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Nuclear Factor-κB Activation in Schwann Cells Regulates Regeneration and Re-Myelination

Paul D. Morton
University of Miami, pmorton15@gmail.com

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NUCLEAR FACTOR-κB ACTIVATION IN SCHWANN CELLS REGULATES REGENERATION AND RE-MYELINATION

By
Paul D. Morton

A DISSERTATION

Submitted to the Faculty of the University of Miami
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Paul D. Morton

Approved:

John R. Bethea, Ph.D.
Professor of Microbiology & Immunology and Neurological Surgery

Terri A. Scandura, Ph.D.
Dean of the Graduate School

Robert W. Keane, Ph.D.
Professor of Physiology and Biophysics

Christine K. Thomas, Ph.D.
Professor of Neurological Surgery

Daniel J. Liebl, Ph.D.
Associate Professor of Neurological Surgery
Director of Neuroscience Program

Robert H. Miller, Ph.D.
Professor of Neuroscience Case Western Reserve University
Schwann cells (SCs) are crucial for peripheral nerve development and regeneration; however, the intrinsic regulatory mechanisms governing post-injury responses are poorly understood. Activation and deacetylation of nuclear factor-κB (NF-κB) in SCs have been implicated as prerequisites for peripheral nerve myelination. Using GFAP-IκBα-dn mice, in which NF-κB transcriptional activation is inhibited in SCs, we found no discernable differences in the quantity or structure of myelinated axons in adult facial nerves. Following crush injury, axonal regeneration was impaired at 31 days and greatly improved at 65 days in GFAP-IκBα-dn mutants. Compact re-myelination and sensory fiber organization were significantly compromised at 31 days and restored by 65 days. Together, these data indicate that NF-κB activation in SCs is dispensable for peripheral nerve myelination in adults, but required for early re-myelination and axonal regeneration. SC myelination during development and following injury in adult mice may hinge on different transcriptional cascades; these findings may offer new therapeutic avenues for PNS and CNS regeneration.
Dedication
This work is dedicated to my mother, sister, and mentor. Together, they taught me to make, shape, and believe in myself.
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## Table of Contents

**List of Tables** .................................................................................................................. ix

**List of Figures** .................................................................................................................. x

**Abbreviations List** .......................................................................................................... xiii

**Chapter 1  Introduction** ................................................................................................. 1

1.1 Peripheral nerve function and structure ................................................................. 1

1.2 Glia in the peripheral nervous system .................................................................. 3

1.3 A brief history of gliogenesis ............................................................................... 4

1.4 Schwann cell origin .............................................................................................. 6

1.5 Schwann cell precursors ...................................................................................... 7

1.6 Immature Schwann cells ..................................................................................... 12

1.7 Myelinating and non-myelinating Schwann cells ........................................... 13

1.8 Positive regulators of myelination ...................................................................... 18

1.9 Negative regulators of myelination .................................................................... 20

1.10 Peripheral nerve injury, regeneration and repair ........................................... 22
Chapter 3  NF-κB activation in Schwann cells is crucial for regeneration and re-myelination

3.1 Background

3.2 Results

3.2.1 GFAP-IkBα-dn transgene expression

3.2.2 Elevated GFAP protein expression in astrocytes following injury

3.2.3 NF-κB activation in GFAP-expressing glia is required for axonal regeneration and motor recovery 1 month following transection

3.2.4 NF-κB activation in GFAP-expressing glia is required for axonal regeneration 1 month following crush injury of the buccal nerve

3.2.5 Tracer studies in various branches of the facial nerve
3.2.6 NF-κB activation in Schwann cells is dispensable for myelination in adulthood...........................................66

3.2.7 Functional inhibition of NF-κB in transgenic, denervated Schwann cells following crush injury..............68

3.2.8 Facial nerve crush injury model exhibits minimal axonal sparing, abrupt axonal degeneration, and detectable GFAP expression.................................................................70

3.2.9 Inhibition of NF-κB activation transiently delays and then promotes regeneration...............................73

3.2.10 Functional inhibition of NF-κB activation in denervated and unmyelinated Schwann cells delays Remak bundle formation.................................................................76

3.2.11 Inhibition of Schwann cell NF-κB activation delays compact re-myelination........................................77

3.2.12 Expression of myelin regulatory transcription factors and structural proteins.......................................81

3.2.13 NF-κB activation in denervated SCs is not required for Wallerian degeneration........................................85

3.2.14 NF-κB activation in SCs does not alter the expression of genes required for myelination or cholesterol synthesis.................................................................88

Chapter 4 Discussion...........................................................................................................................................92

4.1 Conclusion..................................................................................................................................................105

References.......................................................................................................................................................106
List of Tables

Tables

3.2.1 Average number of Fluorogold positive motor neurons, per section, 31 days following facial nerve transection………………………………………………………………………………58

3.2.2 Total number of Cresyl Violet positive motor neurons, 31 days following facial nerve transection…………………………………….59

3.2.3 Raw values of total projected number of fluorogold positive motor neurons, 10 and 31 days following buccal nerve crush injury…………………………………………..63

3.2.4 Raw values of total projected number of fluorogold positive motor neurons, 12, 31 and 65 days following buccal nerve crush injury…………………………………………..75

3.2.5 Average axonal diameter, fiber diameter, gRatio………………80
List of Figures

Figures

1.1 Peripheral nerve structure ......................................................... 3

1.2 Neurulation and neural crest cells .................................................. 8

1.3 Molecular markers of the Schwann cell lineage .......................... 9

1.4 Schwann cell precursors .............................................................. 11

1.5 Radial sorting process ................................................................. 14

1.6 Mature myelinating and nonmyelinating Schwann cells .......... 15

1.7 Chronology of axon-Schwann cell events paired with relative changes of key molecules following various peripheral nerve injuries ......................................................... 30

1.8 Musculotopic organization of mouse facial nucleus ............... 33

1.9 Transgenic approach to study the pathophysiology of NF-κB in GFAP-expressing glia ................................................................. 37

2.1 Schematic of facial nerve transections of the marginal mandibular branch of the facial nerve ......................................................... 40

2.2 Facial nerve crush injury model ...................................................... 41

2.3 Buccal nerve transection model to assess Wallerian degeneration ................................................................. 42

3.2.1 IkBα-dn transgene is expressed in the brainstem of GFAP-IkBα-dn, but not WT, mice ................................................................. 53

3.2.2 Facial nerve axotomy induces GFAP immunoreactivity in astrocytes within the facial motor nucleus ........................................ 54
3.2.3 Glial NF-κB activation is required for axonal regeneration but not motor neuron survival 31 days after facial nerve axotomy

3.2.4 Inhibition of NF-κB activation in GFAP-expressing glia reduces regeneration compared to WT littermates, 31 days following crush injury

3.2.5 Naïve GFAP-κBα-dn nerves display no aberrations

3.2.6 Transgenic inhibition of NF-κB activation in denervated SCs following facial nerve crush injury

3.2.7 Minimal activation of astroglial NF-κB within the facial motor nucleus following crush injury

3.2.8 Facial nerve crush injury model exhibits abrupt axonal degeneration and minimal axonal sparing

3.2.9 GFAP and transgene expression are active in Schwann cells following crush injury

3.2.10 NF-κB activation in denervated SCs significantly influences axonal regeneration

3.2.11 Effects of NF-κB inhibition on Schwann cell remyelination

3.2.12 Functional inhibition of NF-κB activation in denervated and unmyelinated Schwann cells transiently delays Remak bundle formation

3.2.13 NF-κB activation in Schwann cells does not regulate myelin-associated protein expression following crush injury

3.2.14 Myelin-associated protein expression is unaltered in Schwann cells lacking NF-κB activation, as compared to WT, following crush injury
3.2.15 Wallerian degeneration is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells……………..86

3.2.16 Macrophage infiltration is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells……………..87

3.2.17 Myelin degradation is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells……………………88

3.2.18 Myelin gene expression is unaltered between WT and IκBα-dn mice………………………………………………………90

3.2.19 Genes related to cholesterol synthesis are unaltered between WT and IκBα-dn mice……………………………………91

4.1 NF-κB activation in Schwann cells may be required for cholesterol synthesis and compact remyelination……………99

4.2 NF-κB activation in Schwann cells may be required for Schwann cell sorting following crush injury………………..100
**Abbreviations List**

dpi: days post injury

FG: Fluorogold

FMN: facial motor nucleus

GFAP: glial fibrillary acid protein

HDAC: histone deacetylase

MBP: myelin basic protein

MN: motor neuron

MPZ: myelin protein zero

NF-κB: nuclear factor -κB

PNS: peripheral nervous system

PPD: p-phenylenediamine

SC: Schwann cell

TB: toluidine blue

TNF-α: Tumor necrosis factor alpha

WT: wild type
Chapter 1: Introduction

1.1 Peripheral nerve function and structure

Peripheral nerves expand throughout the entire body and provide motor, sensory and/or autonomic information to nearly every tissue and organ. Since they control the homeostatic functions of several tissues, organs and systems, healthy development and maintenance of peripheral nerves is critical for normal body function. Because of their expansive presence throughout the body, these nerves are extremely vulnerable to traumatic injury.

Two categories of peripheral nerves emerge from the CNS: cranial and spinal nerves. Several branches stem from these main trunks and form complex arborizations, immigrating to all areas of the body in a pattern resembling angiogenesis. Peripheral nerves are typically classified by their fiber-type composition: sensory, motor, or mixed (Williams, 1999). Two main components make a peripheral nerve: (i) parenchyma made by axons and Schwann cells, collectively known as nerve fibers, (ii) stroma made of various connective tissue. A nerve fiber is the smallest functional component within peripheral nerves and can be myelinated or unmyelinated; sometimes fibers are further classified by their conduction velocity, diameter or function.
Since nerves residing in the periphery are susceptible to stretch and compression forces during body movement, nerve fibers are intimately encased and supported by a scaffold of connective tissue to ensure flexibility and robust resistance to such challenges. This peripheral nerve sheath is comprised of three distinct parts: endoneurium, perineurium, and epineurium (Fig. 1.1). The epineurium surrounds the entire nerve trunk, originates from mesoderm, and contains fibroblasts, collagen, and fat. The perineurium encloses individual fascicles of nerve fibers and functions as a diffusion barrier, possibly to maintain osmotic and fluid pressures within the innermost endoneurium (Williams, 1999). The perineurium consists of alternating concentrically arranged layers of flattened epithelium-like cells and collagen (Thomas and Jones, 1967; Akert et al., 1976; Thomas and Olsson, 1984); these layers are each enclosed by basal lamina and separated by spaces housing capillaries, collagen fibrils and elastic fibers (Thomas and Jones, 1967). The endoneurium is the innermost component of the peripheral nerve sheath and embeds fascicles with its loose, soft, collagenous matrix, protecting them from trauma (Lundborg, 2004). The endoneurium is primarily filled with Schwann cells and endothelial cells; however, it also contains fibroblasts, macrophages, mast cells, collagen fibers, and capillaries (Causey and Barton, 1959; Thomas et al., 1993). Although the architecture of the peripheral nerve is well described, studies completely documenting the prenatal to postnatal steps in nerve development are lacking, mainly because of the difficulty in locating and working with embryonic nerves (Song et al., 1999).
Figure 1.1. Peripheral nerve structure. Peripheral nerve trunks are filled with axons coursing through the body of the nerve. Axons traverse the nerve in groups known as fascicles and are embedded in the collagenous matrix of the endoneurium. Fascicles are enclosed by the perineurium. The epineurium surrounds the entire nerve trunk as well as groups of fascicles and their respective perineuriums. Axons (enlarged in top box) can be myelinated or unmyelinated. The bottom outset illustrates myelin sheaths, produced by Schwann cells and separated by nodes of Ranvier, enveloping an axon. Figure adapted from “Nerve Anatomy.” A.D.A.M. Anatomy. 2009. http://bme240.eng.uci.edu/students/10s/mklopfer/anatomy.html

1.2 Glia in the peripheral nervous system

The mammalian peripheral nervous system harbors a variety of distinct glia intimately associated with certain types of neurons or with certain parts of neurons. Myelinating Schwann cells are the most renowned glial cells in the PNS; these cells encase large diameter (>~1 μm) axons within nerve trunks which enables efficient electrical conduction and protection from the extracellular environment. Small diameter axons are enveloped, and often sorted into
bundles known as Remak bundles, by non-myelinating Schwann cells. Non-myelinating Schwann cells also associate with autonomic neuron terminals. Terminal glia (teloglia or perisynaptic Schwann cells) cover the synapses of skeletal neuromuscular junctions (for review, see Griffin and Thompson, 2008). Neuronal somas of sympathetic, parasympathetic, and dorsal root sensory ganglia are encompassed by satellite glia with a flattened sheet-like appearance. Axons of the olfactory nerve are encased by olfactory ensheathing cells. In the enteric nervous system, ganglia are closely associated with enteric glia. Several sensory nerve terminals, or mechanoreceptors, in the skin interact with glial cells at the core of a larger structure, such as the Pacinian corpuscle (Jessen and Mirsky, 2005). With such a diverse and multifunctional population of cells, one can appreciate that almost all of these cells share the same cellular origin.

1.3 A brief history of gliogenesis

Although glia greatly outnumber neurons, substantially less is known about these remarkable cells required for normal nervous system development and function; this is primarily because their existence was established much later. In 1839, Theodore Schwann proposed a theory that changed the course of biology forever. He proposed that all living organisms were formed from cells that were single units, in what is now known as the cell doctrine (Schwann, 1847). Shortly following this fundamental theory, it was shown that all organs, except for those composing the central nervous system (CNS), were made from their own unique type of cells (Webster and Astrom, 2009). In 1846, another type of tissue was documented in the CNS by Virchow which he termed “Kitte” or
“neuro-glia” (Virchow, 1858). He, along with his contemporaries, believed that this tissue, in cooperation with connective tissue, formed a “binding substance” or a glue to hold the nervous constituents together while isolating them from other structures such as blood vessels. Deiters, along with his discovery of astrocytes, abolished this idea of a binding substance in the nervous system (Deiters, 1865); however, his method had severe limitations only enabling visualization of single cells picked from their milieu.

In the last decade of the 19th century, the silver impregnation method was conceived (Golgi, 1894; Cajal, 1895) and neuroglial cells were irrefutably visualized. Perhaps the most exciting advancements in the early history of glia were soon after the introduction of the electron microscope in the 1950s. Aided with high-resolution micrographs, it was established that the interstitial material of the CNS was composed of cells instead of an unknown variety of connective tissue (Peters et al., 1976). Following the discovery of these fascinating cells, investigators immediately began elucidating their origins in developing and mature nervous tissue. Several types of glia were discovered, including astrocytes, microglia, and oligodendrocytes in the CNS, and Schwann cells within the peripheral nervous system (PNS). Today, over half a century later, with the use of cell markers and eclectic, advanced techniques, the development, maturation, and function of glial cells are quickly being elucidated and we now know that these cells are involved in essentially every aspect of the nervous system.
1.4 Schwann cell origin

Even though much was known about the process of differentiation and maturation, the experimental methods needed to trace and determine the origin of the cells first described by Schwann were non-existent in the mid-19th century (Schwann, 1847; Causey, 1960; Jacobson, 1978; Webster and Astrom, 2009). In 1924, Harrison showed that most Schwann cells pervading developing nerves were generated from the neural crest. However, many skeptics disregarded the validity of this finding until Johnston and Noden confirmed it by transplanting radiolabeled neural crest cells and tracing their subsequent differentiation in avian embryos (Johnston, 1966; Noden, 1975). Later observations revealed that peripheral ganglia, including dorsal root and sympathetic ganglia, required the presence of both the neural tube and the notochord, as excision of both structures resulted in somitic cell death (Teillet and LeDouarin, 1983). Currently, it is widely accepted that Schwann cells originate from neural crest cells; however, the signaling mechanisms driving an early glial phenotype remain elusive.

During embryonic development the neural plate folds and fuses to produce the neural tube, from which neural crest cells segregate and migrate to later differentiate into a large diversity of cells (Fig. 1.2). Commencing from the dorsal surface of the neural tube, neural crest cells migrate in a lateral or ventral direction. Those that migrate laterally will become melanocytes and those migrating ventrally can generate dorsal root sensory ganglia, glia, autonomic neurons, and chromaffin cells. Neural crest cells in the cardiac crest can give
rise to fibroblasts and smooth muscle cells and those located in the cephalic crest form cartilage and bone cells (for review, see Jessen and Mirsky, 2005). To date, little is known about the process that governs the initial migration of neural crest cells through immature connective tissue flanking the neural tube and how they differentiate into Schwann cell precursors intimately associated with developing axons.

1.5 Schwann cell precursors

Since axonal signals determine the fate of Schwann cells in developing nerves, Schwann cells and their precursors are commonly viewed as passive recipients of extrinsic differentiation signals (Jessen and Mirsky, 1999). However, it is now evident that Schwann cells, along with their precursors, actively regulate the development of all essential peripheral nerve components. During mouse peripheral nerve development, neural crest cells undergo three primary transitions to become mature SCs (Fig. 1.3); first, neural crest cells give rise to Schwann cell precursors (E12-13) which associate with axons and quickly transition into immature Schwann cells (E 15-16) before birth (Jessen and Mirsky, 2005). Although SCPs are a brief phenotype in the Schwann cell lineage, they have several, crucial functions aside from providing immature Schwann cells within perinatal nerves.
Figure 1.2. Neurulation and neural crest cells. During embryogenesis, the neural plate, located on the dorsal surface, will slowly fold in on itself to create a neural groove. The neural folds then join and fuse to form the neural tube. Collectively, this process is known as neurulation. During neural tube formation, neural crest cells segregate from the dorsal surface and migrate in a lateral (1) or ventral (2,3) direction. Cells migrating laterally will become melanocytes, and those heading in the ventral direction will generate several different cell types, such as glia, autonomic neurons, and sensory neurons of the dorsal root ganglia. Figure obtained from Jessen and Mirsky, 2005.
Figure 1.3. Molecular markers of the Schwann cell lineage. The molecular profile of each stage of Schwann cell maturation can be found in the colored boxes. When immature Schwann cells diverge to become myelinating Schwann cells, they up-regulate the expression of molecules involved in myelination (green box) and down-regulate the expression of immature Schwann cell markers. Generation of non-myelinating Schwann cells exhibits very few molecular changes. Abnormal expression of key myelin-associated proteins can result in hereditary demyelinating neuropathies such as Charcot-Marie-Tooth (CMT) neuropathy and Pelizaeus-Merzbacher disease (PMD). *Proteins also present on neuroblasts. †Expression is acutely dependent on axons. §Early expression of GFAP is unknown but significant expression is seen around birth. GFAP is reversibly down-regulated in myelinating cells. ‡S100 can be detected in mouse Schwann cell precursors if the sensitivity of the detection assay is substantially increased. AP2α: activator protein 2α. BFABP: brain fatty acid-binding protein. DHH: desert hedgehog. ErbB3: neuregulin receptor. GalC: galactocerebroside. GAP43: growth-associated protein 43. GFAP: glial fibrillary acidic protein. L1: L1 cell adhesion molecule. MAL: myelin associated lipoprotein. MBP: myelin basic protein. Ncad: N-cadherin. Oct6: octamer-binding transcription factor 6. O4: lipid antigen. PLP: proteolipid protein. PMP22: peripheral myelin protein 22 kDa. P0: protein zero. p75NTR: p75 neurotrophin receptor. Sox10: SRY (sex-determining region Y) box 10. Adapted from Mirsky et al., 2008.
Early embryonic nerves are made of tight columns of axons intimately associated with SCPs (Fig. 1.4) that, remarkably, survive and influence the construction of their milieu without the aid of connective tissue, a protective perineurium, or a blood supply (Jessen and Mirsky, 2005). SCPs are essential for proper nerve fasciculation and provide vital trophic support to developing neurons (Garratt et al., 2000). *In vitro*, SCPs can generate cells from non-glial lineages such as neurons and endoneurial fibroblasts (Joseph et al., 2004). Unlike mature Schwann cells, SCPs lack autocrine survival loops and rely on a β-neuregulin 1 (NRG1) signal, provided by axons, for survival (Meyer, 1995; Riethmacher et al., 1997; Jessen et al., 1994; Dong et al., 1995). Following injury, SCPs are vulnerable to cell death which can be prevented by NRG1 administration (Winsec et al., 2002). Following their short appearance, SCPs give rise to immature Scwhann cells; this transition is currently viewed as an irreversible process (Jessen and Mirsky, 2005) that is likely dependent on NRG1, as is the case *in vitro* (Dong et al., 1995).
Figure 1.4. Schwann cell precursors. Schwann cell precursors can be seen embedded between axon bundles within the hind-limb nerves of a rat 15 days in gestation. The nuclei of three precursors (P₁, P₃, and P₄) are visible whereas only the cytoplasmic processes of P₂ can be seen. Schwann cell precursors form intimate contacts with axons and each other (arrows). Scale bar: 0.7 µm. Adapted from Jessen and Mirsky, 1999.
1.6 Immature Schwann cells

The second transition during the maturation of Schwann cells occurs abruptly in mouse peripheral nerves; SCPs become immature Schwann cells around E 15-16. This process is paralleled with a key phase in peripheral nerve organogenesis, as an increase in connective tissue spacing, vascularization, and the formation of the perineurium ensue (Jessen and Mirsky, 2005). At this stage, immature Schwann cells encase large bundles of axons that they communally sort into large and small diameter axons to achieve a 1:1 relationship between an axon to be myelinated by a Schwann cell; this process is known as radial sorting (Fig. 1.5) and the community of immature Schwann cells along with their processes that group axons are often referred to as “Schwann cell families” (Webster et al., 1973; Jessen and Mirsky, 2005).

Radial sorting continues postnatally and is a fascinating process, the geometry of which was, first described by Webster in the early 1970’s in perinatal rat sciatic nerves. This sorting sequence of axon bundles begins with the initial surface contact of a Schwann cell process, radial progression of axons into a separate furrow at the outer margins of a family sheath, and Schwann cell mitosis to establish a 1:1 relationship with a daughter cell that will later be separated from the family sheath (Webster et al., 1973). During this process, premature myelination is inhibited and the number of Schwann cells must be balanced in order to provide a matching number to accommodate axons. Although the molecular mechanisms governing the sorting process are unknown,
appropriate Schwann cell numbers are achieved by controlling Schwann cell survival and proliferation (Jessen and Mirsky, 2005).

Immature Schwann cells exhibit incredible plasticity and can support their vitality with survival factors including, but not limited to, secretion of neurotrophin 3 (NT3), insulin-like growth factor 2 (IGF2), leukaemia inhibitory factor (LIF), and platelet-derived growth factor-β (PDGFB) \textit{in vitro} (Dowsing et al., 1999; Meier et al., 1999). It is thought that immature Schwann cells acquire this autocrine ability to ensure survival following injury and provide subsequent support to damaged, regenerating axons; whereas, SCPs need to rely on axonal signals in order to pair with axons efficiently.

\section*{1.7 Myelinating and non-myelinating Schwann cells}

Around birth, immature Schwann cells begin to differentiate into myelinating Schwann cells and, subsequently, non-myelinating (unmyelinating) cells (Fig. 1.6). This reversible process takes several weeks to complete in rodent nerves and is believed to be governed by axonal signals, as random association with axons of variable size dictates subsequent maturation; those associated with large diameter axons (>\sim 1 \text{ μm}) will adopt a myelinating phenotype and those associated with small diameter axons mature into non-myelinating SCs (Jessen and Mirsky, 2005). During this time, myelinating Schwann cells undergo extensive biochemical and morphological changes in order to accommodate an immense demand for membrane synthesis and wrapping of lamellae to form myelin sheaths (Jessen and Mirsky, 1992).
Figure 1.5. Radial sorting process. Illustrative diagram (top panel) of radial sorting and subdivision of axon bundles. Schwann cells will guide axons, destined to be myelinated, radially from a bundle to a segregated trough at the outer edges of the Schwann cell and later provide a daughter cell to establish a 1:1 relationship with the sorted axon. At the end of the sorting process, sequential chains of individual Schwann cells generate sheaths to surround the myelinated and unmyelinated axons. A snap shot of the radial sorting process can be seen in the electron micrograph (bottom panel) of a rat sciatic nerve (post natal day 7). B: axon bundle. S: segregated axon. (1:1): axons in a 1:1 pairing with a Schwann cell. Adapted from Webster, 1971; Webster et al., 1973.
Figure 1.6. Mature myelinating and nonmyelinating Schwann cells. (A-B) Transverse electron micrographs of an adult rat sciatic nerve. (A) Myelinating Schwann cells form compact, multi-lamellar sheaths (M) around individual, large diameter axons (Ax). (B) Nonmyelinating Schwann cell (N-M) ensheathing 13 axons (for example, A) in separate furrows at the cell margins. Nonmyelinating Schwann cells can also form a 1:1 relationship with an individual axon (A*). Collagen-rich extracellular spaces separate the axon-Schwann-cell units (C). Scale bar: 0.5 μm. Adapted from Jessen and Mirsky, 2009.

Myelination offers several biological advantages such as a reduction in energy costs to generate an action potential (Wang et al., 2008), as well as a dramatic increase in conduction velocity which directly correlates with total fiber diameter (Smith and Koles, 1970; Waxman, 1980). Each myelinated segment of an axon is referred to as an internode and internodes are separated by nodes. This arrangement increases conduction velocity in two known ways: saltatory conduction and an increase in axon diameter. Nodes, ~1 μm in length, are populated with an abundance of sodium channel clusters separated by internodes which have a larger capacitance granted by an insulating effect from the myelin sheath (Griffin and Thompson, 2008); combined, these features enable saltation from node to node during an action potential. Myelination is
thought to induce changes in the axonal cytoskeleton, including phosphorylation of neurofilament M (medium) and H (heavy) proteins (Julien and Mushynski, 1983) which results in larger interfilament spacing and therefore an increase in axonal caliber (Hisanaga and Hirokawa, 1989). Myelination also constrains collateral sprouting from myelinated shafts (Huang et al., 2005) and offers a fair amount of axonal protection that is relinquished in demyelinated fibers (Griffin and Thompson, 2008). Perhaps a combination of the advantages granted by myelination explains why the mammalian CNS prefers myelinated nerve fibers instead of unmyelinated axons. The proclivity for myelination in the CNS is well illustrated in the transition zone where the small, unmyelinated axons of dorsal roots enter the dorsal horn of the spinal cord where they acquire myelin (Griffin and Thompson, 2008).

In contrast to the CNS, where even the smallest of fibers are often myelinated, the PNS contains large populations of unmyelinated axons. Unmyelinated nerve fibers were first described by Robert Remak, utilizing teased nerve fibers nearly 2 centuries ago (Remak, 1838). Unmyelinated axons, usually 0.5-1.5 μm in diameter, greatly outnumber myelinated axons by a factor of 4 in the PNS (Ochea and Mair, 1969; Dyck et al., 1971; Holland et al., 1998). Additionally, the intercellular spacing of Schwann cell nuclei between adjacent Schwann cells is far less in unmyelinating Schwann cells (<90 μm) than in myelinating Schwann cells whose internodal distance can range from 200 to 1,500 μm (Aguayo et al., 1973; Aguayo and Bray, 1975). Hence, unmyelinating
Schwann cells, or Remak Schwann cells in this context, immensely outnumber myelinating Schwann cells.

Unmyelinating Schwann cells usually ensheath more than one axon, each of which is sunk into individual mesaxons surrounded by Schwann cell processes (Griffin and Thompson, 2008); generation of these independent mesaxons is thought to depend on NRG1 (Taveggia et al., 2005), the same axon-associated signal that, at higher levels, regulates myelination (Birchmeier and Nave, 2008). Interestingly, in rodents, 10-15% of small non-myelinated axons are radially sorted into a 1:1 relationship with an unmyelinating Schwann cell and in humans almost all unmyelinated axons are sorted in this way (Bert-hold et al., 2005; Sharghi-Namini et al., 2006). Remak bundles include postganglionic sympathetic fibers, C fiber nociceptors, and some preganglionic sympathetic and parasympathetic fibers (Griffin and Thompson, 2008). Currently, the biological relevance for retaining non-myelinated fibers in the PNS is fairly controversial.

Since myelin inhibits collateral sprouting, the absence of myelin permits a high degree of plasticity, as axon shafts and terminals associated with Remak Schwann cells retain an ability to sprout and grow; thus making unmyelinated axons suitable for milieus under constant change (Griffin and Thompson, 2008). This robust plasticity enables nonmyelinated axons to respond quickly to local denervation or assault. For example, following injury or denervation, it is more advantageous for nociceptor fibers to quickly sprout and grow into denervated regions in order to communicate mechanical or thermal danger to the CNS, rather than establish a single point of connectivity (Griffin and Thompson, 2008).
Their abundance and ability to act as “first responders” to injury and disease has brought much attention to this class of Schwann cells that must be thoroughly studied in order to fully understand the developmental and post-injury events that enable peripheral nerve growth and regeneration.

1.8 Positive regulators of myelination

Many cell-extrinsic and/or axon-associated signals have been shown to initiate, promote, and control myelination. For example, axon-associated neuregulin-1type III regulates myelin sheath thickness in vivo (Michailov et al., 2004; Taveggia et al., 2005) and many steroid hormones are suspect in myelin promotion, including glial cell-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), and insulin like growth factor (IGF-1/2) (Cheng et al., 1999; Chan et al., 2001; Hoke et al., 2003; Melcangi et al., 2003; Chan et al., 2004; Schumacher et al., 2004). Pro-myelin Schwann cells synthesize three main structural myelin proteins utilized for compaction of myelin sheaths: myelin protein zero (MPZ or P0), myelin basic protein (MBP) and peripheral myelin protein (PMP22) (Jessen and Mirsky, 1999). Recent advances have shown that this process appears to be regulated by a complex gene regulatory network, balancing the transcriptional control of positive and negative regulators of myelination (Jessen and Mirsky, 2008; Svaren and Meijer, 2008).

Both Sox10 and the zinc-finger protein, Krox-20 (Egr-2), are Schwann cell transcription factors required for myelination (Topilko et al., 1994; Britsch et al., 2001; Schreiner et al., 2001; Topilko and Meijer, 2001). Pro-myelin transcription
factors, whose expression in immature Schwann cells are crucial for the right
timing of myelination, have also been reported, such as octamer-binding
transcription factor 6 (Oct-6, SCIP, Tst-1, or POU3f1) and brain 2 class III POU-
domain protein (Brn-2) (Bermingham et al., 1996; Jaegle et al., 1996). Sox-10 is
essential for Oct-6 expression and the activation of myelin genes in immature
Schwann cells, in addition to its crucial role for the generation of SCPs from
neural crest cells (Britsch et al., 2001; Schreiner et al., 2007). Immature
Schwann cells in Krox-20 null mice establish a 1:1 relationship with axons but do
not produce myelin sheaths, suggesting that radial sorting is not part of the
myelin differentiation program (Topilko et al., 1994). Additionally, Krox-20 is
needed for long-term maintenance of myelin (Decker et al., 2006). On the other
hand, deletion of Oct-6 in mice only transiently delays peripheral myelinogenesis
for 7-10 days, presumably because of a functional redundancy between Brn-2
and Oct-6 (Ghazvini et al., 2002; Jaegle et al., 2003). However, since Schwann-
cells from Krox-20\textsuperscript{-/-} and Oct-6\textsuperscript{-/-} halt at the pro-myelin stage, it is unclear whether
these transcription factors are required for the genes required for myelination or if
the cells are impaired in some way making them incapable of responding to the
drastic metabolic and structural challenges imposed by myelination (Jessen and
Mirsky, 1999).

Schwann cell myelination can be mimicked, even in the absence of axons,
in vitro by the addition of cyclic AMP (cAMP), whose signaling activates myelin
genes, to isolated Schwann cells (Lemke and Chao, 1988; Morgan et al., 1991).
Recent in vitro studies have shown that cAMP signaling is mediated, at least in
part, by NF-κB and CREB (Jessen and Mirsky, 2008; Yoon et al., 2008); however, these results have yet to be confirmed in an in vivo model.

1.9 Negative regulators of myelination

Myelination is a complex process that is currently believed to be regulated by a delicate balance between positive and negative signaling systems. In other words, myelination not only requires an elevation of pro-myelin genes, but also hinges on the suppression of genes that inhibit myelin production. These negative regulators are thought to drive the de-differentiation program in Schwann cells, following injury, as they de-differentiate and revert to a phenotype reminiscent of immature Schwann cells and later re-associate with, and re-myelinate regenerating axons (Jessen and Mirsky, 2008). In this relatively new model, which is rapidly gaining supporting evidence, negative transcriptional regulators of myelination are loosely defined by a few characteristics: potentially active in immature Schwann cells prior to myelination; latent in myelinating Schwann cells; activated upon denervating conditions in order to induce Schwann cell dedifferentiation; suppress pro-myelin signals (Jessen and Mirsky, 2008). Although more is known about the positive regulators of myelination, several candidates for negative regulation have been examined, including the transcription factors c-Jun, Pax-3, Sox-2, Krox-24, Egr-3 as well as the transcriptional regulators Notch and Id2 (for review, see Jessen and Mirsky, 2008). Only two of the aforementioned prospects have been confirmed to function as negative regulators of myelination in vivo: c-Jun and Notch.
c-Jun is a crucial component of the AP-1 transcription factor complex and plays a role in a diverse array of cellular processes that typically require N-terminal phosphorylation of c-Jun by Jun N-terminal kinases (JNKs) (Mechta-Grigoriou et al., 2001; Jessen and Mirsky, 2008). Immature Schwann cells during perinatal nerve development express c-Jun which is subsequently suppressed within cells as Krox-20 is activated, driving pro-myelination and then myelination (Parkinson et al., 2004, 2008). Additionally, in Krox-20 null nerves, where myelination is inhibited, c-Jun is robustly expressed (Jessen and Mirsky, 2008). Following nerve injury, c-Jun is quickly upregulated in denervated Schwann cells (De Felice and Hunt, 1994; Shy et al., 1996; Parkinson et al., 2008) and is required for Schwann cell dedifferentiation (Arthur-Farraj et al., 2007); as illustrated by the findings that c-Jun null Schwann cells fail to digest myelin properly, fail to suppress myelin genes, and fail to activate normal amounts of proteins associated with denervated Schwann cells (Arthur-Farraj et al., 2007). Interestingly, myelination is relatively unimpaired in these animals during development, perhaps because c-Jun is normally suppressed during myelination during development.

Notch is a transmembrane receptor whose effects on Schwann cell development and dedifferentiation are strongly dependent upon what stage in the lineage it is expressed. After binding to a ligand, Notch is cleaved and its intracellular fragment, known as the Notch intra-cellular domain (NICD), regulates transcription (Schweisguth, 2004). Notch is required for the correct timing of the transition from SCPs to immature Schwann cells and also controls
proliferation within embryonic nerves (Woodhoo et al., 2007). Postnatally, Notch negatively regulates myelination; in fact, if NICD expression is briefly upregulated near birth, myelination will be delayed (Woodhoo et al., 2007). Levels of NICD substantially rise within distal stumps of transected nerves in order to promote Schwann cell dedifferentiation and effective myelin degeneration (Woodhoo et al., 2007).

1.10 Peripheral nerve injury, regeneration and repair

The dichotomy of the mammalian nervous system is well illustrated in the regenerative capacity of peripheral nerves, unlike axons residing in central nerve tracts. Written descriptions of peripheral nerves can be found as early as the fourth century B.C. in Hippocrates’ manuscripts (Adams, 1868); by the second century A.D., the first descriptions of nerve regeneration and repair had been documented by Galen (Terzis et al., 1997). Since these early anecdotes and reports, scientists have been trying to understand the regenerative potential of the peripheral nervous system in hopes of manipulating and enhancing regeneration within the central nervous system. But peripheral nerve traumas can be devastating themselves.

Although peripheral nerve traumas and diseases usually present no threat to a patient's vitality, they occur rather frequently and often impose immense socioeconomic costs as well as impact the quality of life (Evans, 2001; Ruijs et al., 2005; Midha, 2006). Despite our technical advancements in nerve reconstruction, arguably dating as far back as the sixteenth century in Gabriele
Ferrara’s seminal description of how to suture a severed nerve (Artico et al., 1996), complete recovery and restoration of nerve function is almost never seen and almost always results in a poor clinical outcome (Lundborg, 2002; Gordon et al., 2003; Battiston et al., 2005; Hoke, 2006; Casha et al., 2008). Today, scientists and surgeons are taking many approaches, including reconstructive and tissue engineering, to improve the functional outcome of peripheral nerve trauma that may also elucidate causes of debilitating nerve diseases.

Following traumatic injury of a peripheral nerve, many complex pathophysiologic changes take place at the site of injury and within the nerve segments located proximally (closer to cell body) and distally to the injury site. Additionally, dramatic changes take place in the axonal cell bodies and in terminal muscle endplates and sensory receptors (Geuna et al., 2009). Although the signals that initiate regeneration are unknown (Makwana and Raivich, 2005), extensive studies within each compartment and cell type associated with injury have revealed what a complex multi-faceted process peripheral nerve regeneration entails and has resulted in many enticing theories.

1.11 Intrinsic growth capacity of peripheral neurons

Axons are large cellular structures that require a combined support of their neuronal cell bodies and the glial cells with which they associate (Coleman and Freeman, 2010). Degeneration will occur if axons cannot transport materials from their cell bodies (Coleman, 2005) or in the absence of glial cell support (Nave and Trapp, 2008). Axons are currently thought to initiate their own
degredation and actively regulate their own destruction in a process distinct
from apoptosis (Deckwerth and Johnson, 1994; Buckmaster et al., 1995; Burne
et al., 1996; Finn et al., 2000; Raff et al., 2002).

Transecting an axon amputates a large amount of axoplasmic volume
and, within several hours, results in chromatolysis of the neuron soma; this
process entails soma swelling, nucleolar swelling, and nuclear eccentricity
(Lieberman, 1971). The metabolic machinery of the injured neuron shifts its
concern from generating nerve impulses to creating structural components for
remodeling and reconstructing the injured nerve (Ducker et al., 1969; Lieberman,
1971). Nerve injury also results in the activation of intrinsic growth capacity
which has been extensively studied in the dorsal root ganglion (DRG) which has
two axonal branches that respond differently to injury (Chen et al., 2007).
Peripheral branches spontaneously regenerate and overcome the halting effects
of myelin-associated inhibitory molecules, whereas central branches, which enter
the CNS through the spinal cord, do not regenerate following injury (Chen et al.,
2007).

Several molecules upregulated in neurons following nerve injury are
strong candidates for the initiation of regeneration. For example, cyclic
adenosine monophosphate (cAMP) signaling through protein kinase A (PKA)
activity promotes regeneration and is transcriptionally regulated through cAMP
response element binding protein (CREB) (Cai et al., 2002; Gao et al., 2004).
Conditional knockout of the transcription factor c-Jun in neurons significantly
reduces axonal regeneration (Raivich et al., 2004). Other regeneration
associated genes upregulated in neurons following injury include Arginase 1, GAP-43, Integrin α7β1, CD44, and Galanin (Chen et al., 2007). These genes differentially promote regeneration by enhancing neurite outgrowth (Schreyer and Skene, 1991; Tetzlaff et al., 1991) and cytoskeletal assembly (Tetzlaff et al., 1998; Fornaro et al., 2008). Although the intrinsic growth capacity of peripheral neurons increases their regenerative ability, they must also grow through a permissive environment and must have the appropriate axon guidance cues reach the correct target tissue and restore function (Chen et al., 2007). The environmental factors promoting regeneration are primarily governed by Schwann cells which behave differently depending on whether they reside in an environment proximal or distal to the injury site.

1.12 Post injury events in the proximal nerve segment

Following injury, axons in the proximal nerve segment retrogradely degenerate from the injury site, sometimes over one or several internodal lengths depending on the severity of the injury, and leave behind corresponding endoneurial tubes as empty cylinders (Cajal, 1928). In only a matter of hours, proximal axons generate collateral and terminal sprouts that grow distally/longitudinally inside of the basal lamina (Mira, 1984; Fawcett and Keynes, 1990). These newly sprouted neurites advance through the injury site and are pruned down in numbers when they reach and reinnervate the endoneurial tubes of the distal stump (Donnerer, 2003). This process is facilitated by and rate-dependent upon the extension of elaborate Schwann cell processes that arrange to form physical conduits to guide axons to their final targets (Son and
Thompson, 1995). Regardless of whether an axon was previously myelinated or is currently myelinated in the proximal stump, the regenerating sprouts will lack myelin until a later stage (Flores et al., 2000). Regenerating axons will preferentially reinnervate endoneurial tubes in the distal stump over neighboring tissues (Brushart et al., 1998; Rajan et al., 2003; Redett et al., 2005; Kovacic et al., 2007).

1.13 Post injury events in the distal nerve stump

Shortly after nerve transection, the axonal membranes fuse their ends to protect their intracellular compartments from the extracellular environment (Geuna et al., 2009). Within the first days following transection, the distal segment will undergo degeneration known as Wallerian degeneration, a process originally described by and christened after Augustus Waller’s milestone observations in 1850 documented in his paper entitled *Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres* (Waller, 1850). Wallerian degeneration starts immediately following injury and involves the granular disintegration of axonal cytoskeleton, microtubules, and neurofilaments due to proteolysis (Vial, 1958; Scchlaepfer, 1977; Lubinska, 1982). Axon fragmentation is evident by the characteristic myelin ovoids left behind and can easily be seen on the light level in nerve cross sections. Schwann cells and macrophages phagocytize and clear away all of the myelin and cellular debris over a period of 3-6 weeks; this is a crucial part of Wallerian degeneration that renders a permissive regenerative environment (Geuna et al.,
A similar process occurs with unmyelinated axons in mammals and invertebrates (Macdonald et al., 2006; Ayaz et al., 2008; Avery et al., 2009).

Regenerating axons that have reached the distal stump are aided by neurite outgrowth promoting factors including laminin and fibronectin (Baron-Van Evercooren et al., 1982; Liu, 1996; Hall, 1997). Cell adhesion molecules also play a pivotal role in regeneration, such as N-CAM, L1, myelin associated glycoprotein (MAG), and tumor-associated glycoprotein (TAG)-1 (Daniloff et al., 1986; Walsh and Doherty, 1996). If axons regenerate within basal lamina tubes (Schwann cell columns) they will likely be guided to their correct target, however, axons can also grow into the connective tissue of the nerve (Chen et al., 2007; Geuna et al., 2009). An excessive number of regenerating sprouts invade the distal stump compared to the number of axons in the proximal segment (Sanders and Young, 1946; Aguayo et al., 1973; Povlsen and Hildebrand, 1993). As a result of trophic supply, several axons reaching appropriate distal target organs will mature and enlarge to an almost normal sized diameter (Sanders and Young, 1946). Axons that do not reach their target will be pruned away (Griffin and Hoffman, 1993). A few months after nerve regeneration, the nerve trunk will reorganize into a large number of small compartments, each enveloped with new perineurium (Geuna et al., 2009).

There are several factors involved in peripheral regeneration, such as neurotrophic factors, cytokines, and transcription factors. NGF, NT-3, and BDNF have all been shown to induce axon growth in vitro (Zhou et al., 2004; Yoshimura et al., 2005; Zhou et al., 2006). Although their role in peripheral nerve
regeneration is unclear, NGF and BDNF levels are elevated following injury (Markus et al., 2002; Snider et al., 2002; Makwana and Raivich, 2005). Mice lacking the cytokine interleukin-6 (IL-6) exhibit sensory defects and delayed regeneration of sensory axons following crush injury (Zhong et al., 1999). Additionally, leukemia inhibitory factor (LIF) knockout mice show impaired regeneration after peripheral nerve injury (Cafferty et al., 2001). Signal transducer and activator of transcription 3 (STAT3) is a transcription factor required for the survival of injured motor neurons (Schweizer et al., 2002). During nerve regeneration, STAT3 promotes the transduction of IL-6 and LIF (Chen et al., 2007).

1.14 Post-injury Schwann cell activity

Nerve transection triggers a dramatic series of complex changes in the nerve segment distal to injury (Fig. 1.7). Wallerian degeneration ensues as follows: axons degenerate and die, blood-borne macrophages invade the injured nerve, myelin sheaths collapse and myelin debris is ingested by Schwann cells and macrophages, Schwann cells briefly proliferate and undergo a reversal of their molecular expression from a mature phenotype back to one reminiscent of an immature state (Fu and Gordon, 1997; Scherer and Salzer, 2001; Stoll et al., 2002; Chen et al., 2007; Raivich and Makwana, 2007; Vargas and Barres, 2007; Jessen and Mirsky, 2008). The phenotypic reversion of mature Schwann cells is known as dedifferentiation and is a key feature of successful regeneration.
In myelinating Schwann cells, dedifferentiation involves a downregulation of genes related to myelination, such as cholesterol synthesizing enzymes, myelin structural proteins (e.g., P0, MBP), and membrane associated proteins (e.g., MAG, periaxin) (Nagarajan et al., 2001, 2002; Verheijen et al., 2003; Buchstaller et al., 2004; Leblanc et al., 2005; D’Antonio et al., 2006). This is paralleled by the activation of molecules normally found active in immature Schwann cells during development, such as L1, NCAM, p75 low affinity neurotrophin receptor (p75NTR), and glial fibrillary acidic protein (GFAP) (Scherer and Salzer, 2001). Additionally, de-differentiated Schwann cells activate growth factors (e.g. NGF, BDNF, GDNF) and cytokines (e.g. TNF-α, IL-1β, IL-6, LIF, MCP-1) to aid in the regenerative process (Meyer et al., 1992; Curtis et al., 1994; Shamash et al., 2002; Boyd and Gordon, 2003).
Although de-differentiated Schwann cells and immature Schwann cells share many similarities, their molecular phenotypes are not identical. Immature Schwann cells express extremely low levels of N-Cadherin in vivo, whereas N-Cadherin is robustly activated in injured nerves (Thornton et al., 2005; Wanner et al., 2006). Immature Schwann cells do not express integrin α1β1, but this integrin can be found in de-differentiated Schwann cells (Stewart et al., 1997). In opposition, immature and both classes of mature Schwann cells express the lipid antigen 04 which is suppressed in de-differentiated Schwann cells (Mirsky et al., 1990). Because of these differences, many terms have been used to refer to Schwann cells in an injured environment and are often used interchangeably: denervated Schwann cells, immature-like Schwann cells, activated Schwann cells, and de-differentiated Schwann cells.

The transcriptional regulatory programs responsible for Schwann cell dedifferentiation, activation of injury related genes, and myelin ingestion remain largely unknown. Recent studies have shown that negative transcriptional regulation of myelin-related genes can drive the dedifferentiation program in myelinating Schwann cells upon their loss of axonal contact (Jessen and Mirsky, 2008). Two of the most well described negative gene regulators of dedifferentiation are c-Jun and Notch, both of which are both expressed during development prior to myelination (Jessen and Mirsky, 2008). Expression and activation of transcriptional regulators and transcription factors can be controlled by extrinsic signals, however, very little is known regarding integration of
transcriptional, cytoplasmic, and cell-extrinsic cascades in Schwann cells (Jessen and Mirsky, 2008).

Nerve injury is not the only way to induce Schwann cell dedifferentiation; in fact, adult myelinating Schwann cells dissociated from axons and incubated in simple culture media will halt myelin protein synthesis and re-express many immature Schwann cell markers (Jessen and Mirsky, 2008). Comparisons between Schwann cells de-differentiated in vitro and those de-differentiated following injury in vivo will be invaluable; many conclusions about immature Schwann cells and development are derived from in vitro cultures which may better mirror the mechanisms governing denervated Schwann cells and regeneration in vivo. It seems that mechanical dissociation of adult nerves constitutes an injury.

1.15 Facial nerve injury

The facial nerve, also known as the seventh cranial nerve, contains approximately 3600 motoneurons whose cell bodies reside in one of seven facial motor subnuclei in the mouse brainstem (Moran and Graeber, 2004). Axons within this bilateral nerve exit the skull at the stylomastoid foramen and innervate muscles involved with lip, eyelid, ear, and whisker hair movement (Fig. 1.8). Due to these characteristics, paired with the knowledge that lesioned peripheral nerves show robust regeneration, the facial nerve axotomy model has become a well-established system to study neuronal regeneration.
Figure 1.8. Musculotopic organization of mouse facial nucleus. The adult mouse facial motor nucleus (FMN) is located in the brainstem at the pontine level of the medulla and can be divided into seven distinct subnuclei (top diagram). Facial motor neuron cell bodies reside in the FMN and innervate different regions of the face; these corresponding regions can be seen in the bottom diagram. The size of each innervated region is distorted to indicate the size or amount of neurons that supply it. nl: nasolabial musculature. oo: orbicularis oculi muscle. m/p: mentalis and associated portions of platysma. pa: posterior auricular musculature. pd: posterior belly of digastrics muscle. st: stapedius muscle. Adapted from Ashwell, 1981.
By unilaterally transecting the facial nerve, leaving the contralateral side as an uninjured control, one can study key molecular, ultrastructural, and cellular responses associated with target reinnervation and subsequent functional recovery within the same animal. Peripheral nerve reinnervation is quite rapid following a crush lesion taking approximately 2-3 weeks and an additional week for reinnervation following complete transection (Moran and Graeber, 2004). In this model system, functional recovery is relatively simple to assess by monitoring the return of blink reflexes, vibrissial sweeping functions and loss of vibrissial fibrillations (Hadlock et al., 2009).

The facial nerve regenerates and behaves in a similar fashion to peripheral nerves ensheathed in SCs, despite the facial nerves central glial environment from which it originates (Choi and Dunn, 2001). In fact, data obtained from peripheral nerve studies in the arm and leg are applied to facial nerve repair techniques (Choi and Dunn, 2001). Since the peripheral component of the facial nerve is of primary interest, the facial nerve will be referred to as a peripheral nerve, instead of a cranial nerve, in the studies described in later chapters.

Since the facial nerve has a relatively long anatomical course within the cranium and is located superficially in the face, it is the most liable cranial nerve to damage (Diamond C, 1979; Dai et al., 2000); hence, facial nerve palsy is a rather common clinical problem. Damage to the facial nerve is typically a result
of road traffic-accidents, infectious diseases, intracranial compression from a tumor, or damage during surgical manipulations (Moran and Graeber, 2004). Facial nerve injury is rather debilitating considering its numerous muscular targets. Understanding how glia contribute to regeneration of a motoneuron in a mouse model will lend insight into the regeneration program in humans and hopefully aid in clinical reparations of human injuries.

1.16 Nuclear factor κB (NF-κB) and nerve injury

NF-κB is a ubiquitous transcription factor which regulates many physiological processes, including neuroinflammation, apoptosis, neuronal plasticity, cell survival, growth and proliferation (Pollock et al., 2005). Under normal circumstances, NF-κB remains quiescent in the cytoplasm as homodimer or heterodimer of variable subunits (p65, relB, c-rel, p50, and p52) bound to an inhibitory protein, inhibitor of κB (IκB). Following injury, IκB is phosphorylated and undergoes proteosomal degradation, a process that unmasks the nuclear localization signal of NF-κB enabling translocation to the nucleus and hence regulation of nearly 400 genes (Bethea et al., 1998; Ahn and Aggarwal, 2005). Since p65 knock out animals die before birth (Beg et al., 1995) and p50 knockouts suffer severe immunological complications (Sha et al., 1995), in vivo studies of NF-κB have been fairly limited. However, an upregulation of NF-κB activity following peripheral nerve injuries has been reported (Ma and Bisby, 1998; Fernyhough et al., 2005; Pollock et al., 2005; Fu, et al. 2010). Functional inhibition of NF-κB in GFAP-expressing cells reduces inflammation, improves functional recovery, and increases axonal sparing and sprouting following spinal
cord injury (Brambilla et al., 2005, 2009). Additionally, improved axonal sprouting 3 days following sciatic nerve crush by inhibiting NF-κB (p50) activity with a TNF-α inhibitor was recently demonstrated (Smith et al., 2009). Surprisingly, the long term effects of glial NF-κB activation on peripheral nerve regeneration and remyelination remain unexplored in vivo.

1.17 GFAP-IkBα-dn mice

In order to study the astroglial role of NF-κB following spinal cord injury (SCI), Brambilla et al. generated a line of transgenic mice (Fig. 1.9), whereby classical NF-κB activity was functionally repressed in GFAP-expressing cells. Driven by the human GFAP promoter (gfa2), the cDNA encoding a truncated form of the human IκBα gene was overexpressed in order to prevent NF-κB nuclear localization and subsequent gene regulation. By functionally knocking-down NF-κB activity in CNS astrocytes and non-myelinating Schwann Cells, found within peripheral nerves, the authors reported reduced expression of proinflammatory chemokines, increased neuroprotection and improved functional recovery following SCI.
Figure 1.9. Transgenic approach to study the pathophysiology of NF-κB in GFAP-expressing glia. To study the role of NF-κB in GFAP-expressing glia following nervous system traumas, transgenic mice whereby the classical NF-κB signaling is functionally inhibited in GFAP-expressing cells. Under normal circumstances, NF-κB heterodimers, typically p65/p50 heterodimers, form a complex with the IκBα protein which masks the nuclear localization signal (NLS) preventing nuclear translocation and transcriptional regulation (top pathway of flow chart). Following injury or stimulation by cytokines, neurotrophins, and/or environmental factors, the signalsome (a complex formed by IKKα, IKKβ, and IKKγ) will phosphorylate IκBα resulting in ubiquitination and proteosomal degradation; this unmasks the p65/p50 NLS allowing nuclear translocation and regulation of target gene expression. To generate transgenic mice, cDNA encoding a truncated form of human IκBα, lacking two crucial serine phosphorylation sites, was overexpressed using a human GFAP promoter (gfa2). Following stimulation of canonical NF-κB signaling, IκBα does not get phosphorylated and proteosomally degraded keeping NF-κB heterodimers latent in the cytoplasm (bottom pathway of flow chart). This results in a functional inhibition of NF-κB signaling in GFAP-expressing cells.
Chapter 2: Materials and Methods

2.1 Animals

2-4 month old, male mice were used throughout this study and were generated by breeding heterozygous GFAP-IκBα-dn males with WT females on a C57BL/6 background in order to produce progeny lacking functional activation of canonical NF-κB in GFAP-expressing cells (Brambill et al., 2005). Animals were housed in a virus/antigen-free facility on a 12 hr light/dark cycle with food and water provisions ad libitum. Genotyping of tail DNA was performed as previously described (Brambilla et al., 2005; Bracchi-ricard et al., 2008).

2.2 Surgeries

All surgeries were performed in the Animal and Surgical Core Facility at the Miami Project to Cure Paralysis under protocols approved by the University of Miami Animal Care and Use Committee. All animals were anesthetized with ketamine (.1 mg/g)/xylazine(7.5 µg/g) (University of Miami, Division of Veterinary Resources).
2.3 Facial Nerve Trunk Surgeries

For facial nerve regeneration studies, the right facial nerve was completely severed/transected where it exited the stylomastoid foramen, approximately 1 mm proximal/caudal to trifurcation; or the buccal branch nerve fibers of the right facial nerve were uniformly crushed, ~4 mm distal/rostral to trifurcation from the facial nerve trunk until completely transparent, using ground microforceps. Animals were sacrificed 10 or 31 days following injury.

2.4 Mandibular Nerve Surgeries

For retrograde studies to determine the contributing motor neuron population within the mandibular branch, the following two surgical procedures were performed. The upper marginal mandibular branch of an adult, male, wild-type mouse was unilaterally transected, distal to its divergence from the main mandibular nerve trunk (Fig. 2.1A). The proximal nerve stump was incubated in a 2% (in water) Fluorogold (FG) solution for 40 minutes. Three days later, the brainstem was extracted to quantify the total number of FG+ motor neurons within the facial motor nucleus. Additionally, the marginal mandibular branch of an adult, male, wild-type mouse was unilaterally transected proximal to bifurcation (Fig. 2.1B). The proximal nerve stump was incubated in a 2% fluorogold solution for 40 minutes. Three days later, the brainstem was extracted to quantify the total number of FG+ motor neurons within the facial motor nucleus.
Figure 2.1. Schematic of facial nerve transections of the marginal mandibular branch of the facial nerve. The upper division (A) or the entire (B) marginal mandibular branch was completely transected. TEM: temporal. ZYG: zygomatic. BUC: buccal. MAR MAN: marginal mandibular branches, upper (U) and lower (L) divisions. Diagram (top) adapted from Moran and Graeber, 2004.

2.5 Surgery for Colabeling

For colabeling studies, WT animals were anesthetized with ketamine/xylazine and the right marginal mandibular branches were transected proximal to bifurcation and intubated in Fast Blue (FB) (2% in water) for 40 minutes. Three days later, the buccal nerve of anesthetized mice was uniformly crushed, ~4 mm distal to trifurcation from the facial nerve trunk. The buccal branch was then transected, distal to injury, 21 days after the crush injury. The proximal stump was then incubated in a 2% FG solution. Three days later, animals were sacrificed.

2.6 Pinch Crush Injury

Mice underwent a unilateral crush injury (blue forceps) of the right buccal nerve until completely transparent, ~ 4 mm distal to its diversion from the facial
nerve trunk, using ground microforceps (Fig. 2.2). Animals were sacrificed 1, 2, 4, 12, 31, or 65 days following injury.

Figure 2.2. Facial nerve crush injury model. Schematic representation of facial nerve crush model. For tracer studies, following variable recovery times the mandibular branches were surgically removed and Fluorogold (yellow dye) was administered subcutaneously into the whisker pad.

2.7 Facial Nerve Axotomy to Assess Wallerian Degeneration

WT and transgenic littermates were anesthetized with ketamine/xylazine. Subsequent to facial nerve exposure, the buccal branch of the right facial nerve was completely transected ~4mm distal to trifurcation from the trunk. The proximal stump of the transected nerve was sutured (Ethicon 10-0 monofilament) to adjacent, underlying muscle tissue at a 90-120 angle to prevent reinnervation of the distal nerve segment (Fig. 2.3). Animals were allowed to recover with food and water ad libitum for 4 or 12 days. At each respective time point, animals
were sacrificed with a lethal dose of anesthesia and distal nerve stumps were removed and post-fixed for analysis of Wallerian degeneration. A 3mm segment distal to the site of transection was removed for CD11b (1:100; Serotec) immunostains and the remaining ~2mm nerve segment was collected for TB/PPD(p-Phenylenediamine) histology.

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**Figure 2.3. Buccal nerve transection model to assess Wallerian degeneration.** Schematic representation of facial nerve transection model. The buccal nerve is unilaterally transected (red scissors), ~4 mm distal to its divergence from the facial nerve trunk. The proximal segment is the sutured (blue X) to proximal muscle tissue in order to prevent target regeneration. Following injury, 4 or 12 days, the distal buccal nerve segment was surgically removed. Longitudinal sections spanning the whole distal segment and/or transverse semi-thin sections (blue rectangle), ~4 mm distal, were used to assess Wallerian degeneration.

Semi-thin (1 µm), transverse sections of distal (5mm distal to transection) injured nerve stumps were collected, stained with PPD and counterstained with TB. Using StereolInvestigator, myelin rings undergoing demyelination, and intact myelin rings were counted from several random sites (25x25 µm² counting frame; 75x75 µm² grid). Myelin rings exhibiting severe lamellae in/out-foldings,
tethering, myelin debris, vacuolization, incisures and/or collapsed axoplasm were considered demyelinated. Total population projections of each identifier were compared between WT and transgenic littermates at the respective time points following injury.

2.8 Behavioral Testing

Following facial nerve transection ~1 mm caudal to trifurcation, vibrissae movement was completely abolished ipsilateral to injury and sustained contralateral to injury. Prior to FG administration, vibrissae behavior was carefully assessed 28 days following injury and scored on a scale from 0, indicating no movement, to 3, denoting robust, normal whisker sweeping, as previously described (Raivich et al., 2004). Motor scores were assessed by monitoring whisker movement, in the absence of whisker stimulation, in an open field for 60 seconds. A score of 1 was received for minimal movement, and a score of 2 was received for moderate vibrissae movement. All animals demonstrated normal (3) vibrissae movement on the uninjured side.

2.9 Retrograde Tracing

Gel foam pads, pre-soaked in 20 µl of a 4% FG (Fluorochrom, Denver, CO) solution, were inserted for 20 minutes beneath the ipsilateral and contralateral whisker pads 28 days after unilateral facial nerve transection, as previously described (Werner et al., 2000). Three days later, the total number of FG⁺ MNs within the facial motor nucleus (FMN) were counted in 6-8 sections by a single investigator, blinded to genotype and expressed as a ratio
Images were obtained using a 20X objective on a Zeiss Axiovert 200M fluorescent microscope with Neurolucida software.

Following buccal nerve crush injury, axonal sparing and whisker pad re-innervation were assessed by injecting 2 μl of a 4% FG solution subcutaneously into both whisker pads immediately or 9, 28, and 62 days following injury, respectively (Fig. 2.2). To prevent labeling of non-buccal-associated motor neurons in the lateral and intermediate FMN, the right mandibular branches were surgically removed immediately before injections. After 48 or 72 hours, animals were transcardially perfused with a 4% paraformaldehyde solution in 0.1 M PBS, cryoprotected in 20% sucrose in 0.1M PBS, and cut into 20 μm coronal sections spanning the FMN; fluorescently labeled motoneurons within the FMN were quantified by a single investigator under double blind conditions using unbiased Stereo Investigator software (Stereo Investigator; MicroBrightField, Williston, VT, USA). The total number of FG+ MNs in the FMN ipsilateral to injury were compared following facial nerve crush. Images were obtained using a Leica TCS SP5 Confocal Microscope at 40X.

2.10 Immunohistochemistry

As previously described (Bracchi-ricard et al., 2008), animals were transcardially perfused; brainstems and a ~4 mm segment containing the injury site from the buccal branch of the facial nerve was removed and fixed for 20 min prior to cryoprotection. Longitudinal sections were cut at 16 μm and incubated overnight at 4°C with a mouse antibody against NF-H (1:3000; Covance), p65,
phosphoSer276 (1:400; Millipore), GFAP (1:1000; BD Pharmingen), MPZ (1:100; Abcam) or CD11b (1:100; Serotec) followed by a species specific secondary fluorescent antibody: Alexa Fluor 488 (1:750; Molecular Probes), Alexa Fluor 546 (1:750; Molecular Probes) for 1 hr at room temperature. Confocal images were acquired on a Zeiss LSM 510 confocal microscope with a 20X objective or 40X oil objective and LSM imaging software.

2.11 Facial Motor Neuron Counts

One month following transection, coronal sections spanning the FMN were prepared as described above (see Retrograde Tracing). Sections were incubated overnight in EtOH/Chloroform (1:1), rehydrated and placed in a 0.5% Cresyl Violet acetate (SIGMA) solution for 20 seconds to label MNs. Using Stereo Investigator software, the total number of Cresyl Violet MNs were quantified by a single investigator, under double blind conditions, in both facial motor nuclei and expressed as a ratio (Injured/Uninjured). The average motor neuron cell body size (area) was determined on each MN counted by measuring the soma diameter using the nucleator probe with 4 nodes and expressed in $\mu$m$^2$.

2.12 Macrophage Quantification

To quantify the number of macrophages present in degenerating nerves, longitudinal fresh frozen sections (20 µm), distal to transection, were collected and stained with a rat-anti-CD11b antibody and DAPI. Using StereoInvestigator,
CD11b⁺ cells whose nuclei were contained and/or overlapped with the "acceptance region" of a 25x25 µm² counting frame were counted in 4-9 serial sections/animal/timepoint. The estimated total number of CD11b⁺ cells/mm² were recorded in WT and transgenic animals. Images were acquired with a Zeiss LSM 510 confocal microscope and LSM imaging software.

2.13 Toluidine Blue and Electron Microscopy Tissue Preparation

Following perfusion, a 2 mm nerve segment distal to the crush injury site was fixed in EM fixative (2% Glutaraldehyde/0.05M PO₄/100mM Sucrose) and post-fixed in 2% O₃O₄/0.1M PO₄ for 1 hr at room temperature at the EM core facility in the Miami Project to Cure Paralysis as previously described (Xu et al., 1995). Following dehydration, tissues were placed in fresh resin molds and incubated overnight at 64°C. Using a Leica Ultracut E microtome, semi-thin and ultra-thin sections were cut transversely from the rostral portion corresponding to a cross section of the buccal nerve 4 mm distal to the injury site, and stained with Toluidine Blue. Toluidine Blue images were acquired on a Zeiss Axiovert 200M microscope with a 63X oil objective and Neurolucida Imaging software. Electron micrographs were obtained with a Philips CM-10 transmission electron microscope.

2.14 Morphometric Analysis

The total number of myelin rings were acquired using the optical fractionator probe (Stereo Investigator software) in transverse Toluidine Blue stained sections distal to injury with a 63X, oil objective. All ultrastructural
analysis associated with myelin was determined by hand measurements (mm) from 20-28 randomly selected EM micrographs spanning the nerve and calibrated using an EM ultrastructural calculator. The vertical and horizontal diameter of the inner mesaxon were recorded and averaged to obtain axonal diameter (AxD). Similarly, the vertical and horizontal diameter of the outer lamellae were averaged to determine fiber diameter and ring thickness ((outer sheath diameter-axonal diameter)/2). For each myelinated axon, the gRatio was calculated: (axonal diameter)/(fiber diameter). All other ultrastructure descriptors were acquired by hand from 20-28 EM micrographs, including the number of SC nuclei, Remak bundles, umyelinated axons and organized Remak bundles. Values obtained were normalized to the total number of photos assessed prior to statistical analysis.

2.15 Western Blot Analysis

Distal tissues from the buccal nerve were disrupted in 200 µl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% SDS, 0.5 mM dithiothreitol) containing complete protease inhibitor (Roche, Indianapolis, IN, USA) and phosphatase inhibitors (phosphatase inhibitor cocktails I and II, SIGMA) as previously described (Bracchi-ricard et al., 2008; Monje et al., 2008). SDS sample buffer was added to supernatants followed by 10 min incubation at 100°C. 10 µl of protein samples were resolved on a 12.5% SDS-polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane (BioRad). All membranes were blocked in 5% BSA in TBS-T for 1 hr at room temperature and subsequently incubated
with the following antibodies (1:1000) in TBS-T at 4°C overnight: anti-MBP (monoclonal rat; Millipore), Lamin A/C (polyclonal rabbit; Cell Signalling), c-Jun (polyclonal rabbit; Santa Cruz Biotechnology, Inc.), MPZ (Covance), Krox-20 (rabbit polyclonal; a kind gift from D. Meijer, Erasmus University Medical Center). Blots were incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham Biosciences), anti-rat (SIGMA), or anti-chicken (Jackson ImmunoResearch Laboratories, Inc.) secondary antibodies (1:2000) in 3% BSA diluted in TBS-T for 1 hr at room temperature. Immunoreactive proteins were detected using a SuperSignal West Pico Chemiluminescent kit (Thermo Scientific). Densitometric quantification was performed using Quantity One software (BioRad) and normalized against Lamin A/C and expressed as a percentage of WT naïve. The total number of animals used for this procedure was between 3 and 7 per time point.

2.16 Real-time RT-PCR

Total RNA was extracted from distal, buccal nerve tissues with an RNeasy Micro Kit (Qiagen). For verification of transgene expression within the FMN, total RNA was extracted from brainstem tissue homogenates from the mid-pontine level of the brainstem; the cerebellum and a majority of the brainstem tissue near the FMN was removed. cDNA was generated with a Sensiscript RT Kit (Qiagen), according to manufacturer’s instructions, using total RNA as a template. A Rotor-Gene 3000 Real Time Cycler (Corbett Research) was utilized for Real-time RT-PCR on cDNA, along with a Quantitect SYBR Green PCR kit (Qiagen). Relative gene expression was calculated upon comparison with a
standard curve generated for each respective gene and subsequently normalized to 18S. The following primers were used: 18S (forward: 5'-
GAACTGAGGCCATGATTAAGAG-3'; reverse: 5'-
CATTCTTGCAATGCTTTC-3');

GFAP (forward: 5’- AGAAAGGTTGAATCGCTGGA-3'; reverse: 5’-
CGGCATAGTCTGTTAGCTTCC-3'; SIGMA); IκBα-dn (forward: 5’-
TTCATAAAGCCCTCGCATCC-3'; reverse: 5’-ACAGCCAGCTCCCAGAAGTG-3'). β-actin (forward, 5'-ATG GTG GGA ATG GGT CAG A-3'; reverse, 5’-CAC
GCA GCT CAT TGT AGA AGG-3').

2.17 Statistical Analysis

For single comparisons, a two-tailed, unpaired Student’s t test was applied; p < 0.05 determined significance. A one-way ANOVA was performed for multiple comparisons; p <0.05 determined significance.
Chapter 3: NF-κB activation in Schwann cells is crucial for regeneration and re-myelination

3.1 BACKGROUND

In the peripheral nervous system (PNS), Schwann cells (SCs) myelinate axons which enables efficient electrical conduction and protection from the extracellular environment. During mouse peripheral nerve development, neural crest cells undergo three primary transitions to become mature SCs. Neural crest cells give rise to Schwann cell precursors (E12-13) which associate with axons and quickly transition into immature Schwann cells (E 15-16) before birth (Jessen and Mirsky, 2005). Immature Schwann cells pervading perinatal nerves are partly characterized by expression of glial fibrillary acid protein (GFAP). Random association with axons of variable size dictates subsequent maturation; those associated with large diameter axons (>~1 μm) will adopt a myelinating phenotype and relinquish GFAP synthesis. Immature SCs associated with small diameter axons mature into non-myelinating (unmyelinating) SCs and maintain GFAP expression throughout adulthood (Jessen and Mirsky, 2005).

Following peripheral nerve injury, adult SCs cease myelination, ingest myelin and subsequently de-differentiate into a denervated phenotype permitting
proliferation and re-association with regenerating axons. Denervated SCs adopt a phenotype reminiscent of perinatal, immature SCs and are partially characterized by re-expression of GFAP. SC de-differentiation and subsequent re-myelination is governed, at least in part, by positive (e.g. Krox-20, Oct-6) and negative (e.g. c-Jun, Notch) transcriptional regulators (Jessen and Mirsky, 2008). Perturbances in these regulatory programs can impair nerve regeneration and are associated with several elusive demyelinating neuropathies, including Dejerine-Sottas Syndrome, Charcot-Marie-Tooth disease and Congenital Hypomyelinating Neuropathy (Svaren and Meijer, 2008).

Nuclear factor-κB (NF-κB) is most appreciated for its transcriptional regulation of the immune response and is a target of many approved pharmaceuticals (Miller et al., 2010). Exciting studies suggest that activation of NF-κB is essential for driving immature SCs into a pro-myelinating phenotype to insulate axons during development (Nickols et al., 2003; Yoon et al., 2008; Limpert et al., 2010). Recently, deacetylation of NF-κB was shown to be crucial for SC myelination using histone deacetylase 1/2 double knockout mice (Chen et al., 2011). Although peripheral nerve assault generates a robust upregulation of NF-κB activity, almost nothing is known regarding the effects of NF-κB activation in denervated SCs on peripheral nerve regeneration and re-myelination (Ma and Bisby, 1998; Fernyhough et al., 2005; Pollock et al., 2005; Smith et al., 2009; Fu et al., 2010). Additionally, studies utilizing a genetic, animal model, in which NF-κB activation is inhibited solely in SCs to determine its transcriptional regulation of myelination and post-injury events are lacking.
We sought to address these topics within the facial nerve of our previously described GFAP-IκBα-dn (transgenic) mice, in which NF-κB activity is functionally inhibited in GFAP-expressing cells, including SCs and astrocytes (Brambilla et al., 2005; Bracchi-ricard et al., 2008; Brambilla et al., 2009). Here, we show that activation of NF-κB is required for timely regeneration and remyelination following nerve injury.

3.2 RESULTS

3.2.1 GFAP-IκBα-dn transgene expression

Unlike whole animal knockouts of NF-κB familial subunits, our GFAP-IκBα-dn mice survive into adulthood with no apparent sensorimotor abnormalities (Brambilla et al., 2005). Transgene expression has previously been detected in whole brain and nerve homogenates, from naïve, adult mice, by RT-PCR (Brambilla et al., 2005). In order to determine if the GFAP-IκBα-dn transgene was present within the FMN and neighboring brainstem tissues in transgenic animals, we performed RT-PCR on brainstem homogenates extracted from the pontine level where the FMN is located. Transgene expression was seen (Fig. 3.2.1) in the brainstem region that contains the FMN in IκBα-dn animals and absent in WT controls (n=3 animals/group). This is likely due to the astrocytic expression of GFAP in the white matter and possibly within the FMN.
Figure 3.2.1. **IκBα-dn transgene is expressed in the brainstem of GFAP-IκBα-dn, but not WT, mice.** DNA gel from RT-PCR of RNA extracted from the area of the brainstem corresponding with the facial nucleus of adult (2-4 months) male mice. cDNA was amplified for the IκBα-dn transgene which expressed in transgenic but not wild type mice. As a positive control, amplified β-actin can be seen in both littermates in the bottom gel. –RT represents a genomic DNA control and absence of DNA demonstrates reverse transcription of RNA only. BS: brainstem. These results were repeated on 3 animals/group. IκBα-dn (forward, 5'-TTCATAAAGCCCTCGCATCC-3'; reverse, 5'-ACAGCCAGCTCCCAGAAGTG-3'). β-actin (forward, 5'-ATG GTG GGA ATG GGT CAG A-3'; reverse, 5'-CAC GCA GCT CAT TGT AGA AGG-3').

3.2.2 Elevated GFAP protein expression in astrocytes following injury

It is well established that GFAP protein expression is elevated in astrocytes as early as one day after facial nerve injury (Tao and Aldskogius, 1999); GFAP immunoreactivity within the regenerating FMN marks an important, non-mitotic transformation of astrocytes in injured adult animals. To determine if astrocytic activation in the FMN requires NF-κB activation, GFAP protein expression was assessed (Fig. 3.2.2) by immunohistochemistry, 14 days following a unilateral facial nerve transection, ~1mm proximal to trifurcation (n=2 animals/group). GFAP expression is minimal in the FMN and primarily restricted
to the medullar margins of brainstems corresponding to the uninjured facial nerve from both groups. In both groups, GFAP expression was seen 14 days post axotomy in the FMN corresponding to axotomized nerves.

Figure 3.2.2. Facial nerve axotomy induces GFAP immunoreactivity in astrocytes within the facial motor nucleus. Immunostains of GFAP expression within the facial motor nucleus 14 days following a unilateral facial nerve transection, ∼1 mm distal to stylomastoid foramen exit site. Astrocytic GFAP immunoreactivity is similar in the facial motor nucleus of IκBα-dn mice compared to WT littermates. Co, control or uninjured side. Inj, facial motor nucleus of the injured nerve. Scale bar: 50 µm. These results were repeated in 2 animals from each group.
3.2.3 NF-κB activation in GFAP-expressing glia is required for axonal regeneration and motor recovery 1 month following transection.

In order to determine if NF-κB inactivation in GFAP-expressing glia effects axonal regeneration, the right facial nerve trunk was transected, ~1 mm proximal to trifurcation, in WT (n=5) and GFAP-IκBα-dn (n=4) mice. A 4% Fluorogold (FG) solution was administered beneath the whiskerpads, ipsilateral and contralateral to injury, 28 days after transection, as previously described (Werner et al., 2000); animals were sacrificed 3 days later and retrograde transport via facial motor neurons was quantified within the facial motor nucleus (Fig. 3.2.3A). A ratio of FG⁺ FMNs (ipsilateral to axotomy/contralateral to axotomy) was calculated as an indicator of axonal regeneration and re-innervation into target facial muscles. A drastic reduction (53%) in FG uptake was exhibited in our GFAP-IκBα-dn mice (32 ± 8%) when compared to WT (69 ± 12%) littermates (Fig. 3.2.3B; Table 3.2.1). Immediately prior to FG administration, functional recovery was assessed by scoring vibrissae movements (Raivich et al., 2004) from 0 (no movement) to 3 (normal, robust movement) and yielded a significant decrease in vibrissae movement from an average of 2.0 ± .22 in WT (n=7) to 1.2 ± .20 in transgenic (n=5) mice (Fig. 3.2.3B).

To determine if the reduction in axonal regeneration seen was due to an increase in motor neuron (MN) cell death as opposed to a decrease in axonal elongation, Cresyl Violet stained MNs were quantified within the injured and uninjured (control) FMN and expressed as a survival ratio (injured/control). There were no significant differences in MN cell survival between WT (74 ± 9%, n=5) and GFAP-IκBα-dn (83 ± 16%, n=3) groups (Fig.
by measuring MN cell body area in the injured FMN compared to the contralateral FMN, we found no differences in MN atrophy (WT: $304.4 \pm 23.85 \, \mu m^2$ and GFAP-\textit{IκBα-dn}: $289.0 \pm 43.40 \, \mu m^2$). Together, these data suggest that GFAP-expressing glial activation of NF-κB is required for axonal elongation and re-innervation 1 month following facial nerve transection. However, facial nerve axotomy leads to excessive axonal branching (Kamijo et al., 2003) and misguidance (Ito and Kudo, 1994) or synkinesis (Hadlock et al., 2009) of axonal fibers to new facial targets which may confound tracing data from the whiskerpad loci as well as behavioral assessment (for review, see Raivich and Makwana, 2007).
Figure 3.2.3. Glial NF-κB activation is required for axonal regeneration but not motor neuron survival 31 days after facial nerve axotomy. Adult mice underwent a unilateral transection of the facial nerve, ~1 mm proximal to trifurcation. (A) Fluorogold (FG) labeled facial motor neuron somas within the facial motor nucleus (FMN) of the brainstem 31 days after injury. Co: uninjured side. Ax: injured side. Scale Bar: 100 μm. (B) Quantification of FG⁺ MNs 31 days post injury (left bar graph). Results were obtained by dividing the total number of regenerating axons by the total number of axons from the uninjured facial motor nucleus (Ax/Co). Quantification of vibrissae motor performance scores (right bar graph) on the injured side, assessed 28 days post injury, before FG administration beneath the whiskerpads. The scores range from 0, no movement at all, to 3, robust normal vibrissae sweeps. (C) Quantification of the total number of Cresyl Violet (CV) motor neurons (MN) expressed as a ratio of CV⁺ MNs ipsilateral to injury versus CV⁺ MNs contralateral to injury. Quantification of MN soma area (μm²) measured from CV stains. Data expressed as the mean ± SEM of 4-5 animals/group; * p<.05, unpaired students t-test.

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<tr>
<th>Genotype</th>
<th>FG⁺ MNs (Mean ± SEM)</th>
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<tr>
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<td>Injured</td>
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<tr>
<td>WT</td>
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<td>12</td>
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<td>9</td>
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<td>15 ± 2</td>
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<td>IκBα-dn</td>
<td>8</td>
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<td>8</td>
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<td></td>
<td>10</td>
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Table 3.2.1. Average number of Fluorogold positive motor neurons, per section, 31 days following facial nerve transection. Following unilateral facial nerve transection injury (see Fig. 3.2.3), the number of FG⁺ MNs within the facial motor nucleus were counted in 6-8 sections (20 μm thick). The average number of FG⁺ MNs, per section from the injured and uninjured facial motor nuclei, is listed above.
Table 3.2.2. Total number of Cresyl Violet positive motor neurons, 31 days following facial nerve transection. Following unilateral facial nerve transection injury (see Fig. 3.2.3), the number of Cresyl Violet (CV) positive MNs within the facial motor nucleus were using stereological principles. The average number of CV+ MNs from the injured and uninjured (control) facial motor nuclei are listed above.

3.2.4 NF-κB activation in GFAP-expressing glia is required for axonal regeneration 1 month following crush injury of the buccal nerve.

One month following facial nerve transection, inhibition of NF-κB activation in GFAP-expressing glia results in a drastic reduction, or possibly a delay, in axonal regeneration and recovery of whiskerpad movement; this effect appears to be independent of MN cell death or atrophy (Fig. 3.2.3). However, we can’t rule out the possibility that this reduction is due to an increase in collateral sprouting or misguided re-innervation of multiple muscle groups by single MNs, since our site of FG administration is only directly innervated by the buccal and upper marginal mandibular branches. Hence, the same question was addressed in a pinch crush model where the neural sheath remains intact to allow better
guidance of axons to their original targets. Additionally, only the buccal branch, which innervates the nasolabial musculature, was crushed to restrict the focus of this study.

WT and IκBα-dn animals underwent a pinch crush of the right buccal branch leaving the uninjured, contralateral nerve as an intra-animal control. Three days prior to sacrifice, animals received subcutaneous FG (4%) injections into both whisker pads. Animals were then sacrificed 10 or 31 days post injury. FG positive neurons were then quantified using stereoinvestigator software, paired with a high power objective (63X), to accurately distinguish FG positive MNs from neuronal processes and to prevent counting fragmented MNs more than once (Fig. 3.2.4, Table 3.2.3). MNs have a distinct size and morphology; however, it should be noted that the facial nerve is purely composed of motor fibers at the height of the lesion site (Moran and Graeber, 2004).

Although a large increase in facial nerve reinnervation is often seen near 14 days following a facial nerve crush at the trunk proximal to trifurcation (Werner et al., 2000), a 10 day time point was chosen due to an increase in rostral proximity of our injury site to the whisker pad. Furthermore, the rate of regeneration of the buccal branch is approximately 2.3 mm/day, exceeding the rate of the upper division of the mandibular branch, regenerating at 1.8 mm/day (Hadlock et al., 2005).

Interestingly, IκBα-dn animals exhibit a significant reduction (~25%) in regeneration 31 days post injury compared to WT littermates (Fig. 3.2.4, Table
3.2.3). This is an attenuated result compared to the aforementioned transection data, perhaps due to the reduced severity of the injury. There is no statistical difference in regeneration between WT and \( \text{IkB\alpha-dn} \) animals, 10 days following injury (Fig. 3.2.4, Table 3.2.3); however, more animals need to be analyzed in order to conclude this with confidence. Additionally, there is a significant difference in regeneration in WT animals between 10 and 31 days post injury but no difference in \( \text{IkB\alpha-dn} \) animals between 10 and 31 days following injury, using a one way ANOVA followed by a Tukey Test (Fig. 3.2.4, Table 3.2.3).

Taken together, these data suggest that NF-\( \kappa \)B activation in GFAP-expressing glia is required for regeneration. However, analysis of the raw values, obtained from FG counts, revealed a potential, confounding factor. For example, an estimated total population of \( FG^+ \) MNs in WT facial motor nuclei show an average of 1824 ± 127 \( FG^+ \) MNs residing in the FMN ipsilateral to injury and an average of 1918 ± 125 \( FG^+ \) MNs residing in the FMN contralateral to injury, 31 days following injury (Table 3.2.3). This suggests that nearly all WT axons regenerated successfully or that other, uninjured branches of the facial nerve retrogradely transported FG to their somas. The buccal branch is the primary motor nerve innervating the whiskerpad, where FG was administered; however, the upper division of the marginal mandibular branch partly innervates this region as well; next we devised injury models to address this concern.
Figure 3.2.4. Inhibition of NF-κB activation in GFAP-expressing glia reduces regeneration compared to WT littermates, 31 days following crush injury. Quantification of fluorogold positive neurons within the facial motor nucleus 10 and 31 days following buccal nerve crush injury. Adult, male mice underwent a unilateral crush injury of the buccal branch of the facial nerve, ~4mm distal to trifurcation. Initially, IκBα-dn animals exhibit the same amount of regeneration, 10 days following injury. 31 days post injury, WT animas show a significant increase in regenerating axons that is not seen in IκBα-dn littermates. Co: uninjured side. Inj: injured side. Values are expressed as a percentage obtained by dividing the total number of regenerating axons by the total number of axons from the uninjured facial motor nucleus (inj/co). Results are expressed as the mean ± SEM of 3-10 animals/group. *p<.05, unpaired Student’s t-test. **p<.05, one-way ANOVA.
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<th>Days Post Injury</th>
<th>Genotype</th>
<th>FG⁺ MNs (Mean ± SEM)</th>
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<td>IκBα-dn</td>
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<td>10</td>
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<td><strong>1055 ± 184</strong></td>
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<td>31</td>
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<td>1447</td>
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Table 3.2.3. Raw values of total projected number of fluorogold positive motor neurons, 10 and 31 days following buccal nerve crush injury. Following unilateral buccal nerve crush injury, fluorogold (FG) labeled motor neurons were quantified using stereological principles at two different time points (column 1). The total estimated population of FG⁺ motor neurons from the injured and uninjured (control) facial motor nuclei can be seen in columns 3 and 4, respectively.
3.2.5 **Tracer studies in various branches of the facial nerve**

In order to determine putative contributions of the marginal mandibular branch to our aforementioned FG counts, following buccal nerve crush injury, we implemented two transection injuries. An adult, male, WT mouse underwent a unilateral transection of the marginal mandibular branch of the facial nerve, proximal to its upper and lower division (see Chapter 2). The proximal stump was immediately intubated in a 2% FG solution for 40 minutes to retrogradely label MN somas. Three days following injury, brainstems were removed and sectioned to assess the number of FG+ neurons. Approximately 2500 cell bodies were labeled demonstrating an immense number of mandibular branch neurons residing in several subnuclei of the FMN. These values exceed the range of raw values quantified in Table 3.2.3, following buccal nerve crush injury, suggesting that putative mandibular FG+ MNs may be from a single branch of the marginal mandibular nerve.

Since the upper division of the marginal mandibular branch innervates targets near the site of FG administration in the whisker pads, we isolated and studied this branch. Another adult, male, WT mouse underwent a unilateral transection of the upper marginal mandibular branch of the facial nerve (see Chapter 2). The proximal stump was immediately intubated in a 2% FG solution for 40 minutes to retrogradely label MN cell bodies. Three days following injury, brainstems were removed and sectioned to assess the number of FG+ neurons. Fewer cell bodies were labeled (465) in several subnuclei of the FMN, suggesting a confounding variable within previous regeneration assessment.
following buccal crush injury. However, nerve stump intubation ensures direct contact with the retrograde tracer as opposed to the diffuse or gradient-like exposure of axons to tracers when the tracer is injected into the whisker pad.

A separate procedure was performed to assess the possibility of misguided, regenerating axons that may terminate in different regions from their initial innervation. Adult, male, WT mice (n=2) underwent a unilateral transection of the mandibular branch trunk proximal to its bifurcation. Proximal stumps were intubated in a 2% Fast blue (FB) solution to label neurons of the marginal mandibular branch in the FMN. Three days later, the right buccal branch of the facial nerve was crushed using a pair of uniformly ground micro-forceps. Three weeks later, a 2% FG solution was administered to the whiskerpad subcutaneously. Animals were sacrificed 3 days later and the FMN was analyzed for labeling and colabeling of facial motoneurons. Regenerating motoneurons corresponding to the buccal branch of the facial nerve were FG\(^+\). FB\(^+\) neurons represented the population of mandibular facial nerve cell bodies initially labeled prior to injury. Several co-labeled somas were seen and corresponded to mandibular branches that regenerated, possibly to the whisker pad.

Taken together, these findings demonstrate a shared capability of buccal and mandibular facial nerve axons to retrogradely transport tracers administered in the whisker pad. Additionally, these branches have some overlap in their muscolotopic organization within the FMN. In order to accurately assess regeneration of the buccal branch to the whisker pad using retrograde tracers,
the marginal mandibular branch must be removed upon tracer administration. Another option is to intubate the injured buccal branch directly in tracer solution for assessment; however, this method imposes a second injury (transection) on the regenerating nerve in question.

3.2.6 NF-κB activation in Schwann cells is dispensable for myelination in adulthood

Since activated NF-κB is highly upregulated in premyelinating SCs shortly after birth and required for SC myelination in vitro, we investigated whether inhibition of glial NF-κB affected myelination within the facial nerve of naïve, adult mice (Nickols et al., 2003; Yoon et al., 2008). Toluidine Blue (TB) stained sections and electron micrographs revealed no differences in the quantity (WT: 1108 ± 8; GFAP-IκBα-dn: 1067 ± 38), caliber (WT: 2.28±0.34 μm; GFAP-IκBα-dn: 2.57±0.21μm) or quality of myelinated axons between WT (wild type) and transgenic nerves (Figs 3.2.5B,C, 3.8a,c). Both groups exhibited the same distribution and thickness of compact myelin rings (data not shown), gRatio (axonal diameter/fiber diameter), and expression of myelin structural proteins MBP and MPZ (Figs 3.2.5D, 3.2.11B,D). Interestingly, all nerves contained Remak bundles encasing non-myelinated (unmyelinated) axons. We saw no differences in the total number of Remak bundles, unmyelinated axons, or SC nuclei between groups (Fig. 3.2.12A-C). Taken together, we found no major-morphological or developmental- abnormalities within transgenic nerves that may preclude regeneration studies.
Figure 3.2.5. Naïve GFAP-IκBα-dn nerves display no aberrations. (A) Naive, WT facial nerve schematic showing the buccal (*) and mandibular (upper(U) and lower (L)) branches. Arrow indicates the cranial nerve exit site from the stylomastoid foramen, caudal to trifurcation. (B) Transverse, Toluidine Blue stains (top four panels) and electron micrographs (lower panels) of the buccal branch, 8mm distal to trifurcation from main facial nerve trunk, of adult, male mice. Scale bar: 20, 10, and 5 μm respectively from top to bottom panels. (C) Quantification of the total number of myelin rings within the buccal branch (n=4 animals/group), using Stereoinvestigator software with Toluidine Blue stained, transverse sections. (D) Densitometric quantification of MBP and MPZ protein expression determined by Western Blot analysis (n=3-7 animals/group). MBP: myelin basic protein. MPZ: myelin protein zero. Data are expressed as the mean ± SEM.
3.2.7 Functional inhibition of NF-κB in transgenic, denervated Schwann cells following crush injury

To ensure that expression of our GFAP-IκBα-dn transgene results in functional inhibition of NF-κB activation in Schwann cell glia, we developed an injury model (see Chapter 2) whereby the buccal branch of the right facial nerve was crushed, leaving the epineurial sheath intact to foster regeneration and minimal motor neuron (MN) cell death. Injured facial nerves were immunostained for activated NF-κB [phospho-p65 (Ser276)] and GFAP, one day following crush injury (Fig. 3.2.6). Confocal microscopy revealed clear nuclear expression of phospho-p65 (colocalized with DAPI) within GFAP expressing SCs in WT nerves. In contrast, transgenic nerves lacked NF-κB activation as demonstrated by the absence of nuclear, phospho-p65.
Figure 3.2.6. Transgenic inhibition of NF-κB activation in denervated SCs following facial nerve crush injury. Immunostains of GFAP, phospho-p65, and DAPI within longitudinal, distal buccal nerves 1 day following crush injury (Fig. 3.2). Denervated Schwann cells express GFAP and their cytoplasm’s are seen in green (arrowheads). Nuclear localization of activated p65 ((p)-65), co-localized with DAPI nuclear marker (blue), can be seen in red in WT (arrowheads), but not IκBα-dn, denervated Schwann cells. Merged images can be seen in the rightmost panels. Images were obtained at 40X (oil) using confocal microscopy. Scale Bar: 20 μm.

Since the MN cell bodies of the facial nerve reside in the brainstem, we also assessed injury induced NF-κB activation in astrocytes. Sections from the brainstem immunostained for phospho-p65 and GFAP illustrated nearly no NF-κB activation in astrocytes within the facial motor nucleus (FMN) (Fig. 3.2.7) of both genotypes 1,4 and 31 days post injury (dpi). Hence, we restricted our regenerative studies to the peripheral component of the facial nerve where NF-κB activation is functionally inhibited in SC glia.
Figure 3.2.7. Minimal activation of astroglial NF-κB within the facial motor nucleus following crush injury. Representative immunostains of GFAP (green), phospho-p65 (red), and DAPI (blue) within WT and transgenic (IkBα-dn) nerves 1, 4, and 31 days post buccal crush injury (Fig. 3.2). Note: astrocytic activation (visualized with GFAP) is strongest at 4 dpi and activated p65 is robust at 1 and 31 dpi, but not in astrocytes, in both groups. Activated p65 appears punctate and surrounds the large, distinct nuclei (labeled with DAPI) of motor neurons (see inset in top, right panel). dpi: days post injury. Images were obtained at 40X (oil) using confocal microscopy. White boxed insets represent magnified areas. Scale Bar: 20 μm.

3.2.8 Facial nerve crush injury model exhibits minimal axonal sparing, abrupt axonal degeneration, and detectable GFAP expression

Next, we assessed the efficacy of the crush injury on ablation of axon fibers and axonal retrograde transport. The mouse whiskerpad is innervated by the buccal and upper marginal mandibular branch of the facial nerve, which partially overlap in somatotopic organization within the lateral FMN (Ashwell,
Immediately following injury, the mandibular branches were removed and FG was subcutaneously injected into the whisker pad. At 2 dpi, the facial nerve was stained for neurofilament (heavy isoform) to illuminate the crush site and degenerating axons with punctate staining within an intact nerve sheath (Fig. 3.2.8). No FG staining was observed in the FMN 2 dpi; indicating minimal axonal sparing (Fig. 3.2.8) ipsilateral to injury. As an internal control, the contralateral, uninjured side showed normal FG uptake and labeling of facial motor neurons, indicating that the IκBα-dn transgene does not interfere with FG transport in uninjured nerves. GFAP gene expression was undetectable prior to injury in both groups, but observed at 4 dpi (Fig. 3.2.9), which is indicative of SC de-differentiation into a denervated phenotype (Stoll and Muller; 1999). This explains the GFAP-driven, IκBα-dn transgene expression (undetected in WT) seen in mutant nerves following injury (Fig. 3.2.9).
Figure 3.2.8. Facial nerve crush injury model exhibits abrupt axonal degeneration and minimal axonal sparing. Top: Injured (ipsilateral) and uninjured (contralateral) buccal nerve immediately following crush injury described in Fig. 3.2. Arrow indicates crush site in the buccal branch where the intact epineurium appears opaque. Middle panels: Neurofilament (NF) staining (top panels) of injured (ipsilateral) and uninjured (contralateral) nerves 2 days post injury shows degenerating and intact axons, respectively. Scale Bar: 20 μm. Bottom panels: Fluorogold (FG) labeled motor neurons in the facial motor nucleus 2 days post injury. Immediately following crush injury, the ipsilateral mandibular branch was surgically removed and FG was injected into both whisker pads (Fig. 3.2). A lack of FG⁺ motor neurons within the facial motor nucleus, ipsilateral to injury, demonstrates the efficacy of the crush injury. Scale Bar: 50 μm. These results were repeated with 3 animals per group.
Figure 3.2.9. GFAP and transgene expression are active in Schwann cells following crush injury. Animals underwent the nerve crush injury described in Fig. 2.2. Quantification of gene expression using Real-time RT-PCR on cDNA (normalized to 18S) generated from buccal nerve tissue distal to the injury site. (A) GFAP gene expression is undetectable prior to injury but detectable at 4 and 12 days post injury in WT and transgenic (IκBα-dn) mice. (B) Transgene expression, driven by a GFAP promoter, is detectable and sustained, following injury in GFAP-expressing Schwann cells pervading IκBα-dn nerves. X-axis: number of days post injury. N: naive. Data are expressed as the mean ± SEM of 3 mice/group.

3.2.9 Inhibition of NF-κB activation transiently delays and then promotes regeneration

Using our pinch crush model, we tested the hypothesis that activation of NF-κB is required for peripheral nerve regeneration (Fig. 3.2.10). Brains were removed from sacrificed animals 12, 31, and 65 dpi and FG-positive (FG+) MNs were quantified (Fig. 3.2.10B, Table 3.2.4). There were no difference in regeneration at 12 dpi, but at 31 dpi transgenic animals had significantly fewer FG+ MNs (356.9 ± 56.50; n=7) compared to WT littermates (613.0 ± 27.53; n=5). Similar results were obtained following complete facial nerve transection, with no differences in facial motor neuronal death or cell body size 31 dpi (Fig. 3.2.3). Surprisingly, by 65 dpi transgenic animals had substantially more FG+ MNs (1305 ± 156.5; n=6) than WT (890.4 ± 78.37; n=9).
Figure 3.2.10. NF-κB activation in denervated SCs significantly influences axonal regeneration. (A) Fluorogold (FG) labeling of motor neuron (MN) cell bodies in the facial motor nucleus (FMN) of normal (WT) and transgenic (IκBα- dn) mice 12, 31 and 65 days post crush injury (dpi) of the buccal nerve. Images were obtained at 40X (oil) and insets at 65 dpi represent 20X magnification. Scale Bar: 50 μm. (B) Stereological quantification of the number of FG+ MNs in the injured FMN. Results are expressed as the mean ± SEM of 5-9 animals/group. *p < .05, **p < .01, unpaired Student’s t-test (WT compared to transgenic). #p < .05, ###p < .001, one-way ANOVA.
Table 3.2.4. Raw values of total projected number of fluorogold positive motor neurons, 12, 31 and 65 days following buccal nerve crush injury.

Following unilateral buccal nerve crush injury, fluorogold (FG) labeled motor neurons were quantified using steorological principles at three different time points (column 1). The total estimated populations of FG+ motor neurons from the injured facial motor nuclei are listed.

Electron micrographs (Fig. 3.2.11A-B) revealed significantly less intact axons encased in compact myelin 4 mm distal to the injury site at 31 dpi within GFAP-\(\text{IκBα-dn}\) (41 ± 27/cm²; n=3) nerves compared to WT (210 ± 15/cm²;
Interestingly, at 65 dpi transgenic nerves had dramatically increased to WT levels of intact, myelinated axons. Importantly, at 65 dpi the quantity of intact, myelinated axons in both groups had increased to naïve levels, a finding indicative of successful regeneration. However, the size (Fig. 3.2.11C, Table 3.2.5) and total quantity of visible axons (data not shown) were similar between groups suggesting that fewer transgenic axons had regenerated to the whisker pad at 31 dpi, since there was less FG uptake by MNs (Fig. 3.2.10B). Taken together, these findings demonstrate an initial decrease followed by a significant increase in the quantity of regenerating MNs when NF-κB activation is inhibited in SCs.

Since NF-κB activation within Schwann cells has been implicated in SC survival and proper regeneration (Weinstein, 1999; Boyle et al., 2005), we were surprised to find no significant differences in the number of SC nuclei assessed in electron micrographs between groups, before or after injury (Fig. 3.2.12C). There was, however, a significant increase in the quantity of SC nuclei from naïve levels at 31 dpi in both groups, which likely reflects injury-induced SC proliferation.

3.2.10 Functional inhibition of NF-κB activation in denervated and unmyelinated Schwann cells delays Remak bundle formation

In naïve nerves, unmyelinated axons were clearly sorted and organized into Remak bundles in both groups (Fig. 3.2.11A). Each bundle was well defined and often contained more than 10 axons separated by the cytoplasm of an unmyelinated SC. At 31 dpi, Remak bundles in transgenic mice appeared
unorganized (Fig. 3.2.11A), where the extracellular matrix was greatly compromised and loosely surrounding disrupted cytoplasmic space housing vacuoles. The total number of intact Remak bundles was significantly less in GFAP- IκBα-dn (15 ± 8/cm²; n=3) compared to WT (111 ± 26/cm²; n=3) 31 days following injury (Fig. 3.2.12A). Additionally, there was a significant reduction in the number of organized Remak bundles seen at 65 dpi compared to 31 dpi in WT animals. Interestingly, the number of unmyelinated axons was unaltered between naïve and 31 dpi nerves in both groups; suggesting a reduction in the quantity of unmyelinated axons within Remak bundles. At 65 dpi, both groups had the same number of organized Remak bundles as well as unmyelinated axons (Fig. 3.2.12A,B). However, there were far fewer sorted axons within each bundle (usually 2-3), when compared to naïve conditions which may necessitate the overall increase in the number of SCs seen in injured and restored facial nerves.

3.2.11 Inhibition of Schwann cell NF-κB activation delays compact re-myelination

Next, we sought to determine if there were any changes in re-myelination associated with NF-κB activation. Electron micrographs of cross sections distal to injury revealed a general lack of myelin compaction and adaxonal contact in GFAP-IκBα-dn nerves (Fig. 3.2.11A), although some compact myelin rings were present 31 dpi. Since both groups had the same average axonal diameter (Fig. 3.2.11C, Table 3.2.5), a significant reduction in the gRatio (Fig. 3.2.11D, Table 3.2.5) at 31 dpi supports the lack of ring compaction seen in mutant nerves compared to WT. Additionally, there was a significant reduction in the gRatio of
transgenic, myelinated axons, but not WT 31 dpi compared to naïve. There were no apparent differences in myelin ring size or compaction at 65 dpi between groups. These data suggest that NF-κB activation in SCs is required for timely re-myelination following injury. However, a putative delay in Wallerian degeneration and myelin clearance at 31 dpi can not be ignored.
Figure 3.2.11. Effects of NF-κB inhibition on Schwann cell re-myelination.

(A) Electron micrographs on transverse, ultrathin sections of the buccal branch, 4 mm distal to the crush injury site (described in Fig. 3.2). Left panels show uninjured nerves with intact, myelinated axons (*) and organized Remak bundles (arrowhead) containing several unmyelinated axons with their individual axoplams. Middle panels display regenerating and remyelinating axons 31 days post injury (dpi). Transgenic animals display fewer intact axons and a clear lack of myelin compaction. SC: Schwann cell nuclei. Right panels show complete remyelination and axonal regeneration 65 dpi in both groups. Scale Bar: 5 μm.

(B-D) Quantification of intact, myelinated axons (B), average axonal diameter (C), and gRatios (axonal diameter: fiber diameter) (D). Geometric measurements were taken by hand on all myelin rings encasing an axon, regardless of ring morphology. X-axis: number of days post injury. N: naïve. Data were obtained from electron micrographs and are expressed as the mean ±
SEM of 3-4 animals/group. *p<.05, **p<.01, unpaired Student’s t-test (WT vs transgenic). #p<.05, ###p<.001, one-way ANOVA.

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Table 3.2.5. **Average axonal diameter, fiber diameter, gRatio.** Data values in bold font represent the mean ± S.E.M. before (naive) and after buccal nerve crush injury. Axon diameter was determined by measuring and averaging the vertical and horizontal diameter of each mesaxon displayed in electron micrographs. Fiber diameter was determined by measuring and averaging the vertical and horizontal diameter of the outer most myelin lamellae encasing axons displayed in electron micrographs. gRatio was calculated for each axon by dividing the axonal diameter by the fiber diameter.
**Figure 3.2.12. Functional inhibition of NF-κB activation in denervated and unmyelinated Schwann cells transiently delays sensory bundle formation**

Quantification of organized Remak bundles (A), unmyelinated axons (B) and Schwann cell nuclei (C) before (N) and after injury. Measurements were obtained from transverse electron micrographs (Fig. 3.2.11) of the buccal nerve distal to the site of injury. Transgenic nerves exhibited significantly fewer organized Remak bundles (A), 31 dpi, compared to WT littermates; however, both groups had the similar numbers of unmyelinated axons (B). A significant increase in the number of Schwann cell nuclei (C) was seen 31 dpi in both groups. X-axis: number of days post injury (dpi). N: naïve. Data are expressed as the mean ± SEM of 3-4 animals/group. *p<.05, unpaired Student’s t-test (WT vs transgenic). #p<.05, ##p<.01, ###p<.001, one-way ANOVA.

3.2.12 Expression of myelin regulatory transcription factors and structural proteins

Following loss of axonal-SC contact, effective regeneration in the PNS is heavily reliant on SC dedifferentiation into an immature phenotype (Jessen and Mirsky, 2008). Developmental myelination initiated by immature SCs and re-myelination orchestrated by denervated SCs are thought to utilize similar genetic programs (Stoll and Muller, 1999; Jessen and Mirsky, 2008). SC de-differentiation is dependent on intracellular signaling molecules such as c-Jun and Krox-20 which serve as negative and positive regulators of myelination, respectively. To assess SC-mediated events following injury, we performed
Western blot analysis on harvested distal nerve tissue (Fig. 3.2.13). In both animal groups, uninjured nerves exhibited virtually no c-Jun protein expression (Figs. 3.2.13, 3.2.14); however, by 4 dpi, a strong increase in c-Jun expression was found, indicative of SC de-differentiation. At later time points c-Jun was attenuated, as SCs presumably re-enter the promyelin state (Figs. 3.2.13, 3.2.14).

Krox-20 is a zinc finger protein essential for myelin basic protein (MBP) and myelin protein zero (MPZ) production, two structural proteins involved in myelin sheath formation and maintenance (Topilko et al., 1994; Decker et al., 2006). We found a significant reduction in krox-20 expression in naïve GFAP-\(\text{IκBα-dn}\) nerves when compared to WT littermates, suggesting that NF-κB may positively regulate krox-20 expression under normal physiological conditions (Figs. 3.2.13, 3.2.14). Krox-20 protein expression levels are greatly reduced by 4 dpi in distal nerves as SCs phenotypically revert to an immature/denervated state, and are steadily recapitulated at 12 and 31 dpi in both groups (Figs. 3.2.13, 3.2.14).

Next, we explored the expression levels of myelin structural proteins utilized for lamellae compaction by SCs: MBP and MPZ (Figs. 3.2.13, 3.2.14). We were surprised to find no differences between MBP and MPZ protein expression before or after injury in both groups (n=3/group/time point). Taken together, these data suggest that NF-κB activation in denervated SCs is not required for demyelination or synthesis of key myelin structural proteins critical for re-myelination. It is important to note that the expression of these
proteins does not indicate their cellular location. Cholesterol is required for trafficking of MPZ from the endoplasmic reticulum and inhibition of this process impedes myelin compaction (Saher et al., 2009).

Figure 3.2.13. NF-κB activation in Schwann cells does not regulate myelin-associated protein expression following crush injury. Representative immunoblots of myelin-associated transcription factor (cJun, Krox-20) and structural (MPZ, MBP) protein expression within the distal nerve of normal (WT) and transgenic (IκBα-dn) mice, before (N: naive) and 4, 12, and 31 days after injury. Lamin A/C serves as a nuclear loading control. Krox-20 (Egr-2): early growth response 2. MBP: myelin basic protein. MPZ: myelin structural protein.
Figure 3.2.14. Myelin-associated protein expression is unaltered in Schwann cells lacking NF-κB activation, as compared to WT, following crush injury. Densitometric quantification of protein expression, obtained from immunoblots (Fig. 3.10) normalized to WT naïve (N). Prior to injury, IκBα-dn nerves contain significantly less Krox-20 (myelin maintenance protein) protein expression compared to WT littermates. Following injury, Krox-20 is downregulated to equivalent levels, and later restored, in both groups. c-Jun (negative regulator of myelination) expression levels are minimal in naïve nerves but greatly upregulated 4 days post injury (dpi) in both groups. Myelin structural proteins (MPZ, MBP) are acutely downregulated following injury and later upregulated during remyelination. Krox-20 (Egr-2): early growth response 2. MBP: myelin basic protein. MPZ: myelin protein zero. Each protein band on the Western blots was normalized to a respective Laminin A/C protein band to control for loading variability. AU: arbitrary units. Results are expressed as the mean ± SEM of 3-7 animals/group. *p<.05, unpaired Student’s t-test.
3.2.13 NF-κB activation in denervated SCs is not required for Wallerian degeneration

A delay in axonal degeneration could explain the delay in regeneration and re-myelination seen in mutant mice. Reduced macrophage infiltration into the injured nerve, for example, impedes proper clearance of myelin and axonal debris which serves as an inhibitory substrate to fiber regeneration (Boivin et al., 2007). This is unlikely in our model, since MBP and MPZ expression levels decrease following injury (Figs. 3.2.13, 3.2.14); however, we wanted to carefully assess Wallerian degeneration histologically.

Since degeneration and regeneration occur somewhat simultaneously following nerve crush, we completely transected the buccal branch and sutured the proximal stump to underlying muscle tissue to prevent reinnervation of the distal nerve segment (see Chapter 2). Transverse, semi-thin cross sections of distal nerves stained with p-phenylenediamine (PPD) and TB revealed no differences in the rate of degeneration between groups (Fig. 3.2.15); the number of demyelinating axons was significantly reduced at 12 dpi compared to 4 dpi in both groups. At 4 dpi nerves primarily contained demyelinating axons and myelin debris with few intact axons visible. By 12 dpi both groups had virtually no intact axons or myelin debris and fewer axons undergoing demyelination. Additionally, macrophage infiltration to the injured nerve, determined by quantification of CD11b+ cells, was equivalent between groups (Fig. 3.2.16). MBP immunofluorescence on longitudinal, distal nerve segments demonstrated effective demyelination in both groups (Fig. 3.2.17). Hence, there is little evidence to support the role of Schwann cell NF-κB activation in Wallerian
Figure 3.2.15. Wallerian degeneration is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells. Transverse, semi-thin sections stained for myelin with paraphenylenediamine (PPD) and toluidine blue (TB) can be seen in the left panels. Sections were collected 4 mm distal to transection (Fig. 3.12), 4 and 12 days post injury (dpi). Axons, as well as myelin debris, become consecutively less visible subsequent to transection. Scale Bar: 10 μm. Bar graphs represent the total number of de-myelinating (top) and intact axons (bottom). X-axis: number of days post injury. Data expressed as the mean ± SEM of 4-8 animals/group. WT: wild type. IκBα-dn: transgenic. ###p<.001, one-way ANOVA.
Figure 3.2.16. Macrophage infiltration is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells. Representative immunostains of infiltrating macrophages (labeled in green with CD11b) in the distal facial nerve following transection injury (Fig. 3.12). dpi: days post injury. The rightmost immunostain for CD11b was performed on an adult, naïve sciatic nerve and demonstrates a lack of resident macrophages in the uninjured peripheral nerve. Bar graph represents stereological quantification of CD11b⁺ macrophages obtained from immunostains. Scale Bar: 20 μm. X-axis: days post injury. Results expressed as the mean ± SEM of 3-6 animals/group.
Figure 3.2.17. Myelin degradation is unaltered by transgenic inhibition of NF-κB in denervated Schwann cells. Longitudinal facial nerves, distal to transection site (Fig. 3.12), immunostained with myelin protein zero (MPZ). dpi: days post injury. Naïve: uninjured sciatic nerve from an adult WT mouse, demonstrating normal distribution of MPZ. By 12 dpi, essentially no MPZ is present which supports the myelin degradation seen in Fig. 3.13. Scale Bar: 20 μm.

3.2.14 NF-κB activation in SCs does not alter the expression of genes required for myelination or cholesterol synthesis.

Finally, we wanted to determine if the reduction/delay in compact remyelination was caused by an increase in non-compact myelin proteins or a deficiency in cholesterol synthesis. Preliminary results show a potential reduction of key cholesterol synthesizing and myelin membrane genes in uninjured, adult IκBα-dn mice (Figs. 3.2.18, 3.2.19). Real time RT-PCR was
performed on cDNA generated from RNA extracted from naïve, buccal nerves or
distal, injured buccal nerve segments, 4 and 12 days post injury (n=3
animals/group). The following genes were assessed: Apolipoprotein E, required
for proper expression of lipoproteins; HMG-CoA, the rate-limiting enzyme in
cholesterol biosynthesis (Fu et al., 1998), and myelin and lymphocyte protein
(MAL), a raft-associated membrane protein required for peripheral myelin
membrane formation. Gene expression was downregulated to undetectable
quantities following injury in both groups. In naïve animals, gene expression was
similar between WT and transgenic littermates, although transgenic mice showed
a downward trend for all three genes. Future studies, with more animals, will
likely show a decrease in cholesterol related genes and proteins.

Additionally, we performed RT-PCR on compact (MBP, MPZ, PLP) and
non-compact (MAG, CNPase) myelin structural proteins (Fig. 3.2.18) and found
no significant differences between groups (n=3 animals/group). Again, by
repeating these studies on more animals, we are likely to see a reduction in MAG
(involved in adaxonal contact with Schwann cells) and an elevation of CNPase
(non-compact myelin protein) gene expression in naïve, and possibly
regenerating, transgenic nerves when compared to WT littermates. Future
experiments need to be performed at later time points during remyelination so
see if cholesterol and/or non-compact myelin protein composition is altered. If
naïve transgenic animals maintain myelin with reduced levels of cholesterol,
perhaps these Schwann cells are less prepared to handle remyelination and
plasma membrane restructuring in a timely manner.
Figure 3.2.18. Myelin gene expression is unaltered between WT and IkBα-dn mice. Quantification of gene expression using Real-time RT-PCR on cDNA (normalized to 18S) generated from buccal nerve tissue distal to the injury site. (A-E) Following nerve crush injury, myelin genes associated with compact (MBP, MPZ, PLP) and non-compact (MAG, CNPase) myelin are downregulated in both WT and IkBα-dn mice. MBP: myelin basic protein. MPZ: myelin protein zero. PLP: proteolipid protein. MAG: myelin associated glycoprotein. CNPase: 2', 3'-cyclic nucleotide 3'phosphodiesterase. X-axis: number of days post injury. N: naive. Data are expressed as the mean ± SEM of 3 mice/group.
Figure 3.2.19. Genes related to cholesterol synthesis are unaltered between WT and IκBα-dn mice. Quantification of gene expression using Real-time RT-PCR on cDNA (normalized to 18S) generated from buccal nerve tissue distal to the injury site. (A) An elevation or de novo CD11b (produced by macrophages) gene expression can be seen following injury (4 days) which is later reduced (12 days); this likely corresponds with macrophage infiltration and subsequent departure from the injured nerve. (B-E) Expression levels of genes associated with lipid storage (PPARγ) and cholesterol synthesis (ApoE, HMG-CoA reductase, MAL) are all downregulated in both animal groups following injury. CD11b: PPARγ: peroxisome proliferator-activated receptor gamma. ApoE: apolipoprotein E. HMG-CoA reductase: 3-hydroxy-3-methylglutaryl-Coenzyme A. MAL: myelin and lymphocyte protein. x-axis: number of days post injury. N: naive. Data are expressed as the mean ± SEM of 3 mice/group.
Chapter 4: Discussion

The peripheral nervous system is capable of repair after traumatic or immune-related injuries; regeneration and remyelination is likely contingent upon the coordinated signaling between Schwann cells and regenerating axons. Upon de-differentiation, SCs upregulate expression of the intermediate filament GFAP, re-acquire proliferative capabilities, re-associate with regenerating axons and finally re-differentiate into a mature myelinating or unmyelinating phenotype (Chen et al., 2007). Interestingly, the precise signaling mechanisms underlying these events are largely unknown.

An elegant series of studies have implicated a role for NF-κB signaling in SC myelin induction. At perinatal time points, NF-κB (p65) activation regulates the expression of the pro-myelin transcription factor Oct-6 in dorsal root ganglion-SC co-cultures (Nickols et al., 2003). However, it is also known that deletion of Oct-6 transiently delays peripheral myelinogenesis, in vivo, by 7-10 days, presumably because of a functional redundancy between Brn-2 and Oct-6 (Ghazvini et al., 2002; Jaegle et al., 2003). Furthermore, HDAC1 and HDAC2 were recently shown to regulate SC differentiation and myelination, in part by modifying the acetylation state of NF-κB; however, HDACs are known to deacetylate several transcription factors in addition to their epigenetic regulation of chromatin remodeling (Chen et al., 2011). The transcriptional programs
regulating Schwann cell myelination are complex and sensitive to each milieu in which they are studied; for example, HDAC1/2 double knock-out mice, under the control of a Desert Hedgehog promoter, demonstrated a massive reduction in SC survival and myelination (Jacob et al., 2011), effects that were not seen in another study using the same genetic, Schwann cell specific deletion of HDAC1/2 (Chen et al., 2011). HDAC-regulated deacetylation of SC NF-κB was not explored in our model, since transgenic inhibition of NF-κB activation restricts nuclear translocation of canonical NF-κB hetero- and homodimers.

Our results suggest that NF-κB activation in immature SCs is not required to maintain mature myelination in adulthood in vivo. One possible explanation for the discrepancy between these findings is that SCs behave differently in vivo than they do in vitro. Perhaps dissociation of embryonic dorsal root ganglia and SC isolation impose or reflect an injury event which alters the typical SC signaling ensued during development. In the context of NF-κB myelin regulation, in vitro immature Schwann cell activity resembles that of denervated SCs in vivo. However, in vitro immature Schwann cell differentiation and myelination does not appear to hinge on the same signaling cascades as those utilized by SCs during perinatal nerve development. Further studies on NF-κB regulation of myelination in vivo will help resolve these conflicts.

Currently, studies elucidating the role of activated NF-κB in SCs on regeneration are sparse. Sustained NF-κB activation in immature SCs, expressing high levels of p75NTR, is protective from tumor necrosis factor α (TNF-α)-mediated SC death following neonatal sciatic nerve axotomy (Boyle et al.,
Because p75<sup>NTR</sup> is upregulated in denervated SCs and nerve crush induces elevated TNF-α levels (Jessen and Mirsky, 2008), we were surprised to see no differences in the total number of SC nuclei, between WT and transgenic animals following injury. Additionally, inhibition of TNF-α signaling using etanercept results in less p50 nuclear localization in SCs, promoting axonal growth 3 days after sciatic nerve crush (Smith et al., 2009). In our model, where all canonical NF-κB activation is inhibited, we observed no differences in acute regeneration between normal and transgenic mice at 12 dpi.

Post-injury aberrations in SC differentiation and re-differentiation could explain the delay or impediment in re-myelination exhibited in transgenic nerves 31 dpi. We were surprised to see no differences in the expression pattern of proteins governing SC differentiation (e.g. Krox-20, c-Jun) following injury between groups. However, many other mechanisms, which remain unexplored in our model, can account for a delay in regeneration and/or re-myelination.

In the previous chapter, we showed a novel function for NF-κB signaling in Schwann cells during peripheral nerve injury and repair: without NF-κB activation in GFAP-expressing glia, primarily Schwann cells, regeneration and compact remyelination is transiently delayed 31 days following buccal crush injury. Later, this results in improved regeneration and normal myelin compaction 65 days after injury. These findings were not a result of impaired Wallerian degeneration, macrophage infiltration, Schwann cell de- and/or re-differentiation, or myelin protein synthesis. This phenomenon is probably the result of an increase in axonal regeneration but a decrease in the rate of
regeneration and, therefore, a decrease in the rate of remyelination and myelin compaction. However, there are several, unexplored explanations for this phenomenon: an aberration in axonal-Neuregulin-1 signaling, an ablation or impairment in lipid biosynthesis, perturbed Krox-20 transcriptional regulation, and/or inefficient Schwann cell sorting.

Since developmental myelination requires axonal signaling, it is possible that the delayed myelin compaction, seen 31 days following injury, in our model is a direct result of a delay in axonal regeneration and subsequent exposure/contact with denervated Schwann cells. Axonally derived neuregulin-1 (NRG1) signals through ErbB2 and ErbB3 receptor heteromers on Schwann cells to regulate Schwann cell proliferation, motility, axon ensheathment and myelination during development. Until recently, the role of NRG1 in adult peripheral nerve maintenance and repair was unknown. Using SLICK (single-neuron labeling with inducible Cre-mediated knock-out) mice to ablate NRG1 in a subset of YFP-expressing myelinated motor and sensory axons within the sciatic nerve, Fricker et al. demonstrated that NRG1 is dispensable for the maintenance of the myelin sheath in adult mice. Following sciatic nerve crush injury, NRG1-deficient axons grew at a significantly slower rate and exhibited severe impairments in remyelination: hypomyelination or a complete absence of myelin sheaths (Fricker et al., 2011). Additionally, neuromuscular junctions were still reinnervated but only with excess terminal sprouting (Fricker et al., 2011).

A reduction in axonal-NRG1 could easily explain our findings, in that we see a delay in regeneration and remyelination. However, this does not
necessarily explain the improved regeneration we find at 65 days. If future studies show a significant downregulation of NRG1 in transgenic axons, it may suggest that Schwann cells dictate axonal-NRG1 signaling via NF-κB signaling. Additionally, we see a significant reduction in Remak bundle structure and organization 31 days following injury. This could also be caused by a reduction in NRG1 signaling, since NRG1 is thought to be responsible for the generation of independent mesaxons ensheathing unmyelinated axons (Taveggia et al., 2005). NRG1 signaling needs to be assessed in our model and carefully interpreted.

Myelin is highly enriched in glycosphingolipids and cholesterol; protein trafficking and myelin compaction require cholesterol in Schwann cells. Lipids constitute 70-80% of the dry weight of myelin whereas plasma membranes exhibit a lipid to protein ration of 1:1 (Norton and Cammer, 1984; Saher et al., 2011). To date, cholesterol plays five key roles in myelination: (i) cholesterol is rate limiting for myelination since myelinogenesis requires cholesterol enrichment; (ii) Schwann cells can take cholesterol from their local milieu and enrich it in myelin; (iii) Schwann cells, unlike oligodendrocytes, can synthesize myelin with lower cholesterol content; (iv) cholesterol availability controls transport of myelin protein zero (MPZ) from the endoplasmic reticulum (ER) to myelin membranes; (v) transcription of myelin proteins is coupled to intracellular cholesterol levels (for review, see Saher et al., 2011). Although it is well accepted that cholesterol is important for remyelination, its role in regeneration is still heavily debated.
A reduction in cholesterol synthesis may explain our findings following peripheral nerve injury (Fig. 4.1). Although we see similar temporal expression patterns of myelin structural proteins between WT and transgenic littermates, including myelin basic protein (MBP) and MPZ, we never assessed the subcellular localization of these proteins. A reduction in Schwann cell cholesterol availability may impair MPZ trafficking to the myelin sheath which could explain the delay in myelin compaction seen at 31 days post injury. Additionally, a delay in compaction may facilitate the transport of metabolites, proteins and ions, possibly favoring regeneration (Nave, 2010). Perhaps a reduction in cholesterol supplied by Schwann cells could result in slower neurite and axonal membrane outgrowth following injury, resulting in a ~1:1 pairing of regenerating fibers and Schwann cell columns; in this scenario, axons may reach target tissues in a more effective ratio and require less pruning.

Krox-20 (Egr-2) was recently shown to regulate, at least in part, cholesterol/lipid biosynthetic genes during peripheral nerve myelination in the sciatic nerve (Leblanc et al., 2005). The sterol regulatory element binding protein (SREBP) pathway controls the expression of several cholesterol/lipid synthesis genes. Krox-20 and SREBP transactivators synergistically activate promoters of many SREBP target genes to regulate myelination during development (Leblanc et al., 2005). Immunoblots showed a significant decrease in naïve, krox-20 protein expression levels in IκBα-dn mice, paired with normal myelin structure. This, along with preliminary gene expression studies, suggests that naïve, transgenic animals require less cholesterol for myelin maintenance during
adulthood; peri-natal studies of transgenic animals may exhibit a delay in myelination that is compensated for by adulthood (2-4 months of age).

It was recently shown that ephrin-B/EphB2 signaling between fibroblasts and Schwann cells is required for cell sorting and subsequent directional cell migration out of nerve stumps in order to guide regenerating axons across the site of injury (Parrinello et al., 2011). Loss of EphB2 signaling impairs the organized migration of Schwann cells which results in misdirected axonal regrowth, in vivo. Downstream to EphB2, this process is mediated by the stemness factor Sox2 via N-cadherin relocalization to Schwann cell-cell contacts. An impairment in Schwann cell sorting has been unexplored in our model and may offer an explanation for the delayed regeneration seen at 31 days post nerve injury (Fig. 4.2). Since NF-κB transcriptionally regulates several genes that could be involved in this process, such as extracellular matrix proteins (e.g. fibronectin) and integrins in Schwann cells. Since we see improved regeneration by 65 days following crush injury, a putative aberration in the sorting process may ultimately lead to better axonal guidance in our model.
Figure 4.1. NF-κB activation in Schwann cells may be required for cholesterol synthesis and compact remyelination. Above is a diagram of the events occurring following peripheral nerve crush injury. Shortly following injury, the distal nerve stump undergoes Wallerian degeneration as myelin and axons break down and are primarily cleared away by infiltrating macrophages (1). Axons then send out neurites which are guided across the injury site by Schwann cells (2). Following Schwann cell contact, regenerating axons are re-myelinated, which requires normal to high levels of cholesterol (3). Functional inhibition of NF-κB, however, reduces cholesterol levels impairing the proper trafficking of key myelin structural proteins, such as myelin protein zero (MPZ), from the endoplasmic reticulum (ER) to the myelin membrane impairing or delaying myelin compaction (4). Figure adapted from Saher et al., 2011 and http://imueos.blogspot.com/2010/11/degeneration-regeneration-of-peripheral.html.
Figure 4.2. NF-κB activation in Schwann cells may be required for Schwann cell sorting following crush injury. Diagram of how impaired Schwann cell sorting can reduce or delay regeneration following crush injury. (Top) Three uninjured axons within a nerve fascicle innervating target skeletal muscle. Following crush injury (1) the distal stump undergoes Wallerian degeneration and the denervated muscle begins to atrophy. Schwann cells and fibroblasts coordinate migratory movement to the injury site to guide regenerating axons. (2) Regenerating axons send out neurites which grow through Schwann cell columns, guiding them to their distal target. Axons growing through Schwann cell columns are then re-myelinated and the other axons will be pruned away (3). (?) If Schwann cells are not sorted properly, by fibroblasts, they can’t form Schwann cell columns to quickly guide regenerating axons to their target tissue. Figure adapted from http://imueos.blogspot.com/2010/11/degeneration-regeneration-of-peripheral.html.
Our findings in adult, naïve transgenic mice demonstrate normal myelination at two months of age within the facial nerve. It is possible that there is a developmental delay in myelination that is compensated for by two months of age; this scenario would mirror our post-injury findings and suggest that there is a recapitulation of developmental signaling cascades during regeneration. In order to determine this, transgene expression must be assessed and quantified in perinatal nerves. Since our transgene is under a GFAP promoter and GFAP is not uniformly expressed during development, these studies will be invaluable in elucidating the role of NF-κB in developing Schwann cells.

Our transgene expresses a FLAG tag that is undetectable in immunoblotting and immunohistochemistry assays making it impossible to quantify the number of cells bearing transgene expression. However, a significant reduction in Krox-20 protein expression strongly indicates the efficacy of our transgene in naïve IκBα-dn mice. Additionally, following injury, transgene expression is detectable and effectively inhibits p65 activation in denervated Schwann cells in transgenic mice. However, once denervated Schwann cells begin maturing into a myelinating phenotype they will forfeit GFAP expression; those pursuing a non-myelinating phenotype will retain GFAP expression. Since we only measured transgene expression up to 12 days following injury, it is possible that the transgene is suppressed at the time points used to assess regeneration, 31 and/or 65 days following injury. In this case, the improvements in regeneration and remyelination, seen from 31 to 65 days following injury in transgenic mice, may simply be a result of a lack of transgene expression; this
would imply that NF-κB inhibition in GFAP-expressing Schwann cells impairs regeneration and remyelination and that upregulating NF-κB following this impairment will result in improved regeneration and normal myelination. Hence, future studies to assess the temporal expression pattern of our transgene are forthcoming.

When considering the temporal expression pattern of GFAP during development and following injury, another problem with our model becomes evident. Since GFAP is not expressed in mature, myelinating Schwann cells, we cannot directly understand the initial role played by NF-κB activation in these cells. Since transgenic animals exhibit a normal immune response and normal Wallerian degeneration, it is likely that NF-κB activation is initially unimpaired. There are advantages to the conditional activation of our transgene in that we can study the entire population of denervated GFAP-expressing Schwann cells, as well as immature Schwann cells during development and mature non-myelinating Schwann cells. Furthermore, since GFAP is expressed in astrocytes, it is difficult to completely rule out their contribution in this model. Future studies repeating these results in a different genetic model will be invaluable in elucidating the developmental and regenerative role of NF-κB activation in Schwann cells.

IKKβ is necessary and sufficient for canonical NF-κB signaling (Hayden and Ghosh, 2008); currently we are developing knockout mice (P0ΔSC), lacking IKKβ in mature myelinating Schwann cells. Briefly, using a cre-lox system, mice carrying a floxed IKK-β allele (IKK-βF/F) will be crossed with mice expressing cre
recombinase under the control of a P0 promotor, in order to delete IKKβ in P0-expressing Schwann cells. Since P0 is only expressed by Schwann cells in the peripheral nervous system, we will be able to study the role of NF-κB signaling in Schwann cell precursors, immature Schwann cells, and mature myelinating Schwann cells. Additionally, comparing the outcome of a crush injury to our previous findings with IκBα-dn mice will enable us to determine any weighted contribution of CNS astrocytes in injury and facial nerve repair.

Currently, there are several rodent facial nerve experimental models to ultimately assess regeneration, reinnervation and functional recovery (for review, see Moran and Graeber, 2004). These models implement transection, transection and suture, crush, compression, resection, and/or avulsion injuries. One overarching issue with these models involves the difficulty in accounting for axonal misguidance when assessing regeneration. Here, we report a facial nerve crush injury model that enables the study of a single nerve branch and its target tissue. Although axon regeneration is staggered, occurs at different rates, and axons rarely reinnervate their original tissue, we are able to accurately study the quantity of regenerating axons at static time points. Additionally, our model does not rely on the contralateral facial motor nerve as an intraanimal control; a controversial control to use in regeneration studies since we are unsure of how it may be affected or altered by the ipsilateral injury. Many future studies will benefit from the accuracy of the model developed and utilized in our studies.

Interestingly, the facial nerve is typically treated as a pure population of motor fibers. We found an abundance of unmyelinated fibers within naïve and
regenerating facial nerves that may represent a population of unmyelinated motor fibres or sensory bundles. In the latter case, future facial nerve regeneration studies may need to take extra consideration for the potential pain response during and following injury.

The facial nerve is usually damaged as a result of trauma, inflammation, surgical manipulation or neoplastic disease. Current techniques for nerve repair yield suboptimal results. Among the most successful clinical techniques involve nerve-nerve sutures or nerve-nerve grafts, both of which should be performed within a few weeks of injury (Barr, 1991; Choi and Dunn, 2004). Several different tube, matrix, and guidance structures have been explored in animal facial nerve regeneration models. For example, Schwann cell-seeded nerve grafts can bridge up to 6 mm gaps in the mouse peripheral nerve (Rodriguez et al., 2000). Our studies suggest that manipulating the time and duration of NF-κB activation in SCs may be improve the regeneration capacity of SC-seeded grafts, a finding that would certainly be of clinical interest.

Facial nerve assault evokes a series of responses within injured neurons, microglia, Schwann cells, and immune cells. NF-κB is ubiquitously expressed and is a target of several approved pharmaceutical drugs (Miller et al., 2010), including many non-steroidal anti-inflammatory drugs (NSAIDS). Our findings indicate a role for NF-κB signaling in facial nerve regeneration. Although NSAIDS may reduce inflammation and pain, the acute use of these drugs may delay or impair regeneration. Future studies will likely be therapeutic when
considering the types of pharmaceuticals utilized in the clinic on patients suffering peripheral nerve injuries.

Following spinal cord injury, endogenous Schwann cells can invade the spinal cord and provide a permissive substrate for regeneration and/or remyelination (Gilmore and Duncan, 1968; Hirano et al., 1969; Feigin and Ogata, 1971; Blakemore, 1975; Raine, 1976; Blight and Young, 1989; Bunge, 1994; Bunge et al., 1994; Beattie et al., 1997; Bresnahan, 1978; Takami et al., 2002). For nearly a century, peripheral nerve grafts have successfully been used to promote regeneration within the spinal cord (Ramon Y Cajal, 1928; Richardson et al., 1980; David and Aguayo, 1981; Oudega et al., 1994, 1996; Chenge et al., 1996; Blits et al., 2000). Unfortunately, large pieces of peripheral nerve are needed from the spinal cord injured patient; however, therapeutic cloning techniques are forthcoming and autologous SCs are currently being harvested from embryonic stem cells in vitro (Galli et al., 2003; Oudega and Xu, 2006). Based on our studies, Schwann cell transplants with modified NF-κB signaling may also be therapeutic for spinal cord regeneration.

4.1 Conclusion

NF-κB activation in denervated Schwann cells is required for facial nerve regeneration.
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