Myelination in the Developing Chicken Ciliary Ganglion

by

Alex Tsung-Lin Ho

A DISSERTATION

Presented to the Department of Cell and Developmental Biology
and the Oregon Health and Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

May 2003

School of Medicine

Oregon Health and Science University

CERTIFICATE OF APPROVAL

This is certify that the Ph.D. thesis of

Alex T. Ho

has been approved.

[redacted]

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

[redacted]

Member

[redacted]

Member

This dissertation is dedicated to my maternal grandparents,
Luke Lee and Eng-Er Pan,
who have gone to heaven during my years at O.H.S.U.

Through their hopes, dreams, and beliefs
they taught me
that with faith, anything is possible.

TABLE OF CONTENTS

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

ACKNOWLEDGEMENTS

A warrior once told a king that if an individual saves the life of a dying warrior, then the rescued warrior owes the rest of his entire life to this individual. If that is so, then I owe the rest of my scientific life to my advisor, Dr. Rae Nishi. It has been a great honor and privilege to work with her throughout my graduate education.

I would also like to thank the members of my Thesis Advisory Committee/Exam Committee who spent a tremendous amount of their precious time to help guide me through all of the hurdles that allowed me to complete this dissertation: Dr. Phil Copenhaver, Dr. Bruce Patton, Dr. Karla Kent, and Dr. Gary Banker.

A special thanks goes to Dr. Jan Christian, who came through at the last minute to enable me to retain my residency status at O.H.S.U. while I completed my thesis work at UVM.

I would like to thank the members of the Nishi and Eckenstein labs, past and present, notably Drs. Tom Finn, Diane Darland, Juan Sheu, and Gillian Bunker, who taught me all the important scientific skills through their examples. Dr. Takuya Nakayama, Dr. Sachiko Take, Dr. Anh-Chi Le, Songte Kim, Doris Kulhanek, Greg Smiley, Niki Larson, Ken Coladner, Karen Richer, and many others offered their friendship to make my life as a graduate student memorable.

My greatest appreciation is given to my parents, Kent and Mellisa Ho, who never doubted my decision to come to Oregon to pursue my interest in science. They have always been supportive regardless of whatever I choose to do. I would also like to thank my in-laws, Thomas, Ling, and Elaine Lee, who have been my true family here in Portland, Oregon.

My life would never be complete without my wife, Nicole, who has always been encouraging though the ups and downs of our life together. She has helped me in so many ways that I truly will not be writing this sentence if it wasn't for her love.

And finally, I give my thanks to God for giving me the "touch".....

ABSTRACT

The avian ciliary ganglion (CG) consists of two distinct populations of neurons, ciliary and choroid neurons (Marwitt et al., 1971). Ciliary neurons are myelinated and innervate the iris and the ciliary body (Hess, 1965; Pilar et al., 1987). Choroid neurons are unmyelinated and innervate the vascular smooth muscle of the choroid layer (Meriney and Pilar, 1987). This study addresses how the target tissues of the CG neurons regulate the preferential myelination of ciliary neurons in the developing ganglion. We show that glial cells in the CG differentiate to become either myelinating or non-myelinating Schwann cells between E14 and E18, with the expression of myelin markers Krox20 and P0 starting at E14. At E8, all glial cells express the low-affinity NGF receptor (p75). Schwann cells expressing Krox20 and P0 are detected only in the ciliary nerves and Schwann cells found in choroid nerves are all p75-positive. Unlike the choroid target which consists of only smooth muscles, the ciliary target undergoes a smooth to striated muscle transition between E11 and E17 (Link and Nishi, 1998a). Using *in vitro* methods, we tested whether diffusible factors from different CG targets induce the expression of myelin markers in CG/Schwann cell co-cultures. The data suggest the presence of a target-derived effect that may indirectly regulate the preferential myelination of ciliary neurons. We also show that preferential myelination of ciliary nerves coincides with increased expression of neuregulin in the cell bodies of ciliary neurons. Thus, neuregulin may act as a neuron-derived signal to promote the initial myelination of surrounding Schwann cells. We utilized a replication competent avian retrovirus expressing ribozymes targeted to the neuregulin mRNA (RCASBP(A)-4XRZnrg) to test whether the

expression of neuregulin by ciliary neurons at E14 is necessary to promote myelination processes, specifically the expression of Krox20 and P0. When a suspension of concentrated viral particles was microinjected into the neural tube of stage 9 chicken embryos, the virus infected migrating neural crest cells that eventually formed the CG. Immunoblot analysis of the infected CG showed that there was a 20% to 60% reduction of neuregulin protein in RCASBP(A)-4XRZnrg infected CG, compared to that of control CG. Immunohistological analysis of the infected CG showed an attenuation of neuregulin immunoreactivity in the neurons. In both the ganglia and ciliary nerves Krox20 expression was disrupted in ribozyme infected tissues. However, the expression of P0 in the ciliary nerve remained unchanged. This difference suggested that expression of Krox20 and P0 in chickens during myelination may be regulated independently. These data describe a model that striated muscle of the iris/ciliary body release diffusible factors preferential expression of neuregulin in ciliary neurons which may induce the surrounding Schwann cells to myelinate the ciliary nerve.

INTRODUCTION

I. Myelination – Background

Myelination of neurons allows increased speed in action potentials without increasing the diameter of the axons. During the development of the peripheral nervous system, Schwann cell precursors migrate from the neural crest (Le Douarin et al., 1991) and line up along the axons at equal intervals. The Schwann cells form a 1:1 axon to Schwann cell relationship where, unlike oligodendrocytes in the central nervous system which myelinate multiple neurons, one Schwann cell interacts with only one segment of an axon. As the Schwann cells form a double-membrane structure surrounding the axons in concentric layers, the cytoplasm of the Schwann cells is forced out, and the membranes condense into compact lamellae. Myelinating Schwann cells secrete extracellular matrix molecules and synthesize components of mature peripheral myelin, forming a membrane sheath enriched in lipids and proteins. As many as 500 Schwann cells may line up to myelinate a single axon, each sheath equally spaced apart by the nodes of Ranvier (Bischoff and Thomas, 1975). The saltatory conduction created by myelinated axons may be potentially as fast as 100 times that of non-myelinated axons of an equal diameter (Waxman, 1980).

Early studies of peripheral nerves using electron microscopy identified two types of nerve fibers: myelinated (Bischoff and Thomas, 1975) and non-myelinated (Ochoa, 1975). Myelinated fibers were described as fibers consisting of individual axons wrapped with

Schwann cells that created a myelin sheath. The myelin sheath was characterized as highly organized concentric layers of membrane enriched with alternating cylindrical layers of lipid and protein. When fixed with osmium tetroxide and visualized using electron microscopy, the area of the compact myelin rich in protein appeared as an electron dense structure and the hydrocarbon chains of the bimolecular lipid layer as electron light region. This alternating dark and light region of the Schwann cell membrane wrapping around the axons became the defining mark of myelinated neurons (Bischoff and Thomas, 1975). On the other hand, non-myelinated fibers were composed of Schwann cells that associated with multiple axons, but never developed any myelin sheath. Due to the lack of any dense structures around the axons and the fact that the average non-myelinated neurons were significantly smaller compared to myelinated neurons, characterization of non-myelinated neurons has been difficult without the aid of high-powered electron microscopes. Studies in the ultrastructure of non-myelinated fibers indicated that there are varying degrees of interactions between Schwann cells and non-myelinated axons. Schwann cells in non-myelinated fibers were found to either wrap around a bundle of multiple axons loosely in one big pile, or one Schwann cell interacting with multiple axons so that individual axons in the fiber were independent of each other. In all cases, the ensheathing Schwann cells of non-myelinated fibers lacked any dense myelin deposit. Thus, the characterizations of unmyelinated fibers were defined as any nerve fiber which lacked dense myelin deposits (Ochoa, 1975).

These observations made thirty years ago, characterizing the differences between myelinated and non-myelinated nerve fibers, gave rise to numerous questions, many

which still remain unanswered. For instance, what signals Schwann cells to myelinate or not myelinate an axon? Are all Schwann cells capable of myelinating an axon? Is there any difference in myelination between the peripheral nervous system and the central nervous system? The answers to these kinds of questions have been important in understanding the pathology of many neurological disorders based on defects in the myelinating process.

The availability of a number of mouse mutants with defects in the myelin-forming genes has allowed investigators to dissect out the various molecular components that make up the myelin sheath (Nave, 1996). The three major molecules that make up the myelin sheath in the peripheral nerves are protein zero (P0), myelin basic protein (MBP), and peripheral myelin protein 22 (PMP-22). Myelin in the peripheral nerve is composed of approximately 50% P0, 20% MBP, 5% PMP-22, and 1% myelin-associated glycoprotein (MAG). On the other hand, myelin in the central nervous system is made up of approximately 50% proteolipid protein (PLP), 40% MBP, and a small percentage (< 1%) of MAG.

Deletion mutants of specific myelin genes give rise to mice expressing very specific phenotypes that distinguish one mutant from another. For instance, individuals with a P0 null mutation have a disease known as Charcot-Marie-Tooth type 1b, characterized by the hypomyelination of peripheral nerves (Giese et al., 1992; Martini et al., 1995). P0 is a cell adhesion molecule that belongs in the immunoglobulin gene superfamily (Lai et al., 1987). The Ig domain of the P0 protein has been hypothesized to be involved in

homophilic interactions in both the intracellular and extracellular membrane that aid in myelin compaction. Mutations in the PLP gene result in mice described as *jimpy* mutants, characterized by dysmyelination in the central nervous system (Nave et al., 1986). PLP is co-expressed with DM-20 to form the major integral membrane protein of myelin in the CNS (Milner et al., 1985). The four hydrophobic domains in the PLP gene are homologous to many transmembrane proteins which form pores or channels. The *jimpy* mutation is caused by the truncation of the fourth hydrophobic domain in the PLP gene (Hudson et al., 1987; Macklin et al., 1987; Morello et al., 1986; Nave et al., 1987; Nave et al., 1986). *Shiverer* mutants are caused by the lack of MBP, which affects myelination in both the peripheral nervous system and central nervous system (Popko et al., 1988; Roach et al., 1985). MBP is a group of positively charged molecules that stabilizes the negatively charged head groups in the lipid bilayer of the compact myelin. Though expressed in both CNS and PNS, the expression of P0 in the PNS myelin can compensate for the lack of MBP (Martini et al., 1995). Thus, the severe pathology in *shiverer* mutants is due primarily to the lack of myelin structure in the CNS neurons (Rosenbluth, 1980; Shen et al., 1985). *Trembler* is also a peripheral nervous system specific mutation caused by the deletion in the PMP22 gene (Suter et al., 1992). Similar to the PLP gene, PMP22 is an integral membrane protein that codes for four highly hydrophobic transmembrane domains. However, PMP22 is expressed mainly in the PNS, thus deletion of the transmembrane domain results in the disruption of PNS myelin instead of CNS myelin as seen with PLP mutants (Suter et al., 1992).

Transcription factors that are expressed specifically in Schwann cells during myelination have provided insights into how various myelin genes are regulated. The most prominent and well characterized transcription factor expressed in the Schwann cells during myelination is Krox20 (Stewart et al., 1996). Krox20 is a zinc finger protein that becomes up-regulated in Schwann cells during the early stages of myelination and remains expressed in the myelinated nerve through adulthood. Krox20 knockout mice do not properly myelinate their peripheral nerves (Topilko et al., 1994). The Schwann cells form a 1:1 association with neurons and the expression of P0 and MBP remain low, resulting in a lack of compact myelin. The Schwann cells of Krox20 knockout mice remain at a premyelinating stage, indicating that expression of Krox20 is necessary for the initiation of the myelinating program in the Schwann cells. Another transcription factor expressed in the Schwann cell during myelination is SCIP (also known as Oct-6). SCIP is a cAMP-inducible POU domain gene (Monuki et al., 1989) and, like Krox20, becomes up-regulated at the start of myelination. SCIP knockout mice have a similar phenotype to that of Krox20 null mutants (Zorick et al., 1996). However, in normal individuals, the rapid down-regulation of SCIP after its initial expression is necessary for P0 and MBP to be expressed properly in myelinating Schwann cells (Monuki et al., 1993).

The processes involved in regulating the myelination of neurons by the Schwann cells are extremely complex. Myelination is a multi-step process where Schwann cells undergo a complex transformation to create the myelin sheath, thus requiring precise regulation at each step. First, migrating neural crest cells need to differentiate to form Schwann cell

precursors. Next, the Schwann cell precursors must determine whether or not to myelinate the axons. Then, the myelinating Schwann cells synthesize a specialized membrane to wrap around the neurons multiple times. Once the tight association with the axon is established, the Schwann cells create a thick insulation around the axons to form a myelin sheath. Schwann cells also play an important role in the survival of peripheral neurons both during development and injury. Thus, elucidating the process of myelination in the developing embryo is critical to understanding the development of the nervous system.

II. Development of Schwann cells

The majority of Schwann cells that make up both myelinating and non-myelinating Schwann cells in the peripheral nervous system arise from the migrating neural crest (Anderson, 1993; Bronner-Fraser and Fraser, 1991; Le Douarin et al., 1991). Exceptions include some of the Schwann cells in the ventral root of the spinal cord which arise from the ventral neural tube (Lunn et al., 1987), and a subpopulation of glia in the dorsal root ganglia which originate from neuroepithelial cells in the spinal cord after migration of the neural crests is complete (Sharma et al., 1995). Besides Schwann cell precursors, migrating neural crest cells give rise to neuronal and melanocyte precursors, as well as variety of other non-neuronal cells (Le Douarin and Smith, 1988). Once neural crest cells are specified to follow a glial lineage, Schwann cell precursors receive specific signals from their migrating environment to differentiate into immature Schwann cells. Though still unclear, there is some evidence that certain signals may induce neural crest cells to

choose a Schwann cell fate over a neuronal or melanocyte fate. Two such factors are neuregulin and endothelin. *In vitro*, both neuregulin (Dong et al., 1995) and endothelin (Brennan et al., 2000) have been shown to induce neural crest cells to choose a glial fate and also block their entry into a neuronal lineage. *In vivo*, however, disruption of neuregulin signaling in developing mice does not inhibit formation of immature Schwann cells (Kramer et al., 1996; Meyer et al., 1997), indicating that there are multiple signals other than neuregulin that regulate the differentiation of Schwann cells from the migrating neural crest. On the other hand, a lack of functional endothelin receptors in the developing rat showed accelerated maturation of Schwann cells in embryonic hindlimb nerves, as indicated by an increase of S100 staining in the developing Schwann cells (Brennan et al., 2000). This effect indicates that endothelin signaling may be necessary to maintain the number of immature Schwann cells in the developing nerve prior to the onset of myelination.

Originally discovered as a vasoconstrictor peptide secreted by endothelial cells (Yanagisawa et al., 1988), endothelins have been well characterized as a modulator in neuroendocrine regulation (Kuwaki et al., 1997; van den Buuse and Webber, 2000). Currently there are three members in the endothelin family (ET-1, ET-2, and ET-3), each peptide made up of 21 amino acids joined by two disulfide bonds and related to a family of polypeptide factors related to the sarafotoxins (Hiley, 1997). Analysis of endothelin signaling in primary glial cultures has shown that endothelin increases the level of mitogen-activated protein kinase (MAPK) in Schwann cells (Berti-Mattera et al., 2001; Berti-Mattera et al., 2000), which is known to be a key step during myelination.

However, current *in vitro* and *in vivo* analysis have only shows that endothelin blocks programmed cell death of Schwann cell precursors and delays generation of mature Schwann cells from precursors (Brennan et al., 2000). Hence, there is insufficient evidence to suggest that endothelin directly regulates the formation of either myelinating or non-myelinating Schwann cells.

Current studies in Schwann cell biology advocate neuregulin as one of the key regulators in Schwann cell development (Garratt et al., 2000a; Gassmann and Lemke, 1997). Originally characterized from multiple sources, neuregulin is also known as sensorimotor-derived factor, acetylcholine receptor-inducing activity, heregulin, neu differentiation factor, and glial growth factor. The neuregulin gene gives rise to multiple isoforms of neuregulin protein by alternative splicing, giving the molecule a wide range of possible functions. However, they all contain an epithelial growth factor-like (EGF-like) domain, which is necessary for their activities, thus categorizing neuregulins as a part of the EGF superfamily. The EGF-like domain of neuregulin proteins binds to tyrosine kinase receptors of the EGF receptor family, specifically ErbB-3 and ErbB-4. On the other hand, the ErbB-1 receptor only binds to epidermal growth factor, while ErbB-2 acts as a co-receptor that facilitates neuregulin binding to ErbB-3 and ErbB-4.

Various mouse mutants have been created to address the role of neuregulin signaling in Schwann cell development. Initial knockout studies which disrupted the expression of neuregulin genes (Meyer and Birchmeier, 1995), ErbB-2 (Lee et al., 1995), and ErbB-4 (Britsch et al., 1998) in mice were lethal at embryonic day 10.5 due to lack of

trabeculation in the heart, providing little information about the role of neuregulin in Schwann cell development. The first *in vivo* indication that neuregulin signaling was important in Schwann cell development appeared in ErbB-3 knockout mice (Britsch et al., 1998; Riethmacher et al., 1997). Homozygous ErbB-3 knockout mice that developed to term were motionless and unable to breathe. Histological analysis of the nerves showed that Schwann cell precursors along motor neurons and the enteric nervous system were absent. Interestingly, though sensory neurons in the dorsal root ganglia of the mutants undergo cell death after the ganglia is formed, the glial cells in the dorsal root ganglia and in the CNS appear normal (Riethmacher et al., 1997). Subsequently, various conditional knockout mice which circumvented problems with heart malformation showed that neuregulin is vital in maintaining the survival of peripheral nerves (Morris et al., 1999; Woldeyesus et al., 1999), retaining the number of presumptive glial cells in cranial ganglia (Meyer et al., 1997), and creating the thickness of the myelin sheath (Garratt et al., 2000b). Restoring neuregulin signaling in the hearts of ErbB-2 knockout mice showed that a lack of ErbB-2 signaling in peripheral nerves inhibited migration of Schwann cell precursors along peripheral neurons and caused the motor and sensory neurons projecting from the dorsal root ganglia to defasciculate (Morris et al., 1999; Woldeyesus et al., 1999). Conditional knockout using *Cre-loxP* technology that introduced ErbB-2 ablation in *Krox20-cre* allele created ErbB-2 mutants that lacked ErbB-2 expression in cells expressing the Krox20 transcription factor. The lack of ErB-2 signaling in cells that expressed Krox20 showed two- to three-fold reductions of myelin thickness in the sciatic nerves of 6 month-old mice (Garratt et al., 2000b). These observations indicated that neuregulin is necessary in both the early stages of Schwann

cell development, such as establishing the Schwann cell precursor pool, and the maturation of Schwann cells, such as controlling the myelin thickness of peripheral neurons.

Once neural crest cells become specified as immature Schwann cells, interactions between these cells and adjacent axons determine whether they will differentiate into myelinating or non-myelinating Schwann cells. In rodents, approximately 80% of peripheral neurons are ensheathed in myelin (Bischoff and Thomas, 1975), while the rest remain associated with non-myelinating Schwann cells. Interestingly, myelinated axons are generally larger in diameter than non-myelinated axons (Berthold et al., 1983; Fraher, 1976; Friede and Samorajski, 1967). Furthermore in the PNS, the highest numbers of myelinated axons are found in the somatic motor neurons that innervate skeletal muscle. On the other hand, almost all neurons in the autonomic system are unmyelinated. It is important to note that the autonomic motor neurons that are unmyelinated innervate only cardiac and smooth muscles.

Myelinating Schwann cells may be identified by the increased expression of myelin-specific proteins such as P0, MBP, PMP22, and MAG. However, recent studies suggest that P0 which make up approximately half of the myelin component (Zhang et al., 1995), may be expressed at very low levels in Schwann cell precursors during early development. Thus in some case it is helpful to also analyze the expression of transcription factors such as Krox20 and SCIP that is specific to myelinating Schwann cells, to determine the type of Schwann cells associated with each neurons. On the other

hand, non-myelinating Schwann cells do not express any myelin-specific genes, but they retain a similar phenotype to immature Schwann cells, including expression of neural cell adhesion molecule (NCAM), L1, growth-associated protein 43 (GAP-43), and the low affinity NGF receptor (p75).

Myelinating and non-myelinating Schwann cells interchange their myelin gene expression depending upon the axons with which they associate. Early experiments using cross-anastomosis of nerves revealed that non-myelinating Schwann cells have the potential to myelinate axons when exposed to appropriate environmental cues (Weinberg and Spencer, 1975). When a severed proximal stump of the myelinated sternohyoid nerve (SN) was connected to the distal stump of an unmyelinated cervical sympathetic trunk (CST), the regenerated axons from the SN to the CST became myelinated. Individually radiolabeling each stump with ^{3}H-thymidine demonstrated that the Schwann cells which myelinated the regenerated nerve originated from non-myelinating Schwann cells residing in the CST.

Another example that demonstrates the ability of Schwann cells to switch between myelinating and non-myelinating Schwann cells occurs during peripheral nerve injury (Scherer and Salzer, 1996). When a myelinated peripheral nerve is injured, the severed axon signals surrounding Schwann cells to down-regulate rapidly its myelin genes and revert back to an immature or non-myelinating state, characterized by the increased expression of NCAM, L1, GAP-43, and p75. Schwann cells also proliferate to replace any damaged Schwann cells that are removed by invading macrophages. Once neurons

re-innervate through the damaged nerve, Schwann cells quickly remyelinate the axons. This effect is signified by the down-regulation of non-myelinating markers and up-regulation of myelin-specific genes such as P0, MBP, PMP22, and MAG. Thus, Schwann cells are capable of switching between myelinating and non-myelinating phenotypes in mature nerves, and they can down- or up-regulate myelin specific genes in response to injury. Furthermore, if injured nerves originally contain myelinated neurons, then the Schwann cells along the re-innervated neuron will myelinate the axons. However, the mechanisms of how Schwann cells in the regenerated nerve are able to distinguish between neurons that were previously myelinated or un- myelinated in the injured nerve are still not well understood.

The nerve-derived signals that regulate myelination still remain to be elucidated. One hypothesis suggests that an increase in neuronal activity may promote the myelination of neurons. An *in vitro* analysis using mouse dorsal root ganglia neurons demonstrated that when axons were stimulated for five days with an electrode at either 0.1 Hz or 1 Hz, the formation of compact myelin in the neuron/Schwann cell co-cultures remained unchanged as compared to control cultures. However, in 0.1 Hz cultures, the level of MBP expression decreased by three-fold (Stevens et al., 1998). This decrease indicates that a low stimulation rate (0.1 Hz) in DRG neurons may have an inhibitory effect on their myelination.

Another hypothesis states that myelination may be dependent on the thickness of the axons, stating that myelination is favored in axons that are larger in diameter, while

smaller sized axons tend to remain unmyelinated. Interestingly, analysis of myelin thickness in mice lacking both mid-sized and heavy neurofilament subunits showed that in the PNS, the reduced axon diameter of the L5 ventral root neurons caused by a lack of neurofilament subunits retained their myelin sheath thickness (Elder et al., 2001). Further analysis of the myelin thickness in the mutant nerves showed that even though the axon diameter was reduced by 30%, the thickness of myelin remained constant, causing an over-wrapping of PNS neurons (Elder et al., 2001). Manipulating the axon diameters of sympathetic neurons by surgically altering the size of target tissues has also suggested how certain neurons may become myelinated. In rats, 99.3% of the superior cervical ganglion neurons are unmyelinated and innervate the submandibular salivary gland. However, when one branch of the sympathetic nerve innervating the gland was surgically severed, the increase ratio of target tissues to neurons promoted the axon diameter of the remaining neurons increased by 24%. Upon analysis, 60% of the axons in the manipulated nerve became myelinated (Voyvodic, 1989).

The key mechanism or signal that regulates the commitment of the Schwann cells to choose a myelinating or non-myelinating fate is still unclear. Studies suggest that axon-derived signals are responsible for regulating immature Schwann cells to enter either a myelinating or non-myelinating pathway. Since almost all PNS neurons associate with Schwann cells in one form or another, changes in signals expressed along the axonal membrane must regulate differentiation of Schwann cells. However, the exact type of signal still remains to be elucidated. This signal may be either promoting the Schwann cells to myelinate axons, or inhibiting the differentiation of Schwann cells so that they

remain unmyelinated. The axon bound signal may be embedded in the axon membrane or secreted as a diffusible factor to signal Schwann cells to differentiate. It is important to understand how this axon-derived signal is regulated. For instance, does the decrease in neuronal activity inhibit the myelination of DRG neurons? Or, does the increase in axon diameter promote myelination of sympathetic neurons? Do the targets affect the expression of these inhibitory or promoting signals? In order to understand how the differentiation of Schwann cells are regulated during development, it is necessary to first analyze the signals expressed during myelination.

III. Signals involved in myelination

Understanding the mechanisms of peripheral nerve regeneration has suggested roles for various neurotrophins in regulating myelination (Scherer and Salzer, 1996). During injury, levels of NGF (Heumann et al., 1987; Lindholm et al., 1987), BDNF, NT-3, and NT-4/5 (Funakoshi et al., 1993a; Meyer et al., 1992) in the injured nerve increase over time. These neurotrophins are released by multiple cell types which make up the nerve including the injured axons, the Schwann cells, and the cells which form the outer layers of the nerve. Recent studies show that during the regeneration of axons following injury repair, the low affinity neurotrophin receptor (p75) has been shown to play an important role. During nerve injury, expression of p75 in the Schwann cells is up-regulated (Taniuchi et al., 1986; Taniuchi et al., 1988), indicating that as the nerve becomes injured, the Schwann cells revert from a myelinating to a non-myelinating state. *In vitro*, when p75 signaling was disrupted in DRG neuron/Schwann cell co-cultures with p75

specific blocking antibodies, the accumulation of MAG and P0 protein in cultures was attenuated (Cosgaya et al., 2002). On the other hand, when activation of TrkB receptors was blocked with blocking antibodies or inhibitors, the expression of myelin specific gene increased in DRG neuron/Schwann cell cultures (Cosgaya et al., 2002). Furthermore, Schwann cells in normal peripheral nerve express the truncated form of TrkB and TrkC (Funakoshi et al., 1993b; Sebert and Shooter, 1993) which lack the tyrosine kinase domain. This expression suggested that during re-myelination of injured axons, neurotrophins act through the p75 receptor, rather than the kinase cascade created by activated Trk receptors, to promote myelination of repaired neurons. Furthermore, in mouse mutants lacking the p75 gene, proliferation of Schwann cells is attenuated and ensheathment by Schwann cells is decreased (Bentley and Lee, 2000). However, during development, expression of multiple neurotrophins is detected in multiple types of tissues. For instance NGF is highly expressed in target tissues (Wheeler and Bothwell, 1992), while BDNF and NT3 are expressed at different times during myelination in both neurons and Schwann cells (Chan et al., 2001). Thus, it is still unclear whether the p75-dependent signal during myelination is an axon specific signal or a more global signal that is expressed in the nerve.

Members of the transforming growth factor-β (TGF-β) superfamily have also been shown to mediate many important roles during axon-glial interactions. The TGF-β superfamily includes the three isoforms (TGF-β1, TGF-β2, and TGF-β3), activins, bone morphogenetic proteins (BMP), and glial-derived neurotrophic factor. The effect of TGF-β members is transduced by serine-threonine kinase receptors named as type-I and

type-II (Wrana et al., 1994). Schwann cells express both types of receptors (Einheber et al., 1995; Ridley et al., 1989) and have very different effects depending on the conditions at which they are tested. For instance, in purified Schwann cells TGF-β1 may act as a mitogen (Eccleston, 1992). On the other hand, addition of TGF-β1to neuron/Schwann cell co-cultures inhibits both axon dependent proliferation by Schwann cells and myelination (Einheber et al., 1995). However, since Schwann cells express all three TGF-βs (Einheber et al., 1995; Scherer et al., 1993), activins (Coulombe et al., 1993) and BMPs (Hall et al., 2002) expressed in target tissues, and GDNF in various cell types of the peripheral nerves (Henderson et al., 1994; Springer et al., 1995), it has been difficult to pinpoint which of these molecules act as the key axon-derived molecule during myelination.

Currently, the strongest candidate for an axon-derived signal that regulates the myelinating state of Schwann cells is still neuregulin. The different isoforms of neuregulin may be categorized into three groups (Burden and Yarden, 1997). Neuregulin Type I, which includes neu differentiation factor, heregulin and acetylcholine receptor-inducing activity, is expressed primarily in non-neuronal tissues during early development. Type II, which consists of only the glial growth factor, is expressed mainly in the nervous system. Type III, which was originally known as the sensorimotor-derived factor, is found predominantly in the peripheral sensory and motor neurons. Type I and Type II are also known as Ig-neuregulins because the extracellular domain of these isoforms contain an immunoglobulin-like domain. Type III is also named as CRD-neuregulin because this isoform contains a cystein-rich domain in the N-terminal side of

the molecule instead of the Ig-like domain. The CRD domain is hydrophobic and has been shown to re-insert the neuregulin molecule into the cell membrane, allowing the active EGF domain to be exposed to the extracellular side of the neuronal membrane. Recent *in vitro* experiments have suggested that CRD-neuregulin (Type III) is the isoform responsible for inducing Schwann cell precursors to express SCIP (Leimeroth et al., 2002), one of the myelin-specific genes. Thus, CRD-neuregulin may be the key neuregulin isoform that regulates the myelination of peripheral neurons.

IV. Avian ciliary ganglion and its targets

The chicken ciliary ganglion (CG) is an ideal system for studying the differentiation between myelinating and non-myelinating Schwann cells because the development of this system has been well characterized (Nishi, 1994b). The ciliary ganglion is located behind the eye, where it receives preganglionic input from the accessory oculomotor nucleus (Narayanan and Narayanan, 1976). This parasympathetic ganglion is composed of only two neuronal populations originating from a common precursor, and innervates two specific targets in the chick eye (Marwitt et al., 1971). The ciliary neurons innervate the striated muscles in the iris/ciliary body (Pilar et al., 1987), and the choroid neurons innervate the vascular smooth muscles of the choroid layer (Meriney and Pilar, 1987). The average size of the ciliary neuron is larger than the choroid neurons (Marwitt et al., 1971), and the ciliary neurons are the only population in the adult ciliary ganglion that is myelinated (Hess, 1965). Furthermore, ciliary neurons are unique in that even though

they are part of the autonomic nervous system, they are myelinated and innervate muscles that are striated.

The expression of somatostatin in choroid neurons has been shown to be target-dependent (Darland and Nishi, 1998). Although both CG targets express activin A, the ciliary target expresses another factor, follistatin, which inhibits the expression of somatostatin in ciliary neurons. In contrast, the lack of follistatin in the choroid target allows activin A to induce the expression of somatostatin in choroid neurons. Furthermore, while the expression of somatostatin is induced in choroid neurons, the target tissues of ciliary neurons undergo a smooth muscle-to-striated muscle transition. Thus the dramatic changes that occur between ciliary and choroid target tissues may also play a role in the preferential myelination of the ciliary neurons.

V. Goals of this study

The current study seeks to identify the mechanisms that regulate the myelination of ciliary ganglion neurons. The hypothesis is that the preferential myelination of ciliary neurons is influenced by signals from target tissues. Furthermore, the signal from the target tissue regulates the expression of axon-derived cues, such as neuregulin, in ciliary neurons to promote Schwann cells to myelinate axons. To test this hypothesis, I first characterized the expression of myelin genes in the developing ciliary ganglia to determine whether the myelinating processes correlate with target-dependent differentiation of the ganglia. Next, I tested whether co-cultures of neurons and Schwann

cells with either ciliary or choroid targets influenced the expression of the early myelin-specific transcription factor Krox20. Finally, I tested whether or not neuregulin played a role in regulating the expression of myelinating genes in Schwann cells by using a retroviral gene delivery system to knock down specifically the expression of neuregulin in the developing CG.

My results show that the preferential myelination of ciliary neurons begins at E14 and completed by E18 in the developing CG. This finding is concurrent with the observation that the target dependent neuronal cell death in ciliary ganglion is complete at E14 (Finn and Nishi, 1996a; Nishi, 1994b). Furthermore, the preferential expression of neuropeptide somatostatin in the choroid neurons which is target dependent becomes fully developed by E14 (Coulombe and Nishi, 1991; Darland et al., 1995). Lastly, the smooth-to striated muscle transition in the ciliary targets occurs between E11 and E17 (Link and Nishi, 1998a), where the change in the muscle type in the iris/ciliary muscles may indirectly affect the myelination of ciliary neurons. When co-cultures of CG neurons and Schwann cells are exposed to ciliary target tissues, the expression of myelin specific transcription factor, Krox20, is induced in the Schwann cells. On the other hand, incubating the co-cultures in choroid target tissues does not induce Krox20 expression. Furthermore, the increase of Krox20 is dependent on the presence of CG neurons with the Schwann cells. This increase suggests that Krox20 expression in Schwann cells may be dependent on signals from the neurons, which become altered in the presence of target tissues. Detailed analysis of neuregulin expression pattern in the developing CG also suggests that the axon-derived cue which induces the myelination of ciliary neurons may

be neuregulin. Knock down of neuregulin expression using replication competent avian retrovirus to deliver a ribozyme against the neuregulin mRNA in the developing CG suppresses the expression of Krox20 in the Schwann cells surrounding the ciliary neurons. These observations provide evidence that preferential myelination of ciliary neurons in the developing CG may be regulated in a target dependent manner. The target tissues may be regulating the myelination of neurons in an indirect manner by changing the expression of axon-derived cues which promote the differentiation of Schwann cells to myelinate. Furthermore, in the developing CG, neuregulin may be one of the axon-derived cues which regulate the myelinating state of the Schwann cells.

MATERIALS AND METHODS

Immunohistochemistry

List of antibodies:

The following primary antibodies were used at the indicated dilutions: rat α-somatostatin (1:100, Accurate Chemicals, Westbury, NY), mouse monoclonal antibody (mAb) 1E8, against chicken P0 (1:100, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), rabbit α-neurofilament150 (1:500, Chemicon), mAb 3A10 for neurofilament associated protein (supernatant, DSHB), rabbit α-Krox20 (1:100, Babco, Berkeley, CA), rabbit α-neuregulin (SC-348), (1:100, Santa Cruz Biotech, Santa Cruz, CA), mouse α-human neuronal protein HuD (1:200, Molecular Probes, Eugene, OR), rabbit α-p75 (low affinity NGF receptor), (1:5000, gift from Dr. L. Reichardt, University of Calif., San Francisco), mAb AMV3C2, to detect cells infected with strains of avian sarcoma/leukemia virus complex, (supernatant, DSHB), mouse α-SV2 to detect synaptic vesicles (supernatant, gift from Dr. B. Patton, Oregon Health and Science University), and rabbit α-synaptophysin (1:100, gift from Dr. B. Patton, Oregon Health and Science University). All secondary antibodies were produced in goat (Molecular Probes): α-rat Cy3 (1:200), α-rabbit Rhodamine-X (1:500), α-rabbit Alexa488 (1:500), α-mouse Rhodamine-X (1:500), α-mouse Alexa488 (1:500).

Preparation of cryostat sections:

CG of the appropriate ages were isolated and fixed in Zamboni's fixative (4% paraformaldehyde, 15% picric acid in 0.1M sodium phosphate-buffered saline solution

(PBS; 150mM NaCl, 20mM sodium phosphate, pH 7.4)) overnight at 4°C. The tissues were rinsed 3 times or until any residual yellow color was washed out, and sequentially equilibrated to 1:1 with 30% sucrose:OCT in PBS at 4°C (15% sucrose overnight, 30% sucrose overnight, and 1:1 30% sucrose:OCT overnight). CG were clustered together, mounted in Microm Cryo-embedding compound (Richard Allen Scientific, Kalamazoo, MI) mounting medium, cut in serial sections at 15 μm on a Microm HM560 cryostat at –20°C and collected on gelatin-subbed slides. Slides were air-dried for 30 minutes and post-fixed for 15 minutes in Zamboni's. Slides were rinsed 3 times in PBS and incubated overnight at 4°C in blocking solution (10% (v/v) fetal calf serum (Hyclone, Logan, UT), 10% (v/v) horse serum (Gibco BRL, Grand Island, NY), 0.5% (v/v) triton X-100, and 0.2% (w/v) sodium azide in PBS). The slides were then incubated with the appropriate 1° antibodies diluted in blocking solutions and incubated overnight at 4°C. After rinsing the slides 3 times, 10 minutes each, in PBS they were incubated with the corresponding 2° antibodies for 4 hours at room temperature. The sections were rinsed 3 times in PBS plus 0.5% (v/v) TX-100 (PBST) and mounted onto a coverslip with Permaflour (Thermo Shandon, Pittsburgh, PA) mounting medium. The sections were imaged by using a confocal microscope (Nikon D-Eclipse C-1 confocal attached to a Nikon E800 microscope).

Preparation of whole-mount tissues:

Iris and ciliary muscle were isolated from appropriate stages by removing the eye from the socket and dissecting around the iris with iridectemy scissors. The circular pieces of iris were fixed in Zamboni's solution for 1 hour at room temperature. The tissues were

rinsed in PBS until the yellow dye from the fixative no longer leached out and they were stored at 4°C in PBS + 0.2% (w/v) azide until subsequent processing. In order to stain the tissues, the cornea was removed and a quadrant of the iris were cut and pinned down onto a Sylgard (DuPont Chemical Co., Wilmington, DE) coated 35mm dish with minutein pins. Each iris was divided into three or four quadrants that were pinned separately into different dishes so that they would be stained with different antibodies. The tissues were initially permeablized with DMSO (Sigma Chemical Co., St Louis, MO) and rinsed 3 times with PBS to remove any residual DMSO. The tissues were incubated in blocking solution overnight at at 4°C. The tissues were incubated in 1° antibodies overnight at 4°C, rinsed 3 times in PBST, and incubated in 2° antibodies for 4 hours at room temperature. After the tissues were rinsed 3 times with PBST, they were mounted on a glass slide with Permafluor and imaged by using a confocal microscope.

Preparation of acutely dissociated CG's for immunohistochemistry:

CG from appropriate embryonic stages were dissected out, the nerves trimmed, and incubated in Modified Puck's Glucose containing 0.1% (w/v) trypsin (GibcoBRL) for 20 minutes at 37°C. The tissues were dissociated by trituration through a reduced-bore pasteur pipette. The cell suspension was first washed with DMEM (GibcoBRL) supplemented with 10% FCS (Hyclone) to stop the trypsinization, then centrifuged onto a gelatin-coated slide for 5 minutes at 1000 RPM using a StatSpin 800 Cytospin Centrifuge (StatSpin Inc., Norwood, MA). The slides were air-dried for 5 minutes and fixed with Zamboni's solution for 15 minutes. The slides were rinsed 3 times with PBS, blocked for 2 hours at room temperature, and incubated in 1° antibody overnight at 4°C. The

following day the slides were rinsed 3 times in PBS and incubated with 2° antibody for 4 hours at room temperature. The slides were again rinsed in PBS and mounted with Permaflour containing Hoechst dye.

Tissue Culture

Isolation of E14 sciatic nerve Schwann cells:

Schwann cells from chicken sciatic nerves were isolated as previously described (Bhattacharyya et al., 1993) with the following modifications. 18 sciatic nerves from E14 chicken embryos were dissected out, the outer sheath removed, and the nerves pooled, and then incubated in 300 units/ml of collagenase II (Worthington Biochemical, Lakewood, NJ) in Earle's balanced salt solution (EBSS; GibcoBRL) at 37°C for 40 minutes. The tissues were then incubated in 0.25% trypsin-EDTA (GibcoBRL) at 37°C for 20 minutes. The enzymatic activity was stopped by replacing the medium with cDMEM (DMEM (GibcoBRL), 10% FCS (GibcoBRL), 50 U/ml penicillin, 50mg/ml streptomycin (GibcoBRL)). The tissues were triturated through a reduced-bore pasteur pipette, the suspension centrifuged, and resuspended in 500 µl of cDMEM supplemented with a 1:100 dilution of 1E8 antibody. The cell suspension was incubated on ice for 1 hour with occasional shaking. The cells were washed once with cold cDMEM and resuspended in 10 ml of cold cDMEM. The cold suspension was plated onto a 100 mm^2 bacterial dish coated with 1 µg/ml of goat α-mouse secondary antibody (Boehringer Mannheim). The dish was incubated at 4°C for 80 minutes with occasional shaking. Dishes containing the Schwann cell suspensions were rinsed 5 times with cold cDMEM to remove any unbound cells. The adherent cells were collected using a rubber

policeman, centrifuged, and plated at $1.0x10^4$ cells/cm^2 in serum-free DMEM supplemented with B27 onto a poly-D-lysine (0.5 mg/ml; Sigma-Aldrich) and laminin (1 mg/ml; prepared in the Nishi Lab) coated glass cover slips.

Monitoring P0 and p75 expression in E14 sciatic nerve Schwann cells:

After purified Schwann cells were allowed to adhere to the coverslip overnight, the cells were fixed with Zamboni's solution at different time points ranging from Day 0 to Day 7. The cells were rinsed in PBS and stored in PBS+azide until all of the coverslips were collected. The cells were incubated with antibodies against P0 and p75 simultaneously at the dilutions described above. To visualize the 1° antibodies, an alkaline phosphatase-coupled anti-mouse secondary antibody (Pharmacia) was used for P0, and a biotinylated anti-rabbit antibody (Dako) was used for p75. NBT/BCIP (Gibco/BRL) and ABC staining kit (Dako) were used respectively following the manufacturer's recommendations to give a blue precipitate for P0 and a brown precipitate for p75.

Quantification of Krox20 expression in cultured Schwann cells:

To determine the changes in Krox20 expression in the Schwann cell cultures, the Schwann cells were plated onto a circular glass tissue culture cover slip (Fisher Scientific, Pittsburgh, PA) attached with small drops of paraffin at three corners (Goslin et al., 1998), so that the cells would be inverted and cultured upside down without touching the area beneath the cell monolayer. After the Schwann cells were allowed to attach to cover slips overnight, three cover slips were immediately fixed in Zamboni's solution for 30 minutes, rinsed 3 times with PBS, and stored at 4°C in PBS with 0.1%

azide. The rest of the cover slips were inverted and cultured in DMEM+B27 medium (Day 0). Triplicates of cover slips were fixed, rinsed, and stored at Day 1, 3, and 5 after initial plating. When all time points were collected, the cover slip cultures were incubated for 2 hours in blocking solution at room temperature, and further incubated with α-Krox20 antibody overnight at 4°C. The coverslips were rinsed 3 times in PBS and incubated with secondary α-rabbit Alexa488 antibody for 4 hours at room temperature. The cells were again rinsed 3 times with PBS and mounted onto a slide with Permafluor supplemented with Hoechst dye. Ten fields of view were randomly chosen per coverslip and scored for the number of Krox20 positive cells compared to the total number of cell nuclei.

Isolation and purification of E8 CG neurons:

Cultures of E8 CG neurons were isolated as previously described (Nishi, 1996) with several modifications. After the ganglia were triturated, the suspension was centrifuged through a 30%-60% Percoll (Pharmacia, Piscataway, NJ) step-gradient to separate the non-neuronal cells from the CG neurons. The purified CG neurons were isolated from the 30%-60% interface. This neuronal suspension was then diluted 5-fold with DMEM and centrifuged to pellet the neurons. The neurons were re-suspended in DMEM+B27 supplemented with 10 ng/ml of chicken CNTF ([his]6chCNTFyyy, bacterially expressed and purified in the Nishi Lab) and plated at $1.0x10^3$ cells/cm^2 onto glass cover slips coated with poly-D-lysine (Sigma) and laminin (purified in the Nishi Lab from EHS tumors).

Isolation of choroid smooth muscle cultures:

E14 choroid cells were isolated as previously described (Coulombe and Nishi, 1991). Cells were plated on tissue culture dishes coated with rat tail collagen and incubated in modified L-15 medium CO^2 (Mains and Patterson, 1973) supplemented with 10% chick serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM glutamine, and 6 mg/ml glucose. The cells were cultured for 2 days and the medium switched to DMEM+B27 without any serum.

Isolation of iris/ciliary body muscle cultures:

E11 chick iris cells were isolated as previously described (Link and Nishi, 1998b). Iris cells were plated on tissue culture dishes coated with rat tail collagen (prepared in the Nishi Lab) and incubated in L-15 with 10% horse serum. Myotube differentiation was promoted by replacing the medium with serum-free DMEM+B27 medium after 2 days.

CG/Schwann co-cultures incubated in trans with either choroid or iris cells:

Day 1: Both choroid and iris cultures were isolated and plated onto two separate 24 well culture plates. Day 2: CG neurons were isolated and plated onto glass cover slips as described above. Day 3: Schwann cells were isolated as described above and added into the cultures containing the CG neurons that were plated on Day 2. Day 4: Cover slips containing the CG/Schwann co-cultures were inverted onto wells containing either the choroid monolayer or the iris monolayer. Since the coverslips were pegged with paraffin feet, the co-cultures were able to grow with the target tissues in trans, exchanging soluble factors but not touching the target tissues. Coverslip cultures were then fixed in triplicates

after 1, 4, 7, or 10 days after the co-cultures were inverted onto the target monolayers. As a control, Schwann cell cultures without CG neurons were cultured in parallel with the co-cultures. Thus, each set of experiments included 48 cover slips: 12 CG/Schwann cultures in choroid wells, 12 CG/Schwann cultures in iris wells, 12 Schwann cell cultures in choroid wells, and 12 Schwann cell cultures in iris wells. Three coverslips per condition, per time point, were fixed in Zamboni's for 30 minutes at room temperature, rinsed 3 times in PBS, and stored in PBS/azide until all the coverslips were collected. They were stained for Krox20 and HuD at the same time as described above and mounted with Permafluor containing Hoechst dye. α-rabbit Alexa488 and α-mouse Rhodamine-X were used as secondary antibodies, respectively. Krox20 was visualized under a fluorescence microscope with a FITC filter, HuD with a Rhodamine filter, and Hoechst with a DAPI filter.

Quantification of Krox20 expression in CG/Schwann co-culture:

Ten fields were randomly selected at 400X magnification from each coverslip and the numbers of Krox20-positive Schwann cells were scored, concurrent with the total number of cells in each field. Each field contained approximately 50 cells, thus providing a sampling of approximately 500 cells per coverslip. The ratios of Krox20-positive cells to total number of cells were described as percentages. Staining of CG neuron cell bodies with HuD (a neuronal specific marker) helped determine that Krox20 staining was not associated with the neurons and ensured that the number of neurons on each coverslip remained constant throughout each experiment.

Statistical analysis:

The data from the co-culture experiments were analyzed using a computer-aided SAS GLM procedure provided by the Biometry Department at the University of Vermont. 3-way ANOVA was utilized to compare the data between culture conditions (iris vs. choroid), time points (1 day vs. 7 days), and the reproducibility between each set of experiments.

Knock-down of NRG using RCASBP(A)-4XRZnrg

Virus production:

The viral construct for the neuregulin ribozyme, RCASBP(A)-4XRZnrg, was provided by Dr. G. Lemke (Zhao and Lemke, 1998). High-titer viral stocks were produced as previously described (Morgan and Fekete, 1996). Briefly, the RCASBP(A)-4XRZnrg plasmids were transfected into the DF-1 chicken fibroblast cell line with calcium phosphate (Finn et al., 1998). The infected cell line was passaged until 100% of cells expressed the p27gag protein, as indicated by immunocytochemistry. Culture supernatant containing the virus was concentrated with centrifugation to yield a $2x10^8$ IU/ml viral stock solution. The stock solution was stored at –80°C.

Testing of RCASBP(A)-4XRZnrg virus in vitro:

Schwann cell cultures from E14 chicken sciatic nerves were isolated as described above. The cells were allowed to adhere to a glass coverslip overnight. The following day, the culture medium was aspirated and 50 μl of the viral stock solution was added per cm^2 of coverslip. The cultures were then gently rocked for 30 seconds, and fresh culture

medium was added back to the Schwann cell cultures. The cultures were subsequently isolated at 1, 2, 4, and 7 days, fixed, and stained for neuregulin and AMV to determine whether the virus had infected the Schwann cells and knocked down the neuregulin expression *in vitro*.

In vivo viral injection and tissue isolation:

Concentrated virus was injected into stage 9-10 chick embryos as previously described (Lee et al., 2001). Briefly, 250-350nl of concentrated virus was injected into the lumen of the developing mesencephalon using a Nanoject injector (Drummond Scientific Company, Broomall, PA). The embryos were allowed to develop until stage 40-41 (E14-15), at which time the embryos were removed from the egg. The eyes and the CG's of each embryo were removed. The two eyes and one CG were fixed in Zamboni's solution overnight at 4°C, rinsed with PBS until the yellow stain from the fixative was removed, and stored at 4°C in PBS/azide until processed for immunohistochemistry. The other CG from each embryo was collected into an eppendorf tube and snap-frozen on dry ice. 20 µl of SDS sample buffer was added and the tissues stored at –80°C until subsequent processing for immunoblot analysis.

Analysis of RCASBP(A)-4XRZnrg infected CG tissues using immunoblots:

The efficacy of the neuregulin knock-down in each embryo was determined by immunoblot analysis of CG extracts. The frozen CG samples from each embryo were suspended in 20 µl of SDS sample buffer and heated to 80°C for 30 minutes with occasional vortexing. 10 µl of each extract was loaded onto a 12% SDS-PAGE gel,

separated by size, and transferred onto a PVDF membrane. The membrane was incubated overnight in blocking solution (10% FCS, 10% HS, 0.5% triton X-100, and 0.2% sodium azide in PBS) at 4°C. The membrane was then incubated with α-neuregulin antibody (1:200) overnight at 4°C. The membrane was rinsed 3 times with TBS+0.05% Tween and incubated in α-rabbit Alexa 680 (Molecular Probes) for 4 hours at room temperature. The membrane was rinsed again with TBS+0.05% Tween. Positively stained bands were visualized using the Odyssey Infrared Imaging System. The densities of the bands were quantified using NIH Image.

Immunohistochemical analysis of RCASBP(A)-4XRZnrg- infected embryos:

Based on the results obtained from the immunoblots, samples with higher than a 40% reduction of neuregulin in CG extracts were further analyzed for changes in myelin gene expression. Samples that did not show any reduction of neuregulin in CG extracts were treated as controls. Furthermore, embryos were infected with either RCASBP(A)-vector only virus or RCASBP(A)-GFP (expressing green fluorescent protein) as infected controls.

i) CG sections: The CG's were processed for cryostat analysis as described above. The primary antibodies used in the analysis for the infected CG's were mAb AMV, α-neuregulin, and α-Krox20.

ii) Target tissues whole-mounts: The ciliary nerves of the infected tissues were stained as whole-mount tissues as described above. The primary antibodies used in this analysis were α-Krox20, mAb 3A10, mAb 1E8, and α-NF150.

iii) Cryostat section of iris target tissues: The irises of the infected tissues were processed as described in CG cryostat sections and sectioned perpendicular to the ciliary nerve at 25 μm. The sections were stained for α-synaptophysin (gift from Dr. B. Patton) and incubated with rhodaminated alpha-bungarotoxin (Molecular Probes).

CHAPTER 1

Development of Schwann cells in the avian ciliary ganglion

Alex T. Ho[1,2] and Rae Nishi*[2]

*to whom correspondence should be addressed

[1]Department of Cell and Developmental Biology
Oregon Health and Science University
3181 S.W. Sam Jackson Park Road, L-215
Portland, Oregon 97201

[2]Department of Anatomy and Neurobiology
University of Vermont College of Medicine
149 Beaumont Avenue, HSRF 406
Burlington, Vermont 05403

Summary

The avian ciliary ganglion (CG) consists of two distinct populations of neurons, ciliary and choroid neurons (Marwitt et al., 1971). Ciliary neurons are myelinated and innervate the iris and the ciliary body (Hess, 1965; Pilar et al., 1987). Choroid neurons are unmyelinated and innervate the vascular smooth muscle of the choroid layer (Meriney and Pilar, 1987). In this study, we determined the time course during which ciliary neurons become myelinated. We determined that glial cells in the CG differentiate to become either myelinating or non-myelinating Schwann cells between E14 and E18, with the expression of myelin markers Krox20 and P0 starting at E14. At E8, all glial cells express the low-affinity NGF receptor (p75). At E14, increased expression of Krox20 and P0 is detected only in glial cells surrounding ciliary neurons, while glial cells expressing p75 are found only around choroid neurons. Among the nerves which innervate each target tissue, Schwann cells expressing Krox20 and P0 are detected only in the ciliary nerves and not in the choroid nerves. The Schwann cells found in choroid nerves are all p75-positive. These results suggest that as glial cells differentiate to become myelinating Schwann cells, expression of p75 is replaced with increased expression of Krox20 and P0. In contrast, Schwann cells that remain as non-myelinating Schwann cells retain their p75 expression. We also examined the expression of neuregulin immunoreactivity during CG development to determine whether neuregulin is present at the time that ciliary ganglion neurons are myelinated. At E8, neuregulin is abundant in preganglionic synapses on CG neurons. However, by E14, neuregulin is strongly expressed selectively in ciliary neuron cell bodies. By E18, neuregulin

expression is down-regulated in the neurons, but detected in the ganglionic glia. Thus, the pattern of neuregulin expression by ciliary neurons coincides with the increase of myelin markers in the glial cells of the developing CG, suggesting that neuregulin may play a role in regulating the preferential myelination of ciliary neurons. These observations suggest that the signals controlling myelination of CG neurons may occur in a target-dependent manner, where the ciliary target promotes myelination indirectly by inducing expression of neuregulin in the ciliary neurons.

Introduction

The exact mechanism that instructs Schwann cells to myelinate or not to myelinate an axon is still not well understood. Schwann cells arise as one of the cell types from the migrating neural crest (Le Douarin et al., 1991). The Schwann cell precursors from the neural crest differentiate into immature Schwann cells as they migrate and proliferate along the developing neurons (Jessen et al., 1994a; Jessen and Mirsky, 1991). The immature Schwann cells either differentiate into myelinating or non-myelinating Schwann cells depending on the axons that they associate with (Aguayo et al., 1976). The mature Schwann cells retain their ability to switch between myelinating and non-myelinating phenotype depending on the environment within the nerve.

Early experiments using cross-anastomosis techniques revealed that non-myelinating Schwann cells have the potential to myelinate axons when exposed to appropriate environmental cues (Weinberg and Spencer, 1975). Furthermore, myelinating Schwann cells may revert back to a non-myelinating state during peripheral nerve injury and then re-myelinate the nerve once innervation is restored (Scherer and Salzer, 1996). Thus, Schwann cells have the ability to switch between a myelinating and non-myelinating state, depending on the signals received. Recent studies in Schwann cell biology have shown that the signal that induces Schwann cells to enter their myelinating program is derived from the axons. The elucidation of this axon-derived signal and its mechanism of regulation is important in order to understand the significance of myelination in the developing peripheral nerve.

The chicken ciliary ganglion is an ideal system for studying the differentiation of Schwann cells because it contains both myelinated and non-myelinated neurons that project to two different targets. This parasympathetic ganglion is derived from the cranial neural crest, and is composed of ciliary neurons that innervate striated muscles in the iris/ciliary body and choroid neurons that innervate the vascular smooth muscles of the choroid layer (Marwitt et al., 1971). Unlike other autonomic motor neurons, the cell bodies and axons of ciliary neurons are myelinated. In contrast, choroid neurons remain smaller and non-myelinated and can be distinguished from ciliary neurons by their expression of somatostatin (Epstein et al., 1988). The differentiation of ciliary ganglion neurons has been well studied, and certain aspects such as cell survival (Finn and Nishi, 1996b; Nishi, 1994b) and neuropeptide expression have been shown to be dependent on signals derived from their target tissues (Coulombe and Nishi, 1991; Darland et al., 1995). Thus, axon-derived cues that promote myelination by Schwann cells wrapping ciliary ganglion neurons may also be influenced by signals coming from their targets.

Neuregulin may be one of the key signals that regulate the differentiation of Schwann cells during myelination (Cheng et al., 1998). Originally isolated as a factor which promoted the proliferation of Schwann cells (glial growth factor, GGF), neuregulin has been suggested to be involved in the initial specification of neural crest cells into Schwann cell precursors, proliferation of Schwann cells, and inducing the differentiation of myelinating Schwann cells (Gassmann and Lemke, 1997; Leimeroth et al., 2002; Lemke, 1996; Zorick and Lemke, 1996). Also known as neu differentiation factor,

heregulin, acetylcholine receptor-inducing activity, and sensory and motor neuron derived factor, recent studies looking at different neuregulin isoforms have demonstrated that the membrane-bound neuregulin isoform may induce Schwann cells to express myelin specific genes (Leimeroth et al., 2002). Though neuregulin may regulate the specification, proliferation, and maturation of developing Schwann cells, elucidation of isoform specific functions may clarify the exact mechanisms of neuregulin signaling in different steps of Schwann cell development.

We hypothesized that retrograde signals from target tissues induce neurons to promote myelination. The first step in testing this hypothesis was to determine whether the start of myelination correlated with the period of peripheral synaptogenesis as has been found for other target dependent events (Darland et al., 1995; Finn and Nishi, 1996b). Using both non-myelinating and myelinating Schwann cell markers, we followed the preferential myelination of ciliary ganglion neurons. In addition, the expression of neuregulin was characterized concurrently to determine whether the pattern of neuregulin expression in the ganglion was consistent with a role for this signaling molecule in directing myelination.

Results

Krox20 expression in the ganglia and the ciliary nerve

To determine the stage at which myelination begins in the chicken ciliary ganglion, sections of the ganglia were stained with an antibody to Krox20. Krox20 is a zinc finger transcription factor that has been shown to be up-regulated in the nuclei of Schwann cells destined to myelinate axons (Jessen et al., 1994b). In rodents, selective disruption of the Krox20 gene blocks myelinating Schwann cells from expressing P0 (Topilko et al., 1994), indicating that expression of Krox20 may precede and regulate the expression of P0. CGs from E10, E12, E14, E16, and E18 were isolated, sectioned, and stained for Krox20 and somatostatin (Figure 1). At E10 (Figure 1B) and E12, expression of Krox20 is undetectable. However, by E14 (Figure 1E), Krox20 staining appears as a distinct nuclear stain in glial cells at the periphery of neuronal cell bodies (arrowhead). The nuclear expression of Krox20 in the ganglia continues through E18. Furthermore, the nuclear expression of Krox20 is rarely adjacent to the cytoplasmic expression of somatostatin (Figure 1F), indicating that the Krox20 expression is detected only in glial cells around the ciliary neurons.

To confirm the expression pattern of Krox20 in the nerve, iris tissues were prepared as whole mounts and stained intact with anti-Krox20 (Figure 2B and 2E). To visualize the ciliary nerve, the tissues were also stained with antibodies against neurofilament (Figure 2A and 2D). Similar to the expression pattern in the ganglion (Figure 1), Krox20 immunoreactivity is undetectable at E10 (Figure 2B) and E12, but by E14, the expression

of Krox20 in the ciliary nerve is readily detected as indicated by the arrowheads (Figure 2E). The Krox20 immunoreactivity co-localizes with Hoechst nuclear stain (not shown), indicating that the expression of this protein is in the nuclei of Schwann cells which align along the axons of the ciliary nerve. Furthermore, Krox20 staining expression does not co-localize with neurofilament staining, indicating that the immunoreactivity is not in the axons but in the non-neuronal cells that surround each axon (Figure 2F). In both the ganglia and the ciliary nerve, we were unable to detect whether Krox20 expression precedes P0 expression. Thus, in chicken, the expression of Krox20 in the myelinating Schwann cell occurs at E14.

P0 expression in the developing ciliary ganglion

To confirm that expression of Krox20 in the Schwann cells signifies the start of myelination in the CG, we also characterized the accumulation of a homophilic adhesion molecule highly expressed in myelin, P0 (Bhattacharya et al, 1991; Zhang et al, 1995). . Sections of ganglia were stained with antibody against chicken P0 (1E8) at embryonic days 8, 10, 12, 14, 16, and 18. At E8, E10 (Figure 3B), and E12, expression of P0 in the ganglia was barely detectable. Between E8 and E12, very low levels of P0 expression were detected in the ganglia. However, by E14 (Figure 3E), the expression of P0 in the CG was strongly up-regulated. The cells which express P0 were non-neuronal in their morphology, wrapping around the cell bodies of a subset of the ciliary ganglion neurons. By E18 (Figure 3H), expression of P0 was detected both encircling neuronal cell bodies and ensheathing axons traversing through the ganglion as ciliary nerve is formed.

Because the expression of somatostatin in ciliary ganglion neurons identifies choroid neurons, sections of ganglia were also stained for somatostatin in order to differentiate ciliary neurons from choroid neurons (Figure 3A, 3D and 3G). At E10, expression of P0 in the CG was very faint. Furthermore, lack of Krox20 expression in the nuclei of glial cells (Figure 1) suggested that the Schwann cells were in a non-myelinating state. However, at E14, as the expression of Krox20 was detected, immunoreactivity of P0 around somatostatin-negative neuronal cell bodies increased dramatically (Figure 3F). This effect indicates that only the Schwann cells surrounding the ciliary neurons differentiate into myelinating Schwann cells. The preferential myelination of ciliary neurons became more prominent at E18 as P0 staining intensified around somatostatin-negative neurons (Figure 3I). We did not observe any P0 positive cells surrounding the choroid neurons after E14, confirming that choroid neurons remain unmyelinated.

Expression of P0 in the ciliary nerve

To determine whether myelination of ciliary ganglion axons begins at E14 as observed in the ganglion (Figure 3), the expression of P0 in ciliary nerves was also analyzed. Because nerve fibers located in the iris/ciliary body of the chicken eye are composed only of ciliary nerves, the iris tissues from E10 and E14 chicken eyes were removed and stained as whole mounts with antibodies against the P0 protein (Figure 4B and 4C). To locate the ciliary nerve in the whole mount tissues, antibodies against neurofilament protein (NF150) were utilized (Figure 4A and 4D). Unlike in the ganglia, expression of chicken P0 was undetectable at E10 (Figure 4B), but by E14, P0 expression was observed throughout the ciliary nerve (Figure 4E). Cross sections of the iris were also stained with

α-neurofilament (Figure 5A) and α-P0 (Figure 5B). Similar to the whole-mount staining of the ciliary nerve around the ciliary body (Figure 4), the nerves innervating the iris also expressed P0.

Expression of the low affinity neurotrophic receptor (p75) in non-myelinating Schwann cells

In the developing ciliary ganglion, before the start of myelination (E10), all Schwann cells expressed p75 (Figure 6B), a marker of all neural crest cells. As myelination in the ciliary ganglion began at E14, myelinating Schwann cells down-regulated p75 expression (Figure 6E) and up-regulated P0 expression (Figure 6D). Some overlap in p75 and P0 expression was observed, perhaps as Schwann cells switched their myelinating phenotype (Figure 6E, arrow). Nevertheless, by E18, the segregation between myelinating Schwann cells and non-myelinating Schwann cells was distinct (Figure 6I). Interestingly, at E18, p75 expression is also detected in the cytoplasm of some CG neurons (Figure 6H). Furthermore, the neurons that were positive for p75 did not have any P0 positive glia surrounding them (Figure 6I). In contrast, in choroid nerves, Schwann cells up-regulated their expression of p75 (Figure 7B) and failed to express P0 (Figure 7E). These Schwann cells remained tightly associated with axons(Figure 7C).

Expression of neuregulin in the ciliary ganglion

In order to address the possible roles of neuregulin during the myelination of ciliary neurons, the expression of neuregulin was characterized in the developing CG. Using antibodies that recognized the C-terminal intracellular region of the neuregulin protein,

we stained the CG at E10, E14, and E18 (Figures 8A, 8D, and 8G respectively). The sections were also stained with antibodies against the human neuronal protein, HuD, which labels the cell bodies of CG neurons (Figures 8B, 8E, and 8H). At E10, neuregulin expression was outside the CG neurons, possibly in the presynaptic terminals (Figure 8C) of incoming preganglionic neurons. At E14, the expression of neuregulin was seen in a subset of neuronal cell bodies (Figure 8F). However, by E18, the neuronal expression of neuregulin was attenuated, but intensified in non-neuronal cells wrapped around a subset of CG neurons (Figure 8I)

At E6, neuregulin is expressed in the presynaptic terminal of the CG neurons

As shown in Figure 8C, prior to myelination, expression of neuregulin appeared immediately adjacent to CG neuronal soma, possibly in the presynaptic terminals formed by preganglionic input from the accessory oculomotor nucleus (Narayanan and Narayanan, 1976). To investigate this possibility, E6 CG sections were stained with antibodies to neuregulin (Figure 9A) and the synaptic vesicle specific protein SV2 (Figure 9B). As shown in Figure 9C, there was some overlap between SV2 and neuregulin. Thus, this overlap indicates that at E6, the neuregulin protein is found in the presynaptic terminals innervating the CG neurons

At E14, neuregulin is expressed in ciliary neurons but not in choroid neurons

By E14, the expression of neuregulin switched to a subpopulation of CG neurons (Figure 10F). To determine which neuronal population expressed neuregulin, cells from an acutely dissociated E14 CG were centrifuged onto a glass slide and stained for neuregulin

(Figure 10B) and somatostatin (Figure 10A) to distinguish choroid from ciliary neurons. Figure 10C shows that the neuronal cells that expressed neuregulin did not express somatostatin, indicating that these neurons were ciliary neurons. Combined with the observation that expression of Krox20 and P0 in the ciliary nerve was first detected at E14 (Figure 2 and 4), this result suggests that preferential expression of neuregulin in the ciliary neurons may play an important role in the differentiation of myelinating Schwann cells in ciliary nerves.

At E18, neuregulin is selectively expressed in myelinating Schwann cells

At E18, expression of neuregulin in the CG was detected mainly in myelinating Schwann cells (Figure 11A), as indicated by the co-localization of some neuregulin immunoreactivity with P0 (Figure 11C). As shown in Figure 8I, neuregulin expression in the CG neurons was attenuated by this stage.

Discussion

The data presented here provide evidence that the differentiation of myelinating Schwann cells in the avian ciliary ganglion begins after the neurons in the ganglion have completed the cell death phase and innervated their respective targets at E14. The cell death phase in the developing ciliary ganglion is between E9 and E14 (Landmesser and Pilar, 1974), when the number of neurons in the ganglion is reduced by 50%. This reduction is dependent on trophic factor availability from its targets (Finn and Nishi, 1996b). Thus, the start of myelination in ciliary neurons may be dependent on the populations of ciliary neurons that survived the competition for neurotrophic availability. Furthermore, E14 is an important time point for the target-dependent differentiation of ciliary ganglion neurons. Previous studies in our laboratory have shown that E14 is when target-specific expression of the neuropeptide somatostatin is determined in choroid neurons (Darland et al., 1995). In the ciliary target, the iris/ciliary body undergoes a smooth-to-striated muscle transition (Link and Nishi, 1998a). Thus, the start of myelination in ciliary neurons may also be regulated indirectly in a target-dependent fashion. Because the ciliary target experiences a dramatic change in muscle type, the effects of striated muscle on the ciliary neurons may indirectly promote the Schwann cells surrounding the nerve to start their myelinating program. Alternatively, the smooth muscle target tissues of the choroid neurons may indirectly inhibit Schwann cells in the choroid nerves from switching from a non-myelinating to a myelinating state.

To address mechanisms of preferential myelination in the developing ciliary ganglia, expression of neuregulin, a key regulator in Schwann cell development, was analyzed concurrently. At E14, when myelin markers first appear, the expression of neuregulin in the ciliary ganglia is observed only in ciliary neurons. This observation suggests that the start of preferential myelination of ciliary neurons by the surrounding Schwann cells may be regulated by the specific expression of neuregulin by ciliary neurons. The start of preferential myelination of ciliary neurons in the developing ciliary ganglia at E14 indicates a well orchestrated mechanism where Schwann cells surrounding ciliary neurons receive the signal to start their myelination program, whereas Schwann cells associated with the choroid neurons remain in an undifferentiated non-myelinating state due to a lack of signals from the choroid neurons.

By characterizing the expression of two myelin specific markers Krox20 and P0, we were able to provide some evidence that myelination of ciliary neurons begins at E14. Krox20 is a zinc finger transcription factor that becomes up-regulated in the nuclei of Schwann cells as they enter the myelinating program (Jessen et al., 1994b). The results show that the intense nuclear staining of cells surrounding the ciliary nerve is visible only after E14. Thus, the start of myelination in Schwann cells associated with ciliary neurons begins at E14. Selective disruption of Krox20 in mice blocks the expression of late myelin genes such as P0, suggesting that expression of Krox20 during myelination precedes the expression of P0 (Schneider-Maunoury et al., 1993). Thus, in order to examine whether that increased expression of Krox20 in the CG at E14 lead to the expression of myelin specific genes in Schwann cells, we characterized the expression of P0 during the same

development period. However, in our study, we were unable to detect any sequential expression of Krox20 and P0. Rather, both myelin markers become concurrently up-regulated at E14 in the ciliary ganglia and the ciliary nerves. The 1E8 antibody used in this study to detect changes in chicken P0 expression has been previously characterized (Bhattacharyya et al., 1993; Bhattacharyya et al., 1991; Zhang et al., 1995). Expression of chicken P0 may be detected as early as E4 in neural crest cells as they migrate out from the neural tube (Zhang et al., 1995), some of which differentiate into Schwann cell precursors. In our study, expression of P0 was very faint in the E10 ciliary ganglia, but immunoreactivity increased very rapidly at E14. P0 is a type of cell adhesion molecule in the immunoglobulin gene superfamily (Lai et al., 1987). Thus, the function of early expression of chicken P0 in the migrating neural crest cells may only be to aid in the migration of Schwann cell precursors to their appropriate locations. The expression of P0 during this process may be very low, and once the cells have reached their destination, P0 expression may be further down-regulated until Schwann cells are induced to myelinate their appropriate axons. In order for the Schwann cells to form the myelin sheath, the expression of P0 in the Schwann cells along the ciliary nerve needs to be many-folds higher to create the wrapping of the membrane as seen in all myelinated axons.

The Schwann cells in the developing ciliary ganglia and their nerves behave in a traditional manner, in which there is a switch in the phenotypes of the Schwann cells from non-myelinating to myelinating in ciliary nerves, possibly due to signals received from the axons (Lemke, 1993; Lemke et al., 1990; Zorick and Lemke, 1996). In mature nerves, Schwann cells may be categorized into two different subtypes, myelinating and

non-myelinating. Myelinating Schwann cells are identified by their expression of myelin-specific genes such as Krox20 and P0. In contrast, non-myelinating Schwann cells are identified by their expression of the low-affinity NGF receptor (p75). During the differentiation, Schwann cell precursors express p75 and retain this expression into mature stage of non-myelinating Schwann cells. Thus, our data indicate that as the ciliary ganglia develop, Schwann cells surrounding ciliary neurons switch from non-myelinating to myelinating as indicated by the switch in the expression from p75 to P0. Furthermore, Schwann cells associated with choroid neurons retain their non-myelinating phenotype in both the ganglia and the nerve, indicated by the continued expression of p75 in Schwann cells. This retention may be due to the fact that choroid neurons do not express any myelinating cues throughout their development.

The expression of neuregulin in ciliary neurons at E14 indicates that this neuron-derived signal may induce Schwann cells surrounding ciliary neurons to begin their myelinating program. Recent progress in Schwann cell research has identified neuregulin as a key molecule that regulates the differentiation, proliferation, and maturation of myelinating Schwann cells (Burden and Yarden, 1997; Leimeroth et al., 2002; Lemke, 1996; Zorick and Lemke, 1996). In this study we addressed the possible roles of neuregulin in the preferential myelination of ciliary neurons in the developing ciliary ganglia. Immunohistological results show that between E6 and E12, neuregulin expression is observed only in the extracellular space of the ganglia, possibly playing a role in synaptic formation for the presynaptic innervation of the nerve coming from the brain (Moscoso et al., 1995). At E14, at the start of myelination, neuregulin is expressed only in ciliary

neurons, a key candidate that may act as a neuron-derived cue to promote Schwann cells to myelinate the axons. Furthermore, by E18, expression of neuregulin is seen only in myelinating Schwann cells, as indicated by its co-localization with P0, possibly acting as an autocrine factor for maintaining the survival of differentiated Schwann cells (Rosenbaum et al., 1997). The α-neuregulin antibody used in the characterization recognizes both Ig-neuregulin and CRD-neuregulin isoforms. Thus, we are unable to determine the isoform specific expression of neuregulin in the developing ciliary ganglia. Both isoforms are synthesized with a transmembrane which become inserted in the membrane with the active EGF-domain in the extracellular space (Wolpowitz et al., 2000; Yang et al., 1998). However, Ig-neuregulin is readily cleaved and released from the cellular membrane (Wang et al., 2001). On the other hand, the extracellular domain of the CRD-neuregulin becomes re-inserted into the cell membrane so that the active EGF-domain remains exposed and tethered to the cell membrane (Wang et al., 2001). For synapse formation in the ganglia between E6 to E10, or as an autocrine factor for Schwann cells at E18, the neuregulin expressed in ganglia may be the Ig-isoforms. During myelination the CRD-neuregulin may be the isoform expressed in the ciliary neurons, which promotes the differentiation of myelinating Schwann cells. Thus, neuregulin may have multiple functions in the developing ciliary ganglion, and its localized expression at specific developmental stages ensures that neuregulin signaling is utilized efficiently. It will be necessary to determine which isoforms are expressed at each stage to understand the role of neuregulin in Schwann cell development.

Acknowledgements

The author would like to thank Dr. Bruce Patton for providing us with the antibody supernatant against SV2 and Dr. L. Reichardt for the anti-p75 antibody. We would also like to thank Dr. Jan Christian for the use of the cytospin centrifuge. This work was supported by NIH grant NS25767

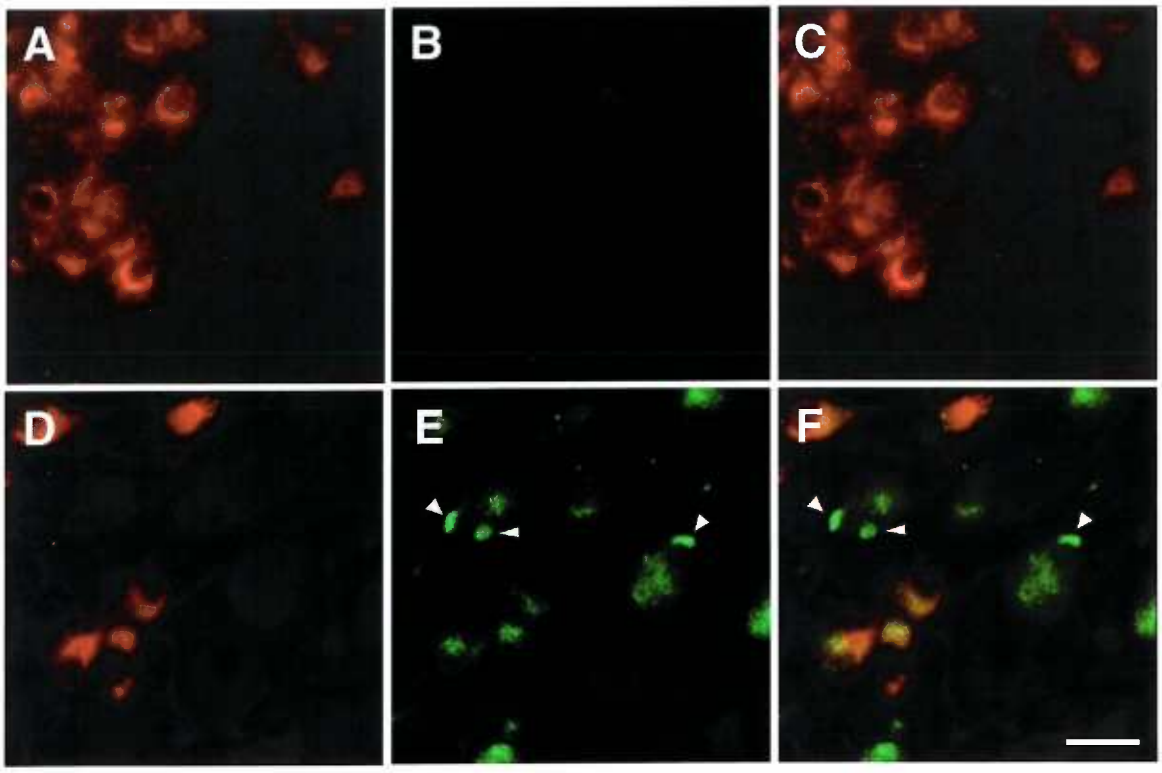

Figure 1. Krox20 expression in the ciliary ganglia.

Cryostat section of E10 (A-C) and E14 (D-F) ciliary ganglion stained for somatostatin (A, D) and Krox20 (B, E). C is an overlay of A and B. F is an overlay of D and E. The intense Krox20 staining in E (arrow) co-localizes with DNA dye (not shown) indicating that the Krox20 immunoreactivity is nuclear. In E14 (Figure 1E), there is a diffuse Krox20 immunoreactivity that does not co-localize with any nuclei stains (not shown). This staining may be a low level expression found in neuronal cytoplasm which is unrelated to the differentiation of Schwann cells during myelination (Herdegen et al., 1994). Bar equals 15 μm.

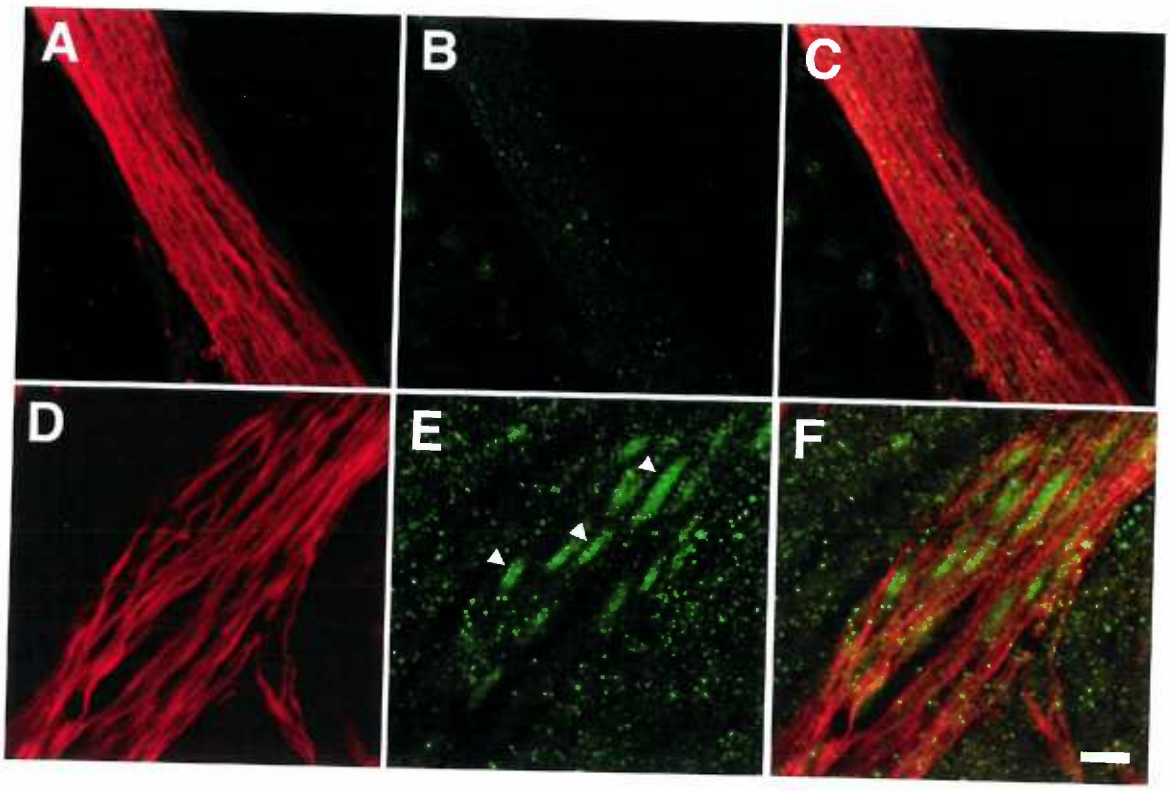

Figure 2. Expression of Krox20 in the ciliary nerve.

Whole mount staining of E8 (A-C) and E14 (D-F) in ciliary nerves, stained for neurofilament (A, D) and Krox20 (B, E). At E8, Krox20 expression is minimal (B), not co-localizing with any nuclear staining (not shown). The intense immunoreactivity of Krox20 in E (arrow) at E14 co-localizes with DNA nuclear dye (not shown) indicating that Krox20 expression in the tissue is nuclear. The nuclear Krox20 staining is interlaced between the neurofilament immunoreactivity in the nerve (F). C is an overlay of A and B, F is an overlay of D and E. Bar equals 10 μm.

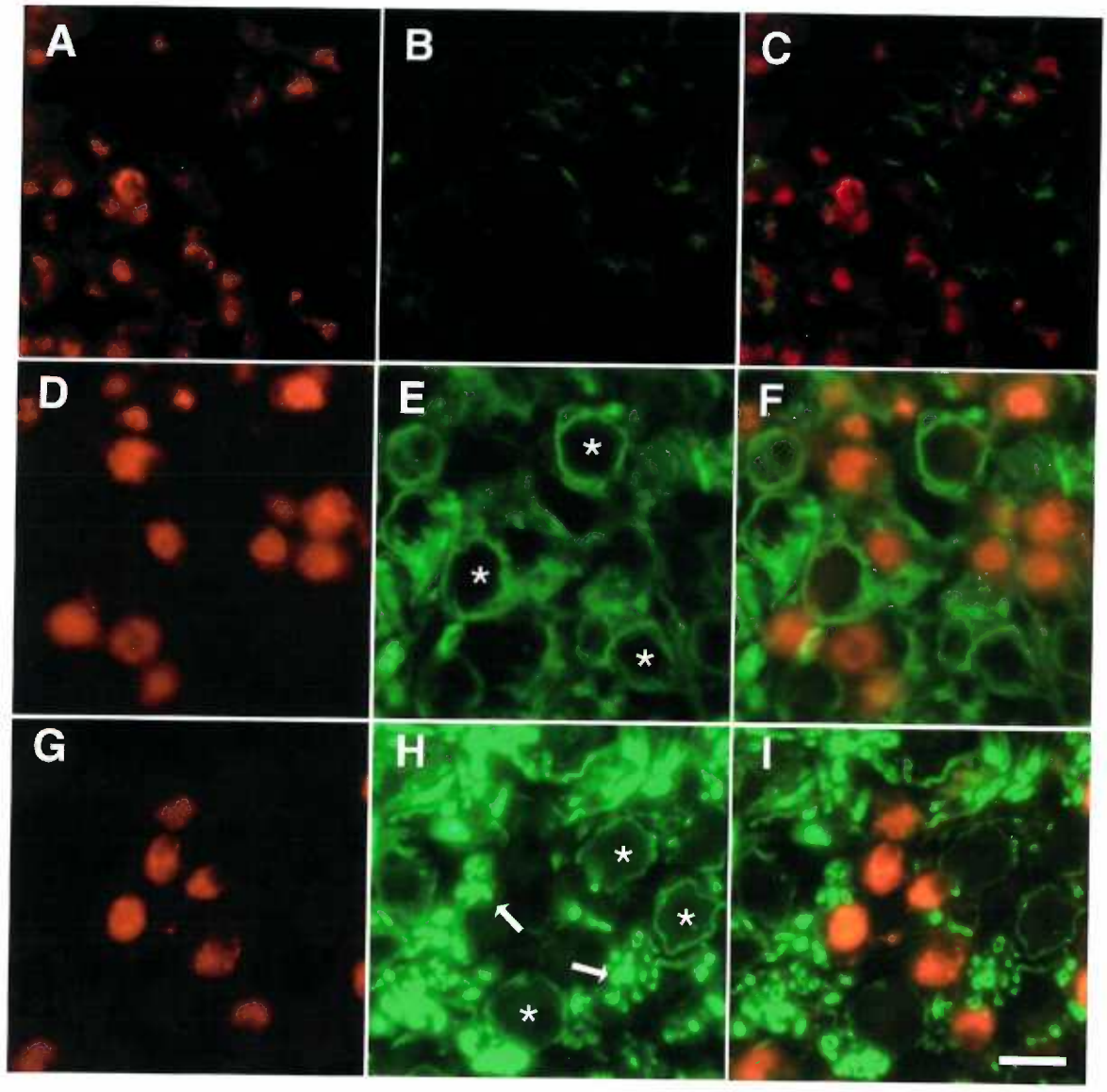

Figure 3. P0 expression in the developing ciliary ganglion.

Cryostat sections of E10 (A-C), E14 (D-F), and E18 (G-I) ciliary ganglion were stained for somatostatin (A, D, G) and P0 (B, E, H). C is an overlay of A and B, F is an overlay of D and E, I is an overlay of G and H. Somatostatin is expressed only in choroid neurons, thus the cell bodies of ciliary neurons are identified by the circular morphology and lack of somatostatin immunoreactivity (*). By E18, the myelinated ciliary nerves are detected traversing through the ganglia (arrow). Bar equals 15 μm.

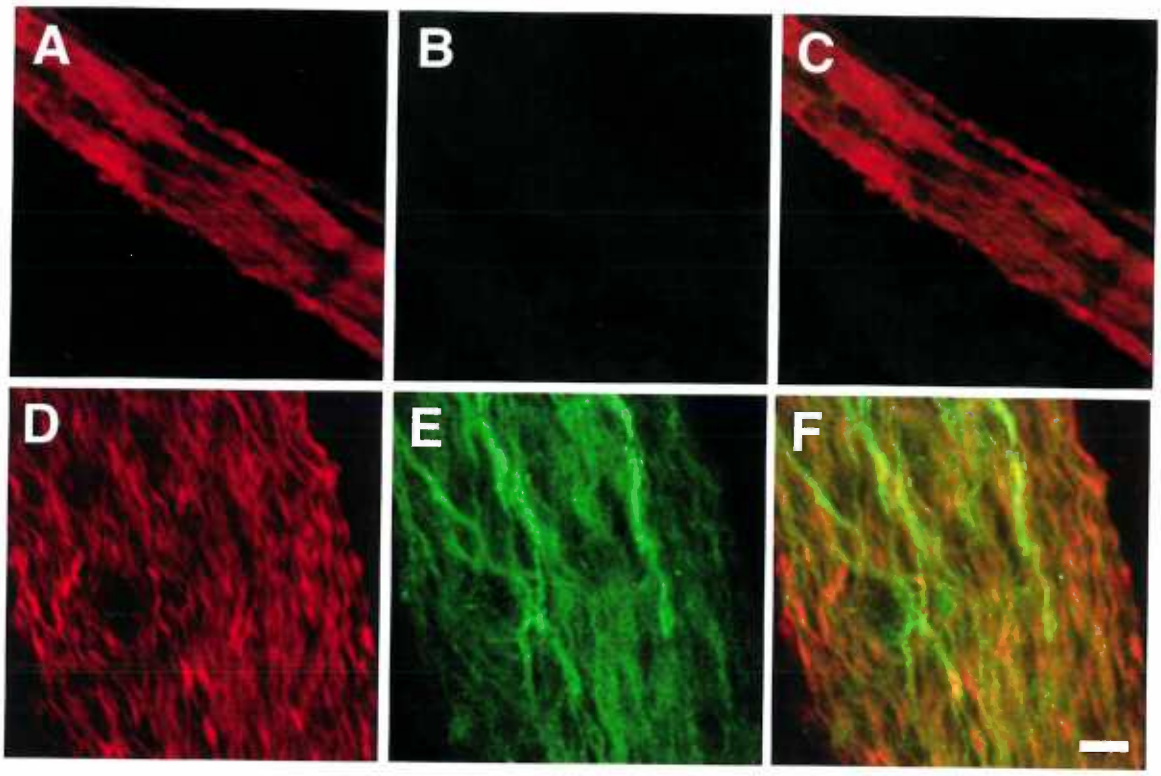

Figure 4. Expression of P0 in the ciliary nerve.

Whole mount staining of ciliary nerves stained for neurofilament (A, D) and P0 (B, E) in E10 (A-C) and E14 (D-F) ciliary nerves. The entire ciliary body of the chicken eye was fixed and pemeablized with DMSO, stained, and the image was captured using a confocal microscope from an intact ciliary nerve lying on top of the ciliary body. Expression of P0 in the ciliary nerve is detected only after E14 (E). C is an overlay of A and B, F is an overlay of D and E. Bar equals 10 μm.

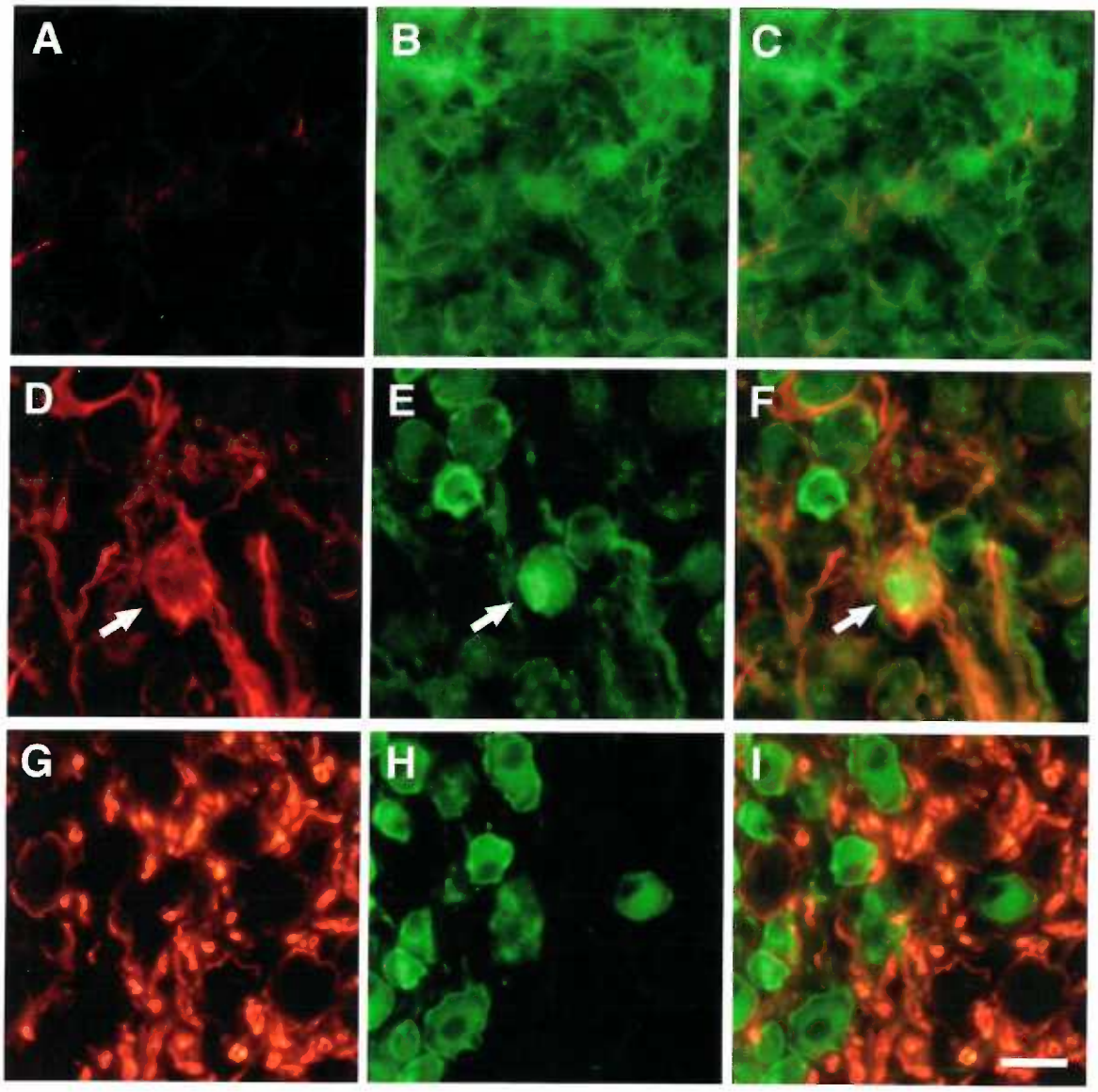

Figure 6. Expression of P0 and p75 in the developing ciliary ganglion.

Cryostat sections of E10 (A-C), E14 (D-F), and E18 (G-I) ciliary ganglion were stained for P0 (A, D, G) and p75 (B, E, H). C is an overlay of A and B, F is an overlay of D and E, I is an overlay of G and H. At E14, there is a P0 positive structure that wraps around a p75 positive structure (arrow). Bar equals 15 μm.

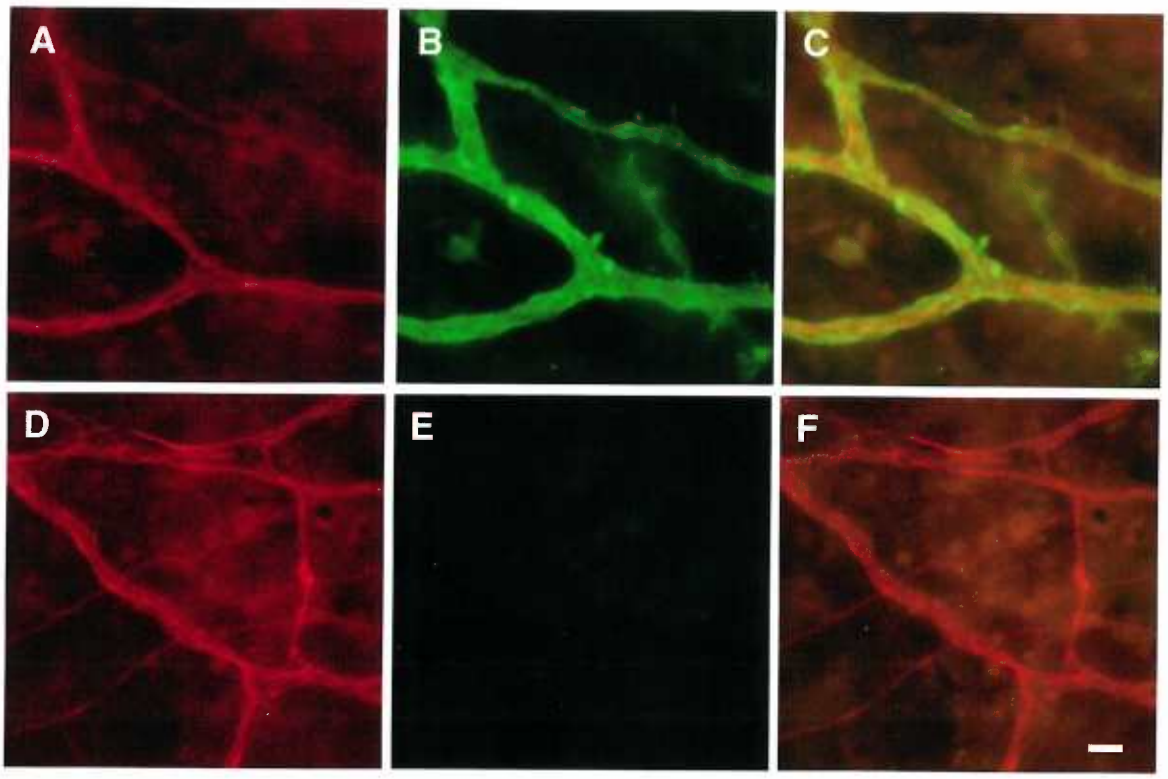

Figure 7. Expression of p75 and P0 in choroid neurons.

Whole mount staining of E14 choroid nerves stained for neurofilament (A, D), p75 (B), and P0 (E). Choroid axons are ensheathed by p75 positive Schwann cells Schwann cells (B). There are no P0 positive Schwann cells in the choroid nerves at any time point during development (E). Bar equals 15 μm.

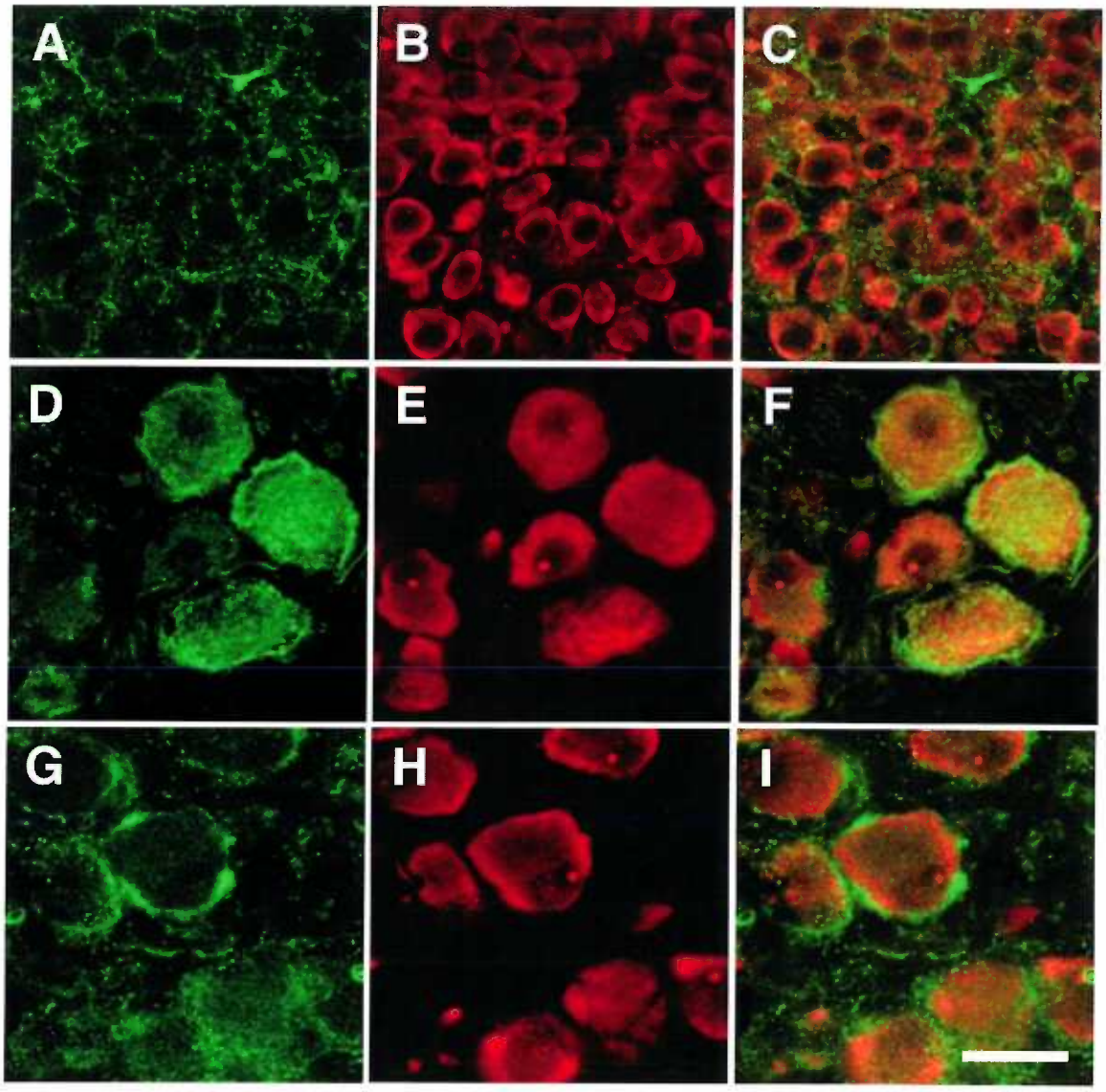

Figure 8. Expression of neuregulin in the ciliary ganglion.

Cryostat sections of E10 (A-C), E14 (D-F), and E18 (G-I) ciliary ganglion stained for neuregulin (A, D, G) and human neuronal protein (Hu) D, a specific marker of neuronal cell bodies (B, E, H). At E10, expression of neuregulin is detected outside of the ciliary ganglion neurons (C). However, at E14, when myelination of the ciliary neurons is first detected, expression of neuregulin switches to a subset of neurons (F). By E18, expression of neuregulin in the neurons is down-regulated and detected only in surrounding non-neuronal cells. Bar equals 15 μm.

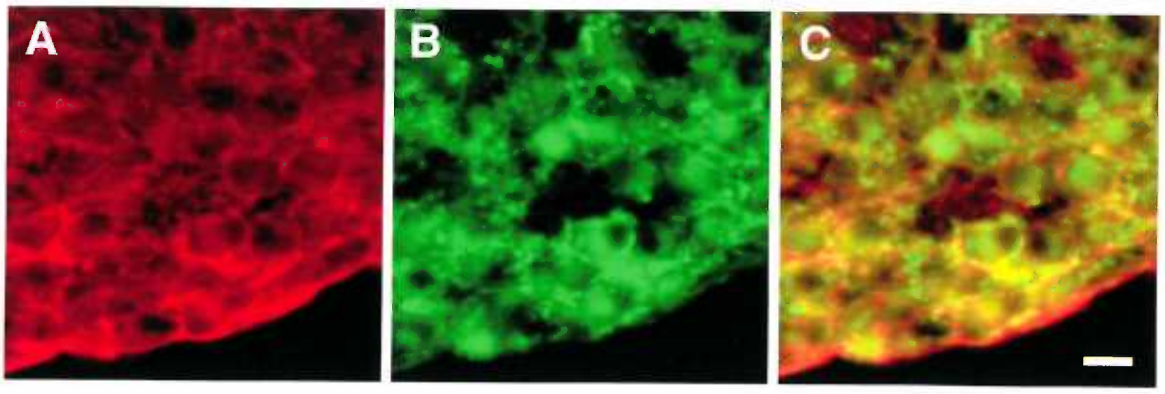

Figure 9. Expression of neuregulin in E6 ciliary ganglion.

Cryostat sections of E6 ciliary ganglion stained with neuregulin (A) and SV2 synaptic vesicle marker (B). C is an overlay of A and B. Almost all of the SV2 immunoreactivity co-localizes with neuregulin; however, neuregulin is also strongly expressed in other structures, perhaps non-neuronal cells, in the ganglion. Bar equals 15 μm.

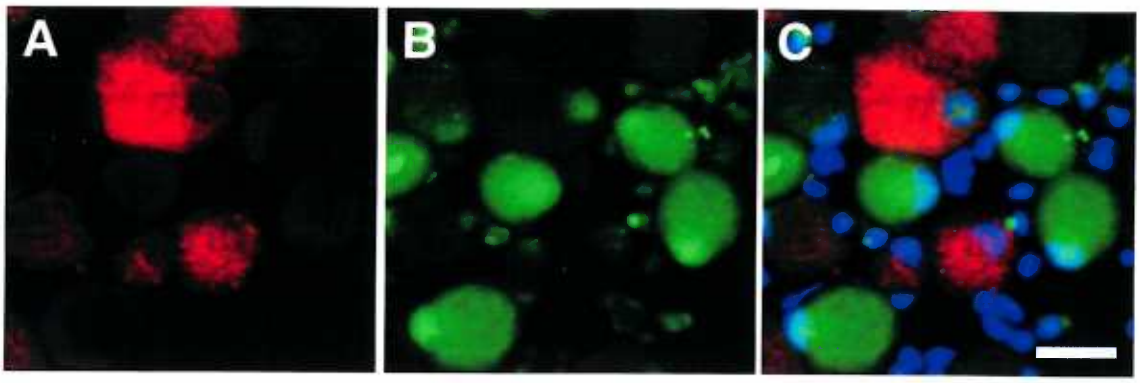

Figure 10. Expression of neuregulin in an acutely dissociated preparation of E14 ciliary ganglion.

Acutely dissociated E14 ciliary ganglion cells centrifuged onto a glass slide and stained for somatostatin (A) and neuregulin (B). Expression of neuregulin at E14 is specific to ciliary neurons, indicated by the lack of co-localization between somatostatin positive choroid neurons and neuregulin expressing neurons (C). The Hoechst DNA dye in C (blue staining) indicates the location of all the nuclei in the dissociated cells. Bar equals 15 μm.

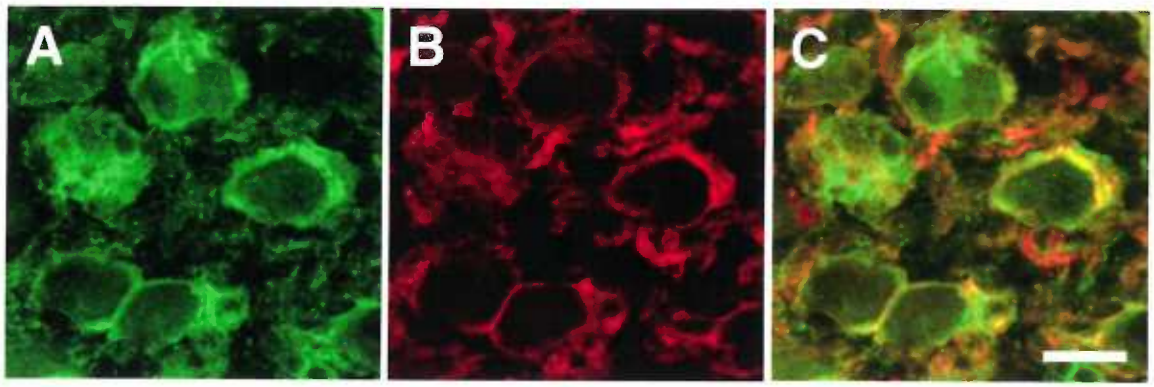

Figure 11. Expression of neuregulin in E18 ciliary ganglion.

Cryostat sections of E18 ciliary ganglion stained for neuregulin (A) and P0 (B). C is an overlay of A and B. Neuregulin expression overlaps with P0 around neuronal cell bodies, but does not seem to be expressed in myelin surrounding nerve bundles passing through the ganglion. Bar equals 15 μm.

Summary

Preferential myelination of the avian ciliary ganglion (CG) begins at embryonic day 14 (E14), as characterized by the increased expression of Krox20 and P0 by Schwann cells (chapter 1). Out of the two neuronal populations, ciliary and choroid neurons, only ciliary neurons become myelinated in the developing CG (Hess, 1965). Unlike the choroid target which consists of only smooth muscles, the ciliary target undergoes a smooth to striated muscle transition between E11 and E17 (Link and Nishi, 1998a). Thus, preferential myelination of ciliary neurons in the developing CG may be regulated in a target dependent manner, where signals from striated muscles of the iris tissue may indirectly promote the myelination of ciliary nerves. Using *in vitro* methods, we have tested whether diffusible factors from different CG targets induce the expression of myelin markers in CG/Schwann cell co-cultures. Cultures of E8 CG neurons and E14 chicken sciatic nerve Schwann cells were incubated together on a glass cover slip and inverted above a monolayer of either iris/ciliary body cultures or choroid smooth muscle cultures. When the co-cultures were grown in the presence of choroid tissues, the ratio of Schwann cells expressing Krox20 in the CG/Schwann cell co-cultures remained low, between 3% to 5%. On the other, hand when the cells were incubated in-trans with iris/ciliary body cultures for 7 days, the ratio of Schwann cells expressing Krox20 in the CG/Schwann cell co-cultures increased from 8% to 20%. In all cases, the number of Schwann cells and CG neurons in each culture remained constant. Furthermore, the increase in Krox20 expression by the Schwann cells was dependent on the presence of

CG neurons in the cultures. The observation suggests a presence of a target-derived effect that may indirectly regulate myelination.

Introduction

The mechanism that regulates the differentiation of Schwann cells into a myelinating or non-myelinating fate is still not well understood. In the PNS, myelination by a Schwann cell is dependent on signals derived from the axon (Aguayo et al., 1976; Weinberg and Spencer, 1976; Weinberg and Spencer, 1975). However, how these axon-derived cues are regulated in the neurons still remains to be elucidated. One common theory hypothesizes that neurons become myelinated when the diameter of an axon increases to a certain thickness. For instance, it has been suggested that in the mouse sciatic nerve, any axon larger than 1 μm will become myelinated (Friede and Samorajski, 1967). The thickness of the myelin sheath is also dependent on the size of the axon (Berthold et al., 1983; Fraher, 1976). However, when mouse mutants with diminished axon diameter due to the lack of mid-sized and heavy neurofilament subunits (Elder et al., 1999) were analyzed for myelin thickness, the amount of myelin wrapped around L5 lumbar roots and neurons in sciatic nerves remained unchanged even when the size of the axon was reduced by 30% (Elder et al., 2001). On the other hand, when the thickness of axons was increased in the rat superior cervical ganglion neurons by 24%, 60% of the neurons became myelinated (Voyvodic, 1989). Normally, 99.3% of superior cervical ganglion neurons are unmyelinated and innervate the submandibular gland (Voyvodic, 1989). Cutting one branch of this sympathetic nerve, allowing the remaining neurons to innervate a larger portion of the target tissues, increased the diameter of the axons. This effect suggested that the increased availability of target derived signals may promote the

myelination of neurons in an indirect manner, such as by increasing the diameter of axons.

Target dependence of peripheral neuron differentiation has long been studied (Barde, 1988; Barde, 1989; Davies, 1988; Hamburger et al., 1981; Oppenheim, 1989). The initial survival of innervated neurons is often dependent on the availability of trophic factors from their targets. Switch in neurotransmitter phenotype (Mains and Patterson, 1973) or onset of neuropeptide expression (Coulombe and Nishi, 1991) by neurons are also dependent on signals released from target tissues. Hence, due to its simplicity and well-characterized biology, the avian ciliary ganglion is an ideal system for studying the mechanisms of target dependent peripheral neuron differentiation. Composed of only two populations of neurons, the myelinated ciliary neurons innervate the iris/ciliary body of the eye, while the non-myelinated choroid neurons innervate the smooth muscles of the choroid vasculature (Marwitt et al., 1971). Our recent inquiry into the development of Schwann cells in the ciliary ganglia has suggested that the preferential myelination of ciliary neurons may also be dependent on target-derived cues.

Our previous studies show that the preferential myelination of ciliary ganglion neurons begins at E14 (see Chapter 1). Furthermore, expression of neuregulin, a potent differentiation factor for Schwann cells, is detected specifically in ciliary neurons at E14. On the other hand, choroid neurons that do not become myelinated do not express neuregulin. Initially, both ciliary and choroid neuron targets are composed mainly of smooth muscles. However, at E11, the ciliary target begins to undergo a transition from

smooth-to-striated muscle phenotype that is complete by E17 (Link and Nishi, 1998a). Thus, changes in muscle type in the ciliary target may induce changes in the ciliary neurons that may in turn promote the myelination of ciliary neurons.

In this study, we have tested whether diffusible factors from target tissues influence the expression of myelinating marker Krox20 in neuron/Schwann cell co-cultures. To do so, we utilized purified neurons from E8 ciliary ganglia and Schwann cells from E14 sciatic nerves co-cultured with target tissues from either the iris/ciliary body or the choroid layer. Our results indicate the presence of a diffusible factor from the ciliary target tissues that promotes the expression of Krox20 in the Schwann cells of neuron/Schwann cell co-cultures. Furthermore, the effect in the Schwann cells is observed only in the presence of neurons, indicating that the diffusible factor is working through neurons to promote the indirect expression of Krox20 in the Schwann cells.

Results

Characteristics of Schwann cells cultured from E14 chicken sciatic nerve

Schwann cells were isolated from sciatic nerves of E14 chicken embryos by immuno-panning using antibodies against P0 antigen on their cell surfaces. Initially most Schwann cells expressed P0 (Figure 1A). However, after 7 days, the expression of P0 was replaced by increased expression of p75 (low affinity NGF receptor) (Figure 1B). The expression of p75 in these Schwann cells indicated that, though initially isolated as myelinating Schwann cells, the cells reverted back to a non-myelinating precursor state. A survey of the long-term Schwann cell cultures (> 7days) shows minimal contamination by non-Schwann cells (i.e. fibroblasts), as indicated by the expression of p75 in 99% of the cells in these cultures (not shown).

To quantify the expression of the myelinating phenotype, we monitored changes in Krox20 expression because the expression of Krox20 is nuclear (Figure 2A, arrows), unlike P0, which resides mainly on cell surfaces (Figure 1). Thus, we were able to compare the distribution of Krox20 expression with the Hoechst nuclear marker. Furthermore, down-regulation of Krox20 expression was more rapid compared to P0 expression For example, Figure 2 shows the changes in Krox20 expression in Schwann cells at progressive times after isolation from the sciatic nerve. After the initial plating of the purified cells, the Schwann cells were allowed to adhere on coverslips overnight and then assayed for Krox20 expression (Figure 2A and 2B), and counter-stained with Hoechst dye (Figure 2C and 2D). The ratio of Krox20 positive Schwann cells to the total

number of cells in each field was determined (Figure 3). Schwann cells isolated from E14 stage sciatic nerve rapidly down-regulated their Krox20 expression within 12 hours (35%), and at 36 hours after the initial plating only 5% of the Schwann cells still expressed Krox20.

Preparation of CG/Schwann cell co-culture

To establish a long term CG/Schwann co-culture, it was critical to separate the neurons and the Schwann cells from any rapidly growing fibroblasts and other contaminating cells that might interfere with neuron-Schwann cell interactions. In order to accomplish this, neurons and Schwann cells were isolated independently, purified, combined, and cultured on glass coverslips. The neurons were purified from E8 CG; the Schwann cells from E14 sciatic nerves. Previous studies have shown that the highest number of CG neurons are most efficiently isolated from the ganglia at E8 (Nishi, 1996; Nishi and Berg, 1979). In culture, ciliary and choroid neurons from E8 CG are indistinguishable. The two neuronal populations both have similar size, require trophic support, and express similar target dependent phenotypes such as somatostatin expression (Nishi, 1994b). When purified using a percoll gradient, E8 CG cultures are composed of 99.9% neurons, characterized as bright round cells with long processes (Figure 4A). Schwann cells were isolated from E14 sciatic nerves because of high yields and efficient purification by panning with P0 antibodies (Figure 4B). As previously described (Bhattacharyya et al., 1993), these cultures are mainly P0 positive, and they retain their Schwann cell phenotypes in long-term cultures as indicated by the expression of p75 (Figure 1). When the two purified

cell types were combined, they associated to form structures in which the Schwann cells bundled along the axons of CG neurons (Figure 4C).

Diffusible factors from target tissues influence Krox20 expression in neuron/Schwann cell co-cultures

In order to expose the neuron/Schwann cell co-cultures to medium conditioned by different target tissues, monolayers of either iris/ciliary body cultures or choroid smooth muscle cultures were first grown in a tissue culture well until the iris/ciliary cultures differentiated into striated muscle cells and the choroid cells were devoid of any pigment cells. Separately, neuron/Schwann cell cultures were grown on glass coverslips with droplets of paraffin attached to form spacers, and the co-cultures were inverted above a monolayer of target cells. The paraffin feet prevented the co-cultures from touching. Day 0 was the day when the coverslip cultures were inverted onto the target cell monolayer. To quantify the induction of the myelin specific gene in co-cultures, coverslips were stained for Krox20 (Figure 5A and 5C) and counterstained for DNA using Hoechst dye (Figure 5B and 5D). Krox20 expression was determined by increased immunoreactivity in Schwann cell nuclei (arrowhead). When co-cultures were incubated for either 1 or 7 days (Figure 6), Schwann cells exposed to the iris/ciliary body monolayer showed a substantial increase in Krox20 expression (from 8% to 20%). However, when co-cultures were incubated together in choroid monolayers, expression of Krox20 in Schwann cells remained low (between 3% and 5%). The total number of cells (approximately 500 per ten fields of view) in each culture condition remained constant, as indicated by the number of Hoechst positive cells in each field (Figure 7). When

Schwann cells were cultured without neurons, the level of Krox20 remained low (below 5%), regardless of time in culture or exposure to different types of target tissue (Figure 8).

Statistical analysis of co-culture data

The data from the co-culture experiments were collected and presented as a ratio estimate: the total number of Krox20 positive cells in ten fields of view were divided by the total number of Schwann cells in ten fields of view to give a ratio in percent form. The number of Schwann cells was determined by the total number of nuclei in each field of view minus the number of neurons as indicated by HuD positive cells. For example, the total number of Krox20 positive cells in co-cultures exposed for 1 day to iris cultures was 101. The total number of Schwann cells in the same condition was 706. Thus the ratio 101/706 = 0.143 was interpreted as 14.3% of Schwann cells expressing Krox20 under this condition. Each condition had three coverslips, thus n=3. Each experiment had two conditions (iris vs. choroid) and two time points (1 day vs. 7 days). This set of experiments was repeated three times.

To determine whether the 3 sets of experiments could be analyzed together or not, the variance of the entire data set was determined using 3-way ANOVA. A computer aided analysis program was used to compare variance between the two conditions, the two time points, and the three data sets obtained from the three experiments. The results of this analysis showed that the variance between each experiment was not significant (p-value = 0.982), meaning that the data from the three experiments could be legitimately combined,

yielding a total n=9 for each condition. Furthermore, the variance between the two conditions (iris vs. choroid) and two time points (1 day vs. 7 days) were significant (p-value < 0.001, p-value $= 0.0016$), indicating that the differences observed between the two conditions and two time points were statistically valid. Table 1 lists the results from the co-culture experiments.

Discussion

The *in vitro* analysis presented in this chapter was conducted to test the hypothesis that preferential myelination of ciliary neurons is influenced by signals from the target tissues. Previous studies suggested that increased axon diameter of rat superior cervical ganglion neurons induced the myelination of these normally unmyelinated sympathetic neurons (Voyvodic, 1989). Partial denervation of the target gland increased the axon diameter, possibly by raising the availability of target-derived molecules for the remaining neurons. However, decreasing the axon diameter by deleting the production of mid-sized and heavy neurofilament subunits did not affect the amount of myelination in developing DRG neurons (Elder et al., 2001). Rather than changes in axon diameter, myelination of PNS neurons may be dependent on changes in neurons based on certain factors released from their target tissues.

Our experiments suggest that diffusible factors from the iris/ciliary body cultures promote the activation Krox20, a myelin specific gene, by Schwann cells in neuron/Schwann cell co-cultures. Furthermore, expression of Krox20 is dependent on co-culturing the Schwann cells with neurons, indicating that the activation of the myelin gene by the target tissues may act indirectly through the neurons. Target-derived factors have been shown to regulate many aspects of neuronal differentiation. For instance, the switch in neurotransmitter phenotype in sympathetic neurons is often dependent on target-dependent factors (Francis and Landis, 1999; Landis, 1990; Landis, 1994). Also, the availability of trophic factors from the target regulates the survival of developing

neurons (Linden, 1994; Nishi, 1994a; Vogel, 1993). Thus, factors from the iris target tissues may be inducing the ciliary ganglion neurons to express myelin-inducing signals such as neuregulin on their cell surface. The expression of axon-derived cues may in turn promote surrounding Schwann cells to begin their myelinating program, including the expression of Krox20.

By using the ciliary ganglion system, we were able to address the role of specific target tissues in myelination, because the targets of both the myelinated and non-myelinated neurons were clearly defined. Previous studies on myelination concentrated on utilizing dorsal root ganglion cultures from rodents in which heterogeneous populations of neurons had multiple target sites that were not readily identifiable (Bunge et al., 1986; Elder et al., 2001; Jessen and Mirsky, 1991; Stevens et al., 1998). Utilizing purified populations of E8 ciliary ganglion neurons allowed the isolation of a large number of homogeneous neurons. Though the ciliary and the choroid neurons begin to innervate their targets at E8, purifying the ciliary ganglia neurons prior to cell death phase allowed the isolation of neurons that are indistinguishable on the basis of known markers. For example, when these neurons are exposed to activin A, which promotes somatostatin expression, all neurons are able to express this neuropeptide (Darland and Nishi, 1998). Thus, E8 ciliary ganglion neurons provide a homogeneous undifferentiated population of neurons that may be readily manipulated with well defined targets.

The ability of the E14 sciatic nerve Schwann cells to switch between myelinating and non-myelinating phenotype was important for this *in vitro* study. The homogeneous

population of P0 expressing Schwann cells were isolated from E14 sciatic nerve by immunopanning (Bhattacharyya et al., 1993). These cells quickly lose their P0 and Krox20 expression in culture and become p75 positive. Since we are selecting for P0 expressing Schwann cells, all isolated Schwann cells must be from the myelinated nerves. 50% of Schwann cells retain their P0 expression *in vitro* for approximately three days (Bhattacharyya et al., 1993). However, Schwann cells isolated from myelinated nerves lose their Krox20 expression very rapidly when separated from the axons. 65% of the Schwann cells isolated for this study lost their Krox20 within 12 hours, indicating that regulation of Krox20 in Schwann cells occurs more rapidly than regulation of P0. The ability of these cells to re-express Krox20 when combined with E8 ciliary ganglion neurons in presence of ciliary target tissues confirmed the principle that Schwann cells switch readily between myelinating and non-myelinating phenotypes. In our hands, we were unable to detect any increase in P0 expression in any of the cultures. When neurons and Schwann cells are combined *in vitro*, they interact to form a bundle-like structure (Figure 4B). In order for these cultures to express P0 and other myelin related structural proteins, it is necessary to add ascorbic acid in the cultures (Eldridge et al., 1987). Furthermore, in rodents, Krox20 expression in myelinating Schwann cells precedes the expression of P0 (Topilko et al., 1994). Thus, monitoring changes in Krox20 allowed us to characterize the co-culture experiment more readily.

The two target tissues may release factors that regulate the myelination of neuron/Schwann cell co-cultures. Both target tissues isolated in this experiment have been optimized to retain their muscle phenotype for long-term cultures (Coulombe and

Nishi, 1991; Darland et al., 1995). Choroid target tissues were isolated from the choroid layer of the eye, which yields a homogeneous population of vascular smooth muscle cells (Coulombe and Nishi, 1991). Ciliary target cultures were optimized to isolate the striated muscle of the iris/ciliary body (Darland et al., 1995). The two cell types both express the chicken ciliary neurotrophic factor, which supports survival of ciliary ganglion neurons in culture (Nishi, 1994a). However, previous studies in our laboratory suggest that diffusible factors from each target tissue can have different effects on the development of the ciliary ganglion neurons (Nishi, 1994b). For instance, the expression of the neuropeptide somatostatin is induced by activin A, which is released from both target tissues (Darland et al., 1995). On the other hand, the ciliary target also produces follistatin, an inhibitor to activin A (Darland et al., 1995). Thus, activin A dependent effects are observed only in choroid neurons (Coulombe and Nishi, 1991). Our results indicate the existence of a diffusible factor from the iris/ciliary muscle which indirectly promote Krox20 expression.

The target-derived factors that promote myelination of CG neurons may be an inductive signal from the iris/ciliary body, rather than an inhibitory one from the choroid target. This is because Krox20 expression remains low in co-cultures without any target tissues. In the adult CG, cell bodies of ciliary neurons are approximately 25-40 μm in diameter, compared to 15-25 μm in choroid neurons (Marwitt et al., 1971). Furthermore, ciliary targets are composed of striated muscle (Pilar et al., 1987) and choroid targets are made of smooth muscles (Meriney and Pilar, 1987). In this study, the size of the CG neurons was not measured. However, if increased axon size promotes myelination (Berthold et

al., 1983; Fraher, 1976; Friede and Samorajski, 1967), then the inductive signal from the ciliary target may induce neurons to produce more neurofilaments. Because striated muscles require higher impulse activity from innervating neurons as compared to smooth muscles, the interactions between ciliary neurons and the ciliary target may promote an increase in neuronal activity. The increase in action potentials in neurons may promote the associating Schwann cells to myelinate axons (Stevens and Fields, 2000; Stevens et al., 1998).

The increase in Krox20 expression observed in our co-culture experiments suggests that target tissues indirectly modulate the myelination of innervated nerves. However, the signals which become up-regulated in neurons of ciliary conditioned cultures still need identification. The immunohistological analysis described in chapter 1 of this thesis suggested that ciliary targets may induce the expression of neuregulin in the ciliary neurons during myelination. Thus, there may be a diffusible factor released from the striated muscle cultures of the iris/ciliary body that modulates an axon derived signal such as neuregulin which in turn induces Schwann cells to myelinate the neurons.

Acknowledgment

The authors thank Dr. Bruce Patton for providing the tissue culture facility for the co-culture experiments. We also thank Dr. Gary Banker for his advice in creating the coverslip cultures utilized in this experiment. This work was supported by NIH grant NS25767

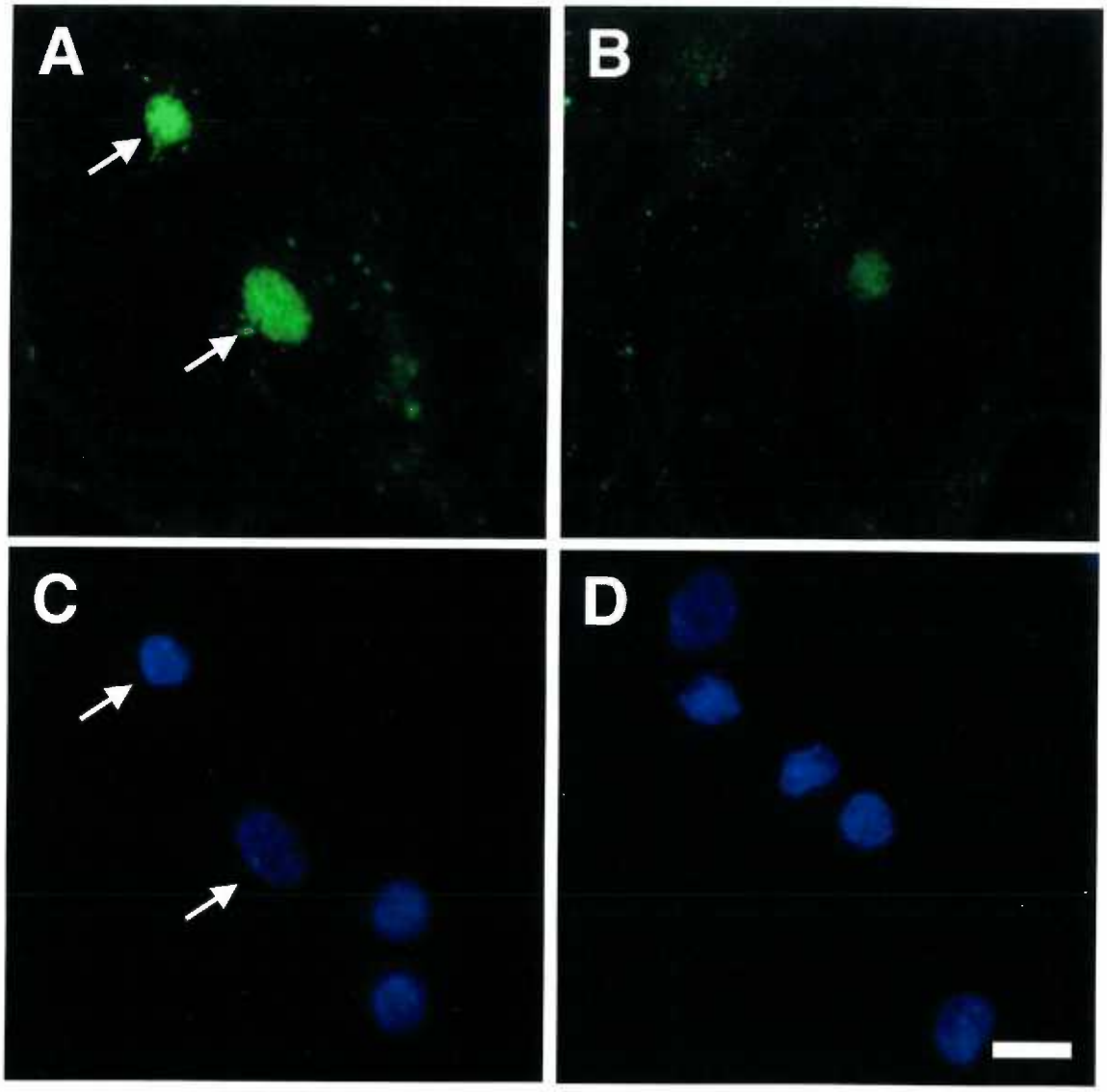

Figure 2. Expression of Krox20 in cultured E14 chicken sciatic nerve Schwann cells.

Cultures of E14 sciatic nerve Schwann cells stained for Krox20 (A, B) and DNA (C, D) after 12 hours (A, C) and 36 hours (B, D). Arrows indicate the nuclei of Schwann cells which express Krox20. After isolation, Schwann cells rapidly down-regulate their Krox20 expression, indicating that the Schwann cells are reverting to non-myelinating Schwann cells. Bar equals 15 μm.

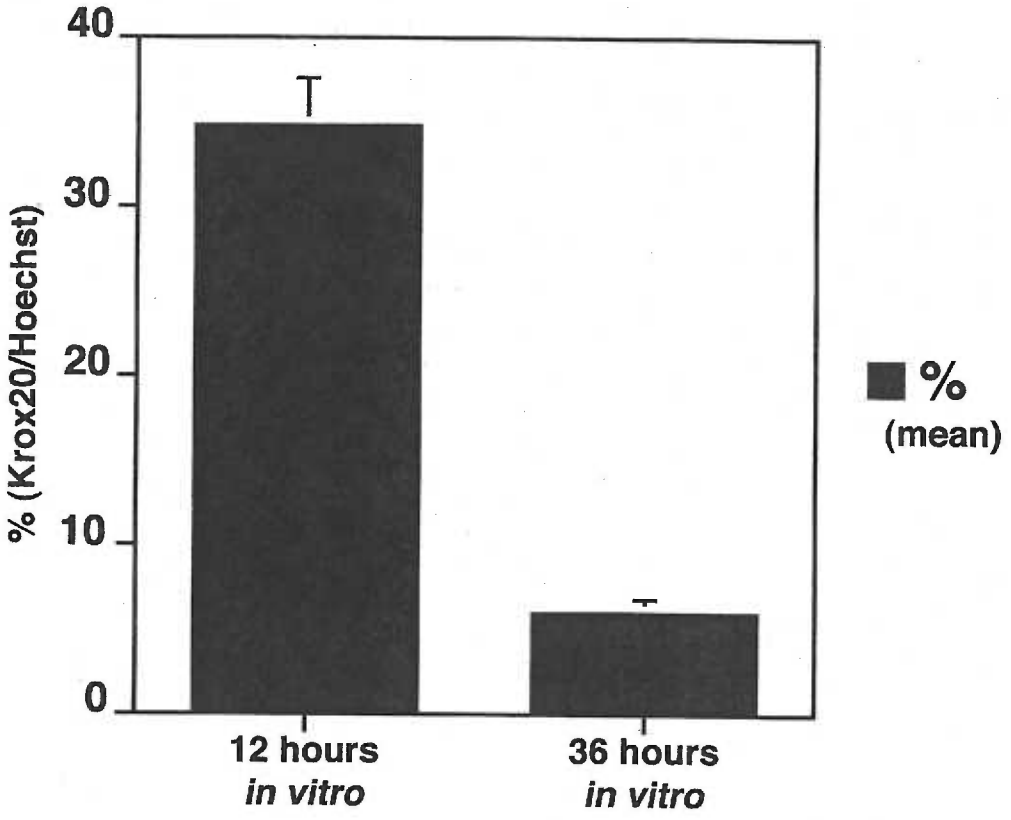

Figure 3. Changes in Krox20 expression of E14 chicken sciatic nerve Schwann cells in culture.

Percent of Krox20 positive Schwann cells isolated from E14 sciatic nerve after 12 hours and 36 hours in culture. Schwann cells lose their Krox20 expression within 2 days in culture, indicating that our default culturing conditions favor expression of the non-myelinating phenotype. $p < 0.0001$

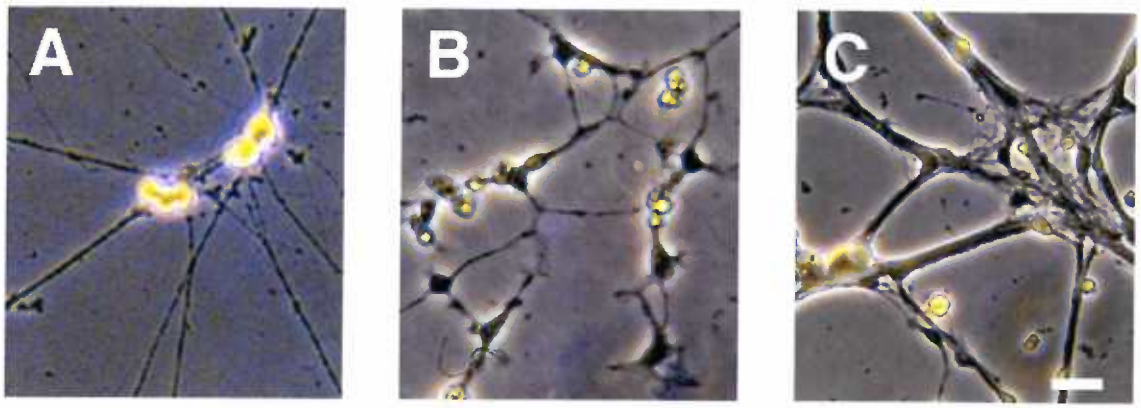

Figure 4. Preparation of co-cultures from purified E8 ciliary ganglion neurons and E14 sciatic nerve Schwann cells.

Cultures of purified E8 ciliary ganglion neurons (A) and purified E14 sciatic nerve Schwann cells (B). When the two cell types are cultured together, the Schwann cells form bundles around the processes of the neurons (C). Days in culture: 3 days (A, B), 10 days (C). Bar equals 15μm.

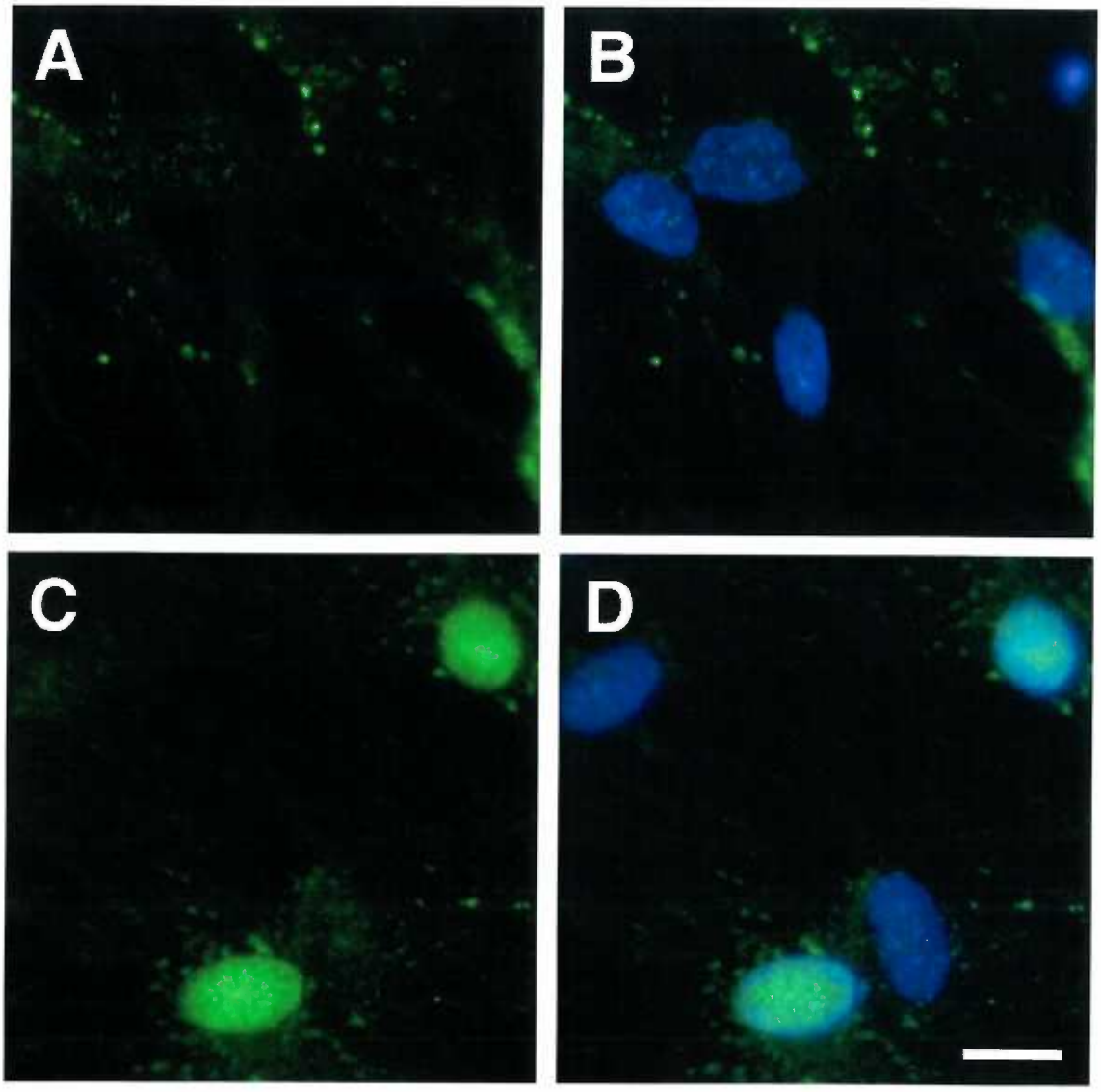

Figure 5. Expression of Krox20 in co-cultures of E8 ciliary ganglion neurons and E14 sciatic nerve Schwann cells incubated in either iris or choroid cultures.

Neuron/Schwann cell co-cultures stained for Krox20 (A, C) or Krox20 and Hoechst nuclear marker (B, D) after they were incubated in trans with choroid target tissues (A, B) or iris target tissues (C, D). Increased expression of Krox20 in CG/Schwann co-cultures are observed only when exposed to iris cultures (C, D). Bar equals 15 μm.

Table 1. Percent Krox20 positive Schwann cells in CG/Schwann co-cultures cultured in CG target tissues.

	1 day in target tissue	7 days in target tissues
Experiment #1		
cultured with iris:	14.3%	14.6%
	15.9%	19.1%
	4.6%	35.4%
cultured with choroid:	5.0%	8.8%
	7.3%	5.5%
	3.4%	9.1%
Experiment #2		
cultured with iris:	4.8%	29.6%
	5.8%	7.4%
	7.8%	10.5%
cultured with choroid:	3.6%	1.3%
	5.3%	3.0%
	2.2%	4.1%
Experiment #3		
cultured with iris:	11.4%	29.4%
	4.5%	24.7%
	7.7%	12.9%
cultured with choroid:	1.6%	4.3%
	0.0%	3.1%
	0.4%	6.8%
Mean ± S.E.		
cultured with iris:	8.53% ±4.33%	20.40% ±9.80%
cultured with choroid:	3.20% ±2.41%	5.11 ±2.67%

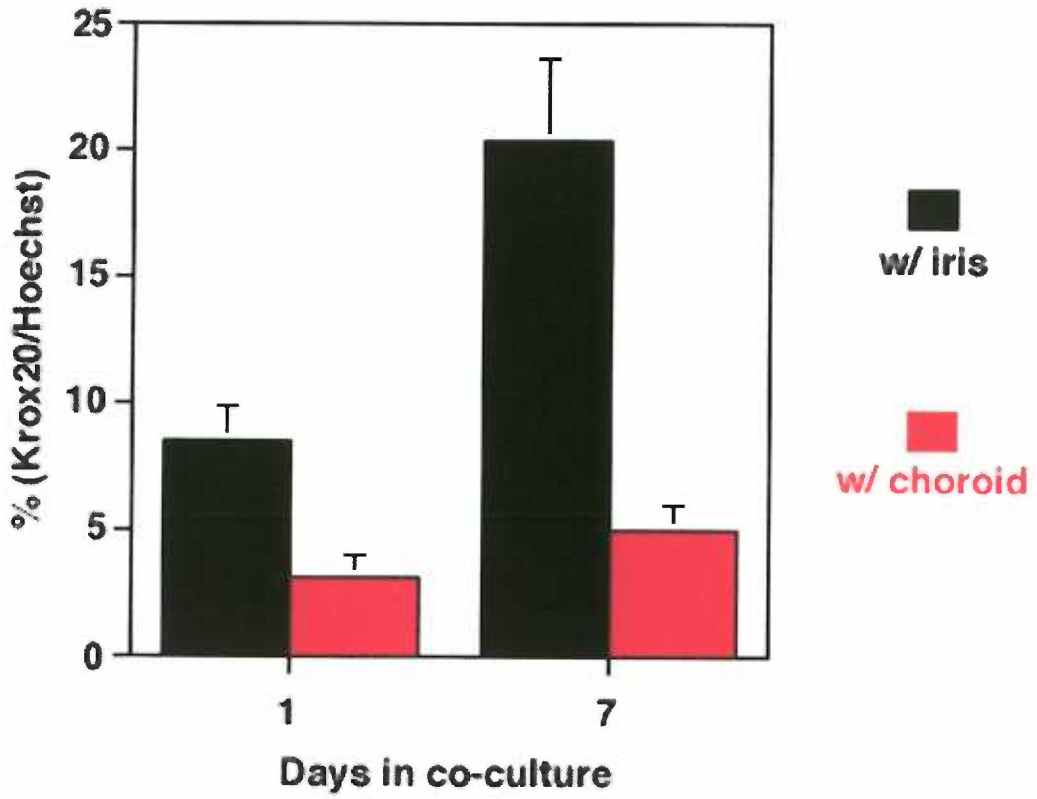

Figure 6. Effect of iris and choroid target tissues on the expression of Krox20 in neuron/Schwann cell co-cultures.

Neuron/Schwann cell co-cultures were incubated with either iris or choroid target tissues. After 7 days, only the co-cultures exposed to iris tissues showed an increase in Krox20 expression. $p < 0.0001$ between iris and choroid conditions.

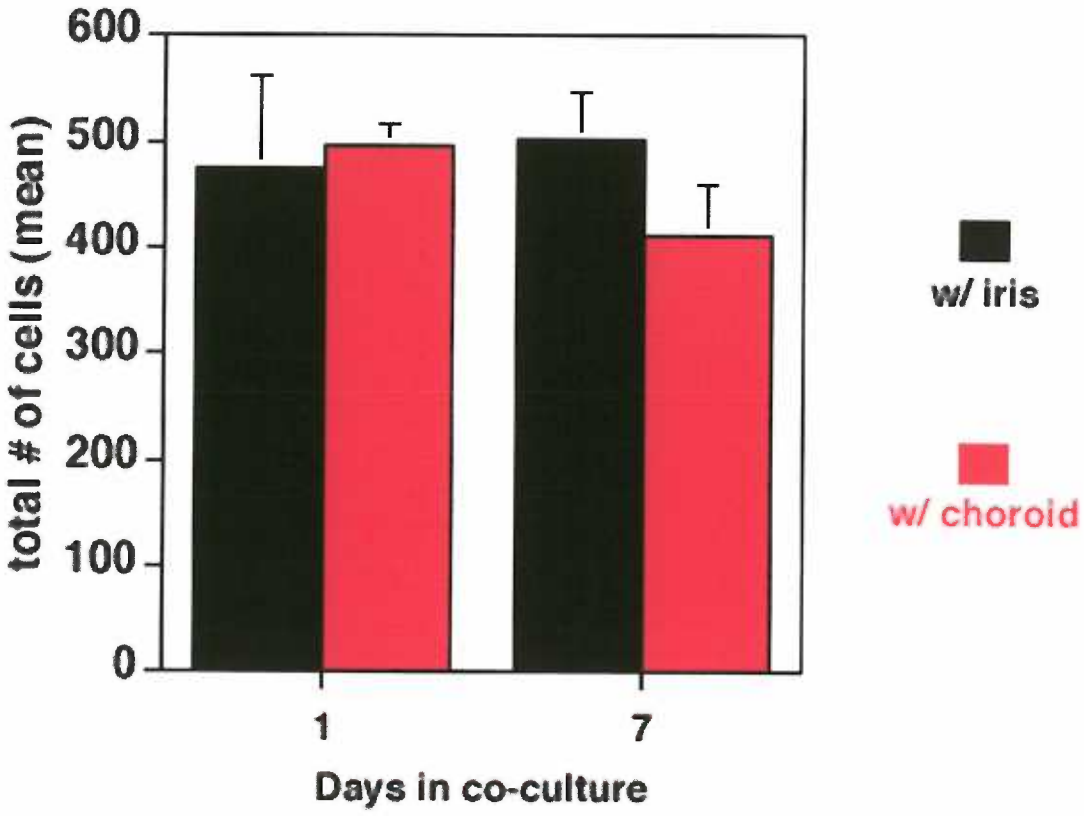

Figure 7. Total number of cells in each neuron/Schwann cell co-culture.

Cultures described in Figure 6 were counted for the total cell number. There were no significant changes in the total cell number between cultures exposed to iris tissues and choroid tissues. $p > 0.05$

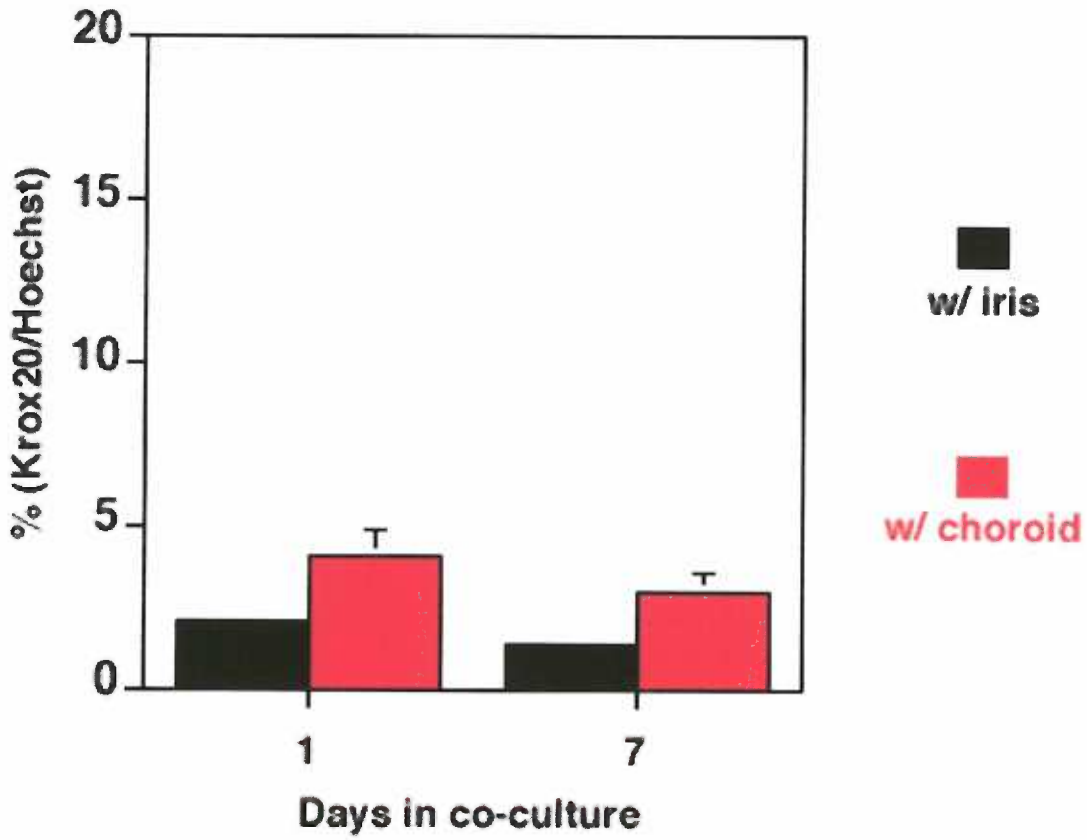

Figure 8. Effect of iris and choroid target tissues on the expression of Krox20 in cultures with only Schwann cells.

Cultures of only Schwann cells were incubated with either iris tissues or choroid tissues. There were no significant changes in Krox20 expression in both conditions, indicating that the increase of Krox20 expression observed in Figure 6 requires the presence of neurons. $p > 0.05$

CHAPTER 3

Knock-down of neuregulin in the developing chick ciliary ganglion disrupts Krox20 expression in Schwann cells of the myelinated ciliary nerve.

Alex T. Ho[1,2], Julie Keefe[2], David Wert[2], and Rae Nishi*[2]

*to whom correspondence should be addressed

[1]Department of Cell and Developmental Biology
Oregon Health and Science University
3181 S.W. Sam Jackson Park Road, L-215
Portland, Oregon 97201

[2]Department of Anatomy and Neurobiology
University of Vermont College of Medicine
149 Beaumont Avenue, HSRF 406
Burlington, Vermont 05403

Summary

Previous studies in myelination demonstrated that signals from the neurons induce Schwann cells to switch from a non-myelinating to a myelinating state. During the development of the avian ciliary ganglion (CG), preferential myelination of ciliary nerves coincides with increased expression of neuregulin in the cell bodies of ciliary neurons. Non-myelinated choroid neurons are devoid of neuregulin immunoreactivity. Preferential expression of neuregulin in ciliary neurons occurs at embryonic day 14 (E14), coinciding with expression of Krox20 and P0 in Schwann cells surrounding the ciliary neurons. However, by E18, the expression of neuregulin in the ciliary neurons becomes down-regulated, concurrent with increased expression in myelinating Schwann cells. Thus, at E14, neuregulin may act as a neuron-derived signal to promote the initial myelination of surrounding Schwann cells. Furthermore, the switch in neuregulin expression from neurons to myelinating Schwann cells may indicate changes in neuregulin function during CG development. We utilized a replication competent avian retrovirus expressing ribozymes targeted to the neuregulin mRNA (RCASBP(A)-4XRZnrg) to test whether the expression of neuregulin by ciliary neurons at E14 is necessary to promote myelination processes, specifically the expression of Krox20 and P0. When a suspension of concentrated viral particles was microinjected into the neural tube of stage 9 chicken embryos, the virus infected migrating neural crest cells that eventually formed the CG. Immunoblot analysis of the infected CG showed that there was a 20% to 60% reduction of neuregulin protein in RCASBP(A)-4XRZnrg infected CG, compared to that of control CG. Immunohistological analysis of the infected CG

showed an attenuation of neuregulin immunoreactivity in the neurons. In both the ganglia and ciliary nerves Krox20 expression was disrupted in ribozyme infected tissues. However, the expression of P0 in the ciliary nerve remained unchanged. This difference suggested that, expression of Krox20 and P0 in chickens during myelination may be regulated independently. Furthermore, the loss of neuregulin eliminated virtually all expression of the synaptic vesicle protein SV2 in the iris, suggesting that ciliary synapses failed to form. This elimination was confirmed by the concomitant loss of another synaptic vesicle protein, synaptophysin. However, nerve axons remained at the target, as observed by neurofilament immunoreactivity, and aggregates of nicotinic acetylcholine receptors on the iris muscle could still be detected by α-bungarotoxin labeling.

Introduction

Mechanisms regarding Schwann cell proliferation, basal lamina synthesis and final differentiation in the peripheral nervous system are poorly understood. Schwann cells are derived from the neural crest (Le Douarin and Smith, 1988) and postmitotically differentiate to either myelinating or non-myelinating. Although axonal signals are required for the expression of the myelinating fate (Weinberg and Spencer, 1976), little is know about the nature of the axonal signal involved. Previously, we demonstrated that neuregulin expression becomes confined to ciliary neurons during the onset of myelination in the developing ciliary ganglion (see Chapter 1). In contrast, the choroid neurons, which do not become myelinated, do not express any neuregulin. Thus, specific expression of neuregulin in the ciliary neurons at E14 may induce preferential myelination.

Neuregulin is a growth factor that has variety of biological actions. Originally characterized from multiple sources, neuregulin is also known as sensorimotor derived factor, acetylcholine receptor-inducing activity, heregulin, neu differentiation factor, and glial growth factor (Garratt et al., 2000a; Gassmann and Lemke, 1997). The neuregulin gene gives rise to multiple isoforms of neuregulin protein by alternative splicing, thus giving the molecule a wide range of possible functions (Meyer et al., 1997). However, all of the isoforms contain an epithelial growth factor-like (EGF-like) domain responsible for their activities, thus categorizing them as part of the EGF superfamily. The EGF-like domain of the neuregulin protein binds to tyrosine kinase receptors of the EGF receptor

family, specifically the ErbB-3 and ErbB-4. In contrast, the ErbB-1 receptor is specific for epidermal growth factor while ErbB-2 acts as a co-receptor to help transmit signals from the other activated ErbB receptors.

Although neuregulin is essential for inducing neural crest cells to become Schwann cells (Garratt et al., 2000a; Gassmann and Lemke, 1997), there is little known as to whether neuregulin also influences the process of myelination. Mice that lack neuregulin or its ErbB receptors are embryonic lethal because neuregulin signaling is essential for cardiac development (Britsch et al., 1998; Lee et al., 1995). When the cardiac defect is rescued in mice lacking ErbB2 receptors, the peripheral nervous system displays a number of abnormalities due to the failure of Schwann cells to differentiate (Morris et al., 1999; Woldeyesus et al., 1999). Thus, the effects of neuregulin on myelination cannot be studied unless the ligand or the gene can be conditionally knocked out after Schwann cells have been generated. However, recent culture studies have shown that one of the neuregulin isoforms, CRD-neuregulin, may induce the expression of myelin associated genes in developing Schwann cells (Leimeroth et al., 2002).

In this study, we tested whether preferential myelination of ciliary neurons is induced by the selective expression of neuregulin in the developing ciliary ganglion. By using an RCASBP(A) virus expressing ribozymes against the neuregulin mRNA (Zhao and Lemke, 1998), we were able to specifically knock down the expression of neuregulin in the developing ciliary ganglion after neural crest cells have become committed to the Schwann cell fate.

Results

RCASBP(A)-4XRZnrg disrupts neuregulin expression

To determine whether neuregulin expression in neurons is necessary for the expression of myelinating genes in associated Schwann cells, we sought to reduce neuregulin expression by using ribozymes targeted against neuregulin mRNA. The construct we used (RCASBP(A)-4XRZnrg) was previously shown to phenocopy the neuregulin knockout mouse when chicken embryos were infected with the virus at the blastoderm stage (Zhao and Lemke, 1998). To test the efficacy of the virus, we infected cultures of E14 sciatic nerve Schwann cells. *In vitro*, all Schwann cells are immunoreactive for the neuregulin protein as detected by the SC-348 antibody (not shown). Figure 1A shows that a subset of Schwann cells was infected successfully with the RCASBP(A)-4XRZnrg virus, as indicated by immunoreactivity to viral particles. Figure 1B shows cells stained for the neuregulin. The lack of neuregulin immunoreactivity in cells expressing viral particles, (Figure 1C) indicated that the ribozyme construct was active. When the experiment was repeated with RCAS virus expressing the GFP protein instead of the neuregulin ribozyme, all Schwann cells retained their immunoreactivity for neuregulin (not shown).

As another means of verifying the efficacy of the RCASBP(A)-4XRZnrg infection, we measured the amount of neuregulin protein using the immunoblot technique. The ciliary ganglia of chick embryos infected with RCASBP(A)-4XRZnrg and with RCASBP(A)-GFP were analyzed for neuregulin protein at stage 40-41 (E14). Preabsorption with the

neuregulin peptide used to generate the antibody blocked the detection of any bands (Figure 2A). Injecting embryos with virus that contained only the vector (RCASBP(A)-control) did not attenuate the expression of the neuregulin protein in the CG (Figure 2B). Reduction of neuregulin expression was specific to the infection with the RCASBP(A)-4XRZnrg virus (Figure 2B). Immunoblots of viral-gag protein (p27gag) confirmed that both experimental and control CGs were infected with the RCAS virus (Figure 2B).

Next, to determine how RCASBP(A)-4XRZnrg infection affects the expression of neuregulin in individual embryos, extracts of ciliary ganglia from each sample were analyzed for neuregulin using immunoblots. Figure 3 shows an example of these immunoblots for various sets of injections, comparing them to non-injected and RCASBP(A)-GFP injected controls. Densitometric analysis with NIH image showed that the relative intensity of the neuregulin bands varied from 4% to 65% when compared to the non-injected control tissue (Figure 3). This result indicated that the viral infection resulted in anywhere from 96% to 35% reduction in the total amount of neuregulin protein in the CG.

In order to determine the exact pattern of viral infection and the region of NRG disruption, immunohistological analysis of CG sections was conducted. CGs were analyzed from both infected and uninfected embryos. Figure 4 is an example of a cryostat section of either non-infected CG or RCASBP(A)-4XRZnrg infected CG. The tissues were analyzed for expression of viral protein (Figure 4A and 4D) and neuregulin (Figure 4B and 4E). In the uninfected CG, neuregulin expression in CG neurons

remained intact (arrowhead) (Figure 4A and 4B). However, in tissues infected with the RCASBP(A)-4XRZnrg virus, neuregulin expression in the CG neurons was undetectable (small arrow). Non-neuronal cells in the CG were also infected. Furthermore, since neuregulin is only expressed in ciliary neurons at E14 (Chapter 1), there are choroid neurons that do not normally express neuregulin regardless of the infection (*).

Effect of RCASBP(A)-4XRZnrg infection in vivo on expression of Krox 20

Table 1 lists the number of injections conducted to determine the effect of RCASBP(A)-4XRZnrg infection on the expression of neuregulin and Krox20. Survival of the injected embryos varied from 50% (15 out of 30 injected in #5) to 10% (3 out of 30 injected in #3). Because multiple factors affect embryo survival until E14 (egg quality, how the eggs were windowed, the amount of damage during the injection process, etc.), we were unable to determine whether the viral infection caused the embryos to terminate prior to E14. In all sets of injections, only a subset of surviving embryos are infected (31% to 75%) as determined by the expression of viral proteins in the ciliary ganglia using immunohistochemistry (Figure 4). Once the viral protein expression was confirmed in each sample, the CG sections were further analyzed for neuregulin expression. Immunohistochemistry of cryostat sections and immunoblots of CG extracts were used to determine the knock-down of neuregulin in the ciliary ganglia. In most cases, the infection of RCASBP(A)-4XRZnrg, as determined by viral protein expression, resulted in the lack of neuregulin expression in the ganglion (Figure 4) (Table 1, injection set #2 and #4). When the knock-down of neuregulin was quantified using immunoblot analysis

(Table 1, injection set #6 and #7), the CG extracts that showed more than 50% reduction in neuregulin showed lack of immunoreactivity in the cryostat sections.

The loss of neuregulin disrupts Krox20 expression in E14 ciliary nerves

After injected embryos were analyzed by neuregulin immunoblots (Figure 3) and immunohistological analysis (Figure 4), we examined the expression of the early myelin gene Krox20 in the ciliary nerves of infected embryos. Iris/ciliary muscles of the infected and uninfected tissues were stained for Krox20 (Figure 5A and 5D) and neurofilament (Figure 5B and 5E). Schwann cells in the ciliary nerves of uninfected tissues expressed Krox20 (Figure 5A). Schwann cells in the ciliary nerves of RCASBP(A)-4XRZnrg infected embryos lacked Krox20 immunoreactivity (Figure 5D). Staining the nerve with Hoechst DNA dye indicated that the Schwann cells remain aligned along the ciliary nerves of the infected preparations (not shown), thus demonstrating that the knock-down of neuregulin expression disrupted Krox20 expression in the infected nerves, but did not affect migration of Schwann cells along the axons. All of the embryos that showed a lack of neuregulin immunoreactivity in cryostat sections lacked Krox20 expression in both the ganglion and the ciliary nerves except for one set of injections (* in Table 1).

RCASBP(A)-4XRZnrg infection does not affect P0 expression

Quadrants from the iris/ciliary bodies of the RCASBP(A)-4XRZnrg infected embryos were also analyzed for expression of another myelin marker, P0 (Figure 6A, 6D and 7B), together with neurofilament (NF150) (Figure 6B, 6E, and 7A). Although the expression

of Krox20 was disrupted by the neuregulin knock-down, expression of P0 remained unchanged relative to controls (Figure 6D, 7B). Thus, these results indicate that expression of Krox20 and P0 in chickens is independent of one other.

RCASBP(A)-4XRZnrg infection affects synapse formation

To determine whether the viral infection disrupted any other neuregulin dependent processes, we characterized synapse formation in the infected iris. Staining the infected tissues with SV2 (Figure 8) and synaptophysin (Figure 9) showed that the viral infection also disrupted maturation of the synapse in the iris, as indicated by the attenuation of these two synaptic markers. However, nerve axons remained at the target as indicated by neurofilament immunoreactivity (Figure 8E), and nicotinic acetylcholine receptor aggregates on the iris muscle remain intact as indicated by the α-bungarotoxin labeling (Figure 9D).

Discussion

The results of the *in vivo* manipulation presented in this work demonstrate that neuregulin is required for the activation of the myelin gene Krox20. However, despite the lack of Krox20, the expression of the late myelin protein P0 was unaffected, suggesting that, in the chick, expression of late myelin genes is not dependent upon the expression of Krox20. This indicates that neuregulin signaling in the developing ciliary ganglion may control only one aspect of the myelination process.

Our results appear to differ significantly from previous work on Krox 20 null mutant mice, in which Schwann cells are blocked at the promyelinating phase (Topilko et al, 1994). In Krox 20 mutants, Schwann cells express early markers such as S100 and myelin associated glycoprotein, but the late myelin markers, P0 and myelin basic protein do not accumulate. However, chick Schwann cells differ from their mammalian counterparts in that they express P0 at low levels early in development, while P0 is expressed only as a late myelin gene in mammals (Cheng and Mudge, 1996). Thus, even though the intensity of P0 staining in our RCASBP(A)-4XRZnrg injected embryos appeared comparable to controls, the formation of compact myelin may still be disrupted. Attempts to stain for compact myelin using histological methods such as Sudan Black were unsuccessful. Unambiguous determination could be achieved by using electron microscopy. Furthermore, disruption of Krox20 by the neuregulin knock-down may have affected the expression of other myelin genes such as myelin basic protein.

The results of the RCASBP(A)-4XRZnrg injection show that there is variability between the infection in the ciliary ganglia, the knock-down levels of neuregulin, and the attenuation of Krox20 expression. The Type A strain of the RCASBP virus used in this study has been shown to infect successfully the cells of the ciliary ganglion (Finn et al., 1998; Lee et al., 2001). In order to determine whether the RCASBP(A)-4XRZnrg virus knocked down the expression of neuregulin successfully in the CG neurons, it was necessary first to confirm the presence of virus in the neurons rather than in the non-neuronal cells of the ciliary ganglion. In some embryos, the expression of the viral proteins was limited to non-neuronal cells. In other samples, the virus infected both the CG neurons and the non-neuronal cells surrounding the neurons. Previous studies (Chapter 1) show that neuregulin expression in E14 ciliary ganglion is detected primarily in ciliary neurons. Thus, the variability of the neuregulin knock-down observed in the cryostat sections and the immunoblot is dependent on the success of the virus infecting the ciliary neurons. The discrepancy between the total number of infected embryos and the number of infected embryos with reduced neuregulin expression in the ganglia may be caused by the inability of the RCASBP(A)-4XRZnrg virus to infect specifically ciliary neurons. This difference may be due to technical difficulty in infecting CG neuron precursors. The window of opportunity for the virus to infect the crest cells that eventually differentiate into CG neurons may be very narrow (between stage 8-9). Viral injection of virus into the neural tube after stage 9 often results in infections found mainly in the non-neuronal cells of the ciliary ganglia (Nishi lab, unpublished results).

In the context of Schwann cell development, neuregulin has been shown to specify Schwann cell lineage (Shah et al., 1994), induce proliferation of both precursor and mature Schwann cells (Dong et al., 1995), and induce the expression of certain myelin genes (Garratt et al., 2000b). However, various neuregulin deletion mutants in mice have often resulted in embryos that died by embryonic day 10.5 due to cardiac malformation (Kramer et al., 1996). Other phenotypes observed by these knockouts include loss of neuronal cell populations (Morris et al., 1999; Woldeyesus et al., 1999), reduced number of glial precursors (Meyer et al., 1997), and lack of Schwann cells along peripheral nerves (Morris et al., 1999; Woldeyesus et al., 1999). Although these experiments demonstrated the importance of neuregulin in early Schwann cell development, the lethality of the embryos prevented previous authors from addressing the role of neuregulin in the differentiation of myelinating Schwann cells. The neuregulin ribozyme utilized in this experiment was designed to knock down all of the different neuregulin isoforms (Zhao and Lemke, 1998). The RCASBP(A)-4XRZnrg virus can phenocopy the neuregulin knockout in the chicken embryo if the virus is infected at an early blastoderm stage (Zhao and Lemke, 1998). Because we were interested only in the development of Schwann cells in the ciliary ganglia and nerves, by injecting the virus at stage 9 into the neural tube, we were able to manipulate neuregulin expression in these embryos without disrupting heart formation.

In this study, the infected embryos that survived to E14 did not show any external deformities. There was no obvious increase in cell death of CG neurons or Schwann cells in both the ganglia and CG nerves as determined by Hoechst DNA dye and P0

expression. The RCASBP virus infects only dividing cells and comes in different strains determined by viral envelope subtypes which limit infection to certain subset of cells (Fekete and Cepko, 1993a; Homburger and Fekete, 1996). Furthermore, it requires approximately 48 hours before the infecting virus replicates and spreads beyond the original infected tissues. Thus, by injecting the RCASBP(A)-4XRZnrg virus at stage 8-9, the infections may have created a conditional knockout that reduced neuregulin expression in our regions of interest without affecting the disruption of neuregulin function in tissues which altered the proliferation and survival of neurons and Schwann cells.

Our results show that the knock-down of neuregulin in E14 ciliary ganglion disrupts Krox20 expression in the ciliary nerve. Interestingly, a lack of Krox20 expression does not affect P0 expression in the ciliary nerve. This observation is the first indication that Krox20 expression by myelinating Schwann cells is dependent on neuregulin expression by the neuron. Furthermore, the lack of coordination between Krox20 and P0 in the myelinating ciliary nerve suggests that regulation of myelin specific genes in chickens may be different from rodents.

Acknowledgement

The author would like to thank Dr. Greg Lemke for providing the RCASBP(A)-4XRZnrg construct and Dr. Bruce Patton for providing the antibodies against SV2. This work was supported by NIH grant NS25767

Injection Set #	# survived to E14 (out of 30)	# expressing viral protein (% infection)	# with NRG knock-down (% embryos)	confirm NRG knock-down with immunoblot	Krox20 expression
1	11	7 (64%)	6 (86%)	No	No
2	5	3 (60%)	3 (100%)	No	Yes*
3	3	1 (33%)	0 (0%)	No	Yes
4	13	4 (31%)	4 (100%)	No	No
5	15	6 (40%)	5 (83%)	No	No
6	12	8 (67%)	6 (75%)	Yes	No
7	8	6 (75%)	5 (83%)	Yes	No

Table 1. Effect of RCASBP(A)-4XRZnrg infection *in vivo* on expression of Krox20.

For each set of experiments, thirty virus-free eggs were windowed and concentrated virus injected into the mesencephalon of stage 9-10 embryo (E1.5). At E14, the ciliary ganglia and eyes were isolated from surviving embryos, and sections of ganglia were analyzed for viral infection (p27 immunoreactivity) and neuregulin expression. Based on the attenuation of neuregulin, Krox20 expression was analyzed in both the ganglia and the ciliary nerves using immunohistochemistry.

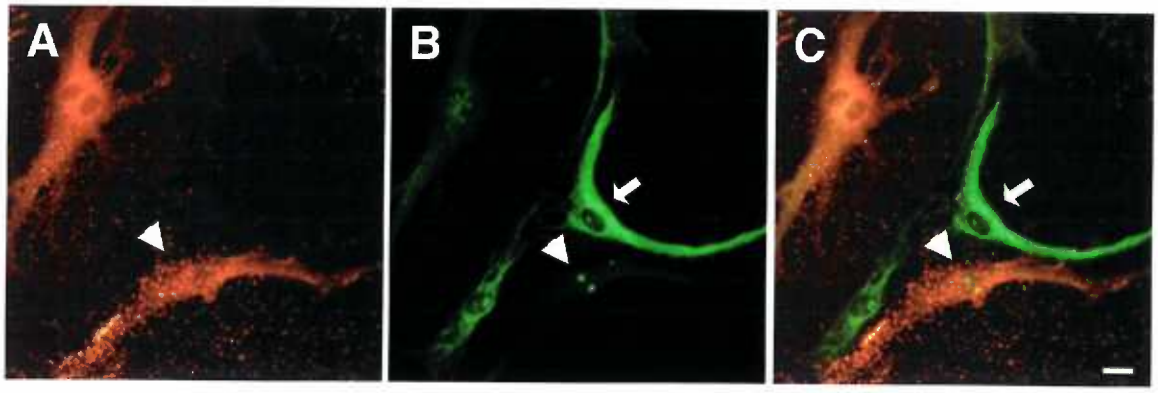

Figure 1. RCASBP(A)-4XRZnrg disrupts neuregulin expression in E14 Schwann cell cultures.

E14 Schwann cell cultures were infected with RCASBP(A)-4XRZnrg. 72 hours after the infection, the cultures were immuno-stained for the presence of viral particles (A) and neuregulin (B). The infected Schwann cells express little or no neuregulin (arrowhead). Schwann cells that remain uninfected retain their neuregulin expression (arrow). C is an overlay of A and B. Bar equals 15 μm.

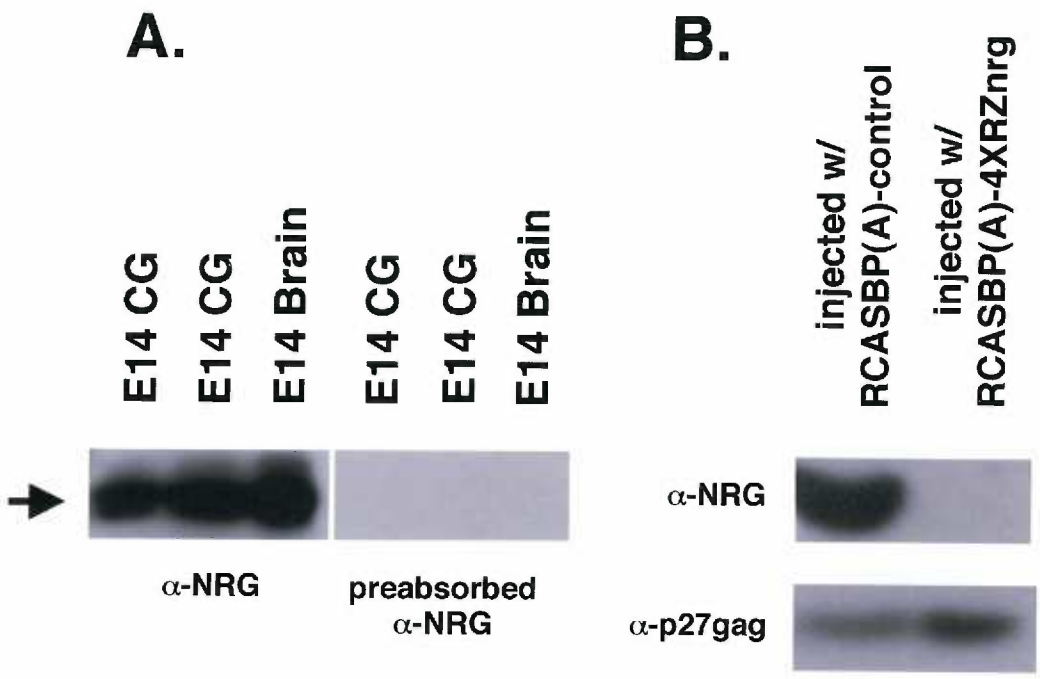

Figure 2. Neuregulin immunoblot of RCASBP(A)-4XRZnrg infected ciliary ganglia.

The anti-neuregulin antibody (SC-348) used to detect neuregulin in CG extracts is specific, as indicated by disappearance of the neuregulin band when preabsorbed with the peptide (A). Infecting the embryo with vector-only virus (RCASBP(A)-control) does not affect neuregulin expression in the ganglia (B). Thus, attenuation of neuregulin *in vivo* is specific to the infection by the RCASBP(A)-4XRZnrg virus (B). In both samples (B), expression of viral protein (p27gag) confirms that the ganglia are infected with the RCAS virus.

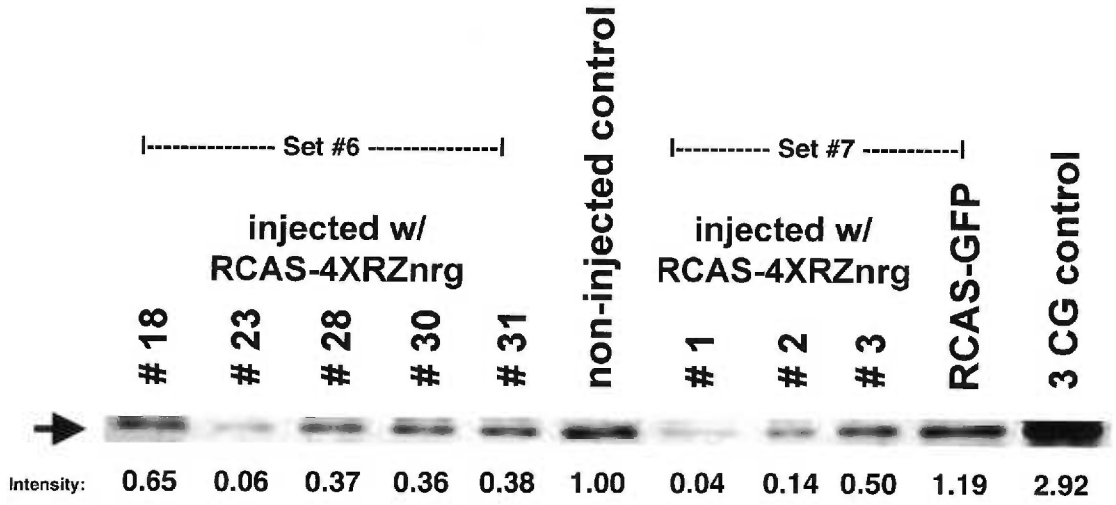

Figure 3. Neuregulin immunoblot of ciliary ganglia infected with RCASBP(A)-4XRZnrg.

Infection of RCASBP(A)-4XRZnrg virus attenuates the expression of neuregulin protein in the developing CG. Densitometric analysis of neuregulin expression in the infected tissues with NIH image shows that the intensity of the bands varies from 4% to 65% when compared to ciliary ganglion of the virus-free (non-injected) embryo.

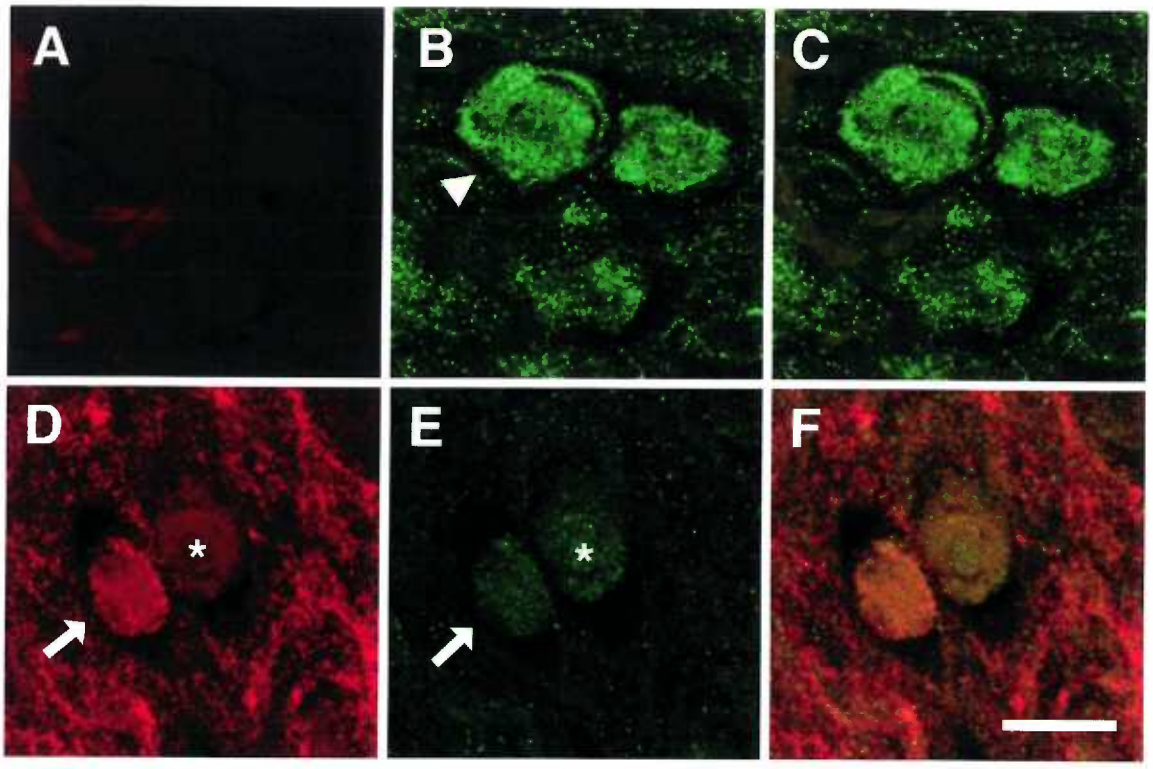

Figure 4. RCASBP(A)-4XRZnrg infection disrupts neuregulin expression.

Cryostat sections of E14 ciliary ganglia from uninfected control embryos (A-C) and infected embryos (D-F) with RCASBP(A)-4XRZnrg. The sections were stained for viral infection (A, D) and neuregulin (B, E). Normally, at E14, neuregulin expression is detected in ciliary neurons (B, arrowhead). However, the infection (D) attenuated neuregulin expression in the ganglia (E). C is an overlay of A and B; F of D and E. Bar equals 15 μm.

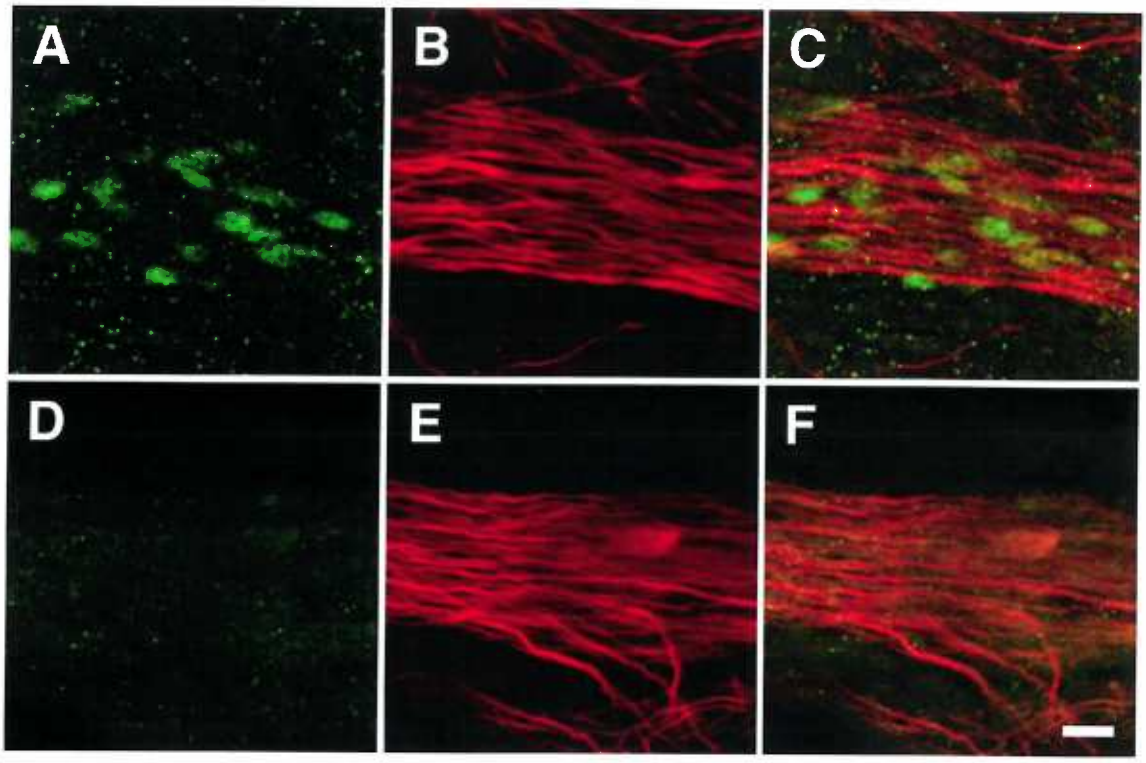

Figure 5. RCASBP(A)-4XRZnrg disrupts Krox20 expression in E14 ciliary nerves.

Whole-mount staining of E14 ciliary nerves from uninfected control embryos (A-C) or infected embryos (D-F) with RCASBP(A)-4XRZnrg immunostained for Krox20 (A, D) and neurofilament (B, E). At E14, Schwann cells in the ciliary nerve express the myelin marker Krox20 (A). The infection of RCASBP(A)-4XRZnrg blocks Krox20 expression in the ciliary nerve (D) indicating that expression of neuregulin in the developing CG is necessary for Krox20 expression. C is an overlay of A and B; F of D and E. Bar equals 10 μm.

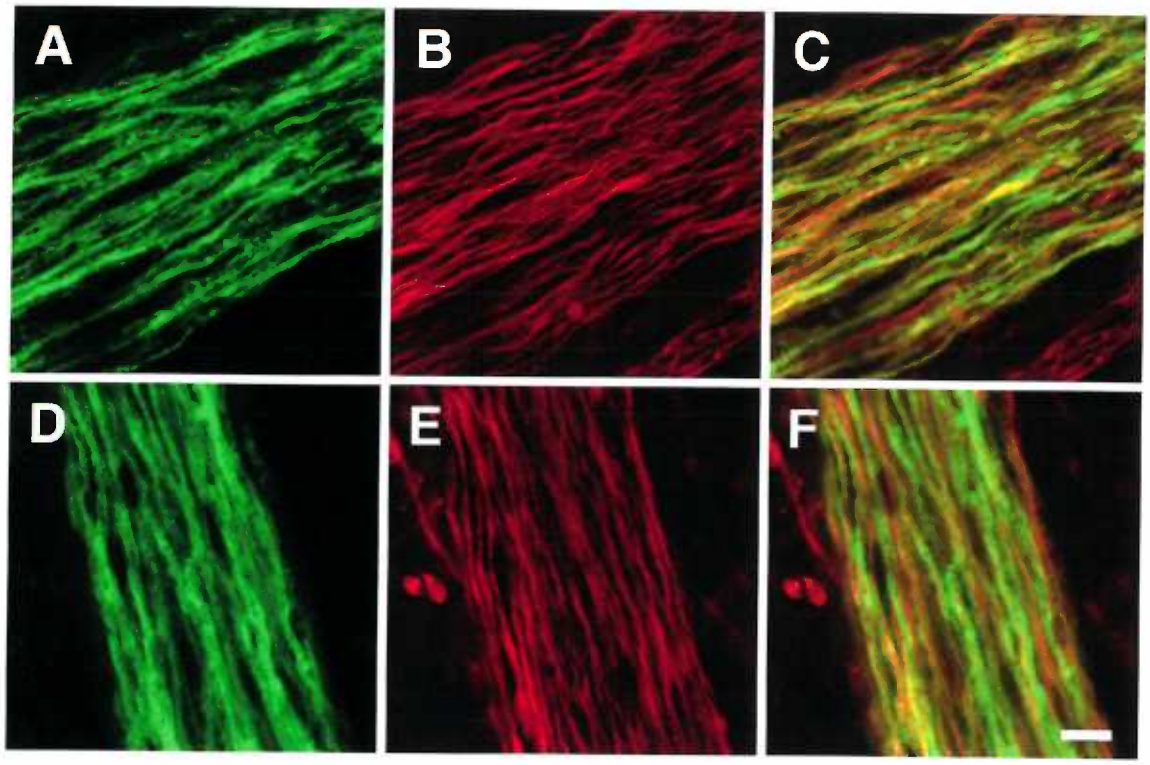

Figure 6. RCASBP(A)-4XRZnrg infection does not affect P0 expression.

Whole-mount staining of E14 ciliary nerves from uninfected control embryos (A-C) or infected embryos (D-F) with RCASBP(A)-4XRZnrg, immunostained for P0 (A, D) and neurofilament (B, E). At E14, Schwann cells in the ciliary nerve express the myelin marker P0 (A). The infection of RCASBP(A)-4XRZnrg does not block P0 expression in the ciliary nerve (D), indicating that neuregulin does not control P0 expression. C is an overlay of A and B; F of D and E. Bar equals 10 μm.

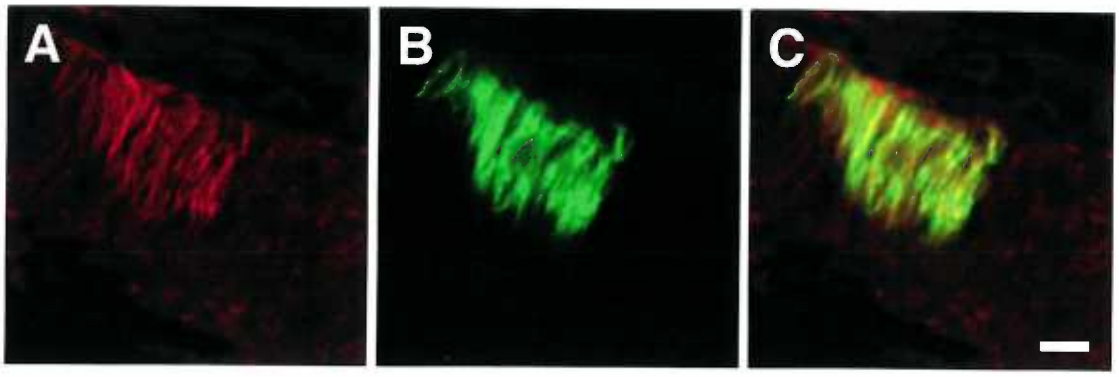

Figure 7. Cross section of iris infected with RCASBP(A)-4XRZnrg immunostained for neurofilament and P0.

Cross-section of E14 iris infected with RCASBP(A)-4XRZnrg. Sections were stained for neurofilament (A) and P0 (B). The infection with RCASBP(A)-4XRZnrg does not affect P0 expression. C is an overlay of A and B. Bar equals 10 μm.

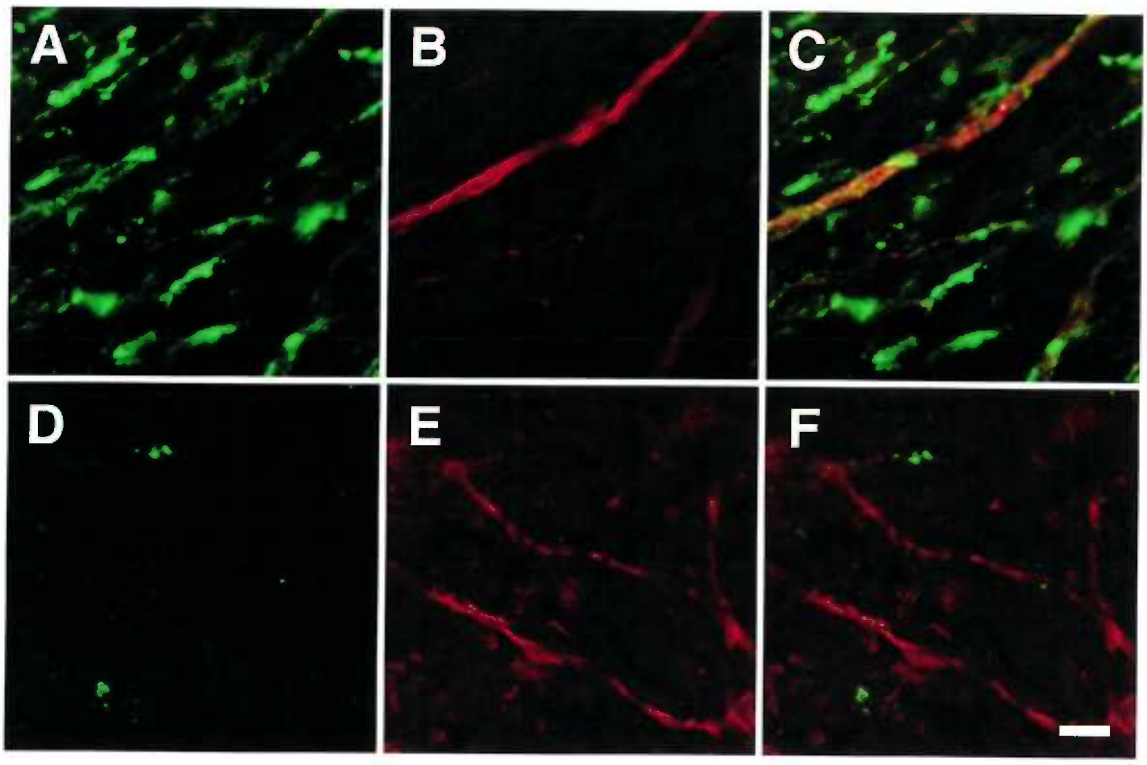

Figure 8. RCASBP(A)-4XRZnrg disrupts SV2 expression in the iris.

Whole-mount staining of E14 iris from uninfected control embryos (A-C) and infected embryos (D-F) with RCASBP(A)-4XRZnrg, immunostained for SV2 (A, D) and neurofilament (B, E). The infection of RCASBP(A)-4XRZnrg attenuates SV2 expression in the iris (D), indicating that synapse formation may be disrupted. C is an overlay of A and B; F of D and E. Bar equals 10 μm.

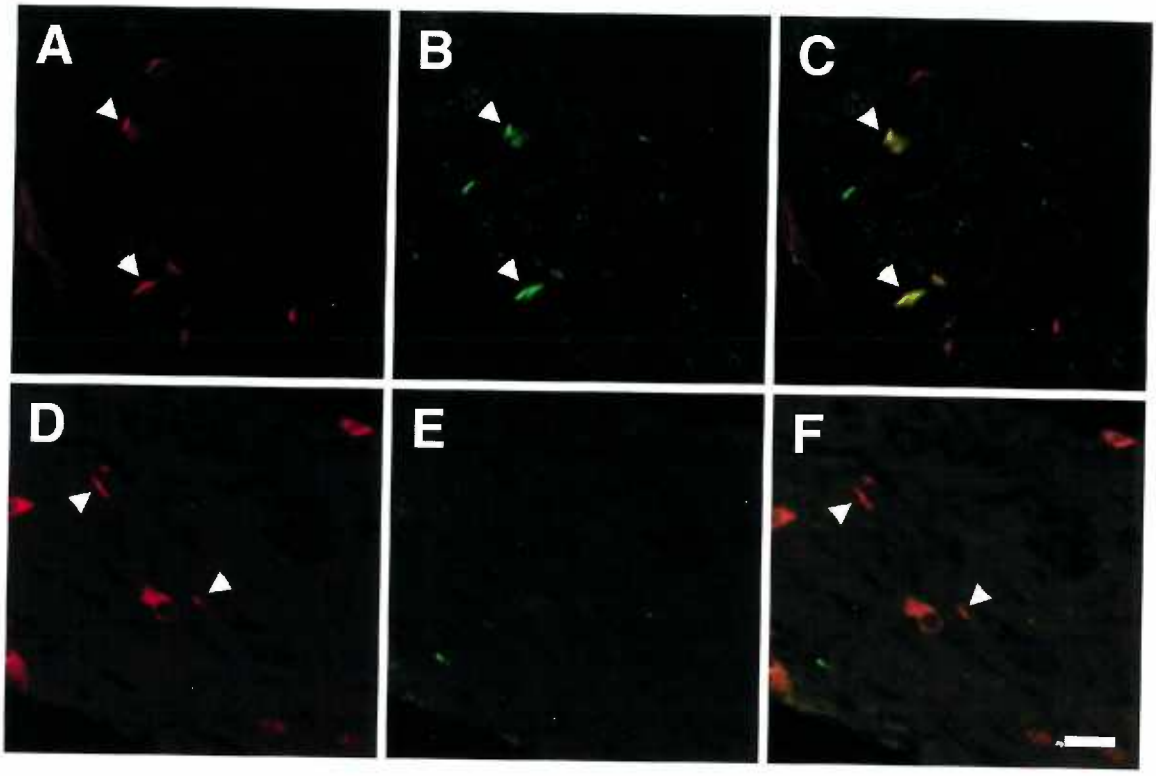

Figure 9. RCASBP(A)-4XRZnrg disrupts synaptophysin expression, but not alpha-bungarotoxin binding sites.

Cross section of E14 iris from uninfected control embryos (A-C) or infected embryos (D-F) with RCASBP(A)-4XRZnrg, immunostained for α-bungarotoxin binding sites with rhodaminated-bungarotoxins (A, D) and synaptophysin (B, E). The infection of RCASBP(A)-4XRZnrg attenuates synaptophysin expression in the iris (E), indicating that synapse formation may be disrupted. C is an overlay of A and B; F of D and E. Bar equals 10 μm.

expression of P0 and other myelin structure proteins. However, in the chicken, Krox20 and P0 expression occur at the same time.

The exact function of p75 in immature or non-myelinating Schwann cells is still unclear. However, recent studies using p75-blocking antibody suggested that p75 signaling is necessary for Schwann cells to switch from non-myelinating to a myelinating state (Cosgaya et al., 2002). p75 receptor activation without Trk receptor signaling with BDNF induced Schwann cells to myelinate DRG neurons *in vitro* (Cosgaya et al., 2002). The function of p75 in Schwann cells may be to monitor neurotrophins expression in the nerve during development and injury, and when given the proper conditions, the surge in neurotrophin levels in surrounding tissues may promote non-myelinating Schwann cells to myelinate associated axons. Other non-myelinating markers include NCAM and L1 adhesion molecules, both of which are required for non-myelinating Schwann cells to associate with multiple neurons, unlike the 1:1 relationship between myelinating Schwann cells and PNS neurons (Daniloff et al., 1986; Martini and Schachner, 1988).

Previous studies showed that signals regulating the differentiation of myelinating versus non-myelinating Schwann cells come from the neurons (Aguayo et al., 1976; Weinberg and Spencer, 1976). Our *in vitro* studies using isolated Schwann cell cultures demonstrated that when Schwann cells lose contact with the neurons, they down-regulate the expression of Krox20 and P0. The decrease of Krox20 occurs very rapidly. Within 12 hours, 65% of the Schwann cells isolated from the myelinated sciatic nerves lose their Krox20 expression. By 36 hours, only 5% of the Schwann cells retain their Krox20

expression. Since Krox20 is a transcription factor specific for myelinating Schwann cells, rapid turnover for this signal in the nuclei may be critical during the switch between myelinating and non-myelinating Schwann cells. Compared to the rapid decline in Krox20, it requires 6 days for 99% of the isolated Schwann cells to lose their P0 immunoreactivity, a result which agrees with that of a previous study (Bhattacharyya et al., 1993). Once myelin specific markers are lost, isolated Schwann cells express p75, the non-myelinating Schwann cell marker. Thus, the Schwann cells used in our culture studies retain the ability to switch between myelinating and non-myelinating characteristics. Without any interactions with the neurons, the Schwann cells *in vitro* lack the ability to maintain their Krox20 and P0 expression.

The results from our co-culture experiments suggest that, in the absence of any target tissues, E8 CG neurons are unable to stimulate the expression of myelin genes in E14 sciatic nerve Schwann cells. Previous studies using cultures from dorsal root ganglia from rodents showed that the culture medium needs to be supplemented with ascorbic acid to aid the formation of myelin structures in neuron/Schwann cell cultures (Eldridge et al., 1987). Furthermore, other studies have shown that the level of cAMP in Schwann cells must be increased by chemical agents such as forskolin or cAMP analogs to induce myelination (Morgan et al., 1991). The culturing medium used in our experiments was serum-free and only contained supplements (B27), and was originally developed for culturing hippocampal neurons (Brewer et al., 1993). Also, *in vivo*, E8 ciliary ganglion neurons are not myelinated. When initially isolated, the ciliary ganglion neurons may lack the ability to induce myelin-associated gene expression in adjacent Schwann cells.

Thus, the increase in Krox20 expression observed in co-cultures incubated in iris-conditioned medium indicates that the ciliary target tissues create an environment that favors the switch from non-myelinating to myelinating Schwann cells. The target tissues may be either priming Schwann cells to accept myelinating signals from neurons or stimulating E8 ciliary ganglion neurons to express phenotypes similar to E14 ciliary neurons, which may promote myelination in surrounding Schwann cells.

Earlier studies have suggested that the thickness of axons may determine whether the neurons became myelinated or remain unmyelinated. Histological analysis of mouse sciatic nerves suggested that the threshold for myelinated axons was anything larger than 1 μm (Friede and Samorajski, 1967). Manipulation of target tissues of rat superior cervical ganglion neurons that altered the axon diameters suggested that sympathetic neurons became myelinated when the diameters of the axons were 1.6 μm or larger (Voyvodic, 1989). However, in mouse mutants lacking the mid-sized and heavy neurofilament subunits, the formation of myelin sheath in the PNS neurons remained unchanged even when the axon diameter was reduced by 20-37% (Elder et al., 2001). Interestingly, reduced axon diameter in CNS neurons showed a decrease in myelination (Elder et al., 2001). Other studies suggest that reduced action potential in neurons may inhibit the myelination of axons (Stevens et al., 1998). Changes in axon diameter and neuronal activity that affect myelination may sometimes depend on signals from target tissues. For instance, increased availability of trophic support from the target may increase the diameter of axons. Or change in muscle type from smooth-to-striated may

increase the impulse activity of neurons. Thus, the target dependent changes in developing neurons may also regulate the myelination of these neurons.

In this study, I was interested in determining how target tissues regulate the myelination of peripheral neurons. Targets of peripheral neurons have been shown to regulate neuronal survival (Barde, 1988; Barde, 1989; Davies, 1988; Hamburger et al., 1981; Oppenheim, 1989), neurotransmitter phenotype (Nishi, 2003), neuropeptide expression (Coulombe and Nishi, 1991), and many other changes during neuronal differentiation. The characterization of myelination in the developing ciliary ganglion showed that target tissues may also regulate the expression of neuron-derived signals that control the myelination of the peripheral nerve. For instance, target tissues may regulate neuregulin expression in developing peripheral neurons, which in turn may regulate the differentiation of surrounding Schwann cells. Specific factors released from the iris/ciliary body may positively induce the expression of neuregulin in the ciliary neurons. Another possibility may be that target tissues release negative effectors. Signals from choroid target tissues may signal choroid neurons to down-regulate signals such as neuregulin, so that the Schwann cells associated with the choroid neurons do not differentiate into myelinating phenotypes.

Change in muscle type in the ciliary target may promote the myelination of ciliary neurons. At E14 when myelination of ciliary neurons is observed, the iris/ciliary muscles are undegoing a smooth-to-striated muscle transition (Link and Nishi, 1998a). At E18 when the myelination of ciliary neurons is complete, the iris/ciliary muscles are fully

composed of striated muscle. On the other hand, the target tissue of the unmyelinated choroid neurons is composed of only smooth muscles (Meriney and Pilar, 1987). When iris/ciliary muscle in the co-culture experiment described in chapter 2 was replaced with chicken pectoral muscle, also striated, Krox20 expression by Schwann cells increased by up to 35% (Ho, unpublished data). Thus, the diffusible factor that induces the expression of Krox20 in the neuron/Schwann co-culture may be specific to striated muscles. This striated muscle specific factor may indirectly affect the differentiation of Schwann cells by inducing neuregulin expression in ciliary neurons, or promote the increase in axon diameter, or even regulate neuronal activity which creates conditions that initiate Schwann cells to start the myelination program.

Another possibility is the presence of an inhibitory factor from the choroid smooth muscles that inhibits the myelination of choroid neurons. *In vitro*, the lack of target tissues in the co-culture experiment described in chapter 2 does not induce the expression of Krox20 in the Schwann cells, indicating that the effect we observe is an inductive signal from the iris/ciliary muscle. However, *in vivo*, the conditions may be quite different. Activin A may be a factor that negatively regulates the myelination of ciliary ganglion neurons. This is because between E9 to E16, expression of activin A is detected in both iris and ciliary targets (Darland et al., 1995). Interestingly, starting at E14 when the myelination of ciliary neurons is first observed, expression of follistatin, an antogonist to activin A, was detected in the ciliary target (Darland et al., 1995). Furthermore, increased follistatin in the iris/ciliary target promoted the emergence of striated muscle during the smooth-to-striated muscle transition (Link and Nishi, 1998b). Thus, *in vivo*,

there may be two opposing signals from the target that regulate the myelination of ciliary ganglion neurons. Signals from the striated muscles may be promoting the differentiation of myelinating Schwann cells indirectly through the ciliary neurons, or inhibitory factors such as activin A may be preventing the choroid nerve Schwann cells from expressing any myelin related genes.

Besides the results described in Chapter 2 of this thesis, which address the role of iris target tissues in promoting Krox20 expression in neuron/Schwann co-cultures, I tested whether activin A, which induces the preferential expression of somatostatin in choroid neurons, has a negative effect on the myelination of ciliary ganglion neurons. By adding activin A into neuron/Schwann cell co-cultures, I detected a slight disruption in the association of Schwann cells and neurons. Members of the TGF-beta superfamily have been shown to disrupt myelination of neuron/Schwann cell co-cultures (Einheber et al., 1995; Guenard et al., 1995). Thus, activin A may also play a role in inhibiting myelination in choroid neurons.

When the lens of the developing chicken eye is removed as early as E3, the eye will develop without a iris/ciliary body (Beebe and Coats, 2000). If the myelination of ciliary neurons is induced by the target, then the surgical removal of the iris/ciliary body may prevent ciliary neurons from becoming myelinated. In a number of attempts (more than 5 sets, 30 eggs per set), I surgically removed the lens from one eye of the developing chick embryo at E3. At E14, the eye and CG were removed from the embryo and processed to analyze for myelination in the ganglia. Unfortunately, in more than 90% of cases, the

surgically altered eye did not develop fully as compared to the control/non-surgical side. Furthermore, because the target tissue also provides trophic support during CG development, the removal of the target tissue results in a reduction of the CG population. These technical difficulties made the interpretation of these experiments very difficult. However, refining this method may help address the question in an *in vivo* setting.

In summary, there may be multiple mechanisms that convey the target-derived signals through the neurons to regulate the differentiation of myelinating and non-myelinating Schwann cells. For instance, signals coming from target tissues that promote myelination may alter neurons to increase their axon diameter in sympathetic neurons (Voyvodic, 1989) or impulse activity in DRG neurons (Stevens et al., 1998), and in turn the neurons express factors that induce surrounding Schwann cells to myelinate. The observation that almost all autonomic neurons, except ciliary neurons, that innervate either smooth or cardiac muscles (with the exception of ciliary neurons) are unmyelinated suggests that target tissues may also have an inhibitory role in maintaining Schwann cells in a non-myelinating state, due to decreased impulse activity (Stevens et al., 1998) or lack of any axon derived cues which promote myelination (Weinberg and Spencer, 1976).

Studies in Schwann cell biology have identified neuregulin as a key regulator controlling multiple functions during Schwann cell development (Garratt et al., 2000a; Gassmann and Lemke, 1997). Neuregulin expression patterns in the developing ciliary ganglion suggest multiple roles for neuregulin in the developing embryo. For instance, at an earlier stage (E6-E12), the neuregulin isoform in the presynaptic region may be type I,

inducing the expression of acetylcholine receptors at the preganglionic synapse. Neuregulin detected in E14 ciliary neurons may be type III, also known as CRD-neuregulin, which is known to induce Schwann cells to express myelin-related genes (Leimeroth et al., 2002). By E18, the neuregulin isoform expressed in the myelinating Schwann cells may be type II, also known as the glial growth factor. This isoform may function as an autocrine factor to support the survival of myelinated Schwann cells (Lemke, 2001; Syroid et al., 1996). In order to address the isoform-specific effects of neuregulin in Schwann cell differentiation, it would be necessary to reinvestigate this issue with isoform specific antibodies or probes to differentiate between the different types of neuregulins.

Our results using the RCASBP(A)-4XRZnrg virus to knock-down neuregulin expression in the developing ciliary ganglion show that reduction of neuregulin in E14 ciliary neurons disrupt the expression of Krox20 in ciliary nerve Schwann cells. Interestingly, the neuregulin knock-down does not alter Schwann cells proliferation, thus keeping the Schwann cells in the ciliary nerve intact. Furthermore, even though Krox20 was attenuated, expression of the myelin protein P0 remains unchanged. These data are the first that correlate neuregulin expression directly with Krox20 regulation in myelinating Schwann cells. Previous conditional neuregulin knockouts in mice showed that neuregulin signaling is important in establishing the Schwann cell precursor pool and proliferation of Schwann cells along the motor and sensory neurons (Meyer and Birchmeier, 1995; Morris et al., 1999; Woldeyesus et al., 1999). Others showed that myelin thickness is dependent on neuregulin in Krox20 expressing cells (Garratt et al.,

2000b). However, because the RCASBP(A)-4XRZnrg infection technique allowed us to knock-down neuregulin expression in neurons of the myelinating nerve without disrupting Schwann cell proliferation, it was possible to characterize specifically the myelination process in developing ciliary neurons.

The lack of Krox20 in the ciliary nerve Schwann cells due to RCASBP(A)-4XRZnrg virus infection does not completely exclude the possibility that neuregulin knock-downs in other cell types may contribute to the disruption of Krox20. For instance, the lack of neuregulin in target tissues disrupted the maturation of the synapse in the iris (Chapter 3). Since Krox20 expression *in vitro* is dependent on diffusible factors from the ciliary target (Chapter 2), alteration of synapse formation in the iris may alter Krox20 expression in the ciliary nerves *in vivo*. Other possibilities include the disruption of neuregulin expression in Schwann cell precursors that do not affect proliferation, but have an effect on the maturation of Schwann cells to express myelinating phenotypes. However, there were no striking anatomical deformities of the infected embryos when isolated at E14. Furthermore, the characterization of neuregulin expression in the ciliary ganglia confirms that neuregulin is expressed only in ciliary neurons at E14 (Chapter 1). Thus, neuregulin may be one of the key signals that regulate Krox20 expression in the myelinating ciliary nerve.

The observations in Chapter 1 demonstrated that expression of Krox20 and P0 occur at the same time, which indicates the start of preferential myelination in the ciliary nerve of the developing ciliary ganglia. Though P0 is one of the key components of the compact

myelin in the myelinated nerve, P0 is also expressed at low levels in migrating avian neural crest cells (Bhattacharyya et al., 1991). Thus, confirming P0 expression with another marker specific to myelinating Schwann cells insures that the intense staining of P0 in the ciliary nerve at E14 is due to the compaction of myelin proteins around the axons. In rodents, Krox20 is expressed in myelinating Schwann cells prior to P0 expression. However, we did not observe this delay in P0 expression in the chicken ciliary ganglion. Rather, Krox20 and P0 are detected together at E14. Analysis of regulatory regions in the P0 gene does not indicate a binding site for Krox20 (Jessen and Mirsky, 1991). Thus, P0 may not be directly regulated by Krox20 expression during myelination. However, the lack of P0 expression in Krox20 knockout mice indicates a tight regulation between these two myelin-specific genes (Topilko et al., 1994). This relationship suggests that regulation of P0 in chickens may be very different from rodents.

When Schwann cells are isolated from E14 chicken sciatic nerves using immunopanning with P0 antibodies, the Schwann cells lose their Krox20 expression within one day, but retain their P0 expression for approximately 6 days. The difference in the turnover between these two markers may be because Krox20 is a transcription factor that needs to be regulated very rapidly in the nucleus. On the other hand, P0 is a cell surface adhesion molecule that may require longer turnover time to be removed from the cell membrane. The rapid regulation of Krox20 is also observed in co-culture experiments where the 25% increase of Krox20 in the Schwann cells by the iris tissues is observed within a week. Though our culture conditions may not have been optimized for P0 expression due to a

lack of ascorbic acid in the medium (Eldridge et al., 1987), in an ideal condition expression of P0 *in vitro* usually requires approximately 14 days (Bunge et al., 1986; Eldridge et al., 1987). Thus, monitoring changes in Krox20 to study myelination of PNS neurons may be more efficient than measuring P0 expression or accumulation of other myelin specific proteins such as MBP, PMP-22, and MAG.

In order to analyze the changes in myelination in RCASBP(A)-4XRZnrg infected embryos in more detail, it is necessary to determine the level of myelin compaction in ciliary nerves. If Krox20 regulates the expression of proteins that comprise the myelin sheath, then the lack of Krox20 expression in embryos with neuregulin knock-down may affect the actual formation of the myelin sheath. Though there was no immunological change in P0 expression in the ciliary nerves of the neuregulin knock-down embryos, there may be a quantitative difference in the accumulation of P0 or other myelin proteins in the myelin sheath. Also, the embryos were isolated at E14, a time when expression of Krox20 and P0 has just started. Thus, to analyze the full myelin ensheathment in the ciliary nerve, the embryos may need to develop until E18, which is more difficult to assess due to significantly decreased rates of survival of injected embryos. Another possibility would be to assess myelin compaction in ciliary nerves at E14 using electron microscopy.

Future experiments

Based on the observations gathered in this study, there are several issues that need to be addressed for future studies. First is the identification of the target-derived factor from the ciliary/iris muscle that promotes Krox20 expression in neuron/Schwann cell co-cultures. Next, though knock-down of neuregulin by RCASBP(A)-4XRZnrg virus suggests that Krox20 expression is depenent on neuregulin expression in the neurons, there is no evidence whether target tissues regulate neuregulin expression in ciliary ganglion neurons. Finally, the lack of neuregulin disrupts Krox20 expression, but the initial expression of P0 at E14 in the manipulated embryo is not affected. It will be necessary to determine whether there is any qualitative difference in P0 expression between the manipulated embryos and the ciliary nerve controls.

Pilot studies using pectoral muscles as target tissues in the neuron/Schwann cell co-culture experiments have suggested that the striated muscle may release factors which promote Krox20 expression in Schwann cells. My initial observation suggests that this effect may be greater when striated muscle cultures are more homogeneous, as indicated by the greater percentage of Krox20 expression in pectoral muscle cultures. Because pectoral muscles are readily isolated in large quantities, characterizing the contents of pectoral muscle conditioned mediums may help elucidate the Krox20 inducing factor. Conditioned medium may be fractionated with chromatography and tested, or blocking antibodies of known factors may be used to identify possible molecules, and other biochemical methods that may help characterize the inductive factor.

To determine whether target tissues may induce neuregulin expression of neuregulin in ciliary ganglion neurons, CG neurons may be cultured alone on glass coverslips and exposed to different target tissue conditioned medium. Expression of neuregulin may be characterized in the neurons using immunohistological methods, immunoblots, or PCR. It will be interesting to determine whether there is isoform specific expression of neuregulin in the developing CG neurons. This expression may be regulated by exposure to either the iris/ciliary muscle or the choroid smooth muscles.

Though knock-down of neuregulin in the developing ciliary neurons disrupts Krox20 expression, it is important to investigate further whether the lack of Krox20 affects the formation of myelin sheath in the ciliary nerve. It may be necessary to determine the formation of myelin sheath in the RCASBP(A)-4XRZnrg infected tissues by histologically analyzing myelin thickness, possibly using electron microscopy. Furthermore, this may require the isolation of the manipulated ciliary nerve beyond E14.

SUMMARY AND CONCLUSION

The data presented in this thesis addresses the basic biological questions of how target tissues may regulate the myelination of developing peripheral neurons. In order to investigate this issue, the avian ciliary ganglion was utilized because of its two well-defined population of neurons, one myelinated and the other unmyelinated. The myelinated ciliary neurons innervate the iris/ciliary muscle and the non-myelinated choroid neurons innervate the choroid smooth muscle. Our initial characterization of these neurons indicated that myelination of ciliary neurons begins at E14, as indicated by the expression of Krox20 and P0 by myelinating Schwann cells. On the other hand, the choroid neurons remain unmyelinated, as indicated by the expression of p75 by non-myelinating Schwann cells in the choroid nerves. Also at this time, expression of neuregulin becomes specified in the ciliary neurons. Based on these observations, two hypotheses were developed to investigate the myelination of ciliary ganglion neurons. The first hypothesis tested whether the preferential myelination of ciliary neurons is influenced by signals from the target tissues. Using *in vitro* methods developed for this study, the results show that induction of the early myelin gene Krox20 in CG/Schwann co-cultures is dependent on signals from iris target tissues, indicating that myelination of ciliary neurons may be target-dependent. Furthermore, Krox20 expression by the Schwann cells is dependent on the neurons. The second hypothesis of this study tested whether neuregulin expression in the ciliary neurons at E14 promotes expression of myelin specific genes in ciliary nerve Schwann cells. Neuregulin disruption by the injection of replication competent retrovirus expressing ribozymes against neuregulin

mRNA into the neural tube at stage 9 in the developing CG shows that the neuregulin knock-down disrupts Krox20 expression of Schwann cells in E14 ciliary nerves. Interestingly, the disruption of Krox20 does not affect P0 expression in the ciliary nerve, indicating that the myelination of ciliary neurons may be regulated at multiple levels.

The model that describes the target dependent myelination of ciliary neurons may be derived from these results. The striated muscle of the iris/ciliary body may release diffusible factors to induce ciliary neurons to express an axon-derived signal which may promote myelination. Neuregulin expressed specifically in E14 ciliary neurons may induce Schwann cells in the ciliary nerve to express myelin specific genes such as Krox20. In conclusion, these results suggest a novel mechanism in the developing ciliary ganglion that may explain how the ciliary target regulates the myelination of ciliary neurons.

REFERENCES

Aguayo, A. J., Epps, J., Charron, L. and Bray, G. M. (1976). Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: quantitative microscopy and radioautography. *Brain Res* **104**, 1-20.

Anderson, D. J. (1993). Cell and molecular biology of neural crest cell lineage diversification. *Curr Opin Neurobiol* **3**, 8-13.

Barde, Y. A. (1988). What, if anything, is a neurotrophic factor? *Trends Neurosci* **11**, 343-6.

Barde, Y. A. (1989). Trophic factors and neuronal survival. *Neuron* **2**, 1525-34.

Bentley, C. A. and Lee, K. F. (2000). p75 is important for axon growth and Schwann cell migration during development. *J Neurosci* **20**, 7706-7715.

Berthold, C. H., Nilsson, I. and Rydmark, M. (1983). Axon diameter and myelin sheath thicness in nerve fibres of the ventral spinal root of the seventh lumbar nerve of the adult and developing cat. *Journal of Anatomy* **136**, 483-508.

Berti-Mattera, L. N., Harwalkar, S., Hughes, B., Wilkins, P. L. and Almhanna, K. (2001). Proliferative and morphological effects of endothelins in Schwann cells: roles of p38 mitogen-activated protein kinase and Ca2+-independent phospholipase A2. *Journal of Neurochemistry* **79**, 1136-1148.

Berti-Mattera, L. N., Wilkins, P. L., Harwalkar, S., Madhun, Z., Almhanna, K. and Mattera, R. (2000). Endthelins Regulate Arachidonic Acid Release and Mitogen-Activated Protein Kinase Activity in Schwann Cells. *Journal of Neurochemistry* **75**, 2316-2326.

Bhattacharyya, A., Brackenbury, R. and Ratner, N. (1993). Neuron-Schwann cell signals are conserved across species: purification and characterization of embryonic chicken Schwann cells. *J Neurosci Res* **35**, 1-13.

Bhattacharyya, A., Frank, E., Ratner, N. and Brackenbury, R. (1991). P0 is an early marker of the Schwann cell lineage in chickens. *Neuron* **7**, 831-44.

Bischoff, A. and Thomas, P. K. (1975). Microscopic anatomy of myelinated nerve fibres. *Peripheral Neuropathy*, 104-130.

Brennan, A., Dean, C. H., Zhang, A. L., Cass, D. T. and Mirsky, R. (2000). Endothelins Control the Timing of Schwann Cell Generation in Vitro and in Vivo. *Developmental Biology* **227**, 545-557.

Brewer, G. J., Torricelli, J. R., Evege, E. K. and Price, P. J. (1993). Optimized Survival of Hippocampal Neurons in B27-Supplemented Neurobasal, a New Serum-free Medium Combination. *J Neurosci Res* **35**, 567-576.

Britsch, S., Li, L., Kirchhoff, S., Theuring, F., Brinkmann, V., Birchmeier, C. and Riethmacher, D. (1998). The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev* **12**, 1825-36.

Bronner-Fraser, M. and Fraser, S. E. (1991). Cell lineage analysis of the avian neural crest. *Development* **Suppl**, 17-22.

Bunge, R. P., Bartlett-Bunge, M. and Eldridge, C. F. (1986). Linkage between axonal ensheathment and basal lamina production by Schwann cells. *Annu Rev Neurosci* **9**.

Burden, S. and Yarden, Y. (1997). Neuregulins and their receptros: A versatile signaling pathways in the nervous sytem. *Current Opinion in Neurobiology* **11**, 287-296.

Chan, J. R., Cosgaya, J. M., Wu, J. Y. and Shooter, E. M. (2001). Neurotrophins are key mediators of the myelination program in the peripheral nervous system. *Proc Natl Acad Sci U S A* **98**, 14661-14668.

Cheng, L., Esch, F. S., Marchionni, M. A. and Mudge, A. W. (1998). Control of Schwann cell survival and proliferation: autocrine factors and neuregulins. *Mol Cell Neurosci* **12**, 141-56.

Cosgaya, J. M., Chan, J. R. and Shooter, E. M. (2002). The Neurotrophin Receptor p75NTR as a Positive Modulator of Myelination. *Science* **298**, 1245-1248.

Coulombe, J. N. and Nishi, R. (1991). Stimulation of somatostatin expression in developing ciliary ganglion neurons by cells of the choroid layer. *J Neurosci* **11**, 553-62.

Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P. and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid cell-conditioned medium. *Neuron* **10**, 899-906.

Daniloff, J. K., Levi, G., Grumet, M., Reiger, F. and Edelman, G. M. (1986). Altered expression of neuronal cell adhesion molecules induced by nerve injury and repair. *J Cell Biol* **103**, 929-945.

Darland, D. C., Link, B. A. and Nishi, R. (1995). Activin A and follistatin expression in developing targets of ciliary ganglion neurons suggests a role in regulating neurotransmitter phenotype. *Neuron* **15**, 857-66.

Darland, D. C. and Nishi, R. (1998). Activin A and follistatin influence expression of somatostatin in the ciliary ganglion in vivo. *Dev Biol* **202**, 293-303.

Davies, A. M. (1988). Role of neurotrophic factors in development. *Trends Gen.* **4**, 139-143.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G. and Mirsky, R. (1995). NDF is a neuron-glia signal and regulates survival proliferation, and maturation of rat Schwann cell precursors. *Neuron* **15**, 585-596.

Eccleston, P. A. (1992). Regulation of Schwann cell proliferation - mechanisms involved in peripheral nerve development. *Exp Cell Res* **199**, 1-9.

Einheber, S., Hannocks, M. J., Metz, C. N., Rifkin, D. B. and Salzer, J. L. (1995). Transforming growth factor-B1 regulates axon-Schwann cell interactions. *J Cell Biol* **129**, 443-458.

Elder, G. A., Friedrich, V. and Lazzarini, R. A. (2001). Schwann cells and oligodendrocytes read distinct signas in establishing myelin sheath thickness. *Journal of Neuroscience Research* **65**, 493-499.

Elder, G. A., Friedrich, V., Pereira, D., Tu, P. H., Zhang, B., Lee, V. M. and Lazzarini, R. A. (1999). Mice with disrupted mid-sized and heavy neurofilament genes lack axonal neurofilaments but have unaltered numbers of axonal microtubules. *Journal of Neuroscience Research* **57**, 23-32.

Eldridge, C. F., Bunge, M. B., Bunge, R. P. and Wood, P. M. (1987). Differentiation of axon-related Schwann cells in vitro. Ascorbic acid regulates basal lamina assembly and myelin formation. *J Cell Biol* **105**, 1023-1033.

Epstein, M. L., Davis, J. P., Gellman, L. E., Lamb, J. R. and Dahl, J. L. (1988). Cholinergic neurons of the chicken ciliary ganglion contain somatostatin. *Neuroscience* **25**, 1053-60.

Fekete, D. M. and Cepko, C. L. (1993a). Replication competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo. *Mol. Cell Biol.* **13**, 2604-2613.

Finn, T. and Nishi, R. (1996a). Does ciliary neurotrophic factor serve a different function in the rat versus the chicken? *Perspect Dev Neurobiol* **4**, 91-9.

Finn, T. P., Kim, S. and Nishi, R. (1998). Overexpression of ciliary neurotrophic factor in vivo rescues chick ciliary ganglion neurons from cell death. *J Neurobiol* **34**, 283-93.

Finn, T. P. and Nishi, R. (1996b). Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell-death phase. *J Comp Neurol* **366**, 559-71.

Fraher, J. P. (1976). The growth and myelination of central and peripheral segments of ventral motoneurone axons. A quantitative ultrastructural study. *Brain Res* **105**, 193-211.

Francis, N. J. and Landis, S. C. (1999). Cellular and molecular determinants of sympathetic neuron development. *Annu Rev Neurosci* **22**, 541-66.

Friede, R. L. and Samorajski, T. (1967). Relation between the number of myelin lamellae and axon circumference in fibers of vagus and sciatic nerves of mice. *J Comp Neurol* **130**, 223-231.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V. M. and Persson, H. (1993a). Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J Cell Biol* **123**, 455-65.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V. M. K. and Persson, H. (1993b). Differential expression of messenger RNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J Cell Biol* **123**, 455-465.

Garratt, A. N., Britsch, S. and Birchmeier, C. (2000a). Neuregulin, a factor with many functions in the life of a schwann cell. *Bioessays* **22**, 987-96.

Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P. and Birchmeier, C. (2000b). A Dual Role of erbB2 in myelination and in expansion of the Schwann cell precursor Pool. *Journal of Cell Biology* **148**, 1035-1046.

Gassmann, M. and Lemke, G. (1997). Neuregulins and neuregulin receptors in neural development. *Curr Opin Neurobiol* **7**, 87-92.

Giese, K. P., Martini, R., Lemke, G., Soriano, P. and Schachner, M. (1992). Mouse P0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. *Cell* **71**, 565-76.

Guenard, V., Rosenbaum, T., Gwynn, L. A., Doetschman, T., Ratner, N. and Wood, P. M. (1995). Effect of transforming growth factor-beta 1 and -beta 2 on Schwann cell proliferation on neurites. *Glia* **13**, 309-18.

Hall, A. K., Burke, R. M., Anand, M. and Dinsio, K. J. (2002). Activin and bone morphogenetic proteins are present in perinatal sensory neuron target tissues that induce neuropeptides. *J Neurobiol* **52**, 52-60.

Hamburger, V., Brunso, B. J. and Yip, J. W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J Neurosci* **1**, 60-71.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A., Simpson, L. C. et al. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062-4.

Herdegen, T., Kiessling, M., Bele, S., Bravo, R. and Gass, P. (1994). The Krox-20 transcription factor in the rat central and peripheral nervous systems: a novel expression pattern of an intermediate early gene encodeed protein. *Neuroscience* **57**, 41-52.

Hess, A. (1965). Developmental changes in the structure of the synapse on the myelinated cell bodies of the chicken ciliary ganglion. *J Cell Biol* **25**, Suppl:1-19.

Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E. and Thoenen, H. (1987). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development,

degeneration, and regeneration: role of macrophages. *Proc. Natl. Acad. Sci. USA* **84**, 8735-8739.

Hiley, C. R. (1997). Endothelin receptors: pharmacological subtyping, agonists, and antagonists.: CRC Press, Boca Raton.

Homburger, S. A. and Fekete, D. M. (1996). High efficiency gene transfer into the embryonic chicken CNS using B- subgroup retroviruses. *Dev Dyn* **206**, 112-20.

Hudson, L., Berndt, J. A., Puckett, C., Kozak, C. A. and Lazzarini, R. A. (1987). Aberrant splicing of proteolipid protein mRNA in the dysmyelinating jimpy mutant mouse. *Proc Natl Acad Sci U S A* **84**, 1454-1458.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y. and Gavrilovic, J. (1994a). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in the rat embryonic nerves. *Neuron* **12**, 509-527.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y. and Gavrilovic, J. (1994b). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron* **12**, 509-27.

Jessen, K. R. and Mirsky, R. (1991). Schwann cell precursors and their development. *Glia* **4**, 185-194.

Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E. and Theill, L. E. (1996). Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci U S A* **93**, 4833-8.

Kuwaki, T., Kurihara, H., Cao, W. H., Kurihara, Y., Unekawa, M., Yazaki, Y. and Kumada, M. (1997). Physiological role of brain endothelin in the central autonomic control: from neuron to knockout mouse. *Progress in Neurobiology* **51**, 545-579.

Lai, C., Watson, J. B., Bloom, F. E., Sutcliffe, J. G. and Milner, R. J. (1987). Neural protein 1B236/myelin-associated glycoprotein (MAG) defines a subgroup of the immunoglobulin superfamily. *Immunol. Rev.* **100**, 129-51.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. *Trends in Neurosciences* **13**, 344-50.

Landis, S. C. (1994). Development of sympathetic neurons: neurotransmitter plasticity and differentiation factors. *Prog Brain Res* **100**, 19-23.

Landmesser, L. and Pilar, G. (1974). Synaptic transmission and cell death during normal ganglionic development. *J Physiol* **241**, 737-49.

Le Douarin, N., Dulac, C., Dupin, E. and Cameron-Curry, P. (1991). Glial cell lineages in the neural crest. *Glia* **4**, 175-84.

Le Douarin, N. M. and Smith, J. (1988). Development of the peripheral nervous system from the neural crest. *Annual Reviews in Cell Biology* **4**, 375-404.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-8.

Lee, V. M., Smiley, G. G. and Nishi, R. (2001). Cell death and neuronal replacement during formation of the avian ciliary ganglion. *Dev Biol* **233**, 437-48.

Leimeroth, R., Lobsiger, C., Lussi, A., Taylor, V., Suter, U. and Sommer, L. (2002). Membrane-Bound Neuregulin1 Type III Actively Promotes Schwann Cell Differentiation of Multipotent Progenitor Cells. *Developmental Biology* **246**, 245-258.

Lemke, G. (1993). The molecular genetics of myelination: an update. *Glia* **7**, 263-71.

Lemke, G. (1996). Neuregulins in development. *Mol Cell Neurosci* **7**, 247-62.

Lemke, G. (2001). Glial control of neuronal development. *Annu Rev Neurosci* **24**, 87-105.

Lemke, G., Kuhn, R., Monuki, E. S. and Weinmaster, G. (1990). Transcriptional controls underlying Schwann cell differentiation and myelination. *Ann N Y Acad Sci* **605**, 248-53.

Linden, R. (1994). The survival of developing neurons: a review of afferent control. *Neuroscience* **58**, 671-82.

Lindholm, D., Heumann, R., Meyer, M. and Thoenen, H. (1987). Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* **330**, 658-659.

Link, B. A. and Nishi, R. (1998a). Development of the avian iris and ciliary body: mechanisms of cellular differentiation during the smooth-to-striated muscle transition. *Dev Biol* **203**, 163-76.

Link, B. A. and Nishi, R. (1998b). Development of the avian iris and ciliary body: the role of activin and follistatin in coordination of the smooth-to-striated muscle transition. *Dev Biol* **199**, 226-34.

Lunn, E. R., Scourfield, J., Keynes, R. J. and Stern, C. D. (1987). The neural tube origin of ventral root sheath cells in the chick embryo. *Development* **101**, 247-254.

Macklin, W. B., Gardinier, M. V., King, K. D. and Kamp, K. (1987). An AG->GG transition at a splice site in the myelin proteolipid protein gene in jimpy mice results in the removal of an exon. *FEBS Letter* **223**, 417-421.

Mains, R. E. and Patterson, P. H. (1973). Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J Cell Biol* **59**, 329- 345.

Martini, R., Mohajeri, M. H., Kasper, S., Giese, K. P. and Schachner, M. (1995). Mice doubly deficient in the genes for P0 and myelin basic protein show that both proteins contribute to the formation of the major dense line in peripheral nerve myelin. *J Neurosci* **15**, 4488-95.

Martini, R. and Schachner, M. (1988). Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve. *J Cell Biol* **106**, 1735-1746.

Marwitt, R., Pilar, G. and Weakly, J. N. (1971). Characterization of two ganglion cell populations in avian ciliary ganglia. *Brain Res* **25**, 317-34.

Meriney, S. D. and Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid coat of the chick eye and its development. *J. Neurosci.* **7**, 3827-3839.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L. E. and Birchmeier, C. (1997). Isoform-specific expression and function of neuregulin. *Development* **124**, 3575-86.

Meyer, M., Matsuoka, I., Wetmore, C., Olson, L. and Thoenen, H. (1992). Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *J Cell Biol* **119**, 45-54.

Milner, R. J., Lai, C., Nave, K. A., Lenoir, D., Ogata, J. and Sutcliffe, J. G. (1985). Nucleotide sequences of two mRNAs for rat brain myelin proteolipid protein. *Cell* **42**, 931-939.

Monuki, E. S., Kuhn, R. and Lemke, G. (1993). Repression of the myelin P0 gene by the POU transcription factor SCIP. *Mech Dev* **42**, 15-32.

Monuki, E. S., Weinmaster, G., Kuhn, R. and Lemke, G. (1989). SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* **3**, 783-93.

Morello, D., Dautigny, A., Pham-Dinh, D. and Jolles, P. (1986). Myelin proteolipid protein (PLP and DM-20) transcripts are delted in jimpy mutant mice. *Embo J* **5**.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol* **51**, 185-218.

Morgan, L., Jessen, K. R. and Mirsky, R. (1991). The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (O4+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-Receptor-) depends on growth inhibition. *J Cell Biol* **112**, 457-467.

Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. and Lee, K. F. (1999). Rescue of the Cardiac Defect in ErbB2 Mutant Mice Reveals Essential Roles of ErbB2 in Peripheral Nervous System Development. *Neuron* **23**, 273-283.

Moscoso, L. M., Chu, G. C., Gautam, M., Noakes, P. G., Merlie, J. P. and Sanes, J. R. (1995). Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev Biol* **172**, 158-69.

Narayanan, C. H. and Narayanan, Y. (1976). An experimental inquiry into the central source of preganglionic fibers to the chick ciliary ganglion. *J Comp Neurol* **166**, 101-9.

Nave, K. A. (1996). Myelin-specific genes and their mutations in the mouse. *Glial Cell Development: basic principles and clinical relevance.*, 141-164.

Nave, K. A., Bloom, F. E. and Milner, R. J. (1987). A single nucleotide difference in the gene for myelin proteolipid protein defines the jimpy mutation in mouse. *J Neurochem* **49**, 1873-1877.

Nave, K. A., Lai, C., Bloom, F. E. and Milner, R. J. (1986). Jimpy mutant mouse: a 74-base deletion in the mRNA for myelin proteolipid protein and evidence for a primary defect in RNA splicing. *Proc Natl Acad Sci U S A* **83**, 9264-8.

Nishi, R. (1994a). Neurotrophic factors: two are better than one. *Science* **265**, 1052-3.

Nishi, R. (1994b). Target-derived molecules that influence the development of neurons in the avian ciliary ganglion. *J Neurobiol* **25**, 612-9.

Nishi, R. (1996). Autonomic and sensory neuron cultures. *Methods Cell Biol* **51**, 249-63.

Nishi, R. (2003). Target-mediated control of neural differentiation. *Progress in Neurobiology* **69**, 213-227.

Nishi, R. and Berg, D. K. (1979). Survival and development of ciliary ganglion neurones grown alone in cell culture. *Nature* **277**, 232-234.

Ochoa, J. (1975). Microscopic anatomy of unmyelinated fibers. *Peripheral Neuropathy*, 131-150.

Oppenheim, R. W. (1989). The neurotrophic theory and naturally ocurring motoneuron death. *Trends Neurosci* **12**, 252-255.

Pilar, G., Nunez, R., McLennan, I. S. and Meriney, S. D. (1987). Muscarinic and nicotinic synaptic activation of the developing chicken iris. *J Neurosci* **7**, 3813-26.

Popko, B., Puckett, C. and Hood, L. (1988). A novel mutation in myelin-deficient mice results in unstable myelin basic protein gene transcripts. *Neuron* **1**, 221-5.

Ridley, A. J., Davis, J. B., Stroubant, P. and Land, H. (1989). Transforming growth factors-b1 and b2 are mitogens for rat Schwann cells. *J Cell Biol* **109**, 3419-3426.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F. and Hood, L. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* **42**, 149-55.

Rosenbaum, C., Karyala, S., Marchionni, M. A., Kim, H. A., Krasnoselsky, A. L., Happel, B., Isaacs, I., Brackenbury, R. and Ratner, N. (1997). Schwann cells express NDF and SMDF/n-ARIA mRNAs, secrete neuregulin, and show constitutive

activation of erbB3 receptors: evidence for a neuregulin autocrine loop. *Exp Neurol* **148**, 604-15.

Rosenbluth, J. (1980). Central myelin in the mouse mutant shiverer. *J. Comp. Neurol.* **194**, 639-648.

Scherer, S. S., Kamholz, J. and Jakowlel, S. B. (1993). Axons modulate the expression of transforming growth factor-betas in Schwann cells. *Glia* **8**, 265-276.

Scherer, S. S. and Salzer, J. L. (1996). Axon-Schwann cell interactions during peripheral nerve degeneration and regeneration. *Glial Cell Development: basic principles and clinical relevance.*, 165-196.

Schneider-Maunoury, S., Topilko, P., Seitanidou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-1214.

Sebert, M. E. and Shooter, E. M. (1993). Expression of messenger RNA for neurotrophic factors and their receptors in the rat dorsal root ganglion and sciatic nerve following nerve injury. *Journal of Neuroscience Research* **36**, 357-367.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P. and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell* **77**, 349-60.

Sharma, K., Korade, Z. and Frank, E. (1995). Late-migrating neuroepithelial cells from the spinal cord differentate into sensory ganglion cells and melanocytes. *Neuron* **14**, 143-152.

Shen, X., Billings-Gagliardi, S., Sidman, R. L. and Wolf, M. K. (1985). Myelin deficient (shi-mld) mutant allele: morphological comparision with shiverer (shi) allele on a B6C3 mouse stock. *Brain Res* **360**, 235-247.

Springer, J. E., Seeburger, J. L., He, J., Gabrea, A., Blankenhorn, E. P. and Bergman, L. W. (1995). cDNA sequence and differential mRNA regulation of two forms of glial cell line-derived neurotrophic factor in Schwann cells and rat skeletal muscle. *Exp Neurol* **131**, 47-52.

Stevens, B. and Fields, R. D. (2000). Response of Schwann Cells to Action Potentials in Development. *Science* **287**, 2267-2271.

Stevens, B., Tanner, S. and Fields, R. D. (1998). Control of Myelination by Specific Patterns of Neural Impulses. *The Journal of Neuroscience* **18**, 9303-9311.

Stewart, H. J. S., Mirsky, R. and Jessen, K. R. (1996). The Schwann cell lineage: embryonic ad early postnatal development. *Glial Cell Development: basic principles and clinical relevance.*, 1-30.

Suter, U., Welcher, A. A., Ozcelik, T., Snipes, G. J., Kosaras, B., Francke, U., Billings-Gagliardi, S., Sidman, R. L. and Shooter, E. M. (1992). Trembler mouse carries a point mutation in a myelin gene. *Nature* **356**, 241-4.

Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K. F., Lemke, G. and Kilpatrick, T. J. (1996). Cell death in the Schwann cell lineage and its regulation by neuregulin. *Proc Natl Acad Sci U S A* **93**, 9229-34.

Taniuchi, M., Clark, H. B. and Johnson, E. M. (1986). Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc Natl Acad Sci U S A* **83**, 4094-4098.

Taniuchi, M., Clark, H. B., Schweitzer, J. B. and Johnson, E. M. (1988). Expression of nerve growth factor receptros by Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact, and binding properties. *J Neurosci* **8**, 664-681.

Topilko, P., S., S.-M., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B. Y., Seitanidou, T., Babinet, C. and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**, 796-799.

van den Buuse, M. and Webber, K. M. (2000). Endothelin and dopamine release. *Progress in Neurobiology* **60**, 385-405.

Vogel, K. S. (1993). Development of Trophic Interactions in the Vertebrate Peripheral Nervous System. *Mol Neurobiol* **7**, 363-382.

Voyvodic, J. T. (1989). Target size regulates calibre and myelination of sympathetic axons. *Nature* **342**, 430-433.

Wang, J. Y., Miller, S. J. and Falls, D. L. (2001). The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. *J Biol Chem* **276**, 2841-51.

Zhao, J. J. and Lemke, G. (1998). Selective disruption of neuregulin-1 function in vertebrate embryos using ribozyme-tRNA transgenes. *Development* **125**, 1899-907.

Zorick, T. S. and Lemke, G. (1996). Schwann cell differentiation. *Curr Opin Cell Biol* **8**, 870-6.

Zorick, T. S., Syroid, D. E., Arroyo, E., Scherer, S. S. and Lemke, G. (1996). The Transcription Factors SCIP and Krox-20 Mark Distinct Stages and Cell Fates in Schwann Cell Differentiation. *Mol Cell Neurosci* **8**, 129-45.

"We choose to go to the moon in this decade
and do the other things,
not because they are easy,
but because they are hard,..."

John F. Kennedy, September 12, 1962