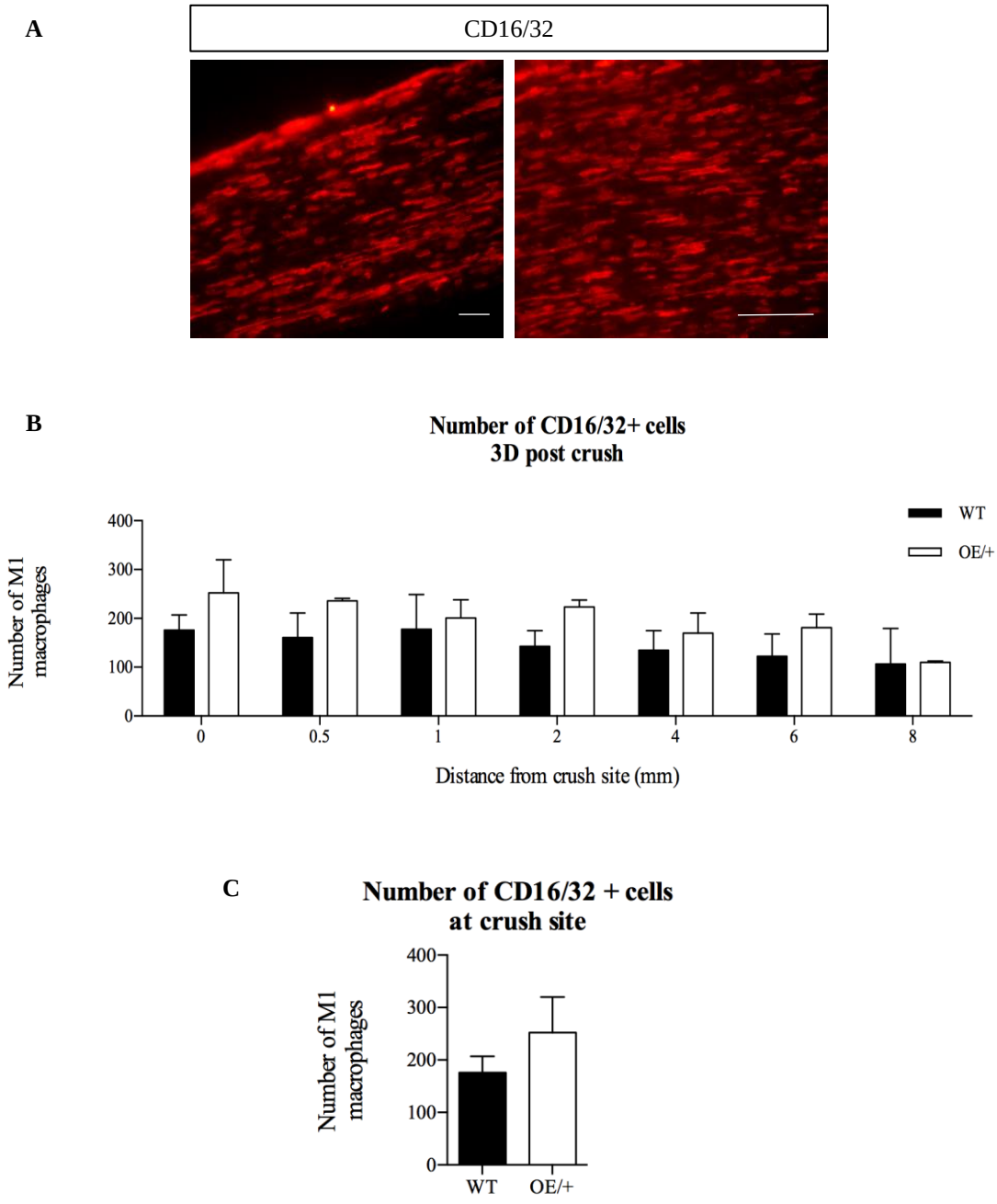


Figure 4.12 M1 macrophages are present in the sciatic nerve post injury

(A) Respresentative CD16/32 immunolabelling in the sciatic nerve 3 days after crush **(B)** Counts of CD16/32 positive macrophages in the nerves of WT and OE/+ 3 days after crush. OE/+ nerves were observed to have slightly more M1 macrophages present in the nerve closer to the injury site. **(C)** Counts of CD16/32 positive macrophages at the crush site. OE/+ nerves attracted slightly more M1 macrophages to the injury site compared to WT nerves, however the small sample size prevents confirmation of this result. Graphs of means ± SEMs, n=2. Scale bars: 100µm.

4.13 Discussion

This chapter aimed to examine if c-Jun maintenance during chronic denervation can be achieved through the use of the OE/+ mouse. The OE/+ mouse was generated by breeding the $OE^{fl/fl}$ mouse with a P_0-cre line. To initially examine the effect of c-Jun over-expression on Schwann cells, cultures from the $OE^{fl/fl}$ mouse were generated. Infection of $OE^{fl/fl}$ Schwann cell cultures significantly increased levels of both c-Jun protein and mRNA (Figures 4.1 and 4.2). Work by Huang et al (2015) has previously shown that over-expression of c-Jun in cultured Schwann cells increases secretion and expression of trophic factors GDNF and BDNF. Here however no such increases were seen *in vitro* despite a 3- fold increase in c-Jun protein and RNA expression (Figure 4.1 and 4.2). However, this increase may not be enough to elevate trophic factors. Cultured Schwann cells already express high levels of GDNF and BDNF (Acheson et al., 1991; Meyer et al., 1992; Springer et al., 1995). It may not be possible to elevate levels further. The previous study also fails to report the quantitative increase in c-Jun that is generated through their viral infection, only that it is successful. As the level of c-Jun increase required to elevate trophic factors is not reported, the data presented here cannot be compared to this study as the elevation of c-Jun required may be significantly higher than that reported here (Huang et al., 2015). Although the results from these experiments (Figure 4.2) were contradictory to the literature and the desired increases in repair cell markers were not observed, these experiments did demonstrate that the transgene in the $OE^{fl/fl}$ mouse is functional. Experiments were therefore carried out to examine the effect of c-Jun over-expression and maintenance *in vivo* by crossing the $OE^{fl/fl}$ mouse with a P_0-cre line to generate a mouse that over expressed c-Jun specifically in Schwann cells.

Although expression of c-Jun in uninjured nerves of mouse model of CMT1A neuropathy has previously been found to be neuroprotective (Hantke et al., 2014), OE/OE mice exhibit disrupted myelination and Schwann cell proliferation (Fazal et al., 2017). The uninjured nerves of these mice at postnatal day 7 express 19 times more c-Jun protein than WT nerves. Here, it was found that Schwann cells in uninjured nerves from these mice express high levels of repair cell markers, such as *Shh* and *GDNF*. This data further contributes to the abnormal characterisation of this mouse. The results here however indicated that OE/+ nerves do not express higher

levels of *c-Jun* mRNA despite a significant elevation in c-Jun protein. *c-Jun* RNA expression may be different between the two genotypes, but the method might not be sensitive enough to detect it as c-Jun protein expression is low in WT nerves (Hantke et al., 2014). The most important finding from these experiments was that uninjured OE/+ nerves do not have markers of the repair cell phenotype elevated, further enforcing the previous findings that the nerves of these mice are largely normal. These mice can therefore be used to examine the effect of c-Jun over-expression on acute and chronic injury.

It was found that following short term injury (1-3 weeks), WT and OE/+ Schwann cells express equal levels of c-Jun. However, 10 weeks after injury, whilst WT Schwann cells down-regulated c-Jun, OE/+ Schwann cell levels were maintained. These mice can thus be used to examine if c-Jun maintenance during chronic denervation maintains the repair cell phenotype.

p75 NTR was described previously as a maker of the repair cell phenotype and it was shown that it is not maintained during chronic denervation (Figure 3.4) (You et al., 1997). Surprisingly, p75 NTR decreased in the distal nerve stumps of OE/+ mice during chronic denervation despite the maintenance of c-Jun. c-Jun has previously been shown to control p75 NTR protein expression, with c-Jun cKO nerves showing suppressed elevation of p75 NTR after injury (Arthur-Farraj et al., 2012). It was therefore expected that p75 NTR would be maintained in the distal nerve stump of OE/+ nerves. However other studies into peripheral nerve injury have found no correlation between c-Jun and p75 NTR protein levels. Stat3 cKO mice have a Schwann cell specific deletion of Stat3. Following chronic denervation, Stat3 cKO mice have significantly lower levels of c-Jun protein and RNA expression compared to WT mice. p75 NTR protein levels however remain consistent between the two genotypes. This suggests there is not always a direct correlation between c-Jun and p75 NTR protein levels (Benito et al., 2017).

Suppression of p75 NTR may be beneficial to regeneration. Studies on *p75 NTR*$^{-/-}$ mice demonstrate that although p75 NTR is important for the development of the PNS, its activation during injury may be detrimental (Lee et al., 1992; Barrett & Bartlett, 1994; Ferri et al., 1998; Boyd & Gordon, 2001). The role of p75 NTR signalling has been suggested to involve apoptosis of neurons and oligodendrocytes

(Rabizadeh et al., 1993; Barrett & Bartlett, 1994; Casaccia-Bonnefil et al., 1996; Frade et al., 1996). Blockade of p75 NTR signalling after axonal injury in *p75 NTR*$^{-/-}$ mice found a decrease in Schwann cell apoptosis, demonstrating that the p75 NTR also mediates apoptosis in Schwann cells (Ferri et al., 1998; Syroid et al., 2000). Axon regeneration after injury has also been examined in *p75 NTR* $^{-/-}$ mice. Following peripheral nerve crush motor neuron regeneration and survival is enhanced in *p75 NTR* $^{-/-}$ mice (Ferri et al., 1998). Further studies examining motor neuron regeneration into repaired chronically denervated common peroneal nerves, found that motor neuron regeneration was greatly increased in *p75 NTR* $^{-/-}$ mice compared to WTs (Boyd & Gordon, 2001). The benefits of low p75 NTR expression in glia cells have further been demonstrated in olfactory ensheathing cells (OECs). In the olfactory bulb, there are two population of OECs, those expressing high p75 NTR levels and those expressing low levels. Low p75 NTR expression in OECs results in activation of genes that control cell adhesion and axon guidance (Honoré et al., 2012). Repression of p75 NTR maintenance may therefore improve nerve regeneration and Schwann cell survival.

Other markers of the repair cell phenotype can be examined by qPCR. Experiments demonstrated that *c-Jun* and *Shh* are elevated in OE/+ nerves following chronic denervation however trophic factors *GDNF* and *BDNF* are not. GDNF and BDNF both enhance axon regeneration following chronic denervation (Boyd & Gordon, 2003). GDNF is directly controlled by c-Jun (Fontana et al., 2012), it is therefore conflicting that c-Jun is elevated in OE/+ nerves while *GDNF* is even lower in these nerves compared to WT nerves. However *c-Jun* RNA expression in OE/+ nerves following chronic denervation is decreased by 44%, yet the protein levels are maintained. It is therefore important to also examine the protein levels of GDNF and BDNF. It may also be that the technique used here is not sensitive enough to detect small changes in expression. These samples should therefore be subjected to RNA sequencing to determine changes in these genes and genes other than the ones examined here to provide a more comprehensive method for deciphering significant changes in both WT and OE/+ acute and chronically denervated nerves.

Despite c-Jun maintenance not maintaining some markers of the repair cell phenotype, it may affect other aspects of the deterioration of the distal nerve stump such as Schwann cell death (Abercrombie & Johnson, 1946; Weinberg & Spencer,

1978; Siironen et al., 1994; Hall, 1999; Benito et al., 2017). Schwann cell numbers were counted in the distal nerve stump by EM following acute and chronic denervation. Counts revealed that OE/+ nerves had significantly more Schwann cells present following chronic denervation compared to WT distal nerve stumps. However, Schwann cell numbers counted by electron microscopy did not change between 1 week and 10 weeks in WT nerves. Previous work that has used this method has found that 1 week after injury, almost 100 more cells are present in the WT nerve compared to the numbers reported here (Arthur-Farraj et al., 2012). It is impotant to remember however that although these mice are considered to be WT controls, they are still transgenic mice carrying an inactive form of the transgene. This could account for the discrepancies in Schwann cell numbers seen between Figure 4.9 and those reported by Arthur-Farraj et al (2012), as two transgenic lines are being compared as opposed to pure WT animals. To investigate this issue further, experiments will be repeated on acutely denervated WT nerves from the appropriate strains at 1 week and 2 weeks after injury to confirm these findings. Previous investigations into Schwann cell numbers by this method in 10 week denervated stumps however confirmed numbers similar to those observed here so these data will not be repeated (Benito et al., 2017).

Macrophage numbers were also counted by EM. Macrophages play a key role in regeneration in the peripheral nervous system. Macrophages are recruited to the injured nerves 1-2 days post injury and remain present in the nerve for up to 3 weeks where they play a key role in the later stages of myelin breakdown (Perry et al., 1987; Stoll et al., 1989; Raivich et al., 1991; Perry et al., 1995; Liu et al., 1995; Hirata et al., 1999; Hirata & Kawabuchi, 2002). When macrophage infiltration, migration and polarization in the injured nerve is altered, the rate of axon regeneration is reduced (Dailey et al., 1998; Barrette et al., 2008; Vargas et al., 2010; Chen et al., 2015).

Counts of macrophages in chronically denervated distal nerve stumps of WT and OE/+ mice indicated that OE/+ nerves retained macrophages. It was also found that 3 days after crush, OE/+ nerves recruited more macrophages to the injury site compared to WT nerves. A link between c-Jun and macrophage recruitment has previously been established (Arthur-Farraj et al., 2012), it was therefore expected that OE/+ nerves would attract a greater number to the injury site.

The increased number of M2 macrophages in OE/+ nerves could aid peripheral nerve regeneration. Mokarram et al (2012) polarised macrophages to M1 or M2 populations in the peripheral nerve using a scaffold containing IFN-γ (M1) or IL-4 (M2) to bridge a transected sciatic nerve. M2 macrophage polarisation was found to improve Schwann cell migration into the graft and improve axon regeneration (Mokarram et al., 2012). Similarly, exposure to M2 macrophages increases growth of adults DRGs through inhibitory chondroitin sulfate proteoglycans and promotes cortical neuron growth and survival (Kigerl et al., 2009). Increased number of M2 macrophages observed in OE/+ nerves may therefore enhance axon regeneration.

In contrast to M2 macrophages, M1 macrophages are generally classed as inhibitory. It has been previously suggested that there are no M1 macrophages in the PNS (Ydens et al., 2012). To test this, transverse sections from 3 day crushed nerves were immunolabelled with CD16/32 antibodies. The results showed that there were many CD16/32 positive cells present in the nerve. Other studies have found M1 macrophages present in the PNS after injury. However, representative images of iNOS labelling from this work is weak (CLR De La Hoz et al., 2003). Work for this thesis was unable to detect positive iNOS staining in injured nerves, hence CD16/32 was examined. Although it was hypothesized that OE/+ nerves might attract fewer M1 macrophages to the injury site due to their increased M2 population, more were observed compared to WT. However, this work only had a sample size of 2 so further experimental repeats are required before any conclusion can be drawn. In order to truly confirm macrophage subpopulations in the nerve, multiple markers for each classification should also be tested especially as macrophages can express markers from other subtypes. Arginase for example is induced in M1 macrophages under certain conditions (El Kasmi et al., 2008). Guidelines are now proposed on how to correctly report macrophage activation and polarization, (Murray et al., 2014; Martinez & Gordon, 2014). However, this work so far does dispute the fact that M1 macrophages are not present in the PNS.

Overall, the work in this chapter has found that Schwann cell c-Jun can be maintained during chronic denervation. However, maintenance of c-Jun does not equate to maintenance of all the markers of the repair cell phenotype. Trophic factor expression significantly decreased during chronic denervation in both WT and OE/+ nerves, while *Shh* and *c-Jun* expression was higher in OE/+ nerves. c-Jun

maintenance however resulted in an increase in the number of Schwann cells and macrophages present in the distal nerve stump following chronic denervation. This alone may enhance axon regeneration into chronically denervated distal nerve stumps which will be examined in Chapter 5.

5. The regenerative capacity of WT and OE/+ nerves following acute and chronic denervation

The reduced capacity for chronically denervated distal nerve stumps to promote axon regeneration in the PNS has been a topic of study for many decades (Cajal, 1959); (Holmes & Young, 1942; Sunderland, n.d.).

One of the factors that contributes to this phenomenon is the deterioration of the nerve stump distal to the injury. This is defined as the decline in the ability of the distal nerve stump to support axonal growth due to down-regulation of trophic support for axon regeneration and a gradual decline in cell numbers (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; You et al., 1997; Hall, 1999; Höke et al., 2002; Michalski et al., 2008; Eggers et al., 2010; Benito et al., 2017). As axons regenerate at a rate of 1-2mm per day (Buchthal & Kühl, 1979), the distal nerve stump they are regenerating into has often already lost its ability to support them when they arrive.

The work presented in this thesis has revealed that c-Jun, and the repair cell phenotype, are not maintained during chronic denervation and this is suggested to be the cause of many aspects of distal nerve stump deterioration. I have shown that OE/+ Schwann cells maintain c-Jun expression from acute to chronic injury (Figure 4.5-6). Although trophic factors are not maintained in OE/+ nerves during chronic denervation, *Shh* and *c-Jun* expression is elevated (Figure 4.8). OE/+ nerves also have an increased number of Schwann cells and macrophages present in the nerves following chronic denervation (Figure 4.9). It is therefore hypothesized that c-Jun maintenance may prevent the regeneration deficit observed with chronic denervation.

To examine regeneration into denervated distal nerve stumps, a nerve cross suture model can be used (sometimes referred to as cross-anastomosis). This model has been used extensively to examine regeneration into denervated stumps. Commonly, a branch of the sciatic nerve, the tibial or common peroneal, is transected then later repaired by suturing the denervated distal nerve stump to a freshly cut promixal stump of a neighbouring nerve. This overcomes the issues that would arise if the repair was performed with the nerve's own proximal stump.These include nerve shrinkage and the inhibitory effects of chronic axotomy on nerve regeneration.

(Abercrombie & Johnson, 1946; Holmes & Young, 1942; Fu & Gordon, 1995; Boyd & Gordon, 2002a, 2003)

The regeneration into denervated stumps then must be quantified. One of the most accurate methods for analysis is counting cell bodies of neurons that have been labelled by retrograde labelling. A labelling dye is applied to the distal nerve stump either through injection into the stump or muscle or cutting the nerve. Regenerating axons exposed to the dye will transport the dye retrogradely, resulting in labelling of their somas. Motor neurons can be counted in the spinal cord and sensory neurons in the DRGs. Flurogold is a widely used in retrograde labelling due to its low diffusion and lack of fading. It is also not taken up by undamaged fibres (Schmued & Fallon, 1986; Brushart, 1993; Sagot et al., 1998; Puigdellívol-Sánchez et al., 1998; Sulaiman & Gordon, 2000; Haenggeli & Kato, 2002; Boyd & Gordon, 2002b, 2003; Catapano et al., 2016).

The aims of this chapter are therefore to use nerve-cross suture and retrograde labelling with flurogold to:

- determine that WT and OE/+ nerves have equal numbers of motor and sensory neurons
- establish the regenerative capacity of WT and OE/+ nerves during acute and chronic denervation
- investigate if the regenerative capacity of the distal nerve stump can be restored following chronic denervation

5.1 WT and OE/+ common peroneal nerves have equal numbers of motor and sensory neurons

Before the regenerative capacity of OE/+ nerves following denervation can be examined, it is first important to confirm that WT and OE/+ mice have equal numbers of motor and sensory neurons. No difference has previously been observed in the number of axons present in the tibial nerves of these mice (Fazal et al., 2017). However it is also important to determine that backfilling with flurogold is equally efficient in both genotypes. To confirm these points, both genotypes underwent backfilling of the uninjured common peroneal nerve, as the common peroneal nerve proximal stump will be used to repair the tibial nerve. The branches of the sciatic nerve were separated and the common peroneal was cut and immediately exposed to fluorogold (Catapano et al., 2016). Mice were sacrificed 1 week post-surgery and the number of motor neurons in the spine and sensory neurons in the L4 DRG were counted (Figure 5.1). Brightly labelled cell bodies were found in the spinal cord and DRG (Figure 5.1 A and B). Counts of labelled motor neurons showed no significant difference in cell numbers between WT and OE/+ mice (Figure 5.1 C) ($p=0.7153$). Comparably, counts of cell bodies in the L4 DRG of each genotype showed no significant difference in cell numbers between the two genotypes (Figure 5.1 D) ($p=0.1872$). OE/+ mice did however demonstrate a trend towards lower sensory neuron numbers.

Overall, the results from this experiment demonstrate that there is no difference in the number of motor or sensory neurons present in the common peroneal nerve between WT and OE/+ mice. This result also suggests that backfilling with fluorogold is an effective way to examine cell numbers with bright staining observed in both samples and equal numbers of cell labelled. It can now be confirmed that any differences in regeneration observed between these genotypes is a true observation.

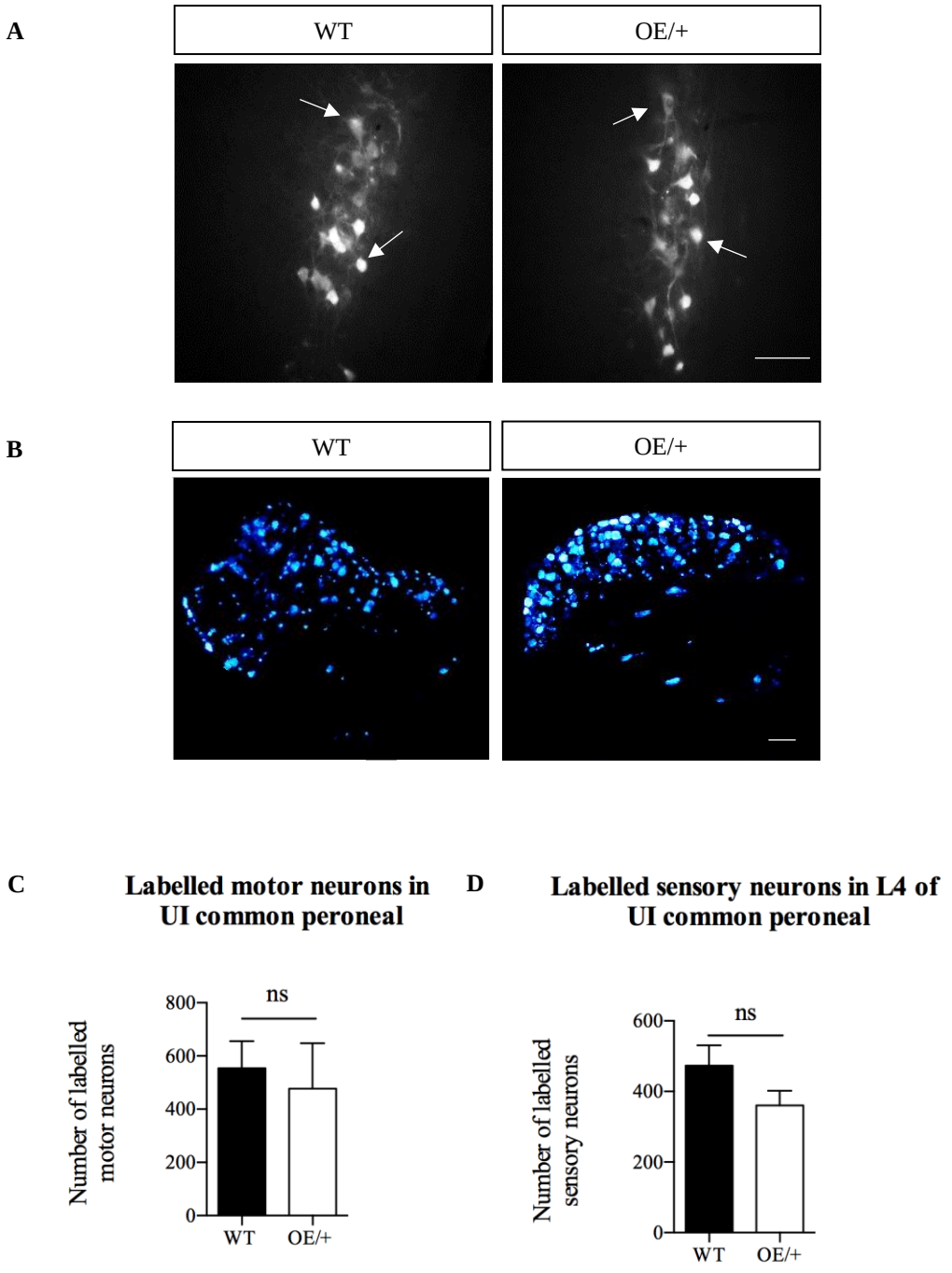


Figure 5.1 | WT and OE/+ uninjured common peroneal nerves have equal numbers of motor and sensory neurons

(A) All labelled cell bodies in 50µm sections of the spinal cord were counted (arrows). **(B)** Sensory neuron cell bodies were labelled in the L4 DRG. **(C)** The Abercrombie correction was applied to the counts from the spinal cord to prevent double counting. There was no significant difference in the number of labelled motor neurons between genotypes (p=0.7153). **(D)** Every 5th section collected from the L4 DRG was counted to prevent double counting. Although OE/+ DRGs had a lower number of sensory neurons labelled, no significant difference was found between the two genotypes (p= 0.1872). Graphs of means ± SEM, n=3, Student's unpaired T-test. Scale bars: 100µm.

5.2 Selecting a model of acute denervation

Before the effect of chronic denervation on regenerative capacity in WT and OE/+ mice can be examined, it is first important to establish a time point during acute injury where regeneration readily occurs. Previous studies have examined various timepoints. Many use immediate repair as a model, whereby a branch of the sciatic nerve is cut and immediately repaired by attaching the distal nerve stump to the proximal stump of a neighbouring nerve (Bain et al., 2001; Boyd & Gordon, 2002a, 2003; Willand et al., 2016). Other models allow a short period of denervation to occur (1-4 weeks) before repairing the nerve (Sulaiman & Gordon, 2000).

As it has been shown that c-Jun is highly up-regulated in Schwann cells 1 week after injury (Chapter 3, Figure 3.1 and 3.3), it was decided to examine 2 acute injury paradigms: Immediately repairing the nerve following injury or cutting the nerve and repairing it after 1 week of denervation. As the paradigm selected is to be directly comparable to chronically denervated nerve repair, it was decided that the tibial nerve distal stump would be sutured to the common peroneal proximal stump. As the nerve shrinks during chronic denervation, the tibial nerve distal stump is likely to be better size-matched with the common peroneal nerve than if the reverse was performed (Abercrombie & Johnson, 1946; Fu & Gordon, 1995; Vuorinen et al., 1995; Bain et al., 2001; Willand et al., 2016). 6-10 week old WT mice underwent surgeries in which their tibial nerves were cut. Either the distal nerve stump was immediately sutured to the proximal stump of the common peroneal nerve (Figure 5.2 A), or the distal nerve stump was denervated for 1 week before suturing to the freshly cut proximal stump of the common peroneal nerve (Figure 5.2 B). Backfilling with fluorogold was then performed 2 weeks after repair to examine regeneration.

Counts of motor neurons showed that no significant difference was observed between these two paradigms (Figure 5.2 C). The regenerative capacity of the nerve at both acute injury time points was equal. It was therefore decided to adopt immediate repair as the method for quantifying regenerative capacity during acute denervation. Not only did this method demonstrate the strong regenerative capacity of the nerve during acute denervation, but using this method also allows the mice to undergo fewer surgeries, decreasing pain experienced by the animals.

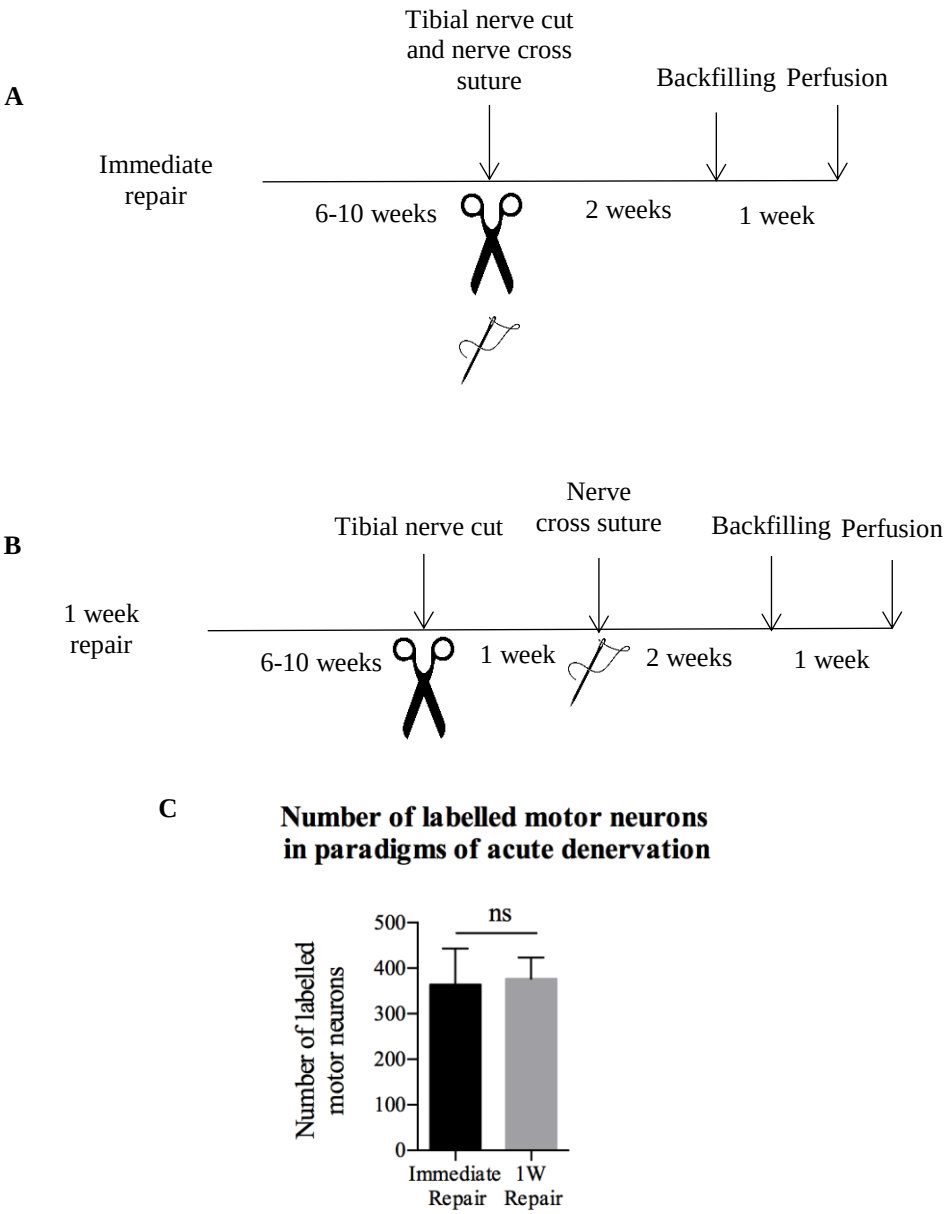


Figure 5.2 | No difference in motor neuron regeneration is observed in immediate and 1 week repair models of acute denervation

(A) Summary diagram of the immediate repair model timeline. 6-10 week old mice underwent a tibial and common peroneal nerve cut. The distal stump of the tibial nerve was then immediately repaired to the proximal stump of the common peroneal nerve. After 2 weeks, the distal nerve stump of the repaired nerve was cut 4mm from the repair site and exposed to fluorogold. Mice were perfused 1 week later. **(B)** Summary diagram of the 1 week repair model. Mice underwent a tibial nerve cut. The nerve was denervated for 1 week then sutured to the proximal stump of the freshly cut common peroneal nerve. After 2 weeks, the distal nerve stump of the repaired nerve as cut 4mm from the repair site and exposed to fluorogold. Mice were perfused 1 week later. **(C)** Counts of motor neurons in the spinal cords showed no significant difference in regeneration between the 2 paradigms ($p=0.9$). Graph of means ± SEM, $n = 3$, unpaired Student's T-test.

5.3 WT and OE/+ display similar rates of regeneration during acute denervation

Now the paradigm for examining regeneration in acute denervation had been established, regeneration could be compared between WT and OE/+ nerves (Figure 5.3). WT and OE/+ mice underwent tibial nerve transection followed by immediate repair to the common peroneal. Backfilling with fluorogold was then performed 2 weeks later to examine regeneration of axons from the common peroneal into the tibial nerve distal stump.

Counts of labelled motor neurons in the spinal cord showed no difference in cell numbers between WT and OE/+ mice (Figure 5.3 C) ($p=0.1698$). In parallel, no significant difference in sensory cell numbers was observed in the L4 DRG between the genotypes (Figure 5.3 D) ($p=0.9195$). These results show that during acute denervation, the regenerative capacity of WT and OE/+ nerves is equal.

This result correlates with the c-Jun expression in the Schwann cells of these nerves. In Chapter 4 it was shown that 1 week after injury, WT and OE/+ distal nerve stumps express equal levels of c-Jun. It should be noted, however, that after chronic denervation c-Jun levels differ in the distal nerve stumps of these mice since they are higher in OE/+ mice (Chapter 4 Figure 4.5-6).

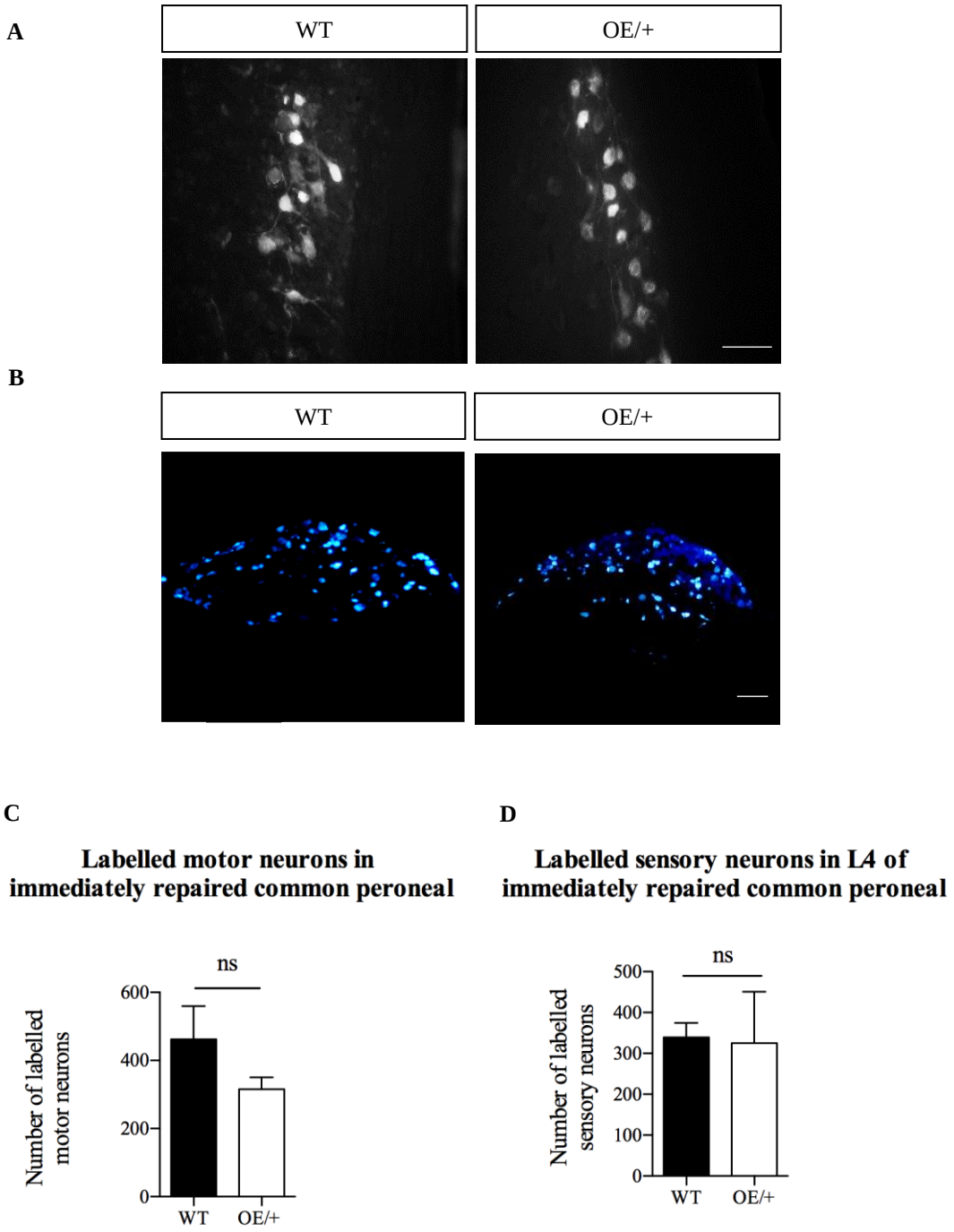


Figure 5.3 | WT and OE/+ nerves have similar rates of regeneration during acute denervation

(A) Cell bodies of motor neurons in the spinal cord of WT and OE/+ mice. **(B)** Sensory cell bodies were labelled in the L4 DRG of WT and OE/+ mice. **(C)** All labelled cell bodies in the spinal cord were counted. The Abercrombie correction was applied to account for double counting. Although OE/+ had fewer regenerating motor neurons compared to WT, this was not found to be significant ($p=0.1698$). **(D)** Every 5th section from the L4 DRG was counted. No difference in the number of regenerating sensory neurons was observed between WT and OE/+ nerves ($p=0.9195$). Graphs of means ± SEM, n=3, Student's unpaired T-test. Scale bars: 100µm.

5.4 The regenerative capacity of OE/+ nerves is maintained during chronic denervation

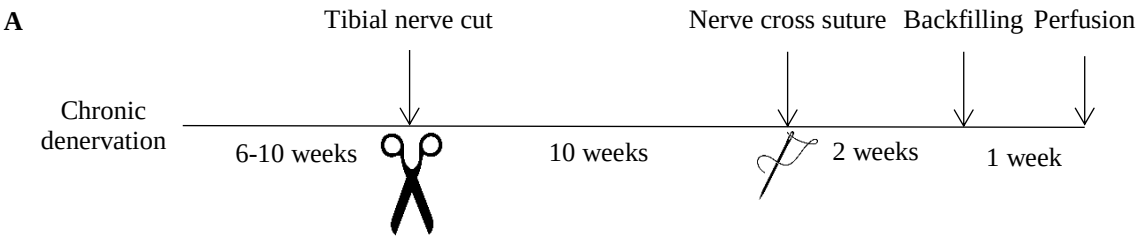
It has previously been widely reported that the regenerative capacity of the nerve decreases with time post injury.

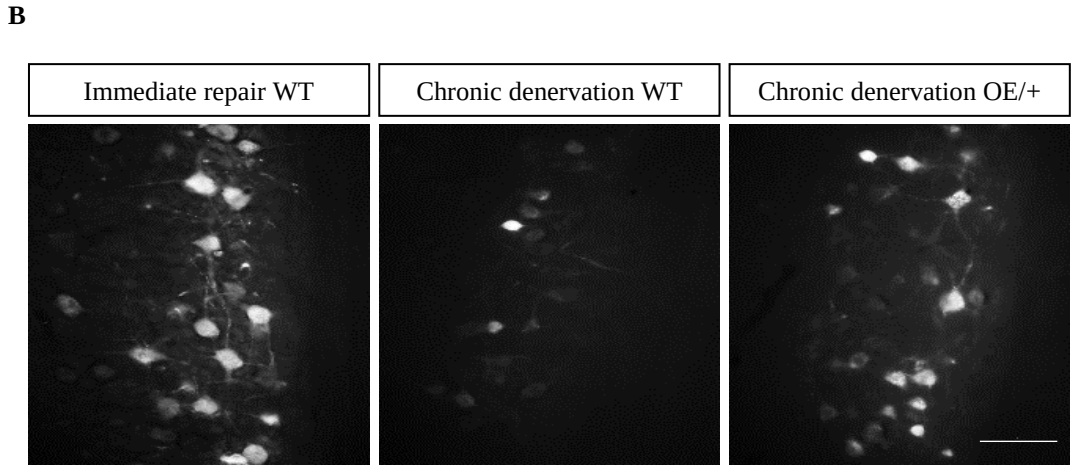
In order to examine if Schwann cell c-Jun maintenance during chronic denervation maintains the regenerative capacity of the nerve, WT and OE/+ mice underwent a tibal nerve cut followed by 10 weeks chronic denervation. The deteriorated distal nerve stump was then sutured to the freshly cut common peroneal proximal stump. 2 weeks later, backfilling with fluorogold was used to measure axon regeneration into the tibial nerve distal stump (Figure 5.4 A).

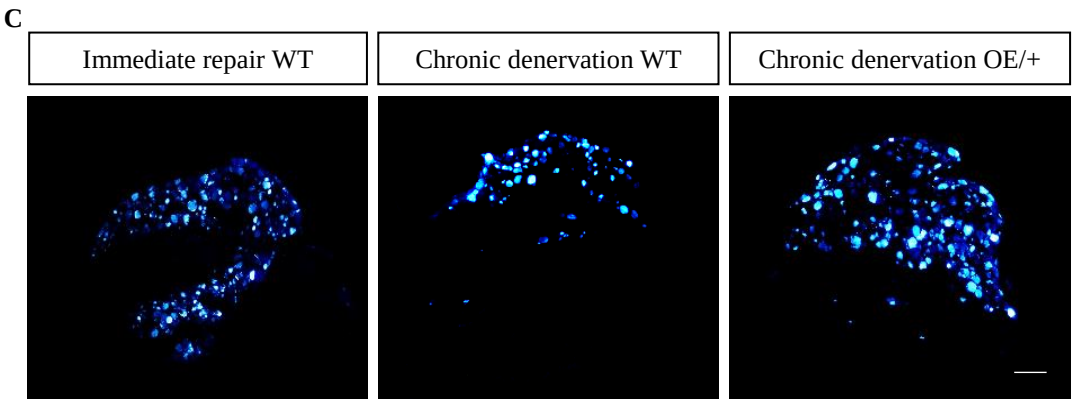
To determine if any decrease in regenerative capacity in the nerves had occurred, the chronically denervated repaired nerves were compared to the immediately repaired WT nerves. Counts of motor neurons in the spinal cord revealed that the regenerative capacity of WT nerves significantly decreases following chronic denervation (Figure 5.4 D). Following chronic denervation there was a 48% decrease in the number of motor neurons regenerating into the tibial nerve. However, when c-Jun was maintained in the Schwann cells of the distal nerve stump during chronic denervation, as in the nerves of the OE/+ mice, no significant difference in motor neuron cell numbers was observed between acute or chronically denervated nerves. OE/+ nerves had significantly more motor neurons regenerating into their chronically denervated distal nerve stumps compared to WT nerves.

To further examine the restorative capabilities of c-Jun maintenance in the distal nerve stump during chronic denervation, labelled sensory cell bodies in the L4 DRG were counted (Figure 5.5). Similarly to the counts of motor neurons, a 47.3% decrease in the number of sensory neurons regenerating into the tibial nerve from the common peroneal nerve was observed between immediately repaired and chronically denervated WT nerves (Figure 5.5 E). No significant difference however was observed between WT acute denervation and chronic denervation in OE/+ nerves. Following chronic denervation, OE/+ nerves had significantly more sensory neurons from the common peroneal nerve regenerating into the chronically denervated tibial nerve compared to WT.

Overall, these experiments confirm that the regenerative capacity of the nerve is reduced following chronic denervation. However, if c-Jun is maintained during chronic denervation, the regenerative capacity of the nerve is also maintained.


A
Tibial nerve cut
Nerve cross suture
Backfilling
Perfusion
Chronic denervation
6-10 weeks
10 weeks
2 weeks
1 week


B
Immediate repair WT
Chronic denervation WT
Chronic denervation OE/+


C
Immediate repair WT
Chronic denervation WT
Chronic denervation OE/+

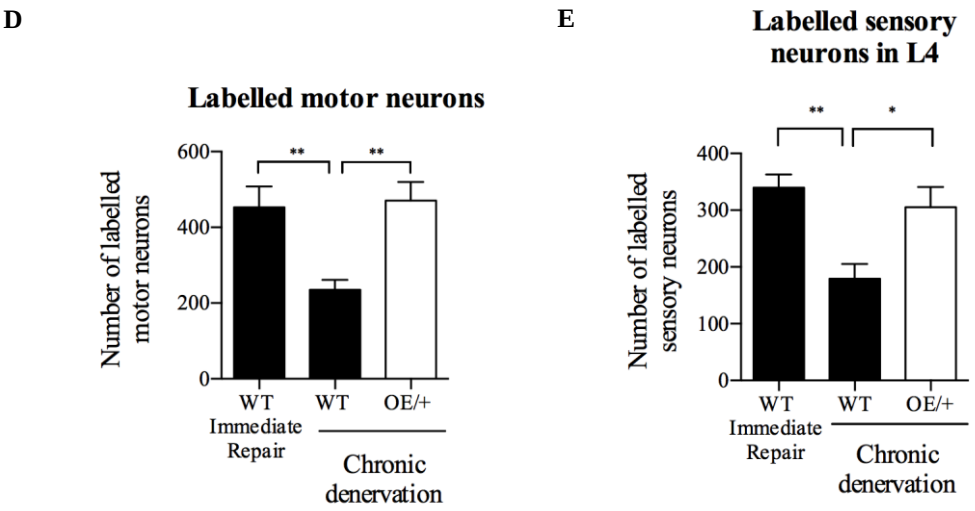

D
Labelled motor neurons
Number of labelled motor neurons
600
400
200
0
**
**
WT Immediate Repair
WT
OE/+
Chronic denervation
E
Labelled sensory neurons in L4
Number of labelled sensory neurons
400
300
200
100
0
**
*
WT Immediate Repair
WT
OE/+
Chronic denervation

Figure 5.4 | OE/+ nerves maintain regenerative capacity during chronic denervation

(A) A summary diagram of chronic denervation followed by repair experimental timeline. Mice underwent a 10 week tibial nerve cut. The tibial nerve distal stump was then repaired by suturing to the freshly cut common peroneal nerve. After 2 weeks, the repaired nerve was then cut 4mm from the repair site and exposed to fluorogold. 1 week later the mice were perfused. **(B)** Many bright motor neuron cell bodies were observed in the spinal cords of immediately repaired nerves. However, following chronic denervation and repair the number of visible cells was reduced. In OE/+ spinal cords however many brightly labelled cells were seen. **(C)** Sensory neuron cell bodies were brightly labelled in the DRGs of immediately repaired nerves. Fewer labelled cells were however seen in WT nerves following chronic denervation. This difference was not observed in the L4s of OE/+ mice, with many labelled cells observed throughout the DRG. **(D)** All labelled cells in the spinal cords were counted and the Abercrombie correction was applied to prevent double counting. WT nerves had significantly fewer regenerating motor neurons as demonstrated by the lower cell body counts ($p=<0.005$). OE/+ nerves however maintained their regenerative capacity with no difference observed between the number of regenerating motor neurons compared to immediately repaired nerves. OE/+ nerves had significantly more regenerating motor neurons compared to chronically denervated WT littermates ($p=<0.005$). WT immediate repair n=5, WT and OE/+ chronic denervation n=8. **(E)** Every 5^{th} section from the L4 DRG was counted. Counts showed that significantly fewer sensory neurons regenerated into the repaired tibial nerve following chronic denervation ($p=<0.05$). The number of sensory neurons regenerating into OE/+ following chronic denervation was significantly higher ($p=<0.05$). Although the number of sensory neurons was not as many as those observed regenerating into immediately repaired nerves, no difference was found between immediately repaired and chronically denervated nerves ($p=>0.05$). WT immediate repair and chronic denervation n= 6, OE/+ chronic denervation n=8. Graphs of means ± SEMs, one-way ANOVAs with Tukey's test. Scale bars: 100µm.

5.5 OE/+ nerve area does not significantly decrease following chronic denervation

It is well established that during chronic denervation, nerve size is reduced (Abercrombie & Johnson, 1946). As mentioned above, it was decided that the tibial nerve would be chronically denervated and the common peroneal nerve would be used as the repair nerve, as their sizes would be more compatible. Nerves of similar size will fit together better when sutured. As previously observed however, OE/+ nerves have a greater number of Schwann cells present during chronic denervation (Chapter 4, Figure 4.8). An increased number of cells could result in a greater nerve size. If OE/+ nerves maintain cell numbers during chronic denervation, they may also maintain nerve area. To examine this, transverse sections of nerves 1 and 10 weeks after cut were stained with DAPI (Figure 5.5 A). The tibial nerve was identified and the area measured. The results indicated that 1 week after injury there was no difference in nerve area between genotypes (Figure 5.5 B). Following chronic denervation, the area of WT nerves was significantly reduced compared to WT ($p=<0.005$) and OE/+ ($p=<0.05$) acutely injured nerves. Although a decrease in area was observed in OE/+ nerves, this result was not significant. OE/+ nerve area was twice as large as WT following chronic denervation. These findings further reinforce that over-expression of c-Jun prevents the deterioration of the distal nerve stump by reducing the extent to which nerve size decreases.

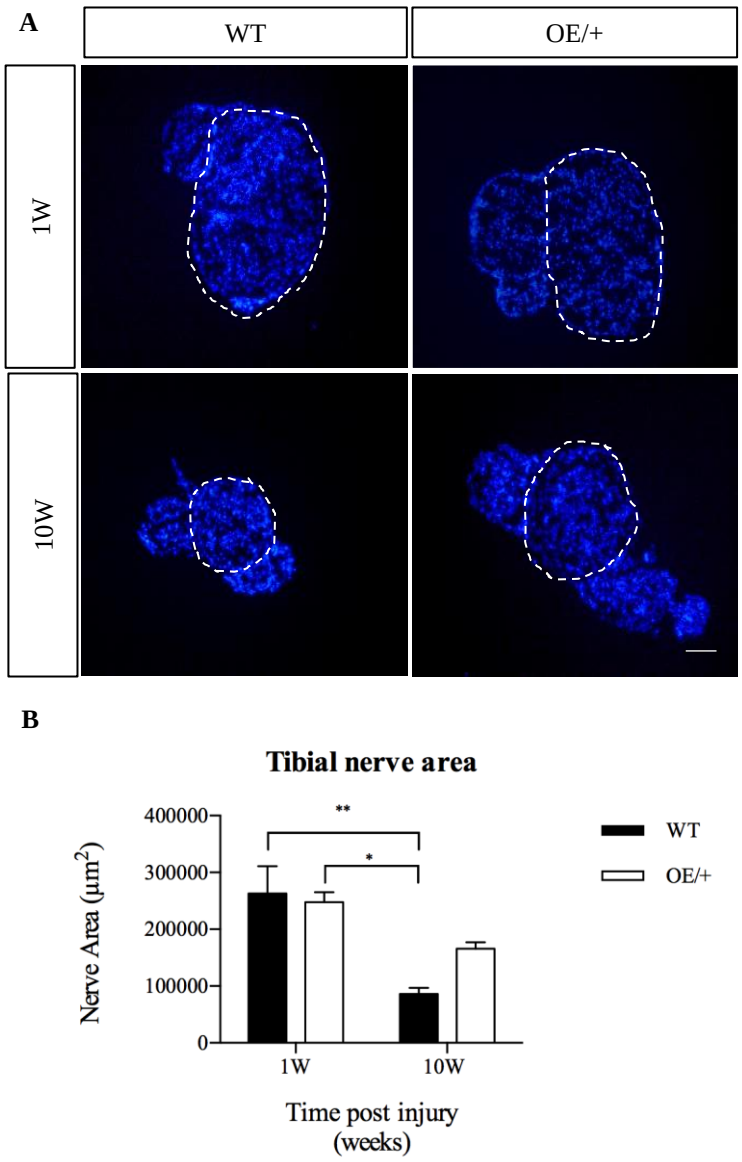


Figure 5.5 | The area of OE/+ tibial nerves does not significantly decrease following chronic denervation

(A) DAPI staining of transverse sections of WT and OE/+ nerves following acute and chronic denervation. The area of the tibial nerve (highlighted, white dotted line) was measured. **(B)** Following chronic denervation, WT tibial nerves were 3 times smaller than acutely denervated nerves ($p=<0.005$). OE/+ nerves however were only 1.5 times smaller and the difference between the acute and chronically denervated nerves was not significant. Graph of means ± SEMs, n=3, two-way ANOVA with Tukey's test. Scale bar: 100µm.

5.6 Chronic denervation of the distal nerve stump cannot be reversed

Results earlier in this chapter demonstrated that maintenance of Schwann cell c-Jun expression maintains the regenerative capacity of the distal nerve stump during chronic denervation. It was thus hypothesized that restoration of Schwann cell c-Jun levels following chronic denervation might restore the regenerative capacity of the nerve. This can be examined using an inducible transgenic mouse model. A PLPCreERT2 OE/+ mouse was generated which, once injected with tamoxifen, was able to over-express c-Jun specifically in Schwann cells (Figure 2.2). During chronic denervation, the distal nerves of these mice down-regulate c-Jun as previously observed in WT nerves. However, if tamoxifen is administered after 8 weeks of chronic denervation, by 10 weeks c-Jun levels are significantly elevated (J.A Gomez-Sanchez, Appendix 1).

These mice can therefore be used to examine the effect of restoring c-Jun levels in chronically denervated Schwann cells. Mice underwent chronic denervation of the tibial nerve for 8 weeks. Mice were then injected with 170mg/kg tamoxifen or vehicle for 5 days. 10 weeks after tibial nerve transection, the nerve was repaired by suturing to the freshly cut common peroneal nerve. 2 weeks post repair, tibial nerve stumps were exposed to fluorogold and mice were sacrificed 1 week later (Figure 5.6 A).

Counts of labelled motor neurons in the spinal cord showed no significant difference in motor neuron regeneration between vehicle (Veh) and tamoxifen (TMX) injected mice (Figure 5.6 B) ($p=0.9485$). Counts of labelled sensory neuron cell bodies in the L4 DRG further showed no significant difference between vehicle and TMX mice (Figure 5.6 C) ($p=0.59$).

These results demonstrate that increasing c-Jun expression following chronic denervation does not restore the regenerative capacity of the peripheral nerve. c-Jun expression must be maintained from the initial injury during chronic denervation to maintain the regenerative capacity of the peripheral nerve.

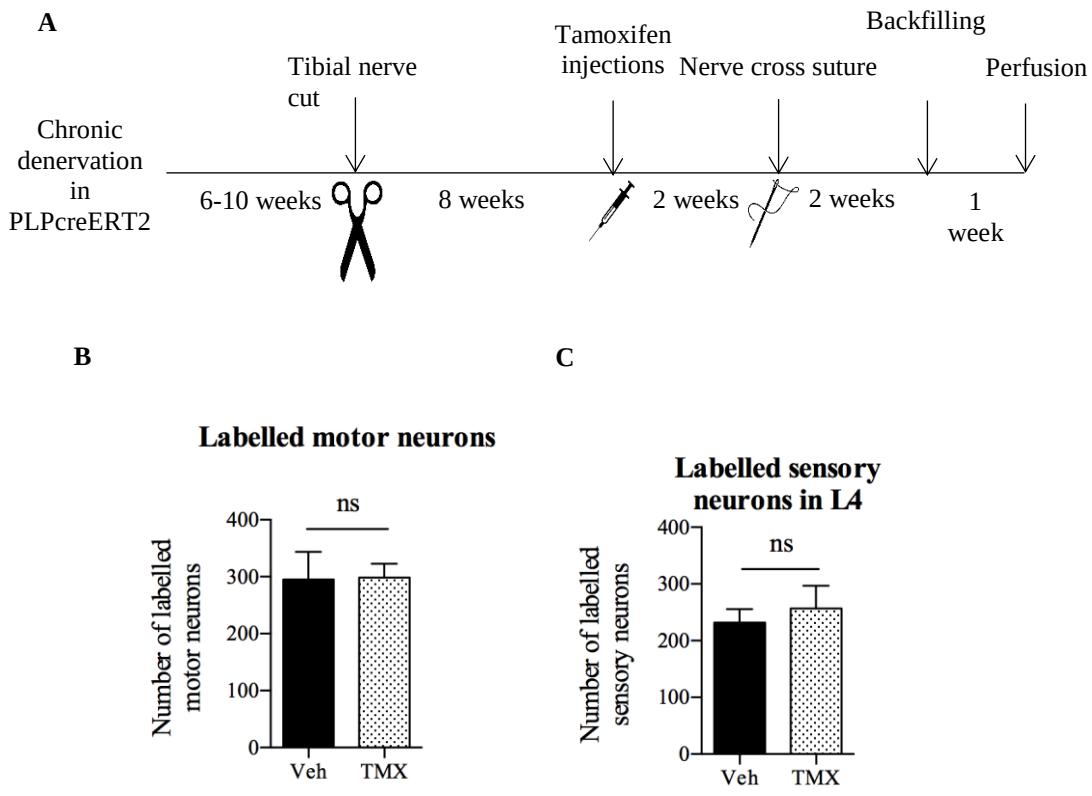


Figure 5.6 | The regenerative capacity of a chronically denervated nerve cannot be restored by c-Jun activation

(A) A summary diagram of the experimental timeline. 6-10 week old mice underwent a tibial nerve cut. 8 weeks later mice received 170mg/kg of tamoxifen or vehicle for 5 days. 10 weeks after the initial cut the tibial nerve was repaired using the proximal stump of the freshly cut common peroneal nerve. After 2 weeks of regeneration, the repaired tibial nerve was cut 4mm from the repair site and exposed to fluorogold. After 1 week the mice were perfused. **(B)** Counts of labelled motor neurons in the spine showed that there was no difference between vehicle and tamoxifen injected mice ($p=0.9485$). Veh n= 5, TMX n=6. **(C)** Counts of labelled sensory neuron cell bodies in the L4 DRG count showed no difference between vehicle and tamoxifen injected mice ($p=0.59$). Veh n= 6, TMX n=5. Graphs of means ± SEM, unpaired Student's T-test.

5.7 Discussion

The results described in previous chapters of this thesis have shown the ability of OE/+ Schwann cells to maintain c-Jun during chronic denervation. This chapter therefore aimed to determine whether c-Jun maintenance equated to maintenance of regenerative capacity.

This was examined with nerve cross-suturing, the method of suturing a proximal stump of one nerve to the distal nerve stump of another. Here, as described previously, the denervated distal tibial nerve was sutured to the proximal stump of the common peroneal nerve (Holmes & Young, 1942; Sadr, 1946; Fu & Gordon, 1995; Sulaiman & Gordon, 2000; Boyd & Gordon, 2001; Bain et al., 2001; Willand et al., 2016).

Regeneration into distal nerve stumps was then examined by retrograde labelling using fluorogold as described previously. The repaired nerve was transected 4mm from the repair site and exposed to a reservoir of fluorogold as described by (Catapano et al., 2016) This method was selected, as although time consuming, it is reliable and commonly used (Gordon et al., 1991; Richmond et al., 1994; Garrett et al., 1991; Schmued & Fallon, 1986; Catapano et al., 2016). Injecting dyes can produce variable results. If dye is injected into muscle, the exact location of the injection is very important. If injections are done incorrectly, the dye can leak, producing non-specific results (Janjua & Leong, 1981; Tosolini et al., 2013). Therefore fluorogold solution was applied directly to the sutured tibial nerve stump as described in the materials and methods section (Chapter 2). Although it has been suspected that fluorogold may be toxic in long-term studies (Garrett et al., 1991), these claims are not widely reported (Novikova et al., 1997) and here tissue was harvested 1 week after labelling so would not be affected by this.

Before experiments into the regenerative capacity of chronically denervated nerves could commence, an acute denervation time point had to be selected. When time points of immediate repair and repair following 1 week denervation were examined, no difference was found between the two. Repairing the nerve after 1 week might be expected to result in increased regeneration compared with immediate suture since during this time myelin clearance and repair cell activation occurs (Perry et al., 1995; Arthur-Farraj et al., 2012), although in previous studies no differences between

immediate and short term repair were seen (Holmes & Young, 1942; Bignotti et al., 1986; Gattuso et al., 1989). This finding was confirmed here since no differences between the time points chosen were found in my experiments, and therefore to reduce the number of surgeries and thus post-operative pain experienced by the mice, immediate repair was selected as the acute denervation model.

Results in this chapter showed no difference in the regenerative capacity WT and OE/+ nerves during acute denervation. It was hypothesized in Chapter 4 that due to the increased number of macrophages attracted to the injury site in OE/+ nerves, an increased rate of regeneration might be observed. However, these macrophages counts were performed 3 days after crush and EM counts found no difference in macrophage numbers between genotypes at 1 week. It is likely, as in c-Jun cKO nerves, the difference observed in initial macrophage recruitment may not be observed at later time points and may be seen only close to the injury site (Arthur-Farraj et al., 2012). WT and OE/+ nerves also express similar levels of c-Jun 1 week after injury. A difference in regeneration is therefore only likely to be seen when differences in c-Jun expression are seen between the 2 genotypes, such as following chronic denervation (Figure 4.5-6).

The reduced regenerative capacity of the nerve during chronic denervation is well established (Holmes & Young, 1942; Bolesta et al., 1988; Fu & Gordon, 1995; Sulaiman & Gordon, 2000). Here, when WT nerve regeneration was compared between acute and chronic denervation, a significant decline was observed. This was not conserved between genotypes, since OE/+ nerves showed significantly more motor and sensory neuron regeneration than WT nerves following chronic denervation. This suggests that maintaining c-Jun during chronic denervation also maintains the regenerative capacity of the nerve. The mechanism behind this however may not be as simple as previously hypothesized.

In Chapter 4 it was shown that OE/+ tibial nerves have significantly more Schwann cells present in the nerve following chronic denervation (Figure 4.9). It could therefore be argued that the maintained regenerative capacity of the nerve is due to the increased Schwann cell number in OE/+ nerves compared to WT following chronic denervation. OE/+ nerves also contain a higher number of macrophages following chronic denervation which could also contribute to enhanced regeneration.

Despite OE/+ nerves containing more Schwann cells and maintaining c-Jun, trophic factor expression is not maintained. Therefore despite an increase in the quantity of Schwann cells in OE/+ nerves, the quality of the cells are similar to WT. In order to elucidate the mechanism behind the enhanced regeneration observed in OE/+ nerves, an inducible mouse line is required.

Despite the temporal up-regulation of c-Jun in the chronically denervated nerves of TMX mice, no improvements in nerve regeneration were seen. This could be due to various factors. Firstly, as the tamoxifen has to be administered for 5 days to take effect, c-Jun is only fully elevated for 9 days before the nerve repair is performed. It is unknown if other repair cell markers are also elevated in this time as no RNA has been extracted from these nerves. Although it is unlikely that trophic factors will be elevated, based on the experiments in Chapter 4.8, *Shh* elevation may be observed. It is likely that 9 days does not allow sufficient time to restore the regenerative capacity of the nerve. Future work could examine nerve regeneration at later time points from tamoxifen administration. However, it could be argued that this work demonstrates that elevation of c-Jun is not solely responsible for the effects seen in OE/+ nerves.

The number of cells present in TMX and vehicle treated mouse nerves was not determined. Schwann cell proliferation may or may not occur following tamoxifen injection and macrophages may not be recruited to the nerve. If counts of these cell types were performed it may be possible to determine if the enhanced regeneration observed when c-Jun is maintained is due to the number of Schwann cells and macrophages present in the nerve.

It is important to examine all the factors that may have contributed to the enhanced regeneration. The fact that OE/+ nerves have a larger area following chronic denervation may have contributed to this result. It may improve size matching with the common peroneal. When selecting nerves for graft repairs, the diameter of nerve is one of the considerations with nerves of a similar diameter being preferable (Sunderland & Ray, 1947). For example, in humans, repairs of the inferior alveolar nerve (diameter: 2.4 mm) are performed with nerve graphs from the sural nerve (diameter: 2.1 mm) (Svane et al., 1986; Brammer & Epker, 1988). However, as the area of the common peroneal was not measured here, this assumption cannot be clarified.

It has previously been reported that the longer a nerve is denervated, the worse the union with another nerve upon its repair. It was noted that this may be due to the surgical technique. As the nerve is known to decrease in size post-injury, it is implied that larger nerves are easier to suture (Holmes & Young, 1942; Abercrombie & Johnson, 1946). The improved regenerative capacity of the nerve measured here could therefore be due to improved surgical technique in these samples. As surgeries were performed blind, this cannot be confirmed, however no difference was noted at the time of surgery in the ease of some surgical procedures compared to others.

It is important to note that regeneration was only assessed in this chapter by one technique. Although retrograde labelling is a very reliable method for examining regeneration, the other hypotheses of this thesis have been tested using a variety of techniques to fully establish the result. Neurofilament staining can be used to examine regenerating axons. Cutting longitudinal sections of the repaired nerves was found to be very difficult due to the formation of fibrotic tissue around the nerves and repair site. This made embedding the nerve flat extremely difficult. Transverse sections taken at various distances from the repair site could overcome this issue and potentially enforce the findings observed here.

Although it is has not been fully determined how the maintenance of c-Jun restores the regenerative capacity of the nerve, the results presented in this chapter demonstrate the importance of c-Jun in the regeneration deficit observed following chronic denervation.

6. Restoring the regenerative capacity of aged nerves with increased c-Jun up-regulation

This thesis has thus far demonstrated that the maintenance of Schwann cell c-Jun prevents the regeneration deficit observed in chronically denervated distal nerve stumps. It was therefore decided to examine if regeneration deficits observed in other systems could be restored through the over-expression of c-Jun.

It is well established that the regenerative capacity of peripheral nerves declines with age (Pestronk et al., 1980; Tanaka & Webster, 1991; Tanaka et al., 1992; Vaughan, 1992; Verdú et al., 1995; Graciarena et al., 2014).

The slow rate of regeneration has been believed to be in part due the diminished regenerative capacity of the neurons. Aged regenerating axons for example have a reduced ability to transport cytoskeletal proteins and to respond to tropic support (Brunetti et al., 1987; Uchida & Tomonaga, 1987; Tashiro & Komiya, 1994). However, as described in the introduction, recent work suggests that it is not the axon's response to injury that is impaired with age. Painter et al (2014) performed a detailed study into the regeneration differences between young and aged mice and the potential causes of these disparities. Transcriptional profiling of DRGs from young and old mice found no difference in the expression in regeneration associated genes and cultures of dissociated DRGs displayed no differences in neurite outgrowth. These findings were further backed up through transplantation studies in which young mice received a nerve graft from old mouse and vice versa. Analysis of axon regeneration through these grafts found that young mice with old grafts regenerated at the same rate as old nerves and that the regeneration deficit observed in old mice could be restored with a young graft. These findings suggest that the regeneration deficit observed in age is not due to the axons diminished regenerative capacity. Painter et al (2014) go on to confirm that it is the Schwann cells response to injury that is impaired with age. Aged nerves have disrupted activation of markers of the repair cell phenotype and reduced c-Jun activation in the days immediately following injury (Painter et al., 2014). Together, the findings of this study demonstrate that the regeneration deficits observed with age are largely due to the Schwann cells inability to activate the repair cell phenotype.

Based in these findings, it was hypothesized that the regeneration deficit observed with age could be ameliorated through the increased activation of c-Jun.

The aims of the chapter are:

- To demonstrate that the reduced c-Jun activation observed previously is Schwann cell specific.
- To examine c-Jun expression in injured old OE/+ Schwann cells.
- To determine if improved c-Jun activation restores the regenerative capacity of aged nerves.

In this chapter, 6-10 week old mice are referred to as young mice whereas old mice are 1 year old.

6.1 Reduced c-Jun expression is Schwann cell specific and is not observed in aged OE/+ nerves

To determine the c-Jun levels in young and old Schwann cells following injury, young and old WT and old OE/+ mice underwent a 3.5 day sciatic nerve cut. Transverse sections of the nerves 2 mm from the injury site were dual immunolabelled with antibodies for Schwann cell marker Sox 10 and c-Jun (Figure 6.1). Labelling showed a decrease in Sox 10 positive c-Jun positive cells between young and old WT nerves despite the many Sox 10 positive cells present in the nerves of old WT mice (Figure 6.1 A). The majority of young Sox 10 positive cells expressed c-Jun. However, no such difference was observed between the young WT nerves and old OE/+ nerves. Bright c-Jun labelling was observed in aged OE/+ nerves which was almost indistinguishable from that seen in young WT samples. Counts of Sox 10 positive c-Jun positive cells were performed and the % of c-Jun Sox 10 positive cells was calculated (Figure 6.1 B). Calculations showed that, after injury, 86% of Schwann cells in young WT nerves expressed c-Jun. However, only 35.23% of Schwann cells in old WT nerves expressed detectable levels of c-Jun, demonstrating impaired activation in response to injury. This confirms the previous findings that old nerves activate lower levels of c-Jun following injury (Painter et al., 2014). These observations were not conserved in old OE/+ nerves. 82.36% of Schwann cells in these nerves up-regulated c-Jun following injury. This was seen to be a significant increase relative to old WT Schwann cells. No difference however was observed between young WT and old OE/+ Schwann cells, demonstrating that c-Jun activation in these Schwann cells is equal. OE/+ Schwann cells are therefore concluded to be immune from the effects of aging on Schwann cell c-Jun activation.

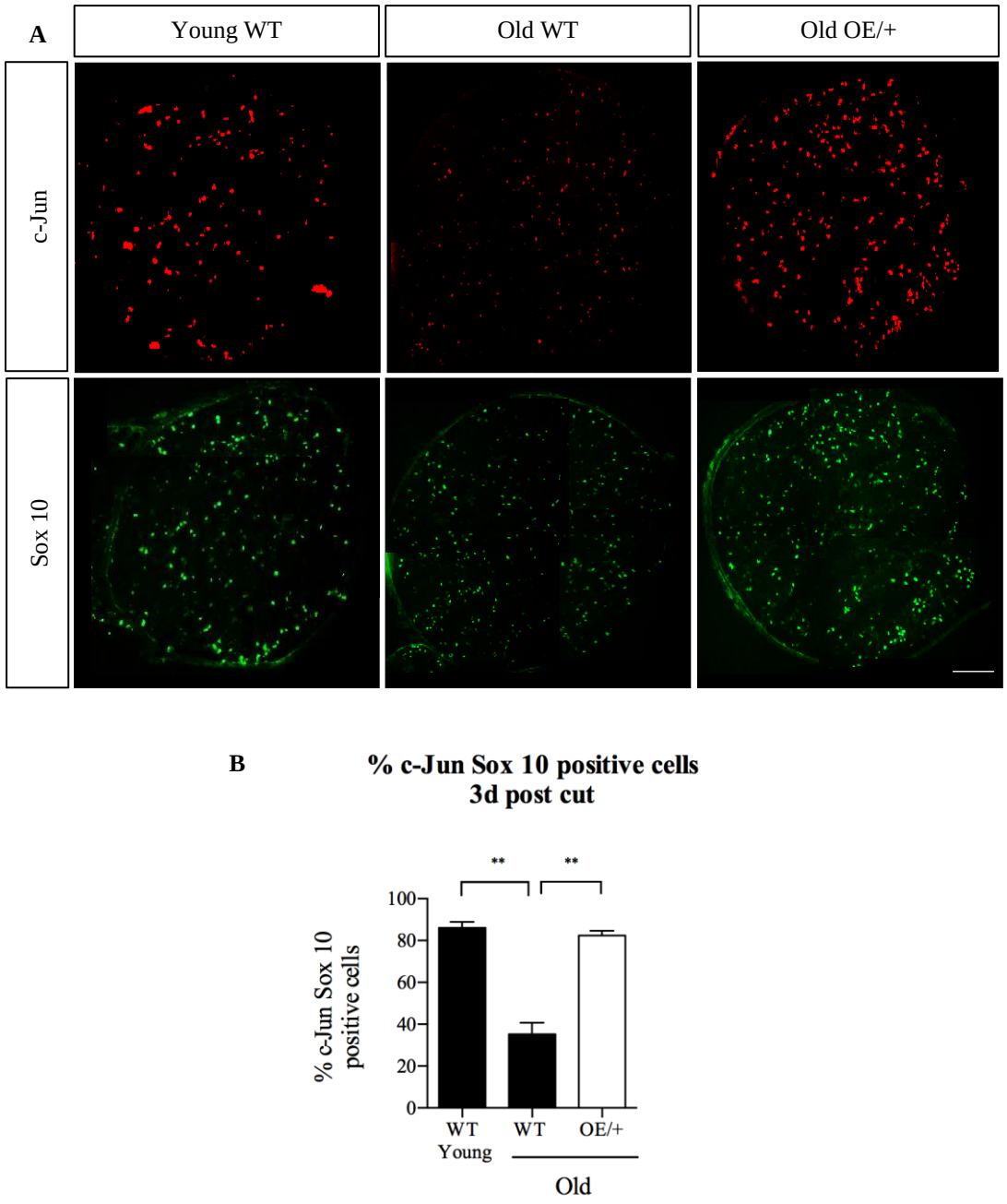


Figure 6.1 | c-Jun activation is impaired in old WT but not OE/+ Schwann cells following injury

(A) c-Jun Sox 10 dual immunolabelling of transverse sections of distal tibial nerve 3.5 days after cut injury. The majority of Schwann cells were c-Jun positive in young mice. In contrast, very low c-Jun expression was observed in aged WT nerves. OE/+ Schwann cells in aged nerves were predominately brightly labelled with c-Jun. **(B)** Counts of the total number of Sox 10 positive cells and the number of those cells that were c-Jun positive were used to calculate the percentage of c-Jun positive Schwann cells (c-Jun Sox 10 positive). A significant reduction in c-Jun activation in response to injury was observed in old WT Schwann cells compared to young. No difference however was observed in c-Jun activation between young and old OE/+ Schwann cells with old OE/+ Schwann cells demonstrating a significant increased response to injury compared to old WT Schwann cells. Graph of means ± SEMs, n=3, two-way ANOVA with Tukey's test. Scale bar: 100µm.

6.2 Young WT and OE/+ nerves regenerate equally well following crush injury

Work in Chapter 5 (Figure 5.3) demonstrated that during acute injury, WT and OE/+ nerves were equal in their regenerative capacity. This however was demonstrated using nerve cross suture as a model to study regeneration. As the nerves examined in this chapter are not chronically denervated, a single crush injury can be used to examine axon regeneration. It is therefore important to determine if any differences in regeneration are observed between these two genotypes using this model. To examine this, young WT and OE/+ mice underwent a sciatic nerve crush at the location of the sciatic notch. 5 days thereafter, the whole nerve was cut 10mm away from the crush site and exposed to a reservoir of fluorogold for 1 hour. A week later the mice were sacrificed and the tissue harvested. Counts of labelled motor neurons in the spinal cord found no difference in the number of regenerating motor neurons between the two genotypes (Figure 6.2). It was therefore concluded that at least initially, young WT and OE/+ nerves regenerate equally well following nerve crush.

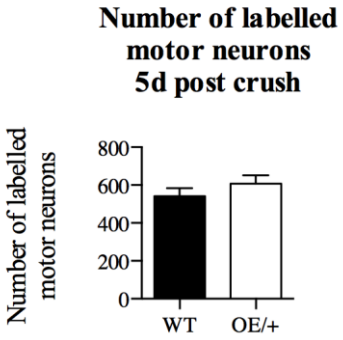


Figure 6.2 | Young WT and OE/+ nerves display equal numbers of regenerating motor neurons following crush injury

Counts of labelled motor neurons in the spinal cord showed no significant difference between the 2 genotypes 5 days after crush (p=0.3128). Graph of means ± SEMs, WT n=5, OE/+ n=4, unpaired Student's T-test.

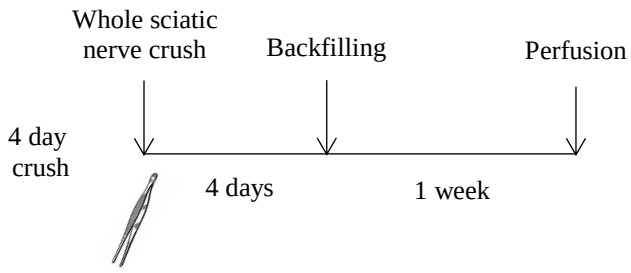
6.3 Enhanced c-Jun expression restores the age-related regeneration deficit

To examine the effect of increasing c-Jun expression in old Schwann cells on peripheral nerve regeneration, young and old WT and old OE/+ mice underwent a whole sciatic nerve crush. Nerves were backfilled after 4 days to examine the number of regenerating axons 7mm from the crush site (Figure 6.3.1 A).

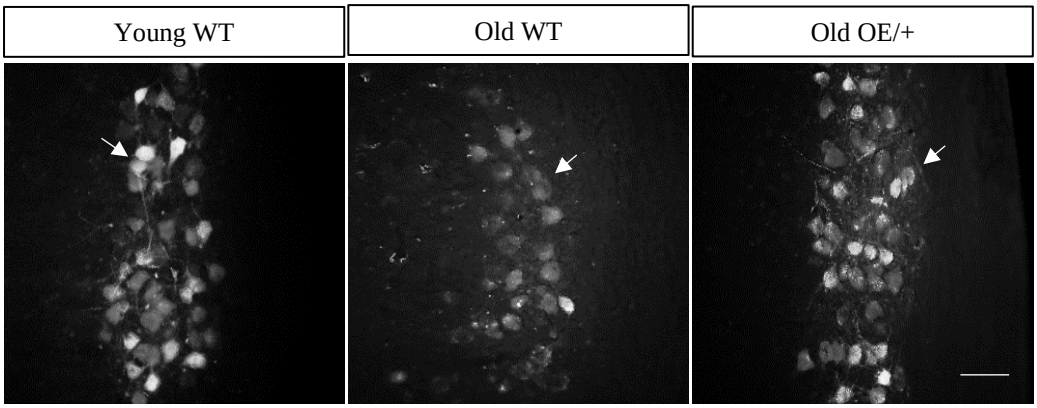
To examine motor neuron regeneration 4 days after crush, labelled cell bodies in the spinal cord were examined (Figure 6.3.1 B). Young WT mice were found to have high numbers of labelled cells suggesting a high rate of axon regeneration. Old WT mice however had low numbers of labelled cell bodies in line with the poor regeneration reported previously with age (Painter et al., 2014). In contrast, OE/+ mice were found to have higher numbers of labelled cells suggesting regeneration in these mice is enhanced compared to that seen in old WT mice. Counts of these cell bodies confirmed these observations (Figure 6.3.1 C). A significant decrease in labelled motor neuron cell numbers was observed between young and old WT mice. Old mice had 50.37% fewer motor neurons regenerating after crush injury. Old OE/+ mice motor neuron regeneration only decreased by 15.7% which was found not to be significantly different to young mice. These mice had a significantly higher number of motor neurons regenerating post crush injury compared to old WT mice.

To further examine the decline in regenerative capacity of WT nerves with age, sensory neuron cell bodies labelled in the L4 DRG were counted 4 days after crush (Figure 6.3.2 B). Counts showed that there was a significant difference in sensory neuron regeneration between young and old nerves ($p=0.0309$). Age decreased the number of regenerating sensory neurons by 43%. When this data was compared to the number of sensory neurons regenerating in old OE/+ nerves, no decline in regeneration was observed with old OE/+ and young nerves supporting the growth of equal numbers of sensory neurons (Figure 6.3.2 C). Old OE/+ nerves showed double the number of sensory neurons regenerating compared to old WT nerves ($p=<0.05$).

A


Whole sciatic nerve crush
Backfilling
Perfusion
4 day crush
4 days
1 week

B


Young WT
Old WT
Old OE/+

C

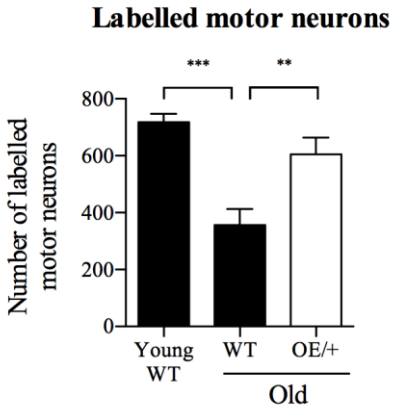

Labelled motor neurons
***
**
800
600
400
200
0
Number of labelled motor neurons
Young WT
WT
OE/+
Old

Figure 6.3.1 | The age-associated regeneration deficit is not observed in motor neuron regeneration in OE/+ nerves

(A) Summary diagram of surgery timeline. Young and old mice underwent a 4 day sciatic nerve crush. The nerve was then cut 7mm from the injury site and backfilled with fluorogold. The mice were perfused and the tissue harvested after 1 week. **(B)** Motor neurons labelled in the spinal cord. Many bright cells were observed in young WT mice compared to old WT mice, which had few, faintly labelled cells. OE/+ spinal cords however contained many brightly labelled cell bodies (arrows). **(C)** Counts of motor neuron cell bodies labelled in the spinal cord showed a reduction in regenerative capacity between young and old WT nerves. With age, the number of regenerating neurons growing past 7mm from the crush site, was half of that in young nerves ($p<0.001$). There was no age-related decline in regeneration observed however in OE/+ nerves with no significant difference in the number of regenerating motor neurons between young WT and old OE/+ nerves. OE/+ nerves had significantly more motor neurons regenerating past 7mm compared to old WT nerves. Graph of means ± SEMs, n=6, one-way ANOVA with Tukey's test. Scale bar: 100μm

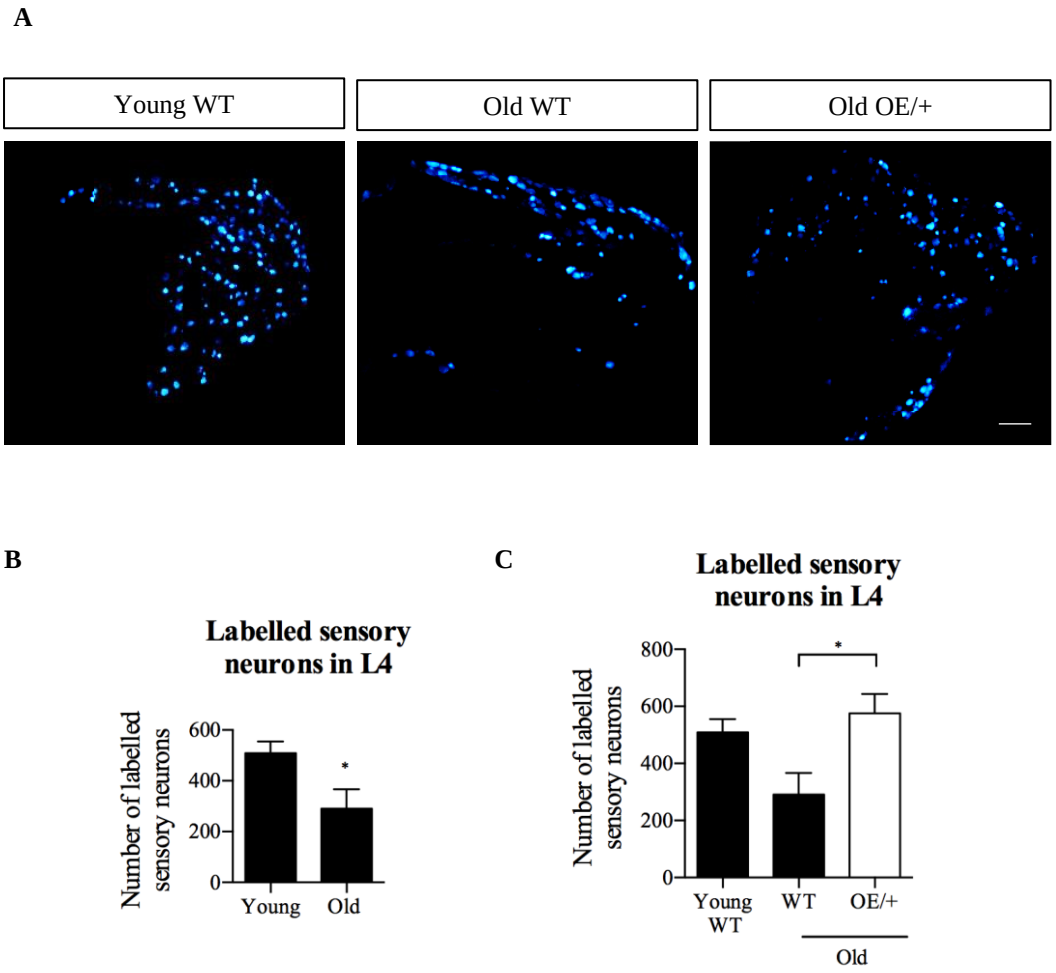


Figure 6.3.2 | The age-associated regeneration deficit is not observed in sensory neuron regeneration in OE/+ nerves

(A) Sensory neuron cell bodies labelled in the L4 DRG of young WT and old WT and OE/+ mice. **(B)** Counts of labelled sensory neuron cell bodies highlighted a significant decrease in the regenerative capacity of nerves with age ($p=0.0309$), unpaired Student's T-test. **(C)** When the data from (B) was compared with sensory neuron regeneration in OE/+ nerves, OE/+ nerves were found to support regeneration in significantly more sensory neurons than old WT nerves. No difference in the number of regenerating sensory neurons was observed between young WT and old OE/+ suggesting these nerves maintain their regenerative capacity throughout life. One-way ANOVA with Tukey's test. Graphs of means ± SEMs, Young WT n=6, Old WT n=5, Old OE/+ n=6. Scale bar: 100µm.

6.4 Discussion

The reduced regenerative capacity of the peripheral nervous system with advancing age has been well documented (Pestronk et al., 1980; Tanaka & Webster, 1991; Tanaka et al., 1992; Vaughan, 1992; Verdú et al., 1995; Graciarena et al., 2014). However, it is only recent work that is establishing the role of Schwann cells in this deficit. A recent study has highlighted the role of Schwann cell c-Jun and the repair cell phenotype. It was demonstrated that in the immediate days after injury, activation of c-Jun and other markers of the repair cell phenotype was reduced (Painter et al., 2014). It was therefore hypothesized here that restoring the injury induced activation of c-Jun in aged nerves would reverse the effects of aging on regeneration. This could be examined using nerves from old OE/+ mice, where it is known that at 1 year old, normal myelin sheaths are maintained, with no tumour development and stereotypical nerve architecture (Fazal et al., 2017).

To confirm the previous findings that c-Jun activation is repressed in old mice following injury and that this is a Schwann cell specific response, c-Jun Sox 10 dual labelling was performed on transverse sections of young and old WT nerves 3.5 days after nerve transection. The percentage of c-Jun expressing Schwann cells was calculated, showing a significant decline in c-Jun activation in aged nerves, confirming previous findings. This observation was in contrast to that shown in OE/+ nerves, the Schwann cells in which expressed c-Jun to the same extent as young nerves. These mice could therefore be used to examine the effect of restored c-Jun expression in aged nerves on nerve regeneration. Further work could confirm these findings with Western blot analysis and examine c-Jun phosphorylation to determine changes in the activation as well as expression of c-Jun with age and in these mutants.

Regeneration was examined by retrograde labelling with fluorogold which has been utilised to study regeneration throughout this thesis. Axon regeneration 4 days after crush was compared between old WT and OE/+ and young WT nerves. The age associated decline in regeneration was observed between young and old WT nerves with a 51% and 43% reduction in motor and sensory neuron regeneration respectively. However, no decrease in regenerative capacity was observed in OE/+ nerves with age with motor and sensory neuron regeneration equalling that seen in

young nerves. Although previous studies have reported no major differences in regeneration between young and old mice in the first few days following injury (Black & Lasek, 1979; Pestronk et al., 1980), a significant difference was reported here. This is likely to be due to a difference in techniques used to estimate regeneration. Neuron backfilling is a reliable technique for studying regeneration as it labels cell bodies. Examining axons alone could report different results due to variations in nerve sprouting. This work demonstrates using backfilling with fluorogold, that the age associated decline in regenerative capacity of peripheral nerves can be reversed when injury induced c-Jun expression is restored.

It is important to note that although the mice here are referred to as old for simplicity, they are not really so. Many other studies into the age associated decline use 24 month old mice. 12 month old mice are classified as 'middle aged' however there is little difference in the functional recovery between these two ages, despite 12 and 24 months being the equivalent of 35-40 and 60 human years respectively (Kang & Lichtman, 2013; Painter et al., 2014; Dutta & Sengupta, 2016). Therefore, although the mice used in this study are 'middle aged', they are unlikely to differ from older mice, producing similar results.

It is important to consider the other effectors of c-Jun, not just the repair cell phenotype, that could improve axon regeneration. Myelin debris is a known inhibitor of PNS regeneration (Mukhopadhyay et al., 1994; Schäfer et al., 1996). As previously discussed, although myelin clearance is heavily mediated by Schwann cells in the days immediately following injury, macrophages are recruited to the nerve after 2-3 days. It has been shown that old nerves have reduced myelin clearance following injury. It has been demonstrated that this may be due not only to a failure to recruit macrophages to the nerve but also to the aged Schwann cells inability to break-down myelin (Kang & Lichtman, 2013; Painter et al., 2014). Results in Chapter 4 (Figure 4.9) demonstrated, 3 days after crush, OE/+ nerves recruit more macrophages to the injury site. It is therefore likely that old OE/+ nerves may be able to recruit more macrophages following injury compared to WT counterparts. This could increase the rate of myelin breakdown and aid regeneration. The rate of myelin breakdown could also be restored due to the enhanced activation of myelinophagy. Schwann cells breakdown myelin debris by autophagy, referred to as myelinophagy, which is controlled by c-Jun through the JNK signalling pathway (Gomez-

Sanchez et al., 2015). Although it has been found that young OE/+ nerves do not demonstrate increased autophagic activity (J.A Gomez-Sanchez, unpublished observations), activity may be higher in old OE/+ nerves compared to old WT nerves. Future work into myelin clearance of old WT and OE/+ nerves would confirm if enhanced debris clearance contributes to the enhanced regeneration in OE/+ nerves.

It has further been suggested that old Schwann cells may have a reduced rate of proliferation due to younger nerves highly up-regulating genes associated with the cell cycle, DNA replication, lipid biosynthesis and the extracellular matrix (Painter et al., 2014). Although an age-related decline in proliferation have been documented, this has not been studied in detail in aged mice (Komiyama & Suzuki, 1991, 1992). If old Schwann cell do in fact have a reduced proliferative capacity, old OE/+ Schwann cells are likely to overcome this and proliferate at rate similar to that of young Schwann cells. However this is unlikely to contribute to improved initial axonal growth examined in this chapter because Schwann cell proliferation is not required for axon regeneration (Kim et al., 2000; Yang et al., 2008). The transverse sections taken 3.5 days after cut however could be used to confirm the effect of age on Schwann cell proliferation. Ki-67 is a marker of proliferation, labelling cells during all stages of the cell cycle except G_0. (Gerdes et al., 1984, 1983). Our laboratory has successfully used Ki-67 as a marker for Schwann cell proliferation in transverse nerve sections (Benito et al., 2017). This method could be applied here to examine proliferation in young and aged nerves of both genotypes.

No experiments were performed to examine the expression of repair cell markers in aged mice. As older mice show reduced activation of repair cell markers such as GDNF (Painter et al., 2014), old OE/+ mice may overcome this and activate the repair cell phenotype as efficiently as young animals do. This work is required to demonstrate that not only c-Jun but improved repair cell activation results in restoration of neuron regeneration in old mice. Samples for this work are in preparation and will undergo RNA sequencing to determine the differences in gene expression in old WT and OE/+ nerves 3.5 days post injury.

Overall, the work in this chapter confirms that Schwann cells from old mice fail to up-regulate c-Jun as efficiently as Schwann cells from young mice. This observation is different from that seen in OE/+ Schwann cells from old mice, which up-regulate

c-Jun to a similar level as young Schwann cells. The enhanced expression of c-Jun restores the age-related deficit in regeneration with old OE/+ and young nerves displaying equal regeneration of motor and sensory neurons.

7. The role of hedgehog signalling in c-Jun activation

Thus far, the results of this thesis have demonstrated the importance of c-Jun activation and maintenance in restoring regeneration deficits of the PNS observed during chronic denervation and advanced aging. These results have highlighted c-Jun as a pharmaceutical target for peripheral nerve repair.

The role of hedgehog signalling in the PNS has been previously established. Desert hedgehog (Dhh) signalling is involved in the development and maintenance of peripheral nerves. When *Dhh* is conditionally knocked out from Schwann cells abnormalities are observed in the nerve structure. Changes in epi-, peri-, and endoneurial structures are observed (Parmantier et al., 1999; Sepideh N Bajestan et al., 2006). Humans carrying mutations in the *Dhh* gene present with minifascicular neuropathy, in which many small fascicles are present in the peripheral nerves (Umehara et al., 2002; Sato et al., 2017). Dhh and its receptor patched 2 are rapidly down-regulated following peripheral nerve injury (S N Bajestan et al., 2006) and sonic hedgehog (Shh), is up-regulated by Schwann cells in the distal nerve stump, however its function is unknown (Arthur-Farraj et al., 2012).

It has been suggested that activation of Shh promotes nerve regeneration. Mice given injections of Shh that has undergone polyethylene glycol modification, have increased levels of Shh in their bloodstream (normal Shh has a short half-life). These mice showed improved functional recovery following sciatic nerve crush (Pepinsky et al., 2002). Studies have also suggested that application of Shh can be neuroprotective following crush injury to the cavernous nerve (Bond et al., 2013; Angeloni et al., 2013). In contrast, when the sciatic nerve is continuously exposed to cyclopamine following crush injury, motor neuron survival is impaired (Hashimoto et al., 2008).

Here, it was hypothesized that Shh signalling could play a role in the activation of c-Jun and that this is the reason for the enhanced regeneration previously observed in the literature. Laner-Plamberger et al (2009) have previously demonstrated the link between Hh signalling and Jun. When HaCat cells (an immortal human keratinocyte cell line) were modified to express activated forms of Gli1 and Gli2, Jun mRNA and protein expression increased (Laner-Plamberger et al., 2009). Kudo et al (2012) contributed to our understanding of this connection by transfecting human ovarian

cancer cells with anti-Gli1 shRNA. This was found to reduce c-Jun protein and inhibited phosphorylation of c-Jun at serine 63. A similar result was observed when the cells were incubated with cyclopamine (Kudo et al., 2012). Laner-Plamberger et al (2009) explained these results by investigating the presence of Gli binding sites on the Jun promoter. They found 6 potential Gli binding sites, five clustered upstream of the transcription start site and one downstream. The area of the promoter containing these sites and the AP-1 binding sites were cloned into a luciferase reporter vector (Figure 7.0 A). Each potential binding site was then inactivated by mutation. Only the inactivation of the binding site at -712 base pairs upstream of the transcription start site affected luciferase reporter activity, confirming this site to be the Gli binding site (Figure 7.0 B). They further found that Jun promoter activity was strongly enhanced by the dual binding of Gli and Jun, suggesting Gli binding is required for enhanced Jun activation (Laner-Plamberger et al., 2009).

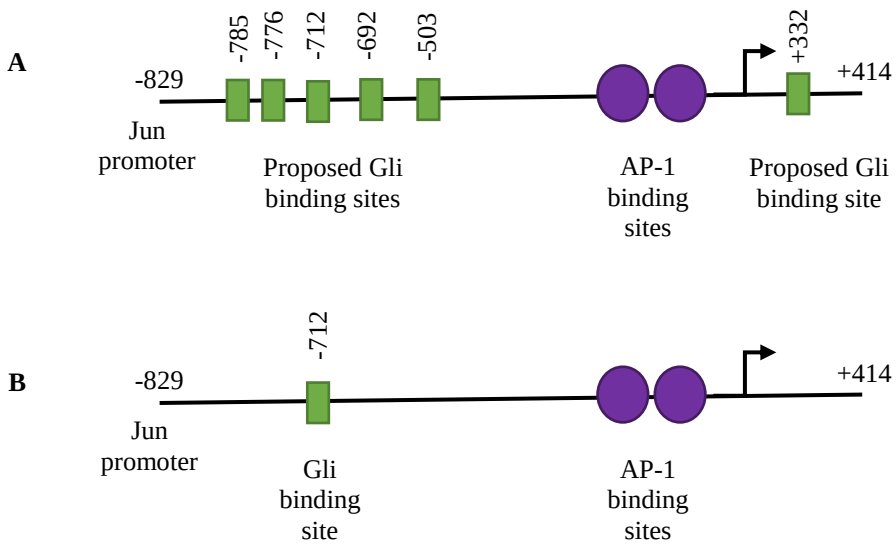


Figure 7.0 | The Jun promoter contains Gli binding sites

(A) The Jun promoter was proposed to have 6 potential Gli binding sites. 5 were located upstream of the transcriptional start site and a single site was positioned downstream. This fragment was cloned into a luciferase reporter vector. **(B)** Mutating each binding site demonstrated that only inactivation of the site at -712 affected reporter activity confirming this to be the Gli binding site on the Jun promoter. Figure adapted from Laner-Plamberger et al., 2009.

In Schwann cells however, no direct link has been made between Hh signally and c-Jun activation. Shh has however been shown to regulate *BDNF* in cultured Schwann cells. When Hh signalling is inhibited in cultured Schwann cells with cyclopamine, *BDNF* expression is decreased. Incubation with the recombinant N-terminal peptide of Shh results in increased *BDNF* expression. *GDNF* expression however is unaffected by Hh signalling (Hashimoto et al., 2008). As *BDNF* expression have previously been shown to be regulated by c-Jun (Arthur-Farraj et al., 2012; Huang et al., 2015), this activation may be a c-Jun mediated response.

This chapter therefore aimed to examine the relationship between Hh signalling and c-Jun activation. If a link between these pathways is established, the effect of Hh signalling on the markers of the repair cell phenotype will also be investigated.

These investigations will utilise:

- A mouse line in which Shh has been specifically knocked out in Schwann cells (Shh cKO), the tissue from which was gifted from the laboratory of John Svaren (USA).
- An *in vitro* model of chronically denervated Schwann cells

7.1 c-Jun activation and p75 are reduced in Shh cKO nerves 7 days post injury

To examine the role of Shh in Schwann cells after injury, 3 and 7 day cut distal nerves stumps from Shh cKO mice were sent from the laboratory of John Svaren. These mice have Shh specifically knocked out in Schwann cells, much like the c-Jun cKO mouse. Western blots of these nerves were run to examine c-Jun expression levels after injury (Figure 7.1.1). Results indicated that 3 days after injury, there was no difference in the level of c-Jun expression between WT and Shh cKO nerves (Figure 7.1.1 A). However, 7 days after injury Shh cKO nerves expressed lower levels of c-Jun compared to WT nerves, with levels similar to those observed at 3 days post injury. Quantification of the data demonstrated that there was no significant difference in c-Jun expression between WT and Shh cKO nerves 3 days after injury, suggesting that initial c-Jun activation is not impaired (Figure 7.1.1 B). However, Shh cKO nerves express significantly lower levels of c-Jun at 7 days. WT c-Jun expression increased 4-fold between 3 and 7 days after injury. Shh cKO c-Jun expression however only doubled during this time. These results suggests that Shh plays a role in the partial activation of c-Jun during the early stages of injury.

To examine the effect on Shh on c-Jun activation further, phosphorylation of c-Jun at serine 63 was examined (p-c-Jun). As no difference in c-Jun expression was observed 3 days after injury, c-Jun phosphorylation was only observed 7 days after injury (Figure 7.1.2). Consistent with previous blots (Figure 7.1.1), total c-Jun expression was lower in Shh cKO nerves. Levels of phosphorylated c-Jun were even lower (Figure 7.1.2A). To quantify this observation, phosphorylated c-Jun was divided by total c-Jun protein demonstrating a 25% difference in c-Jun phosphorylation between WT and Shh cKO nerves (Figure 7.1.2 B). This data further supports the idea that Shh plays a role in the partial activation of c-Jun.

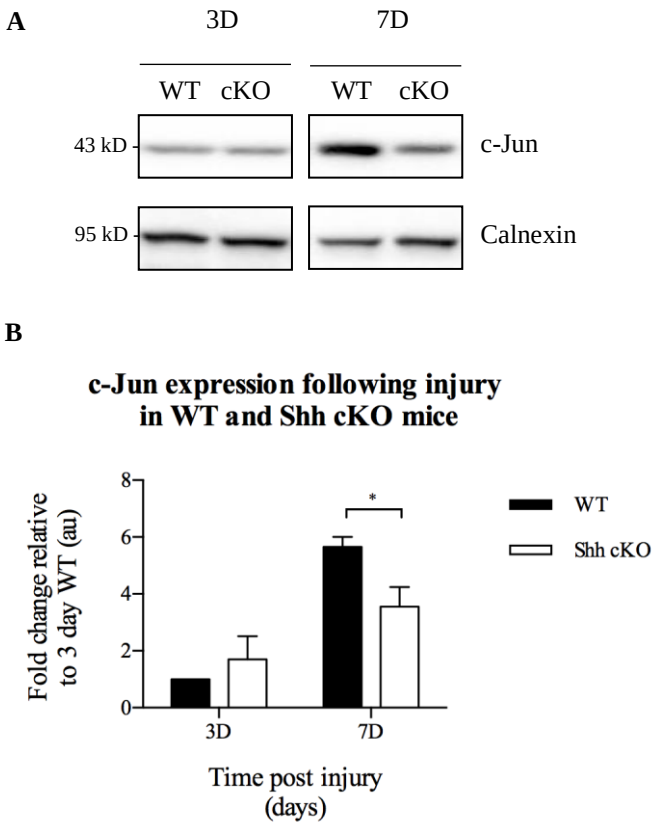


Figure 7.1.1 | c-Jun activation is impaired in Shh cKO nerves 7 days after injury

(A) Western blots of WT and Shh cKO distal nerve extracts 3 and 7 days after injury. No difference in initial c-Jun activation was observed, however 7 days after injury, Shh cKO nerves expressed lower levels of c-Jun. **(B)** Blots were analysed by dividing c-Jun by the house keeping protein calnexin. All data was normalised to 3 day WT. Although no difference in initial c-Jun activation was observed between the 2 genotypes, after 7 days Shh cKO nerves failed to activate c-Jun to the levels observed in WT ($p=<0.05$). Graph of means ± SEMs, n=5, two-way ANOVA with Sidak's test.

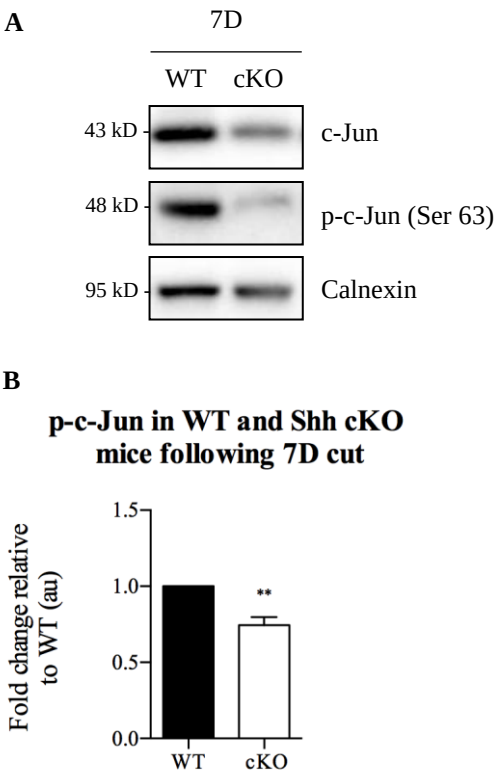


Figure 7.1.2 | c-Jun phosphorylation at serine 63 is reduced in Shh cKO nerves

(A) Shh cKO distal nerve extracts were Western blotted for c-Jun and c-Jun phosphorylated (p-c-Jun) at serine 63 (Ser 63). c-Jun activation was decreased in these nerves in line with previous observations. Phosphorylation of c-Jun was also visibly reduced. **(B)** Blots were quantified by dividing p-c-Jun by total c-Jun protein. Phosphorylation of c-Jun was significantly impaired in Shh cKO nerves 7 days after injury ($p=0.0014$). Graph of means ± SEMs, n=5, unpaired Student's T-test.

Since c-Jun activation is reduced in the nerves of cKO mice, activation of other markers of the repair cell phenotype might also be impaired. Reduced Schwann cell c-Jun has been previously demonstrated to decrease p75 NTR protein expression (Arthur-Farraj et al., 2012). p75 NTR activation in Shh cKO nerves was therefore examined. As has been previously shown here (Figure 3.4) as well as in the literature, p75 NTR expression is low in the early days of injury but high levels are observed 1 week after injury (Taniuchi et al., 1986; Heumann et al., 1987). WT nerves, as expected, followed this expression pattern (Figure 7.1.3 A). Shh cKO nerves, although expressing similar levels of p75 NTR at 3 days compared to WT, displayed lower elevation 7 days after injury. Similarly to c-Jun activation, WT nerves increased p75 NTR expression 6 fold between 3 and 7 days (Figure 7.1.3 B). Shh cKO distal nerves however, only doubled p75 NTR expression between 3 and 7 days, a trend similar to that observed in the activation of c-Jun in these nerves. These results demonstrated that Shh signalling in Schwann cells is not only a marker of the repair cell phenotype, but also plays a role in its activation.

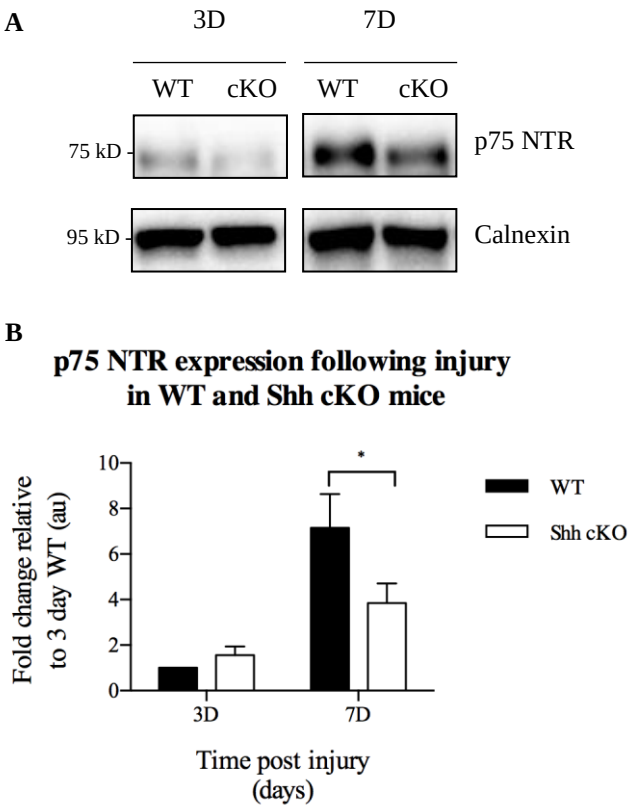


Figure 7.1.3 | p75 NTR protein expression is decreased in Shh cKO nerves 7 days after injury

(A) Western blots of Shh cKO and WT distal nerves 3 and 7 days after injury showed no difference in p75 NTR expression 3 days after injury. Expression was initially very low 3 days after injury but then became elevated after 7 days in WT nerves. Elevation was reduced in Shh cKO nerves at 7 days. **(B)** Blots were quantified by dividing levels of p75 NTR expression by levels of the house keeping protein calnexin. All data was normalised to 3 day WT. Quantification of blots showed no significant difference in the activation of p75 NTR between genotypes. The elevation of p75 NTR was significantly impaired in Shh cKO nerves at 7 days after injury. Graph of means ± SEMs, n=5, two-way ANOVA with Sidak's test.

7.2 *In vitro* modelling of chronically denervated Schwann cells

The results from 7.1 demonstrate the role of Shh signalling in c-Jun and possibly in repair cell phenotype activation. This works highlights the Shh pathway as a potential pharmacological target for c-Jun activation in chronically denervated Schwann cells. However, before this can be examined, it is important to first establish an *in vitro* model of chronically denervated Schwann cells. Although Schwann cell cultures from chronically denervated nerves have been performed in the past, attempts at culturing adult Schwann cells from chronically denervated mouse nerves did not produce high cell yields or pure cultures in my hands. The idea of cell sorting was proposed, however the physical pressures to the cells during this process might lead to the reactivation of c-Jun, invalidating the model. Furthermore, cell surface markers that could be used to identify Schwann cells for cell sorting, such as p75 NTR, are down regulated during chronic denervation (Figure 3.4) (You et al., 1997). When Schwann cells are in culture, they are without axon contact from prolonged periods of time, a situation similar to that seen in chronic denervation. It was therefore hypothesized that long-term cultured Schwann cells might down-regulate c-Jun and not maintain the repair cell phenotype.

To examine this, rat Schwann cells were cultured. At every passage, 1 million cells were removed and cultured separately in DM for 48 hours. Protein was then extracted and stored until all the samples were collected (Figure 7.2.1). Cells underwent a total of 9 passages and were in culture for 6 weeks. A Western blot was run once samples from all 9 passages had been collected (Figure 7.2.1 A). This pilot study showed that c-Jun was activated in Schwann cells by the first passage. Levels increased and remained high during the first 4 passages, equivalent to between 2-3 weeks in culture. c-Jun began to decline however with increasing time in culture. c-Jun levels were found to be lowest at passage 9 where c-Jun decreased 62% relative to passage 4 where c-Jun expression peaked (Figure 7.2.1 B). c-Jun protein levels at passage 9 however were almost half compared to the elevation observed at passage 2, a similar trend that is observed in the nerve between 1 and 10 weeks denervation. It was therefore decided to examine the differences between Schwann cells during the early stages of culture at passage 2 and during long-term culture at passage 9.

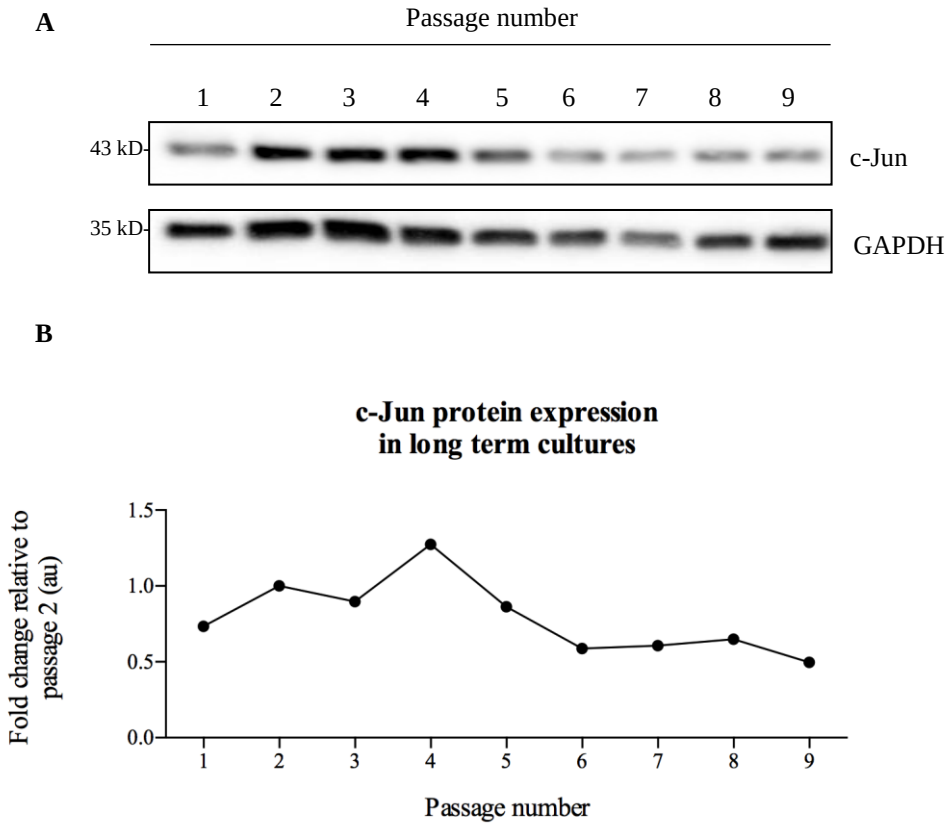


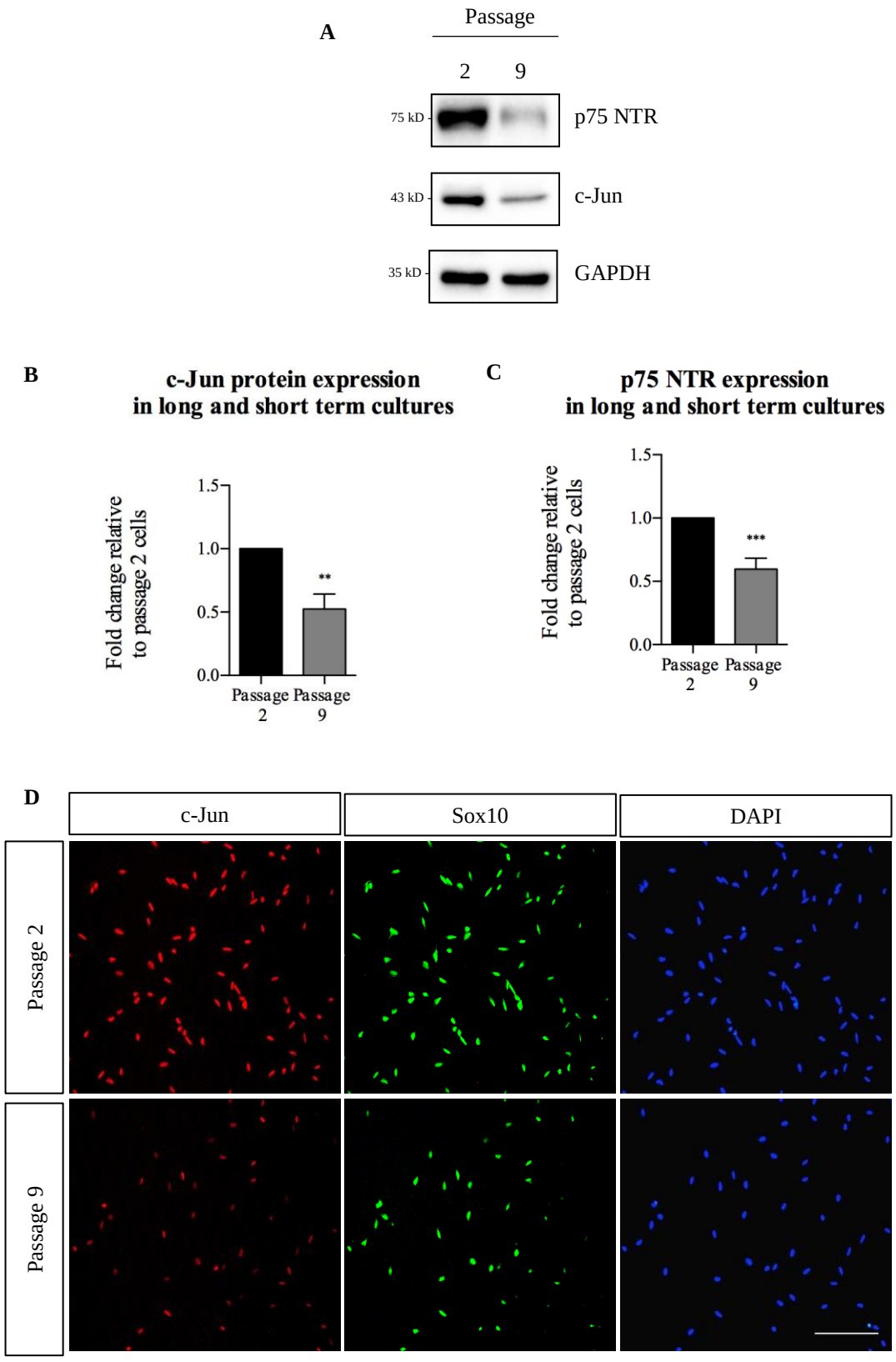
Figure 7.2.1 | c-Jun protein expression decreases during long-term culture of rat Schwann cells

(A) Rat Schwann cells were cultured for 6 weeks. During this time, after each passage, 1,000,000 cells were removed and cultured separately in DM for 48 hours. c-Jun protein levels in these cultures were then examined by Western blot and quantified. **(B)** c-Jun levels increased and remained high during the early passages. c-Jun expression then began to decrease with increasing passage number and time. n=1.

To confirm the results of the pilot study, c-Jun expression was analysed between Schwann cells at passage 2 and 9 (Figure 7.2.2). Western blots confirmed the previous finding with c-Jun expression found to be lower in passage 9 cells compared to passage 2 (Figure 7.2.2 A). As previously observed, this difference in expression in passage 9 cells was found to be half of that at passage 2 (Figure 7.2.2 B).

It was observed that the purity of the cultures decreased with time. Increasing numbers of fibroblasts were present in the cultures. To therefore confirm that the Schwann cells are down-regulating c-Jun, producing these results, instead of the reduction in c-Jun being due to the increasing number of fibroblasts in the culture, a Sox 10 c-Jun dual immunolabelling was performed (Figure 7.2.2 D). Both cultures highly expressed Sox 10, however differences were observed in c-Jun levels. Passage 2 cells expressed high levels of c-Jun with cells brightly labelled. Passage 9 cells however were faintly labelled for c-Jun. The stark contrast in the labelling of c-Jun between these two cultures confirms that Schwann cells down-regulate c-Jun with increasing time in culture.

It was previously found *in vivo*, that decreasing levels of c-Jun lead to a decline in the repair cell phenotype, demonstrated by decreasing levels of phenotypic markers. To examine this phenomenon *in vitro*, markers of the repair cell phenotype were examined. p75 NTR protein expression was examined first (Figure 7.2.2 A). Expression was greatly reduced in long-term cultured cells in comparison to the high levels observed in passage 2 cells (Figure 7.2.2 C). This was a highly significant ($p=0.0007$) decrease, with passage 9 cells expressing half the level of p75 NTR protein as early cultured cells.


A
Passage
2
9
75 kD
p75 NTR
43 kD
c-Jun
35 kD
GAPDH
B
c-Jun protein expression
in long and short term cultures
Fold change relative
to passage 2 cells
1.5
1.0
0.5
0.0
**
Passage
2
Passage
9
C
p75 NTR expression
in long and short term cultures
Fold change relative
to passage 2 cells
1.5
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Passage
2
Passage
9
D
c-Jun
Sox10
DAPI
Passage 2
Passage 9

Figure 7.2.2 | c-Jun and p75 expression decreases in long-term cultured rat Schwann cells

(A) Western blots of c-Jun and p75 NTR after 2 or 9 passages. Both c-Jun and p75 NTR protein levels decreased with time and increasing number of passages. **(B)** Quantification of c-Jun Western blots. The decrease in c-Jun expression observed between short (passage 2) and long (passage 9) term culture was found to be significant ($p=0.0023$). **(C)** Quantification of p75 NTR Western blots showed that the decrease in p75 NTR levels over time was significant ($p=0.0007$). **(D)** Immunofluorescence of c-Jun levels in passage 2 and passage 9 cultures with Sox10 as a Schwann cell marker. Graph of means ± SEM, c-Jun n=6, p75 NTR n=7, Student's t-test. Scale bar: 100μm.

To examine the other markers of the repair cell phenotype such as c-Jun, GDNF, BDNF, Olig1 and Shh, RNA was extracted from passage 2 and 9 cultures. Subsequent qPCR results demonstrated a significant decrease in *c-Jun* mRNA levels, in line with protein levels (Figure 7.2.3 A). Trophic factor *BDNF* was also significantly reduced in passage 9 cultures (Figure 7.2.3 B). *GDNF* expression was reduced following long-term culture, although this was not found to be significant ($p= 0.0889$) (Figure 7.2.3 C). A reduction in *Olig1* was also observed but was similarly found to be insignificant ($p=0.2748$) (Figure 7.2.3 D). *Shh* however was elevated following long-term culture (Figure 7.2.3 E). As Shh has been previously shown to play a role in c-Jun activation (Figure 7.1.1), this may be a compensatory method to elevate or maintain remaining c-Jun levels. This elevation however is not significant ($p=0.4888$).

From these results it can be concluded that c-Jun decreases in long-term cultured Schwann cells, a phenomenon similar to the events in chronic denervation. Some of the markers of the repair cell phenotype also decrease with prolonged culture such as p75 NTR and *BDNF*. p75 NTR decreases in chronically denervated Schwann cells (Figure 3.4) (You et al., 1997) *BDNF* has also previously been to decline in chronically denervated nerves (Michalski et al., 2008). Long-term cultured Schwann cells may therefore be a useful *in vitro* model of chronic denervation and for examining pharmacological activation of c-Jun.

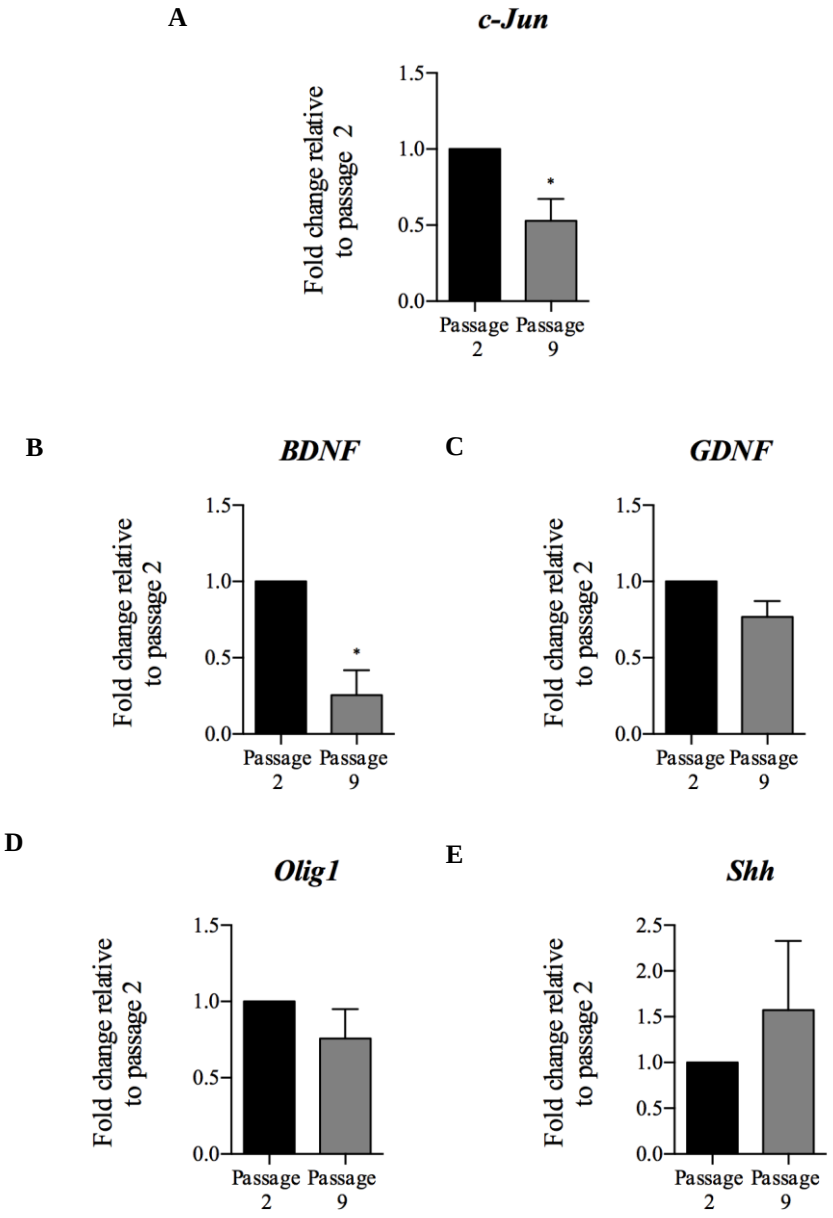

Figure 7.2.3 | mRNA expression of *c-Jun* and *BDNF* decreases in long-term rat Schwann cells cultures

qPCR was used to examine fold changes in RNA expression of repair cell markers in passage 9 cells relative to passage 2 cell cultures. Significant decreases were observed during long-term culture in **(A)** *c-Jun* (p=0.0299) and **(B)** *BDNF* levels (p=0.0105). **(C)** *GDNF* (p=0.0889), **(D)** *Olig1* (p=0.2748) and **(E)** *Shh* (0.4888) levels were not significantly changed. Graph of means ± SEM, n=3, Student's T-test.

7.3 Decreases in c-Jun levels during long-term culture are not associated with cAMP accumulation

cAMP has previously found to be elevated in Schwann cells after prolonged time *in vitro* (Stewart et al., 1991) . This can lead to activation of genes associated with myelin Schwann cells. cAMP is also known to inhibit c-Jun and promote the myelin phenotype (Morgan et al., 1991; Arthur-Farraj et al., 2011; Parkinson et al., 2008). It could therefore be possible that the reduction in c-Jun is due to cAMP accumulation and adoption of the myelin phenotype. This is important to establish to ensure this model is characteristic of chronically denervated Schwann cell as opposed to myelin Schwann cells.

cAMP levels in passage 2 and 9 cultures was measured by ELISA (Figure 7.3). When the concentration (pMol) of cAMP measured in each sample was compared relative to the protein concentration in each sample (mg) as an internal control, it was found that there was no difference in the level of cAMP expressed between the two cultures (p=0.8925). It was therefore concluded that the decrease in c-Jun was not due activation of the myelin phenotype but more likely due to fading of the repair cell phenotype.

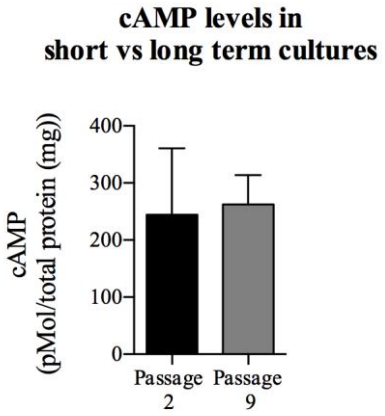

Figure 7.3 | Long-term cultured Schwann cells do not accumulate cAMP

cAMP levels in passage 2 and 9 Schwann cell cultures by ELISA. No difference in cAMP levels were observed following short or long-term culture (p=0.8925). Graph of means ± SEM, n=3, unpaired Student's T-test.

7.4 Agonists of the hedgehog pathway activate c-Jun in long-term cultured Schwann cells

Now that a model for studying chronically denervated Schwann cells *in vitro* has been established, methods of activating c-Jun can be determined. As described earlier in this chapter, Shh signalling may play a role in the partial activation of c-Jun. The Shh signalling pathway could therefore be utilised to activate c-Jun in Schwann cells. To examine this Shh agonists can be incubated with Schwann cell cultures that have undergone 9 passages.

Purmorphamine is an agonist of the hedgehog (Hh) pathway directly targeting smoothened, leading to activation of down-stream targets of the hedgehog pathway such as Gli (Wu et al., 2004; Sinha & Chen, 2006). To examine the effect of stimulating the hedgehog pathway on c-Jun in Schwann cells, rat Schwann cells underwent 9 passages to lower their c-Jun levels. Passage 9 cells were incubated with increasing concentrations of purmorphamine (1, 2.5, 5 or 10µM) (Figure 7.4.1 A). Two controls were established: untreated cells (UT) incubated in DM only and cells incubated with DMSO (Veh). The volume of DMSO added to these was the same as the volume present in cells incubated with 5µM purmorphamine. No difference in c-Jun expression was observed between the controls demonstrating that any changes in c-Jun expression observed are due to Hh signalling. c-Jun was found to increase in cultured cell with increasing concentration of purmorphamine. Although all concentrations of purmorphamine lead to c-Jun activation, only 2.5µM, which elicited a 2.5 fold increase in c-Jun, was a significant result (Figure 7.4.1 B).

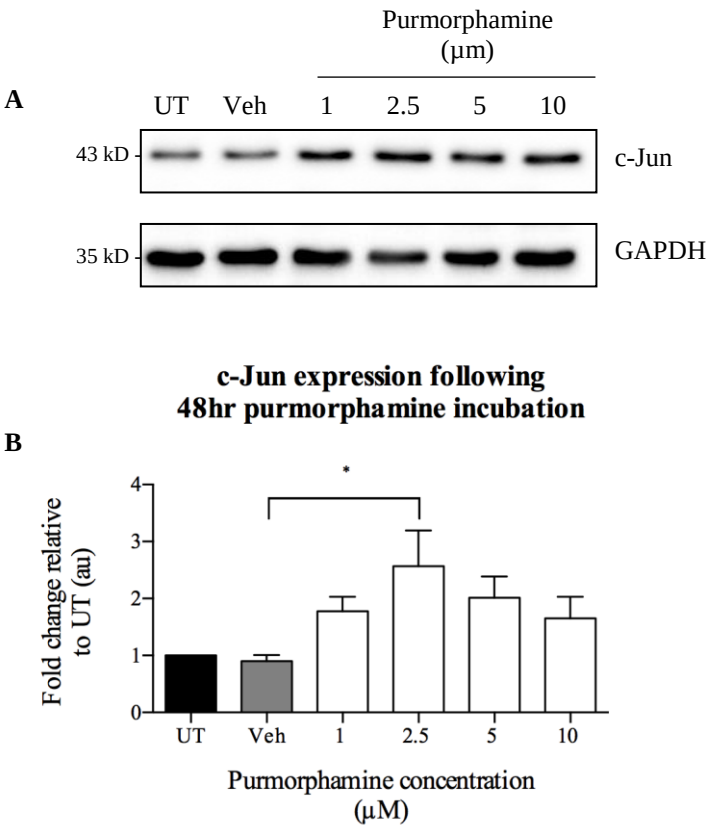

Figure 7.4.1 | Hh agonist purmorphamine activates c-Jun in long-term cultured rat Schwann cells

(A) Western blots of protein lysates showed that all concentrations of purmorphamine activated c-Jun. **(B)** Blots were quantified by dividing c-Jun levels by levels of house keeping protein GAPDH. All blots were normalised to the UT cells and the fold change observed. Although c-Jun was elevated by all concentrations of purmorphamine, 2.5µM was the only one to elicit a significant increase. A decline in cJun expression observed following incubation with 10µM suggests toxicity. Graph of means ± SEM, n=3, one-way ANOVA with Dunnett's test.

To confirm the activation of c-Jun through activation of the Hh pathway, passage 9 Schwann cell cultures were also incubated with smoothened agonist (SAG) for 48 hours. SAG activates the Hh pathway by directly binding to smoothened (Chen, Taipale, Young, et al., 2002).

As it was previously found that DMSO did not activate c-Jun in cultured Schwann cells at the low volumes previously used, only a vehicle control was used in these experiments. The volume of DMSO used was equal to that of cells incubated with 2.5µM SAG. Cells were incubated with 1 or 2.5µM SAG for 48 hours (Figure 7.4.2 A). Both concentrations of SAG were found to significantly elevate c-Jun levels compared to the control (Figure 7.4.2 B).

It was previously observed that long-term cultures decreased in purity. In order to confirm that the changes in c-Jun expression observed were Schwann cell specific, cultures were dual immunolabelled for c-Jun and Sox 10 after 48 hour 2.5µM SAG or DMSO incubation (Figure 7.4.2 C). c-Jun labelling was brighter in the SAG incubated cultures compared to the control. Sox 10 staining demonstrated that this was observed in Schwann cells.

The results from this set of experiments showed that incubation with Hh agonists leads to an increase in c-Jun protein. However to validate these results, the Hh pathway must be inhibited and the effect on c-Jun examined.

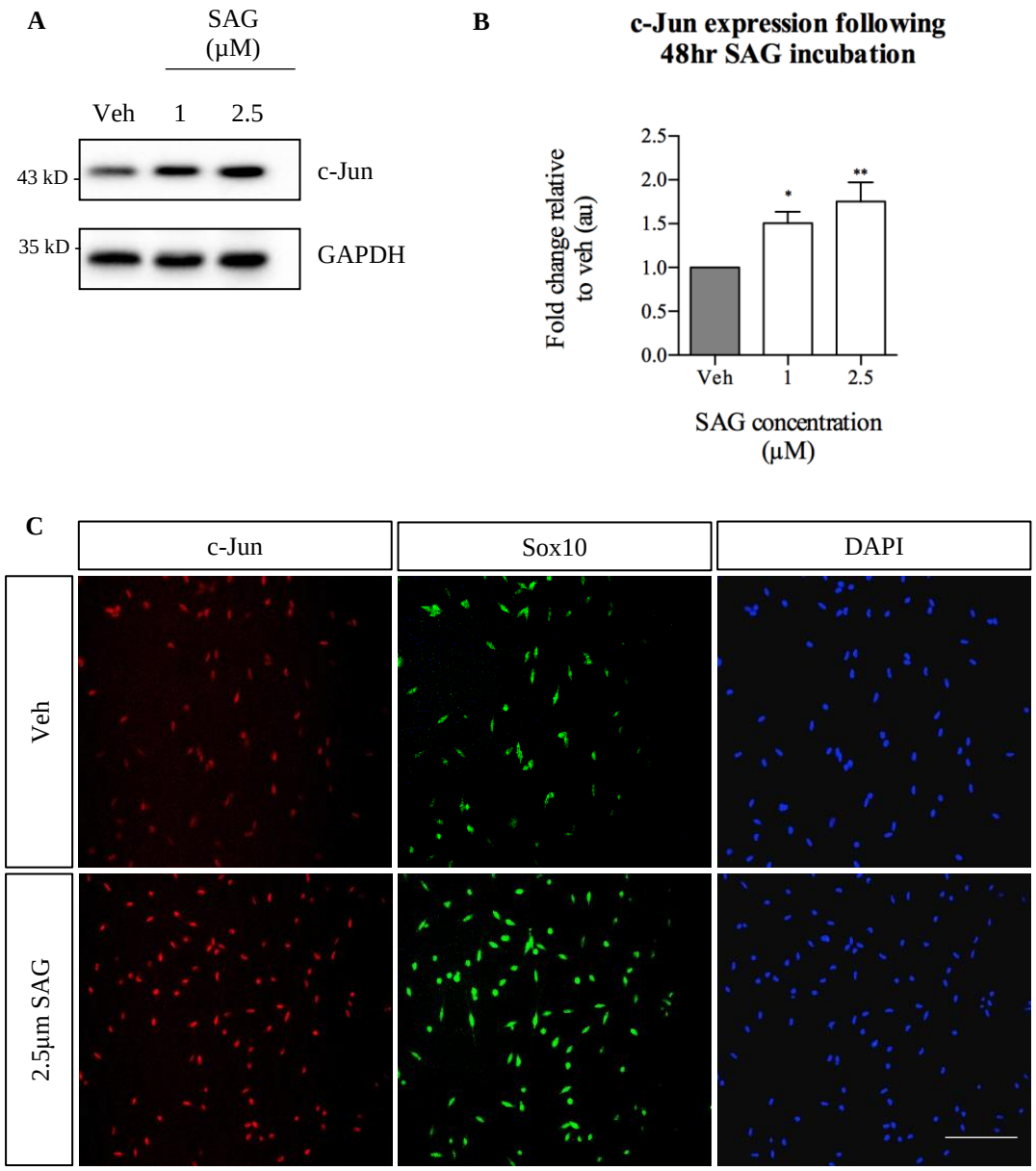

Figure 7.4.2 | Hh agonist SAG activates c-Jun in long-term cultured rat Schwann cells

(A) Passage 9 Schwann cells were incubated with SAG or DMSO (veh) for 48 hours. Western blots of protein lysates show that c-Jun increased with increasing concentration of SAG. **(B)** Blots were quantified and normalised to the vehicle to determine fold change in c-Jun expression. c-Jun was significantly elevated following incubation with SAG with levels increasing with increasing drug concentration. To increase n numbers, 2 data sets obtained from blots run by Liam Wong, a student under the author's supervision, were added to the analysis performed here. **(C)** Dual immunolabelling with c-Jun and Sox 10 antibodies of passage 9 cultures incubated with vehicle or 2.5μM SAG for 48 hours. SAG incubated cells are brightly labelled for c-Jun compared to vehicle control cells. Graph of means ± SEM, n=6, one-way ANOVA with Dunnett's test. Scale bar: 100μm.

7.5 Cyclopamine decreases c-Jun and p-c-Jun in short term cultured Schwann cells

Cyclopamine inhibits the Hh pathway through direct binding with smoothened (Cooper et al., 1998; Incardona et al., 1998; Chen, Taipale, Cooper, et al., 2002). Cyclopamine can therefore be used to examine the effect on c-Jun of inhibiting the Hh pathway. As Shh cKO nerves exhibit impaired c-Jun activation (Figure 7.1.1), cyclopamine is likely to decrease c-Jun expression.

As c-Jun is already low in passage 9 cells, any attempt to lower it further may only be slight and could even result in cell death. Hh inhibition must therefore be examined in conditions were c-Jun is highly elevated, such as during the early stages of culture. Passage 2 Schwann cells were therefore used. Cells were incubated with increasing concentrations of cyclopamine (10-50µM). As higher volumes of DMSO are required for the control cultures under these conditions (DMSO volume = volume for 30µM), the two controls of untreated cells in DM (UT) or DMSO (Veh) where reintroduced in this experiment. No difference was observed between the controls while c-Jun expression decreased with increasing concentrations of cyclopamine (Figure 7.5 A) The higher concentrations of cyclopamine, 40 and 50µM, significantly decreased c-Jun expression by 47% and 52% respectively (Figure 7.5 B).

It was previously found that Shh cKO Schwann cells, as well as having decreased levels of total c-Jun protein, also showed decreased levels of c-Jun phosphorylation (Figure 7.1.1 and 7.1.2). Cyclopamine has previously been shown to decrease phosphorylation of c-Jun at Ser63 (Kudo et al., 2012). The effect of inhibiting Hh signalling on c-Jun phosphorylation in Schwann cells was therefore examined. All concentrations of cyclopamine reduced the phosphorylation of c-Jun at Ser63 (Figure 7.5 A). All concentrations of cyclopamaine halved the levels of phosphorylated c-Jun, with the highest concentrations reducing phosphorylation by two thirds (Figure 7.5 C). The phosphorylation of c-Jun may therefore be a downstream target of Hh signalling and it is through phosphorylation that the Hh pathway activates c-Jun in Schwann cells.

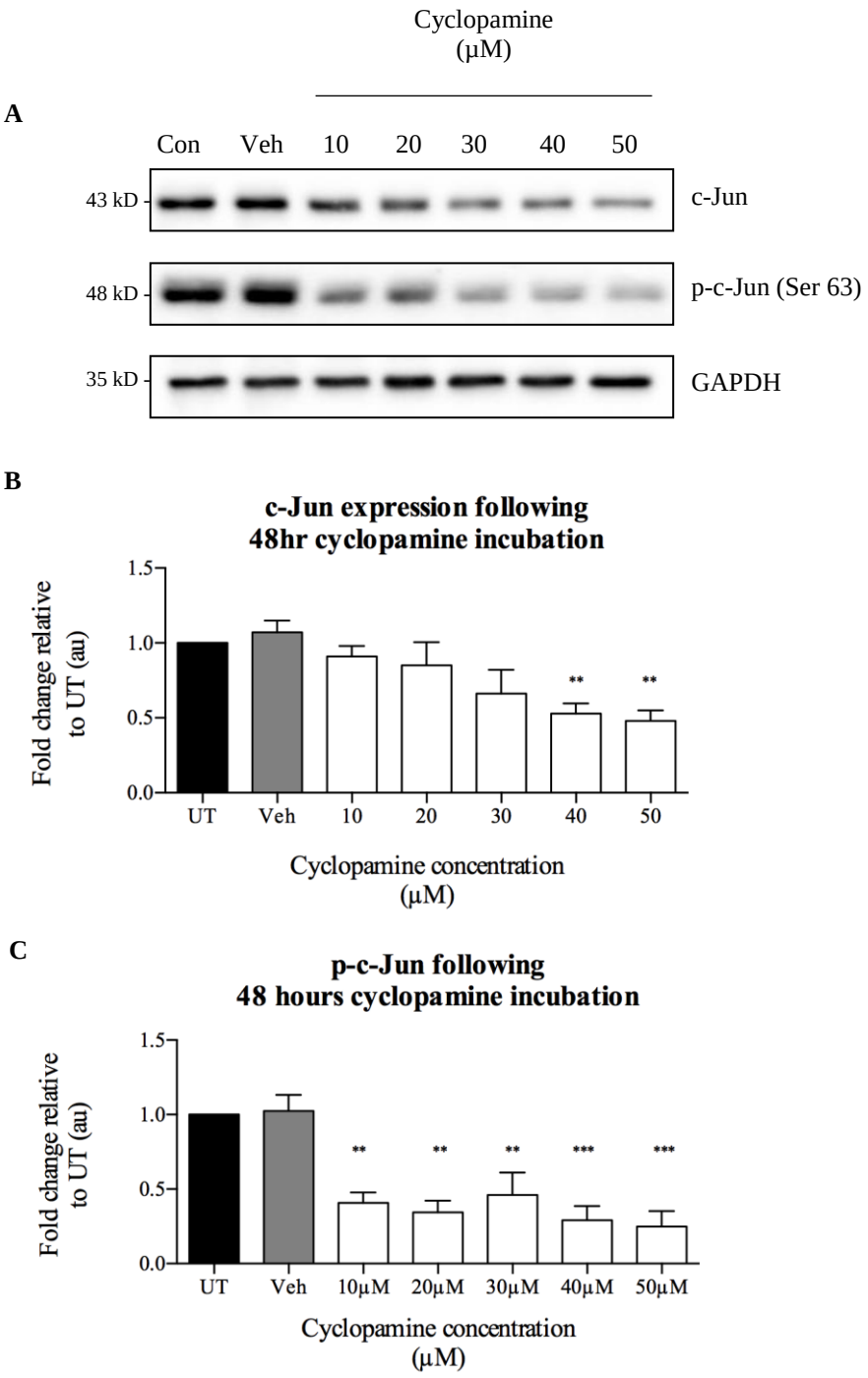

Figure 7.5 | Cyclopamine reduces c-Jun and p-c-Jun expression in cultured rat Schwann cells

(A) Passage 2 Schwann cells were incubated with increasing concentrations of cyclopamine for 48 hours. c-Jun expression gradually decreased with increasing concentration of drug. Cyclopamine dramatically reduced phosphorylation of c-Jun at all concentrations. **(B)** Blot quantification showed that total c-Jun protein is significantly reduced by 40-50μM cyclopamine. **(C)** Cyclopamine inhibits the phosphorylation of c-Jun. Incubation with all applied concentrations of cyclopamine lead to a significant down-regulation of p-c-Jun. Graphs of means ± SEM, n=3, one-way ANOVA with Dunnett's test.

7.6 Hh agonists increase trophic factor expression in long-term cultured Schwann cells

Since it has been shown that the Hh agonists activate c-Jun *in vitro* it is possible Hh signalling is also able to restore the repair cell markers which decrease in long-term cultured Schwann cells. As with previous experiments, passage 9 cultured rat Schwann cells were incubated with 5μM purmorphamine or an equal volume of DMSO and RNA was extracted after 48 hours. The subsequent qPCRs showed that *c-Jun* was highly elevated in cells incubated with purmorphamine (Figure 7.6.1 A). Despite the reduction in *BDNF* expression reported in long-term cultured cells previously (Figure 7.2.3 B), purmorphamine was unable to elevate expression further (Figure 7.6.1 C). *GDNF* however was significantly elevated after incubation with purmorphamine (Figure 7.6.1 B). *GDNF* expression declined during long-term culture (Figure 7.2.3 C). Although this was not significant, incubation with purmorpahmine resulted in a significant 2-fold increase in the trophic factor expression. Olig1 and Shh expression remained unchanged following purmorphamine incubation (Figure 7.6.1 D and E). Neither of these were altered by long-term culturing of Schwann cells and purmorphamine is unlikely to further elevate Shh expression as the Hh pathway is already active.

To further examine the increase in trophic factor expression caused by activation on the Hh pathway, passage 9 cultured rat Schwann cells were incubated with 2.5μM SAG for 48 hours. qPCRs of the extracted RNA found that both *GDNF* and *BDNF* significantly increased in these cultures relative to the control (Figure 7.6.2 A and B).

p75 NTR expression was found to decrease in Schwann cells with increasing time *in vitro* (Figure 7.2.2). Due to Hh signalling increasing other markers of the repair cell phenotype, p75 NTR may also be restored by Hh agonist incubation. Western blots of Schwann cells incubated with SAG for 48 hours were run. Despite the decrease previously observed, p75 NTR expression remained unchanged (Figure 7.6.2 C). Neither concentration of SAG significantly changed levels of p75 NTR (Figure 7.6.2 D).

Together these results demonstrate that activation of the Hh signalling pathway activates c-Jun and some markers of the repair cell phenotype, namely trophic factors GDNF and BDNF.

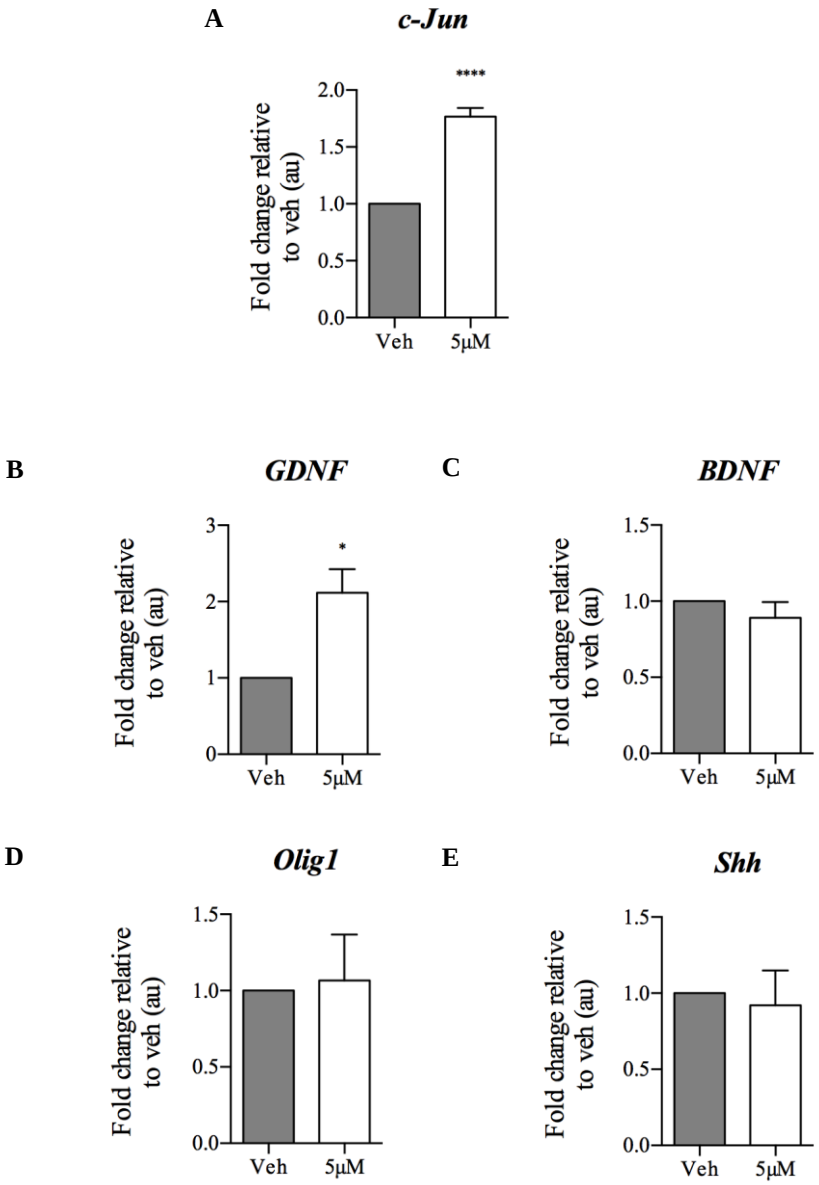

Figure 7.6.1 | Purmorphamine increases mRNA expression of *c-Jun* and *GDNF* in long-term cultured rat Schwann cells

RNA was extracted from passage 9 Schwann cells that were incubated with DMSO (Veh) or 5µM purmorphamine for 48 hours. qPCR results found that **(A)** c-Jun was significantly elevated by purmorphamine (p=< 0.0001). **(B)** *GDNF* expression increased 2-fold following purmorphamine incubation (p=0.0116), however no change in expression was observed in **(C)** *BDNF* (p=0.3344), **(D)** *Olig1* (p=0.8346) or **(E)** *Shh* (p=0.7395). Graphs of means ± SEM, n=4, unpaired Student's T-test.

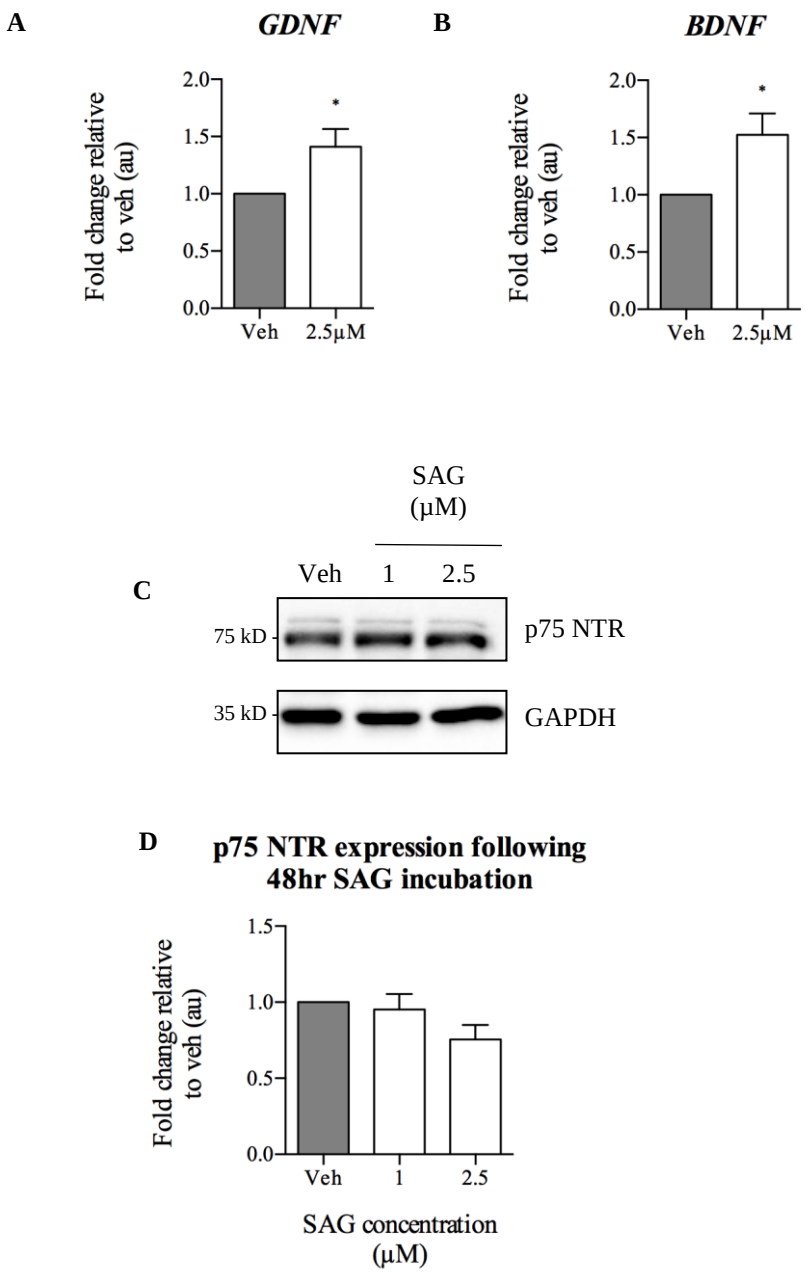

Figure 7.6.2 | SAG elevates trophic factors in long-term cultured rat Schwann cells but not p75 NTR protein expression

RNA was extracted from passage 9 Schwann cells following 48 hours of incubation with 2.5µM SAG. qPCRs found significant increases in both **(A)** *GDNF* (p=0.0382) and **(B)** *BDNF* (p=0.0314) expression. Unpaired Student's T-test. **(C)** Western blots showed no change in p75 NTR expression. **(D)** Quantification of blots even demonstrated a slight decline in levels when cells were incubated with 2.5µM SAG. One-way ANOVA with Dunnett's test. Graphs of means ± SEM, n=4 for all experiments.

7.8 Discussion

Shh is up-regulated by Schwann cells in response to injury. Previous studies have suggested that this provides a neuroprotective effect through the activation of BDNF (Pepinsky et al., 2002; Hashimoto et al., 2008; Bond et al., 2013; Angeloni et al., 2013). Although a link between Hh signalling and Jun activation has been established in various cell lines, this has not previously been established in Schwann cells (Laner-Plamberger et al., 2009; Kudo et al., 2012). Here, the activation of c-Jun in Schwann cells through hedgehog signalling is shown using *in vitro* and *in vivo* models.

To examine the role of Shh signalling in c-Jun activation *in vivo*, sciatic nerves from Shh cKO mice that had undergone a 3 or 7 day cut were gifted from the laboratory of John Svaren (USA). Although c-Jun was expressed equally in WT and Shh cKO nerves 3 days after injury, the mutant displayed reduced levels at 7 days. This suggests Shh signalling, although not responsible for initial activation of c-Jun, is required for its further elevation. This enforces findings previously described in this Chapter that Gli is required for the enhanced activation of Jun (Laner-Plamberger et al., 2009). As previous studies have demonstrated inhibition of Shh signalling reduces phosphorylation of c-Jun at serine 63 (Kudo et al., 2012), it was hypothesised that this might lead to the reduced activation of c-Jun. The phosphorylation of c-Jun at serine 63 was found to be reduced at 7 days after injury. Due to the reduced c-Jun activation observed in Shh cKO nerves, it was considered likely that induction of the repair cell phenotype might also be impaired. To examine this, nerves were extracted and Western blotted for p75 NTR at 3 and 7 days after injury. p75 NTR activation following injury is gradual with levels peaking 1 week after injury (Heumann et al., 1987). It was therefore expected that no difference would be observed between the 2 genotypes at this time. At 7 days however, in line with c-Jun, p75 NTR activation is impaired. Overall these findings demonstrate that Shh signalling in Schwann cells participates in the partial elevation of c-Jun and the repair cell phenotype.

In order to truly establish the impaired induction of the repair cell phenotype, further experiments would be required. qPCRs of repair cell markers could be performed on these nerves at 7 days after injury. Although experiments of this nature have been

performed throughout this thesis, limitations in the amount of tissue supplied prevented these investigations. It would be of further interest to examine the functional implications of Shh inhibition. Although studies described here have examined this, a thorough analysis of regeneration has never been undertaken. These investigations were clearly limited by time considerations and the lack of an established Shh cKO mouse colony in the UK, however if this were not the case many aspects of nerve regeneration could be tested. Retrograde labelling of regenerating motor and sensory neurons could be performed. Work is this thesis has demonstrated a reliable method for this already established in our laboratory. Axon regeneration could further be examined for by immunolabelling transverse sections with calcitonin gene-related peptide (CGRP) or neurofilament (Streit et al., 1989; Dumoulin et al., 1992).

Based on the findings from Shh cKO nerves, the Hh pathway could be activated to maintain c-Jun during chronic denervation or to enhance c-Jun activation in aged Schwann cells. It order to examine this however, it was first important to establish an *in vitro* model in which c-Jun is lowered. As c-Jun is highly elevated in cultured Schwann cells, further elevation might not be possible. It was found that Schwann cells down-regulate c-Jun with increased time *in vitro* similar to during chronic denervation. Markers of the repair cell phenotype such as p75 NTR and BDNF were also shown to decrease. It would have been interesting to establish the functional implications of these results on neurite outgrowth and survival of co-cultures with dissociated DRGs. A previous study examining the effect on long-term culturing of Schwann cells found that Schwann cell growth and proliferation and neurite growth in co-cultures increased with prolonged time *in vitro*. Their classification of short term culture however was 5-11 passages comparing with cells that had undergone at least 20 passages and some of their representative images do not match the quantification in the graphs. Schwann cells that are claimed to be growing well are imaged with short outgrowths and do not display classic Schwann cell bipolar morphology. It was however suggested that the long-term cultured Schwann cells had become tumorigenic (Funk et al., 2007). As many studies have been able to maintain Schwann cell cultures for even longer periods and no difference in the proliferation during prolonged culture has been demonstrated, the results from Funk et al should be viewed with caution (Porter et al., 1987; Mathon et al., 2001).

Application of Hh agonists to long-term cultured Schwann cells significantly elevated c-Jun and trophic factor expression. As with the model, it would be of interest to examine the effect of this elevation on neurite outgrowth and survival in co-cultures with dissociated DRGs. Interestingly, purmorphamine was unable to elicit an elevation in *BDNF*, only in *GDNF*. This is the reverse observation of previous findings which claim Shh activates *BDNF* but not *GDNF in vitro* (Hashimoto et al., 2008). Incubation with SAG however activated both trophic factors. Previous work has demonstrated that the application of both GDNF and BDNF can be more beneficial than activation of a single trophic factor (Boyd & Gordon, 2003). It is possible that the activation of repair cell markers may increase with prolonged exposure to Hh agonists or that their activation occurs only following immediate exposure to the agonist. RNA could be extracted at early and later time points to examine this further.

Although the expression of repair cell markers was examined following incubation with both agonists, it was not examined following inhibition of Hh signalling with cyclopamine. Cyclopamine was found to reduce c-Jun expression and partially inhibit phosphorylation of Ser 63. It is unknown if these reductions are enough to down-regulate the repair cell phenotype or effect the Schwann cells ability to support axonal growth. qPCRs for repair cell markers and Western blots for p75 NTR could examine this as well as co-cultures with dissociated DRGs. Performing neurite outgrowth studies on dissociated DRG cultures would have to be carefully planned. When Shh signalling is knocked out in these cultures, reductions in neurite outgrowth and branching are observed (Martinez et al., 2015). Therefore any alterations in DRG activity as a result of the manipulation of Hh signalling may simply be due to neuronal activation/inhibition of Hh. If activation of Hh improves axon regeneration, it may not be of concern if this is due to a Schwann cell or neuronal activation, the goal is still achieved. However to ultimately examine the effect of Hh and specifically Shh *in vitro,* genetic manipulation is required. For *in vivo* experiments, the Shh cKO mouse could be examined but this mouse can also be used for *in vitro* work. Schwann cells cultured from Shh cKO nerves could be co-cultured with DRGs. To examine activation of Shh expression, a mouse similar to the OE/+ mouse could be created that can over express Shh. It is likely this would be a fl/fl mouse to prevent developmental abnormalities, but Schwann cells cultured

from this mouse, infected with a cre recombinase adenovirus could be used to examine the effect of over-expressing Shh on neurite outgrowth.

Although this chapter has demonstrated the relationship between Hh and c-Jun, this chapter does not demonstrate that that Hh agonists can be used to overcome the effects of Hh inhibition, a crucial experiment in confirming the effects of Hh signalling in Schwann cells. Short term cultured Schwann cells should be incubated with both cyclopamine and a smoothened agonists to confirm this.

Overall the results of this chapter have demonstrated that Shh signalling in Schwann cells plays a role in the elevation of c-Jun. Hh agonists and antagonists were further shown to activate and inhibit c-Jun in Schwann cells *in vitro.* Hh agonists were also shown to increase the activation of trophic factors. This work highlights the Shh pathway as a pharmaceutical target for c-Jun activation and maintenance during times of poor peripheral nerve regeneration such as chronic denervation and advancing age.

8. General discussion

As has been stated previously, the PNS has a remarkable capacity for regeneration. Following nerve injury, myelin and Remak Schwann cells convert to a repair cell phenotype that facilitates axon regeneration. These cells promote axon guidance and regeneration by breaking down myelin through external macrophage recruitment and Schwann cell autophagy. Trophic factors, cell adhesion molecules and cytokines are up-regulated and cells proliferate, elongate, branch and align with each other to form the Bands of Büngner that facilitate axon guidance. c-Jun is a major regulator of this repair phenotype (Arthur-Farraj et al., 2012; Fontana et al., 2012).

Despite the activation of this repair cell phenotype, peripheral nerve regeneration in humans is poor. This is due to a variety of factors, one of which is the deterioration of the distal nerve stump which occurs during chronic denervation. The deterioration of the distal nerve stump is defined by two factors: the gradual decrease of trophic factor expression and a decline in Schwann cell numbers (Abercrombie & Johnson, 1946; Weinberg & Spencer, 1978; Siironen et al., 1994; You et al., 1997; Hall, 1999; Höke et al., 2002; Michalski et al., 2008; Eggers et al., 2010; Benito et al., 2017). This thesis investigated the hypothesis that c-Jun and thus the repair cell phenotype was not maintained during chronic denervation.

Results in Chapter 3 confirmed aspects of this idea. c-Jun protein levels were not maintained in Schwann cells following chronic denervation. p75 NTR protein was similarly found not to be maintained, confirming previous findings (You et al., 1997). qPCR results in Chapter 4 further demonstrated that *c-Jun, GDNF* and *Shh* all significantly decline during chronic denervation (Figure 4.8). *BDNF* expression was also reduced, however this was not a significant finding. GDNF and BDNF have previously been demonstrated to decline in rat models of chronic denervation (Höke et al., 2002; Michalski et al., 2008; Eggers et al., 2010). BDNF elevation is gradual in Schwann cells after injury (Meyer et al., 1992; Funakoshi et al., 1993; Michalski et al., 2008). It may be that no significant decrease is observed in *BDNF* expression as the qPCR only examines two time points. Examining expression at 4 weeks may provide results more in line with the literature. Furthermore, increasing the number of experimental repeats is likely to be beneficial and contribute to these findings.

Overall these data provide strong evidence that c-Jun and the repair cell phenotype are not maintained during chronic denervation.

It was hypothesized that maintaining c-Jun during chronic denervation might maintain the repair cell phenotype, preventing the deterioration of the distal nerve stump. The OE/+ mouse was generated, a heterozygote mouse which over-expresses c-Jun specifically in Schwann cells. During chronic denervation, c-Jun is maintained in the Schwann cells of this mouse (Figures 4.5-6). However, despite the maintenance of Schwann cell c-Jun, the present experiments failed to detect elevation in GDNF and BDNF in OE/+ nerves. Levels of *GDNF* were even lower than those expressed by WT nerves (Figure 4.8). This is an unusual finding as c-Jun has previously been demonstrated to directly regulate GDNF expression (Fontana et al., 2012). Nevertheless, the regenerative capacity of nerves in OE/+ mice is maintained. Motor and sensory neuron regeneration in these mice is equal to that in nerves that have been immediately repaired (Figure 5.4). What therefore, are the possible mechanisms behind this finding?

The reasons for the restoration of regeneration could be complex. c-Jun controls expression of many different genes besides the two trophic factors examined here. c-Jun cKO Schwann cells demonstrate changes in the expression of 172 different genes (Arthur-Farraj et al., 2012). Some of these genes may be maintained by OE/+ Schwann cells during chronic denervation. Furthermore, GDNF and BDNF protein levels were not examined here. It may be possible that maintenance is observed at the protein level. c-Jun protein is maintained by OE/+ nerves during chronic denervation, yet *c-Jun* mRNA expression decreases by half. Discrepancies may therefore also be observed between GDNF and BDNF mRNA and protein levels. More in depth analysis such as whole genome RNA sequencing and proteomic analysis may further explain this result and highlight other Schwann cell genes and proteins, such as other trophic factors and cell adhesion molecules, that might aid regeneration in the chronically denervated nerves of OE/+ mice.

Counts of Schwann cells in the distal nerve stump 1 and 10 weeks after injury showed that OE/+ nerves have an increased number of Schwann cells compared to WT (Figure 4.9). This raises the question of whether cell quality or quantity is an important factor in regeneration. Although the OE/+ nerves do contain more Schwann cells following chronic denervation, and c-Jun protein expression in individual cells is maintained, as demonstrated by immunolabelling (Figure 4.6,) these cells as previously discussed, do not express high levels of trophic factors GDNF and BDNF despite elevated c-Jun levels. Therefore, it could be concluded that by simply increasing the number of cells, regardless of their quality, is enough to enhance regeneration. However, it is been demonstrated that distal nerve stump Schwann cell proliferation is not required for axon regeneration. When proliferation is inhibited, axon regeneration is not impaired following crush injury (Yang et al., 2008). This suggests that an increase in Schwann cell number is not required for axon regeneration which persists regardless.

EM counts also showed that OE/+ nerves contain more macrophages during chronic denervation. It was shown that OE/+ nerves are able to attract more macrophages to the injury site following a short-term crush injury (Figure 4.10), although no difference was found between genotypes at 1 week (Figure 4.9). Therefore the differences between the two genotypes in acute denervation disappears with time, as is observed in the c-Jun cKO (Arthur-Farraj et al., 2012). It is possible however than macrophages do not leave the nerve during the time course usually described. They may even be unable to leave the nerve. A time course study would be needed to determine this. Because this information is not available, it cannot be determined if OE/+ nerves retain macrophages from the outset of denervation or if trafficking is simply increased.

Either way, this increased number of macrophages might contribute to improving axon regeneration. As previously discussed, macrophages normally appear in the nerve 2-3 days after injury where their primary role is to facilitate the secondary stage of myelin clearance. If this process is impaired, axon regeneration is inhibited, partly through the continued presence of myelin debris. (Perry et al., 1987; Stoll et al., 1989; Raivich et al., 1991; Perry et al., 1995; Dailey et al., 1998; Kang & Lichtman, 2013). However, chronically denervated nerves will no longer contain myelin debris. Therefore, how can their presence enhance regeneration? A secondary

function of macrophages is to promote vascularization through secretion of VEGF-A (Cattin et al., 2015). Although this has been solely demonstrated after immediate nerve cut and regeneration across the nerve gap, macrophage retention is likely to result in increased vascularization of the distal nerve stump during chronic denervation, creating a more favourable environment for regeneration. VEGF-A has also been shown to enhance axon regeneration following axotomy (Hobson et al., 2000). The vascularization of nerves from both genotypes following chronic denervation should therefore be examined. Macrophages also secret trophic factors and cytokines to promote axon regeneration (Brown et al., 1991; Reichert et al., 1996; Shamash et al., 2002). Although no changes in trophic factor expression were observed in OE/+ nerves in this thesis, RNA sequencing may highlight the maintenance macrophage specific promoters of regeneration.

The work presented here in aged mice however could contradict the importance of macrophages in regeneration. It was confirmed that up-regulation of c-Jun is impaired following injury in old mice (Figure 6.1) (Painter et al., 2014). This however was not observed in old OE/+ mice, the Schwann cells of which elevated c-Jun to levels similar to those observed in young mice. With the restoration of c-Jun activation, the age-related regeneration deficit was not observed in old OE/+ mice. It is known that macrophage numbers are reduced in old nerves following injury, yet I did not examine if restored c-Jun activation repaired this. This is due to the fact that the regeneration deficit in old nerves was shown by Painter et al., (2014) to be of Schwann cell origin. Parabiosis experiments, in which old nerves are infiltrated with young macrophages following injury, do not restore the age associated regeneration deficit (Painter et al., 2014). This suggests even if old OE/+ nerves recruited more macrophages, this is unlikely to be the cause of the enhanced regeneration observed. It is likely associated with the adoption of the repair cell phenotype, which is inhibited in aged Schwann cells (Painter et al., 2014). To confirm this, qPCR experiments of repair cell markers are required in old WT and OE/+ nerves after injury.

Further work is needed to truly determine the importance cell quality or quantity and cell type in chronic denervation. This issue could be further explored using the c-Jun OE/+ inducible mouse line. The nerves of these mice were allowed to deteriorate for 8 weeks before c-Jun over-expression was induced with tamoxifen. Although the

distal nerve stumps expressed higher levels of c-Jun as a result of this (Appendix 1), 2 weeks later, regeneration was not restored. This suggests that the consequences of c-Jun reduction during chronic denervation are not reversible. Alternatively, this enhances the idea that factors other than c-Jun elevation are required for nerve regeneration in chronically denervated tissue. Nerves from these mice and vehicle controls could be processed for EM and the number of Schwann cell and macrophages counted. Cell quality could also be examined by evaluating repair cell markers by qPCR or RNA sequencing. This could contribute to our understanding of the role of cell numbers in axon regeneration into chronically denervated nerves.

Further experiments with the OE/+ mice are also needed. As previously described in Chapter 4, a decrease in Schwann cell numbers was not observed 10 weeks after injury. It was noted that the numbers counted at 1 week were lower than those previously documented and should therefore be re-examined (Arthur-Farraj et al., 2012). However, as discussed previously in Chaper 4, the difference in cell number could be accounted for by the differences in the mouse strains examined. The mice examined by Arthur-Farraj *et al* (2012) and those used in this work were generated from different backgrounds. Repeating these experiments may therefore not produce different results.

Although the exact mechanism by which c-Jun maintenance and enhanced elevation restores the regeneration deficits observed following chronic denervation and advancing age is still debatable, these regeneration deficits were importantly both restored. This highlights c-Jun has a possible pharmaceutical target. In Chapter 7 it was demonstrated that Hh signalling, particularly Shh, affected c-Jun activation. The hedgehog pathway could therefore be targeted to activate c-Jun and aid peripheral nerve regeneration. The work in this thesis has demonstrated that, if a treatment were to be developed, it would have to be administered as soon as possible. During chronic denervation, the re-activation of c-Jun does not enhance regeneration and is it the early activation of c-Jun that restores the age related decline. Once administered however, the course of drug treatment would have to be very specifically defined. c-Jun is an inhibitor of myelination (Parkinson et al., 2008) and following a crush injury, functional recovery is impaired in young OE/+ mice (Fazal et al., 2017) no difference is observed in motor or sensory neuron regeneration between genotypes following acute injury (Figure 5.3, 6.2), mild and transient

deficits are observed in both motor and sensory function tests such as toe pinch and spread. This is due to a delay in remyelination of the regenerating fibres (Fazal et al., 2017). Drugs would have to be administered long enough to promote axon regeneration then halted to allow for remyelination. However, if the drug was administered at a low dose, axonal signals may override the effects of the drug and promote remyelination.

The over-expression of c-Jun would also have to be confined to the injured nerve. Work in this thesis and by Fazal *et al* (2017) has demonstrated that moderate expression of c-Jun does not have substantial effects on uninjured nerves. c-Jun however is an oncogene. c-Jun is highly expressed in breast cancer (Vleugel et al., 2006) and over-expression of c-Jun in breast cancer cell lines promotes tumorigenesis and cell motility (Smith et al., 1999). c-Jun expression has also previously been observed in 31% of primary and metastatic lung tumours (Szabo et al., 1996). In liver cancer, it is proposed that c-Jun expression prevents apoptosis which is likely to contribute to tumour development (Eferl et al., 2003). The inhibition of c-Jun is now proposed as a potential target for the treatment of cancers (Gurzov et al., 2008). It is therefore unlikely that a treatment that would have effects outside the nerve would be approved.

Overall, the work in this thesis has demonstrated that the maintenance of Schwann cell c-Jun during chronic denervation restores the regeneration deficit observed with increased time after injury. It has also been demonstrated that restoring the activation of c-Jun after injury in old Schwann cells prevents the regeneration deficit observed with advancing age. c-Jun has therefore been highlighted as a pharmaceutical target for improving peripheral nerve regeneration which could potentially be targeted through the Hh signalling pathway.

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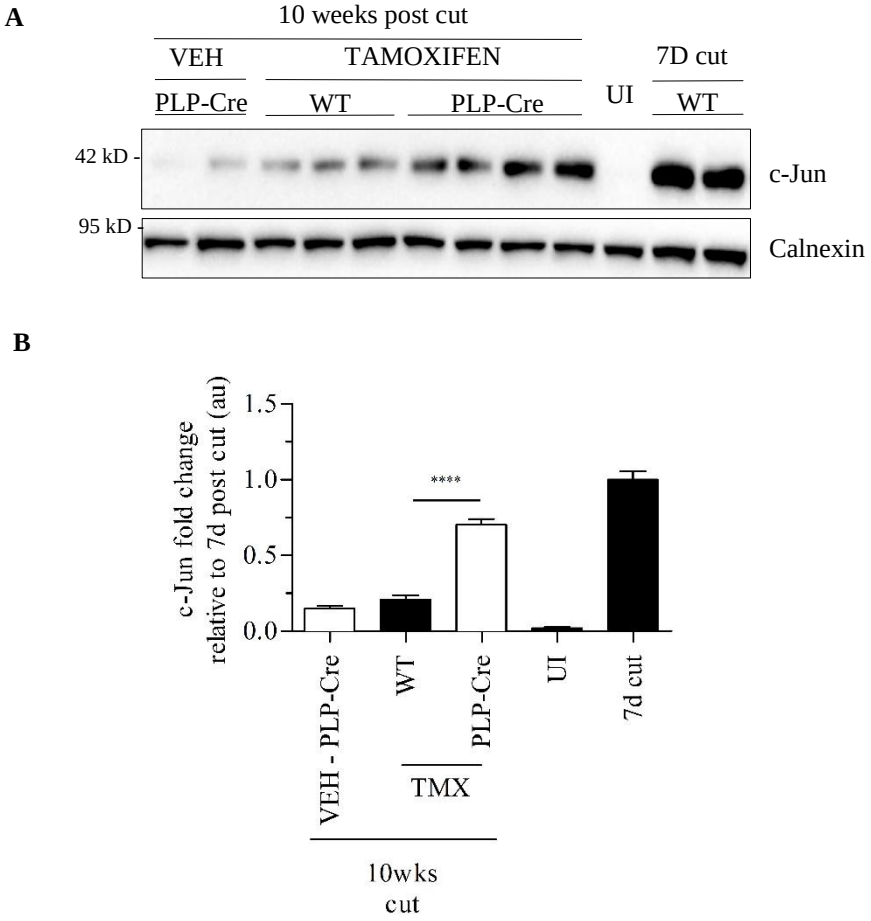
10. Appendix



Appendix 1 | c-Jun protein expression is increased in chronically denervated distal nerve stumps of PLP-Cre mice following tamoxifen administration

(A) A Western blot of c-Jun protein expression in chronically denervated distal nerve stumps of WT and PLP-Cre mice following tamoxifen administration. Tamoxifen had no effect on WT nerve c-Jun levels following chronic denervation, however PLP-Cre nerve c-Jun levels increased. Administration of the vehicle (VEH) did not elevate c-Jun expression in PLP-Cre nerves. **(B)** Tamoxifen administration significantly increased c-Jun protein expression in chronically denervated PLP-Cre nerves in comparison to WT. Administration of the vehicle (VEH) did not significantly alter c-Jun expression in these nerves Graphs of means ± SEM, Veh-PLP-Cre and 7 day cut n= 4, UI n= 3, WT and PLP-Cre n= 6, one-way ANOVA with Tukey's test, p=<0.00001. This experiment was perfomed by J.A Gomez-Sanchez, who also provided the figure.