IMMUNOHISTOCHEMICAL ANALYSES OF NERVOUS SYSTEM STRUCTURE, DEVELOPMENT AND REGENERATION

by

Jeremy Steven Toma

B.Sc.(Honours), The University of British Columbia, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

December, 2006

Abstract

Specific aspects of nervous system structure, development, and regeneration were investigated in two separate studies. The first study was concerned with development of sensory root entry zones. Sensory information enters the central nervous system (CNS) via root entry zones where sensory axons span a glial environment consisting of Schwann cells in the peripheral nervous system (PNS) and astrocytes and oligodendrocytes in the CNS. Little is known about the postnatal development of the glial elements of many root entry zones. I sought to establish a comparative developmental timecourse of the glial elements in the postnatal (P0, P3, P7, P14) and adult rat of three root entry zones: the spinal nerve dorsal root entry zone, the trigeminal root entry zone, and the vagal dorsal root entry zone. I compared entry zone development based on the expression of antigens in peripheral glia, central glia, and the PNS extracellular matrix. While all three root entry zones had reached maturity by P14, the glial elements comprising the PNS-CNS interface of the trigeminal root entry zone and the vagal dorsal root entry zone matured earlier than those of the spinal nerve dorsal root entry zone.

This study revealed unexpected expression patterns of certain glial antigens. For example, the antibody used to label mature oligodendrocytes (RIP) labelled Schwann cell cytoplasm. I sought to follow up on this observation and characterized RIP immunoreactivity in peripheral glia in the second study. In uninjured rats, RIP demarcated paranodal regions of myelinated axons and clearly defined Schmidt-Lantermann incisures. Robust RIP immunoreactivity was present in Remak bundles.

Low levels of RIP immunoreactivity were detectable in satellite cells surrounding dorsal root ganglion (DRG) neurons and in terminal Schwann cells at neuromuscular junctions. These results suggested a correlation between RIP immunoreactivity and amount of axoglial contact. Injury induced sympathetic sprouting and pericellular basket formation in the DRG was conducted to further examine this correlation. All perineuronal sympathetic sprouts infiltrated heavily RIP-immunoreactive satellite cell sheaths. RIP immunoreactivity was absent from placodal-derived olfactory ensheathing cells, suggesting that correlation between axoglial contact and RIP immunoreactivity is confined to peripheral glia of neural crest origin.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BC	boundary cap
BMP	bone morphogenetic protein
Caspr	contactin-associated protein
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CSPG	chondroitin sulphate proteoglycan
DREZ	dorsal root entry zone
DRG	dorsal root ganglion
E	embryonic
GFAP	glial fibrillary acidic protein
ICP	inferior cerebellar peduncle
L	lumbar
NMJ	neuromuscular junction
NRG1	neuregulin-1
OEC	olfactory ensheathing cell
P	postnatal
PB	phosphate buffer
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PNS	peripheral nervous system
PTI	peripheral tissue insertion
SLI	Schmidt-Lantermann incisure
TREZ	trigeminal root entry zone
TZ	transitional zone
vDREZ	vagal dorsal root entry zone
VRTZ	ventral root transitional zone

Acknowledgments

I would like to thank everyone who helped me along the way, but of course those people are too numerous to name here. So I must reserve special thanks for a few select individuals. First and foremost, I would like to thank Dr. Matt Ramer, whose scientific sagacity and technical mastery were greatly appreciated by me. I learned a lot from him, and I am grateful to have had the experience of working in his lab. It was also a pleasure to see him sing and dance. I would also like to thank Dr. Lowell McPhail, who was a constant source of guidance and support in every respect (but especially regarding surgeries and manuscript preparation) and without whose generous offerings of citrus fruits during the winter months, I surely would have succumbed to scurvy long ago. Many thanks go out to other members of the Ramer Lab for all of their moral support and unflagging tolerance of me throughout the years. Their names are listed here in no particular order: Angela Scott, Andrew Gaudet, David Hampton, Lesley Soril, and Leanne Ramer. Special thanks to Leanne Ramer for providing rat DRGs (both injured and uninjured). I thank the newest addition to the lab, Jessica Inskip, for intelligently questioning some of the statements I made in one of my manuscripts which prompted me to think more about that work long after I thought it necessary. I have to thank Dr. Victoria Claydon of the Krassioukov Lab as well for all of her much appreciated inter-laboratory spirit. To all of the friends I leave behind in the lab, a most heartfelt thanks. I will miss all of you. I must also thank my committee members Dr. Vanessa Auld and Dr. Bill Milsom for first of all being on my committee and offering helpful advice at committee meetings and, second of all, for voluntarily subjecting themselves to the

reading of my thesis. And of course, extra special thanks go out to the now infamous Friday afternoon Ramer Lab meetings. Those were sacred times.

Dedication

To my family. Hey, look what I did!

Co-Authorship Statement

The manuscripts presented in Chapter 2 and Chapter 3 were prepared with the aid of co-authors whose contributions were as follows:

Chapter 2 Contributions: This study was conducted by Jeremy Steven Toma under the supervision of Dr. Matt Stephen Ramer. Dr. Lowell Thomas McPhail aided in manuscript preparation.

Chapter 3 Contributions: This study was conducted by Jeremy Steven Toma under the supervision of Dr. Matt Stephen Ramer. Dr. Lowell Thomas McPhail provided technical contributions (i.e. performed the majority of surgeries) and aided in manuscript preparation.

Chapter 1

Introduction

The nervous system of vertebrates is composed of a complex cellular environment. One cell type, the neuron, is responsible for conducting electrical impulses which are important for relaying information throughout the body. Surrounding the neurons are structures which aid in their stabilization, such as elements of the extracellular matrix. The nervous system also contains other cells referred to as glia that are responsible for a host of different functions, but these cells are most renowned for their supporting roles in aiding neuronal function. Studies suggest that 90% of vertebrate nervous tissue is glial (reviewed in Rowitch, 2004); however, the vast majority of literature concerned with nervous system function is focussed on neurons. Today, the relative importance of glia in nervous system function is becoming clear. It is now known that glia not only provide structural support for neurons, as was thought previously to be their sole function (glia literally means "glue" in Medieval Greek), but they also serve more active roles involving the uptake of neurotransmitters and influencing neurotransmission at synapses via direct signalling with neurons (Lin and Bergles, 2004; reviewed in: Kirchhoff et al., 2001; Vesce et al., 1999).

The work presented in this thesis is mainly concerned with the rather underrepresented glial cells. Although much of the recent work on glia has been directed towards understanding the active roles of these cells in neural functioning, the present studies examine some of the more classical structural/supportive aspects of glia. The

focus of the first study presented is glial development in one specialized anatomical region where the central nervous system (CNS) and peripheral nervous system (PNS) are in direct contact with one another: the sensory root entry zone. Glia play a very important structural role at this junction and the array of glial populations present are defining features of sensory root entry zones. The first study (Chapter 2) explores the development of these glial cells and their interactions at sensory root entry zones.

Outside of their roles in providing anatomical structure, glia are involved in a wide variety of other, more intimate interactions with neurons, and these are explored in more detail in the second study (Chapter 3) presented in this thesis. In Chapter 3, the immunoreactivity of the RIP monoclonal antibody (which is used to identify oligodendrocytes of the CNS) was studied in various populations of peripheral glia following the observation that RIP immunoreactivity was present in Schwann cell cytoplasm in the PNS compartment of developing sensory root entry zones. The present chapter begins with a discussion of glial function and development and then continues on with descriptions of glial interface structures (with a focus on the glial interface present at sensory root entry zones).

Overview of glial cell function and development in the CNS and PNS

"Glia" is an umbrella term used to describe all of the cells of neural origin in the nervous system that are non-neuronal. Due to the broad nature of this term, the cells that fall under the glial category perform a wide range of different functions necessary for nervous system function and survival.

In the CNS (which encompasses all of the nervous tissue of the spinal cord and brain), oligodendrocytes produce myelin, the insulating sheath required for maintaining a high conduction velocity in certain types of axons. Recent work suggests that myelination, at least in the CNS, is initiated by signals deriving from neurons which cause the exocytosis of myelin membrane components from internal storage sites within oligodendrocytes (Trajkovic et al., 2006). Other CNS glial cells known as astrocytes are traditionally renowned for their role in providing structural support for axons. More recently, these cells were shown to promote myelination in response to indirect effects of electrical stimulation. Ishibashi et al. (2006) demonstrated that electrical impulses stimulate astrocytes (via ATP released from stimulated neurons) to secrete a molecule known as leukemia inhibitory factor which promotes myelination through signalling with oligodendrocytes. Other functions of astrocytes include involvement in ion buffering and the taking up of excess neurotransmitters at sites of synaptic release (reviewed in: Kirchhoff et al., 2001; Vesce et al., 1999). Oligodendrocytes and astrocytes are the most prevalent and largest glial cell types in the CNS; they are termed macroglia. Another CNS glial type, microglia, has roles in the inflammatory response and immune function following injury (reviewed in Rowitch, 2004).

In addition, there are other types of glia – radial glia and oligodendrocyte precursor cells – that are associated with the developing vertebrate CNS which decrease in number upon maturation. The immature CNS contains glial cell populations that form progenitor pools for glial types important for the mature CNS: for example, oligodendrocyte precursor cells differentiate into functional, myelinating

oligodendrocytes in the developing CNS. In addition, radial glial cells are important for producing and guiding immature neurons in the developing CNS, and later differentiate into astrocytes (reviewed in Rakic, 2003; McDermott et al., 2005).

In the PNS (which encompasses all of the nervous tissue outside of the CNS), Schwann cells are the most prominent glial cell type. Unlike oligodendrocytes, which myelinate several axons/oligodendrocyte, Schwann cells provide myelin for certain small and large-diameter axons and associate with axons in a 1:1 ratio. The sheaths of non-myelinating Schwann cells form Remak fibres with unmyelinated axons so that the axons in most major nerves of the periphery, whether myelinated or not, are ensheathed by Schwann cells.

Other non-myelinating glial cell types exist in the PNS in addition to the Schwann cells of Remak fibres. Satellite cells surround and support neuronal somata in peripheral ganglia, terminal Schwann cells ensheathe axons at neuromuscular junctions and olfactory ensheathing cells (OECs) associate with olfactory sensory neurons. These non-myelinating glia are discussed in more detail below and in Chapter 3.

Development of CNS glia – radial glia and astrocytes

The macroglia of the CNS and Schwann cells of the PNS all arise from neurogenic precursor cells of one form or another that are derived from the neuroepithelium, which itself is of ectodermal origin in the embryo. The first glial cells that are able to be detected from the developing embryonic neuroepithelium are radial

glia (reviewed in McDermott et al., 2005). Radial glia are essential for neural development in the CNS. These cells give rise to neurons as well as providing the scaffolding needed for the migration and growth of these neurons throughout neurogenesis in the developing cortex (reviewed in Rakic, 2003). Radial glia also generate astrocytes following neurogenesis (Voigt, 1989; reviewed in: Rakic, 2003; McDermott et al., 2005). Although most radial glia have differentiated into either neurons or astrocytes upon maturation of the brain, some remain in the adult subventricular zone of the mouse brain and act as progenitors of adult neural stem cells (Merkle et al., 2004). In the spinal cord, astrocytes are the only known cell types to be derived from radial glia; it appears that radial glia do not generate neurons. The majority of astrocytes in the spinal cord are generated from radial glia (reviewed in McDermott et al., 2005). Following neurogenesis, the developmental progression of neuroepithelium to radial glia to astrocytes occurs in a ventrodorsal direction in the spinal cord (Barry and McDermott, 2005; reviewed in McDermott et al., 2005). Cells that expressed the intermediate filament protein glial fibrillary acidic protein (GFAP), which is commonly used to identify astrocytes in vivo, and vimentin, which is expressed in radial glia, were first present in the ventrolateral rat spinal cord by embryonic day (E)16 (Barry and McDermott, 2005).

Barry and McDermott (2005) identified other sources of astrocytes in the spinal cord that were not derived from radial glia. Developing white matter astrocytes that expressed GFAP were identified that did not co-express the neuroepithelial (and radial glial) marker proteins vimentin and nestin (Barry and McDermott, 2005). A possible

source of these astrocytes is cells located in a very specific area of the early spinal cord. In vivo, evidence indicates that there are astrocyte precursors likely derived from the p2 domain of the developing neural tube (neural tube domains are discussed in further detail below); however, astrocyte precursor cells have been difficult to identify due to the paucity of reliable molecular markers (reviewed in Rowitch, 2004). Other work carried out in vivo and in vitro has investigated possible molecular signals involved in promoting astrocytic differentiation. Astrocytes arise from neural progenitors with epidermal growth factor treatment at the expense of neuronal differentiation in the brain (Kuhn et al., 1997; Burrows et al., 1997; Sanes et al., 2006). In vitro, treatment with ciliary neurotrophic factor promotes astrocytic differentiation in cultures of neural progenitors as it results in GFAP upregulation (Bonni et al., 1997; Sanes et al., 2006). Barnabe-Heider et al. (2005) demonstrated that treating cultures of cortical precursor cells with the neurotrophic cytokine cardiotrophin-1, which is produced by developing cortical neurons, results in the generation of astrocytes. In addition, cardiotrophin-1-null mice displayed a deficit in astrocytes compared to wild-type mice. Both ciliary neurotrophic factor and cardiotrophin-1 signal through the gp130/LIFRβ receptor complex on astrocytes and activate the JAK/STAT pathway, demonstrating that the activation of this pathway is important for astrocyte differentiation. Whether astrocytes are formed from radial glia or arise from other precursors, they are all involved in forming a complex cytoarchitectural framework within the CNS that will be discussed below with respect to its role in forming the glial barrier that is present in sensory root entry zones.

Development of CNS glia – oligodendrocytes

Oligodendrocytes are derived from neurogenic precursor cells separate from those of astrocytes. Even though myelination is initiated late in fetal development and continues well after birth in vertebrate animals (e.g. in humans, myelination occurs into adolescence), the developmental processes that first give rise to oligodendrocytes in the CNS (and Schwann cells in the PNS) and later prime these cells for myelination occur much earlier in development (reviewed in Baumann and Pham-Dinh, 2001). In the embryonic CNS, oligodendrocytes are derived from oligodendrocyte precursor cells that arise from specific neurogenic tissue. In the developing neural tube that eventually forms the spinal cord, expression patterns of a variety of transcription factors and proteins in exclusive domains dictate oligodendrocyte development (reviewed in detail by Rowitch, 2004). Gradients of transcription factors and secreted proteins, and the regulatory interactions between them, define particular domains of the developing neural tube. For example, the secreted protein sonic hedgehog, which is most heavily expressed in the floor plate of the developing neural tube, is responsible for signalling to cells of various regions of the neural tube which results in the expression of transcription factors such as Olig2, Nkx2.2 and Pax6; all of these factors then partake in regulatory interactions with each other that progressively define discrete domains of the ventricular zone of the neural tube. These domains are responsible for giving rise to particular neurogenic progeny. An example of one of these domains is the p2 domain, from which astrocytes and a subpopulation of interneurons are derived. Motoneurons of the spinal cord (both visceral and somatic) are derived from the pMN domain, which is located immediately ventral to p2. The progenitor cells of the pMN also give rise to oligodendrocyte precursor cells,

although later on in development. The progenitor cells of the pMN are able to give rise to both neurons and oligodendrocyte precursor cells because of changing expression patterns of transcription factors over time within this domain, resulting in a "switch" from motoneuron generation to oligodendrocyte precursor generation.

During motoneuron development, the pMN expresses the transcription factors Olig1/2 (which is necessary for both motoneuron and oligodendrocyte development) and neurogenin1/2, the latter being a proneural transcription factor (reviewed in Rowitch, 2004; Sanes et al., 2006). While the pMN is solely generating motoneurons, the Delta/Notch signalling pathway is necessary for maintaining a source of Olig2-positive cells that are to become oligodendrocyte precursors at a later time. Experiments have shown that when the Notch receptor (which inhibits proneural gene expression when activated by the Delta ligand expressed by neurons) is mutated, all Olig2-expressing cells generate neurons. Alternatively, if Notch is over-expressed and constitutively-activated in the pMN, excess oligodendrocytes are generated and neuronal development is prevented (reviewed in Rowitch, 2004). Concomitant with Notch/Delta signalling and neurogenin1/2 and Olig1/2 expression in the pMN, the Nkx2.2 transcription factor represses Olig1/2 expression in the p3 domain (which is ventrally adjacent to the pMN). In Nkx2.2-null mice, the pMN domain is expanded ventrally and more motoneurons and oligodendrocyte precursor cells are produced; however, these precursors fail to properly mature into myelinating oligodendrocytes, demonstrating the importance of Nkx2.2 expression in oligodendrocyte development. The switch to oligodendrocyte precursor generation occurs once neurogenin1/2 expression declines in the pMN (~ E10 in the

mouse) and Nkx2.2 expression expands dorsally from p3 into the pMN (~ E13 in the mouse). The coexpression of Nkx2.2 and Olig1/2 is necessary for oligodendrocyte maturation during oligodendrocyte precursor migration outward from the pMN (reviewed in Rowitch, 2004).

Interestingly, oligodendrocyte progenitors develop and migrate in a fashion very similar to that of neurons in the spinal cord. At E9-10.5 in the mouse, the pMN produces motoneurons which then migrate outward from this domain. A similar migration occurs from this domain at E12.5 in the mouse and E6 in the chick, as oligodendrocyte precursor cells progressively radiate to all areas of the neural tube (reviewed in Rowitch, 2004). Migration of these precursors is at first restricted to the ventral neural tube (at E14.5 in the mouse) resembling the distribution of motoneurons in the spinal cord, but by E18.5, precursors have reached all areas of the developing spinal cord (reviewed in Rowitch, 2004). This migration is dependent on the guidance molecule netrin-1, as the oligodendrocyte precursors of netrin-1-null mice do not spread from the ventral midline (Tsai et al., 2006). The ventrodorsal direction of oligodendrocyte development observed in the mouse resembles that of astrocyte development in the rat spinal cord (reviewed in Rowitch, 2004; Barry and McDermott, 2005). In the forebrain of the mouse, a similar ventral origination and migration of oligodendrocyte precursors occurs. Characteristic gene expression profiles throughout the CNS define this population of glial progenitors; oligodendrocyte precursors express Olig2, platelet-derived growth factor (PDGF) receptor α, and Sox10 (reviewed in Rowitch, 2004).

Post-migratory oligodendrocyte precursors, which are often identified by their expression of the chondroitin sulphate proteoglycan NG2 (reviewed in Polito and Reynolds, 2005), gradually differentiate into myelinating oligodendrocytes that express myelin basic protein in the late embryonic and postnatal animal. The cell cycle inhibitor $p27^{Kip1}$ is integral for the process of oligodendrocyte differentiation. In culture, consecutive rounds of oligodendrocyte precursor cell divison is promoted by treatment with mitogenic factors released by astrocytes such as PDGF and neurotrophin-3 – this results in an accumulation of $p27^{Kip1}$ in the precursor cells. Once a threshold level of $p27^{Kip1}$ has been reached, oligodendrocyte differentiation occurs while proliferation of oligodendrocyte precursors is halted (Durand et al., 1997; Sanes et al., 2006).

Development of PNS glia and neurons: the role of neural crest cells

In the trunk, all peripheral neurons and glia are formed from cells of the neural crest, which are formed from neural ectoderm at the dorsal neural tube (Sanes et al., 2006). Following peripheral migration from the neural tube, neural crest cells give rise to many cell types in the periphery. In addition to nervous tissue, neural crest cells are progenitors of a variety of cell types including (but not limited to) "chromaffin cells of the adrenal medulla, smooth muscle cells of the aorta (and) melanocytes" (Sanes et al., 2006). Because the fate determination of these cells is determined by cues derived from the tissue located at the final destination of the migrating neural crest cells as well as cues that are provided during migration, the multipotency of these cells must be maintained until migration is complete and all of the necessary molecular signals are available to promote appropriate cellular fates (Le Douarin et al., 1975; Sanes et al., 2006; Kleber et

al., 2005). Kleber et al. (2005) demonstrated that bone morphogenetic proteins (BMPs) and Wnt proteins are involved in maintaining a multipotent fate in neural crest cells. During early neural crest development, Wnt signalling through β-catenin promotes differentiation along the sensory lineage (Lee et al., 2004; Bronner-Fraser, 2004). However, nearly all early neural crest cells are sensitive to Wnt signalling (Lee et al., 2004), but only a subpopulation of these cells develops along the sensory lineage, meaning that other signalling is involved in preventing sensory differentiation in the remaining neural crest cells.

In contrast to Wnt signalling, BMP signalling promotes neuronal development along an autonomic lineage, but this occurs later in neural crest cell migration, even though early neural crest cells are exposed to BMP from the dorsal neural tube (Reissmann et al., 1996; Shah et al., 1996; Liem et al., 1995). It is the BMPs (specifically BMP-7) secreted by the dorsal aorta (not the dorsal neural tube) that activate the expression of the Phox2b transcription factor in neural crest cells; this converts these neural crest cells into adrenergic neurons of the sympathetic nervous system, as Phox2b promotes the transcription of tyrosine hydroxylase, an enzyme necessary for noradrenaline synthesis (Reissmann et al., 1996; Pattyn et al., 1999; Sanes et al., 2006). In addition to the functions of BMPs mentioned above, Kleber et al. (2005) demonstrated that BMP signalling is important for antagonizing Wnt signalling in early neural crest cells and vice versa. This mutually antagonistic signalling results in the maintenance of multipotency in early neural crest cells and allows for differentiation into multiple

lineages, such as sensory and autonomic, at appropriate stages of crest cell migration (Kleber et al., 2005).

The glial fate of neural crest cells is determined after sensory and autonomic lineages have been established. The expression of the transcription factor Sox10 is essential for generating peripheral glia from migrating neural crest cells of the trunk. Whether Sox10 is expressed or not is determined by signalling within newly formed peripheral ganglia (such as the sensory and sympathetic ganglia). The growth factor neuregulin-1 (NRG1) is expressed by neurons in these peripheral ganglia (Britsch et al., 2001; Leimeroth et al., 2002; Sanes et al., 2006). NRG1 promotes glial fate specification, as cultured neural crest cells become glia in response to NRG1 treatment. All neural crest cells are sensitive to NRG1 signalling after they have migrated peripherally from the neural tube and have begun to form peripheral ganglia but only neuronally differentiated cells express NRG1. However, only those neural crest cells that receive high amounts of NRG1 (provided by neuronally differentiated cells within the ganglia), which are the last ones to migrate into the ganglia, become glia (satellite cells); therefore, glial differentiation within peripheral ganglia is dependent on neuronal differentiation, and thus glia arise after neurons in the PNS.

Glial fate specification within peripheral ganglia is also established through the activity of the Delta/Notch signalling molecules; this activity provides another example of how glial differentiation is dependent on neurons. The Delta ligand is expressed by newly-differentiated neurons and by signalling through the Notch receptor on

neighbouring neural crest cells, gliogenesis is promoted as proneuronal gene expression is inhibited by Notch signalling. The importance of Delta/Notch signalling in establishing glial fate was demonstrated by treating cultures of DRG neural crest with Delta. This resulted in the majority of the cultured cells taking on a glial fate (Morrison, 2001; Sanes et al., 2006).

Development of Schwann cells

During embryonic development, Schwann cells arise from neural crest cells that migrate peripherally from the central neural tube. Migrating neural crest cells (at E10-11) first give rise to *Schwann cell precursor cells*, which can be isolated from the E14-15 spinal nerves of rats (Jessen et al., 1994). These eventually go on to form *immature unmyelinating Schwann cells* (at E15-17) which then differentiate into either *mature non-myelinating Schwann cells* such as those of Remak fibres (which associate with only small-diameter axons) or *myelinating Schwann cells* (reviewed in Jessen and Mirsky, 2005).

The Schwann cell lineage can be traced by a transient expression profile of particular proteins that alters as development proceeds (reviewed in Jessen and Mirsky, 2005). *Schwann cell precursors* are identified by the expression of brain fatty acid binding-protein, protein 0, cadherin-19 and desert hedgehog; none of which are expressed in multipotent migrating neural crest cells. The *Schwann cell precursor* stage represents the earliest stage of development where cells of the Schwann cell lineage interact with axons in the periphery (E14-15 in the rat, E12-13 in the mouse; reviewed in Jessen and

Mirsky, 2005). *Schwann cell precursors* give rise to *immature Schwann cells* by E15-17 in the rat and E13-15 in the mouse (Jessen et al., 1994; reviewed in Jessen and Mirsky, 2005). Immunohistochemical methods have been used to demonstrate that *immature Schwann cells* begin expressing S-100, a calcium binding protein that is also expressed by *mature Schwann cells*. *Mature Schwann cells* (either myelinating or non-myelinating) are then generated from *immature Schwann cells* around birth (which occurs about E20 in the rat; reviewed in Jessen and Mirsky, 2005). Finally, *mature myelinating Schwann cells* are characterized by expression of myelin-specific proteins such as myelin-associated glycoprotein.

It is the *mature Schwann cells* that will either myelinate axons or ensheathe unmyelinated axons. Whether an axon is myelinated or unmyelinated is correlated with the diameter of the axon that is enveloped by the maturing Schwann cell. Generally, small-diameter axons (less than 1 μm) are unmyelinated while axons greater than 1 μm are myelinated (reviewed in: Jessen and Mirsky, 2005; Sherman and Brophy, 2005). Recently, Taveggia et al. (2005) elucidated a molecular mechanism that accounts for these observations. As in the CNS, axons initiate myelination in the PNS via signalling to myelinating glia. It was revealed that small-diameter axons destined to be unmyelinated express lower levels of the growth factor NRG1 type III (which is expressed on the axonal surface) than large-diameter axons that become myelinated. Virally-induced expression of functional NRG1 in sensory neurons that were removed from NRG1-null mice rescued deficient levels of both myelinating ensheathment and non-myelinating-ensheathment. In addition, normally unmyelinated sympathetic axons

(that contained low levels of NRG1 and normal levels of non-myelinating-ensheathment) became myelinated when they were infected with NRG1-expressing lentiviruses. NRG1 interacts with erbB receptors on Schwann cells which then activate PI 3-kinase; this pathway is important for inducing myelination. These results suggest that NRG1 is essential for axonal ensheathment by Schwann cells and that a threshold level of NRG1 is necessary for determining whether or not myelination takes place (Taveggia et al., 2005).

While all Schwann cells are derived from the neural crest, a very specific type of neural crest cell, known as the boundary cap (BC) cell (which will be discussed in detail below), is thought to provide most, if not all, of the Schwann cells present in the dorsal roots of the spinal nerve, where sensory information enters the spinal cord (reviewed in Jessen and Mirsky, 2005).

Primary sensory neurons and sensory root entry zones

The sensory component of the nervous system is critical for survival, as the detection of both external and internal stimuli allows animals to respond appropriately to varying environmental conditions and maintain homeostasis. Primary sensory neurons are the cells responsible for relaying sensory information that is detected in the periphery to the CNS. The transmission of temperature, noxious stimuli and mechanical displacement by somatic primary sensory neurons results in the perception of heat/cold, pain and touch/limb position, respectively. Visceral sensory neurons convey additional information regarding the state of cardiovascular (e.g. blood pressure and CO_2 content) and gastrointestinal (e.g. hunger and satiety) systems. The cell bodies (somata) of

neurons that carry sensory information reside in ganglia located outside of the CNS in the PNS. For example, the somata of sensory neurons that innervate the trunk and limbs are situated in DRGs. Sensory neurons are pseudounipolar; they have a single axon that bifurcates in the ganglion, with one branch extending into the periphery and one branch projecting into the CNS. The requirement that sensory neurons transmit information from the periphery to the CNS means that their centrally-projecting axons are situated within peripheral and central nervous tissue – these represent two very distinct environments. At sensory root entry zones, an abrupt glial barrier exists that separates the PNS from the CNS.

Sensory root entry zones as a focus of study

Sensory root entry zones contain divisions known as glial barriers or glial interfaces that separate the PNS from the CNS. Glial interfaces are of interest to researchers investigating neural regeneration and development because they represent an area where two different physiological and structural nervous environments (i.e. the CNS and the PNS) come into direct contact with one another. The close apposition of these two discrete nervous system environments at sensory root entry zones allows for the convenient comparison of the dynamic physiological and morphological events that occur during neural regeneration and development in the PNS with those events that occur in the CNS. For example, when the dorsal root of the spinal nerve is injured, different responses occur in the PNS compartment of the dorsal root entry zone (DREZ) of the spinal nerve compared to the CNS compartment (reviewed in Cafferty and Ramer, 2002). In the PNS compartment, axons will regenerate up to the glial interface but not beyond,

as the PNS compartment is amenable to axonal regeneration whereas the CNS compartment is generally considered to be inhibitory (reviewed in Fenrich and Gordon, 2004). In addition to being able to observe spontaneous and naturally occurring changes in both the CNS and PNS following injury at sensory root entry zones, researchers can also simultaneously study the reactions of both nervous system environments to experimental treatments. For example, neurotrophic factor treatments are used in attempts to increase the regenerative ability of injured axons through both the PNS and CNS compartments of the DREZ and into the spinal cord (reviewed in Cafferty and Ramer, 2002). While much interest in sensory root entry zones revolves around neural regeneration, the work conducted in this thesis examines the developmental changes that occur in the early CNS and PNS that form sensory root entry zones.

The nerve transitional zone: structure and development

The remainder of this chapter discusses structural and developmental aspects of sensory root entry zones. In order to relay a comprehensive description of the structural components of sensory root entry zones, it is necessary to discuss some key structural and developmental features of what are referred to as transitional zones (TZs). Sensory root entry zones are only one example of a TZ, the latter being a general term describing a region of a nerve containing both PNS and CNS that possesses a glial barrier flanked on one side by peripheral tissue and by central tissue on the other side. All nerves that are attached to the CNS possess a TZ, and most of the TZs of these nerves (with the exceptions of the vomeronasal and olfactory nerves) in vertebrates are characterized by a glial barrier separating the CNS from the PNS. A considerable body of work has been

done elucidating the ultrastructural organization of many of these TZs (reviewed in Fraher, 1992; 2002). For example, studies of the ventral root transitional zone (VRTZ), which is present where axons of motoneurons exit the ventral horn of the spinal cord, have revealed that myelinated axons are ensheathed by Schwann cells and endoneurial connective tissue in the PNS compartment of the VRTZ while being mainly supported by astrocytes in the CNS (reviewed in Fraher, 2002). At the CNS-PNS interface of the VRTZ, the density of nodes of Ranvier is higher than that of the nervous tissue on either side of the interface (Fraher and Bristol, 1990). High nodal density is a defining feature of the CNS-PNS interface because of the sharp transition that separates the myelinated segments of axons – oligodendrocytes myelinate the central portion of the axon on one side of the interface and Schwann cells myelinate the peripheral portion on the other side of the interface.

Ultrastructural (and some immunohistochemical) studies of the development of nerve transitional zones have been heavily focused on efferent nerves such as the TZ present in the VRTZ (Fraher and Kaar, 1982; Fraher, 1992, 1997; O'Brien et al., 1998, 2001). A study by O'Brien et al. (2001) established E18 (which is roughly 2 days before birth) as a critical time point in the development of the VRTZ in the rat. It is at this time that astrocytic processes begin to associate with axons at the edge of the spinal cord at the root exit zone. Immunohistochemically, these astrocytes upregulate the mature astrocytic marker protein GFAP and downregulate the early glial marker vimentin. By P2, the astrocytic segregation of axons is complete and a solid glial barrier has formed, delineating a sharp boundary between the CNS and the PNS at the developing TZ.

"Cluster cells" present at the proximal root adjacent to the spinal cord (i.e. the PNS component of the developing TZ) begin to associate with axon bundles and segregate axons at E14 until E18, after which time cluster cell-mediated axonal segregation decreases. These cells then become undetectable at the root exit zone as they decrease their expression of adhesion molecules such as neural cell adhesion molecule. It has been hypothesized that the matrix formed by cluster cells prevents Schwann cells from invading the spinal cord until the glial barrier is formed postnatally. Cluster cells are also BC cells similar to those found at the developing dorsal root (see discussions of BC cells below and in Chapter 4). The morphometric and immunohistochemical results of the study by O'Brien et al. (2001) complement previous ultrustructural studies of VRTZ development (Fraher, 1992, 1997; O'Brien et al., 1998).

Transitional zones: comparing structures across species

Most of the studies conducted on the structure and development TZs have been focused on mammals such as the rat or cat. Some interesting comparative work has been undertaken in order to characterize the TZs of different vertebrate species. Fraher and Cheong (1995) found that the ultrastructure of TZs present in the lamprey *Petromyzon* differ from those present in mammalian nerves. Although a glial barrier is present, such as in the mammalian TZ, the structural organization of the lamprey TZ does differ from the mammalian TZ in some key ways. For example, the glial extent of the CNS that is formed of astrocytes and covered by basal lamina, known as the glia limitans, is thicker at the CNS-PNS interface of mammalian TZs than anywhere else, whereas the thickness of the glia limitans at the lamprey TZ is consistent with the glia limitans elsewhere. As

well, the glial processes present at the lamprey TZ contain microfilamentous bundles that are attached to hemidesmosomes that connect these bundles to the glia limitans. These associations are absent in the mammalian TZ (Fraher and Cheong, 1995; Fraher, 2002). Further work was conducted for the purpose of determining the prevalence of glial barriers in the TZs of different vertebrate species. In the marine chordate *Amphioxus*, for example, it was discovered that the glial structure of the TZs differed from those of both mammals and the lamprey, as they were found to lack the prominent glial demarcation present in the PNS-CNS interface of the TZs of all other known vertebrates (Fraher, 2002). However, a gradual transition between PNS and CNS glia was present (Fraher, 2002). In addition, research on the presence of glial barriers in the TZs of the invertebrate nervous system has demonstrated that, while there is a clear transition between CNS and PNS tissue, there is no glial barrier present (Fraher, 2002).

Sensory root entry zone structure and development

Comparative work is the focus of the next chapter, as the study presented is a comparative analysis of the development of three specific sensory transitional zones in the rat: the dorsal root entry zone (DREZ), the trigeminal root entry zone (TREZ), and the vagal dorsal root entry zone (vDREZ). These three root entry zones are all unique in one way or another (as is described below) and I investigated whether these structural differences were associated with any differences in glial development (Chapter 2). The remainder of this chapter covers what is known about the structure and development of the DREZ, TREZ and vDREZ.

Glial structure of the spinal dorsal root entry zone

In vertebrates, the point of entry into the CNS for primary afferent axons is known as the DREZ. The DREZ is the portion of the sensory root that contains both PNS and CNS tissue along with the actual interface between the PNS and CNS. Primary afferent axons that project into the CNS are supported by BC cells during development and grow through tubes of the basal lamina in the periphery. These tubes are continuous with the covering of basal lamina over the peripheral boundary of astrocytic processes. One characteristic of the DREZ is the expression of laminin in the peripheral portion by Schwann cells, while laminin is only expressed in the pia mater and on blood vessels in the CNS. The central portion of the DREZ is characterized by a dome of astrocytic tissue within the most central portion of the root, which can be identified immunohistochemically by immunoreactivity to GFAP. This central projection also includes oligodendrocytic processes, and together the astrocytes and oligodendrocytes form a solid "glial dome" (reviewed in: Fraher, 1992, 1999; Cafferty and Ramer, 2002; Figure 1-1).

The actual PNS-CNS interface of the DREZ is formed by astrocytes that are a peripheral extension of the glia limitans of the CNS into the root. At this point, sensory axons projecting into the spinal cord are myelinated on the peripheral side of the DREZ by transitional Schwann cells and on the central side by oligodendrocytes. This region of the DREZ has a very high nodal density because the transition from PNS to CNS is abrupt; it occurs over one node of Ranvier, similar to that present at the VRTZ (reviewed in: Fraher, 1992, 1999; Cafferty and Ramer, 2002; Figure 1-1). Unmyelinated axons projecting into the CNS exist in bundles associated with transitional Schwann cells in the

periphery and are ensheathed by astrocytic processes centrally (reviewed in: Fraher, 1992, 1999; Cafferty and Ramer, 2002; Figure 1-1).

Development of the spinal dorsal root entry zone

The glial elements of the spinal DREZ are formed from an interaction between three distinct components: late-surviving transient neural crest-derived cells, the basal lamina of the neural tube, and neuroepithelial cells (reviewed in Cafferty and Ramer, 2002; Golding et al., 1997). The cells derived from the neural crest are known as BC cells. These cells migrate to distinct points along the dorsolateral surface of the spinal cord that eventually form the entry points for sensory axons into the spinal cord (i.e. the DREZ) (Golding and Cohen, 1997). BC cells are characterized by the expression of a member of the cadherin family of cell adhesion molecules: c-cad7 (reviewed in Golding et al., 1997). They are also identified by early expression of Krox-20 (or Egr-2), a transcription factor demonstrated to be important for myelination in the PNS (Golding and Cohen, 1997; Murphy et al., 1996; Topilko et al., 1994).

BC cells play critical roles in the development of the DREZ. BC cells may express inhibitory cues for inhibiting astrocytic migration from the CNS into the proximal portion of the root, as migration was halted in the presence of BC cells in vivo (Golding and Cohen, 1997). BC cells express the Ca^{2+}-binding cell adhesion molecule N-cadherin in the embryonic rat and this may allow for the glioglial interactions important for simultaneously preventing astrocytic invasion of the dorsal root and forming an environment amenable to sensory axon growth into the CNS (Wanner et al.,

2006; Golding and Cohen, 1997; discussed in detail in Chapter 4). Cryoculture experiments have shown that neurite outgrowth from DRGs was enhanced on tissue containing BC cells but not on tissue lacking BC cells (Golding and Cohen, 1997). Importantly, they are present at the developing prenatal DREZ in time to act as a conduit for growth of sensory axons through the PNS-CNS interface and into the spinal cord (see below for timing of sensory axon entry into the spinal cord). Interestingly, BC cells prevent the migration of motoneurons out of the spinal cord and into the ventral root while maintaining a permissive growth substrate for motor axons. Vermeren et al. (2003) demonstrated that ablation of BC cells does not affect motor axon outgrowth but does allow motoneuron somata to migrate out of the cord. Thus, BC cells are integral for nervous system compartmentalization at both the DREZ and the VRTZ.

In addition to providing a favourable growth environment for sensory axons, BC cells also generate glia within the peripheral compartment of the DREZ. A recent study conducted in mice by Maro et al. (2004) traced the fate of Egr-2-expressing progeny of BC cells along the dorsal root. It was found that these cells provide most (if not all) of the Schwann cells along the dorsal root as well as satellite cells and nociceptive neurons within the DRG (Maro et al., 2004). The developing Schwann cells in the dorsal root mature into either myelinating or non-myelinating mature Schwann cells around birth (which occurs about E20 in the rat) which then go on to interact with sensory axons as described above (reviewed in Jessen and Mirsky, 2005; Fig. 1-1). More recent studies of BC cells have revealed that they possess the properties of neural stem cells as they give

rise to numerous subtypes of sensory neurons when grown in culture (Hjerling-Leffler et al., 2005) as well as Schwann cells upon treatment with neuregulin (Aquino et al., 2006).

In addition to studies focussing on the importance of BC cells in aiding axonal outgrowth during the development of the vertebrate DREZ, the necessity of PNS glial cells in guiding sensory axons through the PNS-CNS interface early on in development was demonstrated in a study conducted in the invertebrate *Drosophila* (Sepp et al., 2001). This study showed that when peripheral glial cells were ablated early in development, the central projections of sensory axons were disrupted.

Peripheral glia and neural crest derivatives are not the only sources of appropriate guidance cues for growing sensory axons. There are molecular signals that have been shown to play a role in guiding sensory axons to central targets. Axons extending from DRG neurons are susceptible to chemorepellant cues that are secreted by "non-target" tissues, such as the dermamyotome, the notochord, and the ventral spinal cord. This susceptibility aids in directing sensory axons towards the DREZ and into the spinal cord (reviewed in Masuda and Shiga, 2005). One of these chemorepellant cues, Semaphorin 3A, is derived from the dermamyotome and notochord and acts through the Neuropilin-1 receptor on sensory axons. Other chemorepellant cues derived from the notochord and ventral spinal cord mediate chemorepulsion through interactions with the axonin-1/SC2 receptor on sensory axons (reviewed in Masuda and Shiga, 2005).

The extension of sensory axons into the CNS from the DRG begins early on in development, as axons in mouse embryos have been shown to have reached the dorsolateral wall of the spinal cord by E10.5 and the gray matter of the dorsal horn by E13 (Ozaki and Snider, 1997). In the rat, primary afferents reach the spinal cord surface by E13 and the gray matter by E15 (Mirnics and Koerber, 1995). In both rats and mice, the timing of axonal extension from the DRG into the spinal cord depends on the neuronal subclass of the sensory axons. For example, large-diameter A-fibres extend into the spinal cord before small-diameter C-fibres in the rat, as the former reach the dorsal horn by E14 and the latter reach the dorsal horn by E18 (Jackman and Fitzgerald, 2000).

After innervation of the spinal cord occurs, the axons are then separated at the DREZ by Schwann cells and astrocytes (reviewed in: Fraher, 1992, 1999; Cafferty and Ramer, 2002). Myelination of axons at the peripheral portion of the DREZ has been shown to occur 1.5 weeks before birth in the cat sacral (S1) dorsal root (Nilsson et al., 1998). The formation of the glial dome of the DREZ occurs later on and involves invasion of the root by astrocytic processes and oligodendrocytes which is then followed by astrocytic cell bodies. In the rat, astrocytic invasion is initiated postnatally. Fraher and Sheehan (1987) have demonstrated that projections of CNS tissue into rat cervical dorsal roots are not present before postnatal day 3 and take on mature morphological features by postnatal day 20. Histochemical studies of the S1 dorsal root of the cat have shown that CNS tissue is present in the DREZ by the second postnatal week (Berthold and Carlstedt, 1977). A later ultrastructural study by Carlstedt (1981) further established that CNS tissue protrudes distally along the root during the first postnatal week in the S1

dorsal root of the cat and that this protrusion continually migrates at an average rate of 5 micrometers/day throughout the first postnatal month. These data demonstrate that the development of the glial dome present on the CNS side of the DREZ does not take place until after birth.

Glial structure of cranial sensory root entry zones

The root entry zones of certain cranial nerves have unique structural qualities that differentiate them from the DREZ. For example, the entry zone of cranial nerve I, the olfactory nerve, contains a unique cell type that co-exists with axon bundles through the PNS-CNS interface of the entry zone. These are known as OECs and they are immunohistochemically and structurally similar to both Schwann cells and astrocytes (reviewed in Fraher, 1999; Doucette 1984, 1991). The PNS-CNS interface of the olfactory nerve entry zone lacks a proper glial barrier, unlike the DREZ, vDREZ and TREZ. This is likely due to the fact that all olfactory axons are unmyelinated (OECs ensheathe olfactory axons but do not myelinate them) and cross the entry zone in bundles, whereas myelinated axons that exist in other root entry zones are individually ensheathed by endoneurial tubes and myelinated by Schwann cells; therefore the olfactory entry zone lacks a significant glial demarcation due to the absence of a substantial contribution of peripheral glia at the PNS-CNS interface that would be required for a tight glial barrier between PNS and CNS glia (Doucette, 1991; reviewed in Fraher, 2002).

The vagus nerve (cranial nerve X) is responsible for transmitting sensations from the viscera; the somata of this nerve reside in the nodose ganglion. Vagal afferents are both myelinated and unmyelinated although the majority is the latter. This nerve has a root entry zone which is structurally unlike any other. The vDREZ is characterized by the presence of peripheral tissue insertions (PTIs) that contain Schwann cells that penetrate deep within the surrounding CNS tissue of the brainstem (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991; Figure 1-2). These intercalated Schwann cells differ from the transitional Schwann cells found in the PNS at the distal portion of the root entry zone in that they are completely surrounded by CNS tissue both proximally and distally along the root entry zone. The internodal length along the axons is shorter in the PTIs than in either the distal PNS portion of the root or the CNS, as intercalated internodes are approximately 60% that of both transitional Schwann cell and oligodendrocytic internodes (Fraher and Rossiter, 1991; Figure 1-2).

The structure of the vDREZ is much more complex than that of the DREZ. Serial rostrocaudal transverse sections through the vDREZ reveal areas where peripheral tissue is actually isolated proximally along the root from other PNS tissue, forming islands of peripheral tissue located either rostrally or caudally adjacent to sections where PTIs are present. The vDREZ does not have a sharp boundary demarcating the PNS from the CNS like the DREZ, but rather a zone of staggered transition exists (Figure 1-2). This means that centrally-projecting axons must cross multiple PNS-CNS interfaces before reaching their targets within the CNS in areas of the vDREZ where either PTIs or islands of peripheral tissue exist (Fraher and Rossiter, 1991).

Sensory information from the face and head is transmitted to the pons, medulla and cervical spinal cord along a pathway that includes the three main branches of the trigeminal nerve (cranial nerve V), the trigeminal ganglion, and the trigeminal sensory root. The root contains both centrally-projecting axons of trigeminal ganglion neurons that enter the pons at the TREZ, and peripherally projecting sensory (proprioceptive) axons originating from the trigeminal mesencephalic nucleus in the brainstem. The TREZ is structurally quite similar to the DREZ as it is typified by a glial dome of astrocytic and oligodendrocytic processes extending peripherally within the root forming a PNS-CNS interface (Fraher, 1992). In addition, the high nodal density at the PNS-CNS interface of the TREZ resembles that of the DREZ (Henry et al., 2005).

Development of the cranial sensory root entry zones

The developmental processes involved in the formation of the vDREZ are not well understood, but they may involve the invasion of Schwann cells early in development along blood vessels that lead deep into the brainstem, forming PTIs and islands of peripheral tissue (Rossiter and Fraher, 1990). Alternatively, PTI Schwann cells could have derived from the neural tube or resulted from an overgrowth of tissue from a nearby developing region of the brainstem, such as the inferior cerebellar peduncle, into the vagal rootlet (Rossiter and Fraher, 1990).

Many aspects of the development of the TREZ remain unkown; however, it is known that both the sensory and motor systems of the face and the viscera are functional earlier in development than those of the extremities, as behaviours mediated by facial

sensation in the mammal, such as suckling, and internal homeostatic mechanisms involving appropriate sensory feedback from the viscera mediated by the vagus are more integral to the survival of newborn altricial mammals than trunk and limb somatosensory feedback. This difference could be mediated by the different developmental origins of cranial sensory neurons versus the sensory neurons that innervate the trunk and limbs.

The sensory neurons that are associated with cranial root entry zones are of similar developmental origin. The sensory ganglia of cranial nerves are derived from what are known as neurogenic placodes (reviewed in: Graham and Begbie, 2000; Graham et al., 2004). These are formed from thick areas of the embryonic ectoderm and are not associated with other embryonic sources of neurogenic tissue such as the neural crest. The majority of the neurons in the sensory ganglia of the trigeminal nerve are derived from placodes, although some neurons are of neural crest origin (Sanes et al., 2006; reviewed in Graham and Begbie, 2000). The nodose ganglia of the vagus nerve is entirely of placodal origin. In the olfactory system, both the neurons and the associated OECs arise from the neurogenic placodes (Doucette, 1991). The sensory neurons that reside in DRGs, innervate the trunk and limbs and associate with the DREZ are of neural crest origin. It is possible that the onset and completion of root entry zone development may be correlated with the developmental origin of the neurons associated with these entry zones (i.e. placodal vs. neural crest origin).

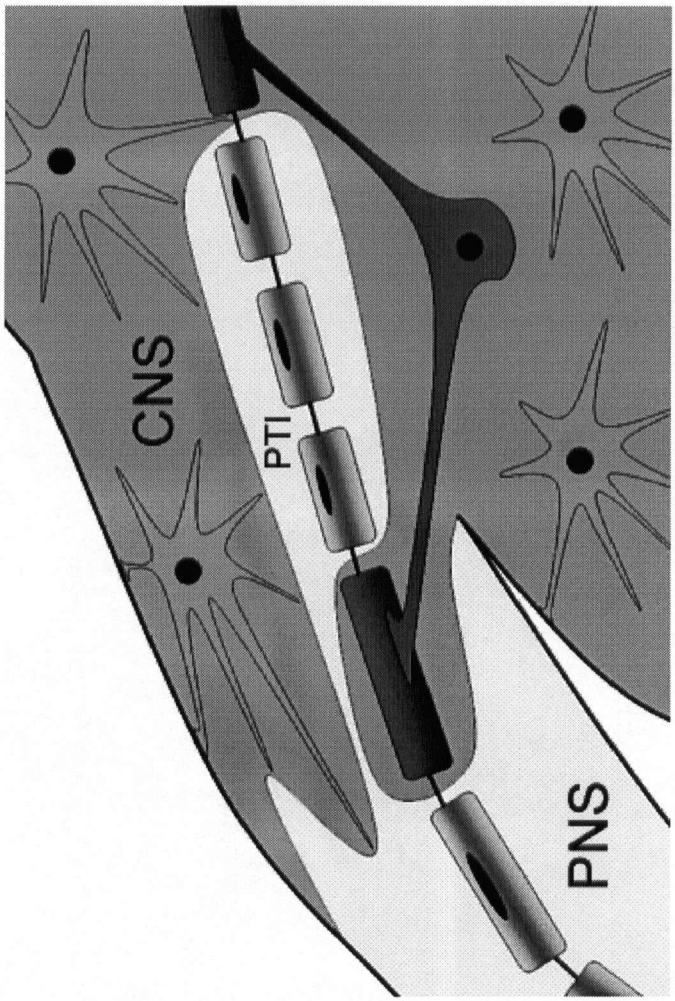
Objective of Chapter 2

The adult structures of the DREZ, TREZ and vDREZ have been extensively characterized (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991; reviewed in: Fraher, 1992, 1999; Cafferty and Ramer, 2002) and much work has been done establishing the morphological events involved in the glial development of the DREZ (Berthold and Carlstedt, 1977; Carlstedt, 1981; Fraher and Sheehan, 1987). As well, much is known about the early developmental events that result in the formation of both peripheral and central glia. However, much remains unknown about the glial development of the TREZ and vDREZ. As glia define both the central and peripheral structural components of all sensory root entry zones, tracing the glial development (from onset to maturity) of the DREZ, TREZ and vDREZ and comparing this progression across these three root entry zones will greatly increase our understanding of how these entry zones are formed – this is the objective of the study presented in Chapter 2.

Figure 1-1. The dorsal root entry zone (DREZ) in cross-section. The defining feature of the DREZ is the distal extension of CNS tissue into the dorsal root, where a glial dome is present. The glial dome is largely composed of astrocytes and oligodendrocytes. Myelinated axons cross the PNS-CNS interface at the transitional node (arrow); here, axons are myelinated by Schwann cells on the peripheral side and oligodendrocytes on the central side. Unmyelinated axons are ensheathed by non-myelinating Schwann cells in the PNS and astrocytes in the CNS. Astrocytes also contact myelinated axons at nodes of Ranvier. At the peripheral extent of the CNS tissue projection, astrocytes form the glia limitans, which lies directly adjacent to the basal lamina. Tubes of the basal lamina cover Schwann cells as well as the glia limitans.

CNS
PNS

Figure 1-2. The vagal dorsal root entry zone (vDREZ) in cross-section. The complex structure of the vDREZ is illustrated here. Unlike the DREZ, which contains a solid glial dome of central tissue, the vDREZ is composed of both extensions of central tissue and peripheral tissue insertions (PTIs). Vagal afferent axons may be situated across multiple PNS-CNS interfaces resulting in multiple transitional nodes. The internodal lengths along myelinated axons in PTIs are much shorter (around 60%) than those of the root or the brainstem proper.


CNS
PTI
PNS

Bibliography

Aquino JB, Hjerling-Leffler J, Koltzenburg M, Edlund T, Villar MJ, Ernfors P (2006) In vitro and in vivo differentiation of boundary cap neural crest stem cells into mature Schwann cells. Exp Neurol 198: 438-449.

Barnabe-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, Miller FD (2005) Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 48: 253-265.

Barry D, McDermott K (2005) Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. Glia 50: 187-197.

Baumann N, Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 81: 871-927.

Berthold CH, Carlstedt T (1977) Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. II. General organization of the transitional region in S1 dorsal rootlets. Acta Physiol Scand Suppl 446: 23-42.

Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD, Greenberg ME (1997) Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278: 477-483.

Britsch S, Goerich DE, Riethmacher D (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. 15: 66-78.

Bronner-Fraser M (2004) Development. Making sense of the sensory lineage. Science 303: 966-968.

Burrows RC, Wancio D, Levitt P, Lillien L (1997) Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron 19: 251-267.

Cafferty WBJ, Ramer MS (2002) Promoting Sensory Axon Regeneration Across the PNS-CNS Interface. In: Glial Interfaces in the Nervous System: Role in Repair and Plasticity (Aldskogius H, Fraher JP, eds), pp 23-40. Amsterdam: IOS Press.

Carlstedt T (1981) An electron-microscopical study of the developing transitional region in feline S1 dorsal rootlets. J Neurol Sci 50: 357-372.

Doucette JR (1984) The glial cells in the nerve fiber layer of the rat olfactory bulb. Anat Rec 210: 385-391.

Doucette R (1991) PNS-CNS transitional zone of the first cranial nerve. J Comp Neurol 312: 451-466.

Durand B, Gao FB, Raff M (1997) Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. EMBO J 16: 306-317.

Fenrich K, Gordon T (2004) Canadian Association of Neuroscience review: axonal regeneration in the peripheral and central nervous systems--current issues and advances. Can J Neurol Sci 31: 142-156.

Fraher J, Cheong E (1995) Glial-Schwann cell specialisations at the central-peripheral nervous system transition of a cyclostome: an ultrastructural study. Acta Anat (Basel) 154: 300-314.

Fraher J (2002) Axons and glial interfaces: ultrastructural studies. J Anat 200: 415-430.

Fraher JP, Kaar GF (1982) The maturation of the ventral root-spinal cord transitional zone. Part 2. A quantitative ultrastructural study of the dynamics of its early development. J Neurol Sci 53: 63-75.

Fraher JP, Sheehan MM (1987) The CNS-PNS transitional zone of rat cervical dorsal roots during development and at maturity. A morphological and morphometric study. J Anat 152: 189-203.

Fraher JP, Bristol DC (1990) High density of nodes of Ranvier in the CNS-PNS transitional zone. J Anat 170: 131-137.

Fraher JP, Rossiter JP (1991) Myelin-axon relationships established by rat vagal Schwann cells deep to the brainstem surface. J Comp Neurol 304: 253-260.

Fraher JP (1992) The CNS-PNS transitional zone of the rat. Morphometric studies at cranial and spinal levels. Prog Neurobiol 38: 261-316.

Fraher JP (1997) Axon-glial relationships in early CNS-PNS transitional zone development: an ultrastructural study. J Neurocytol 26: 41-52.

Fraher JP (1999) The transitional zone and CNS regeneration. J Anat 194(Pt 2): 161-182.

Golding J, Shewan D, Cohen J (1997) Maturation of the mammalian dorsal root entry zone--from entry to no entry. Trends Neurosci 20: 303-308.

Golding JP, Cohen J (1997) Border controls at the mammalian spinal cord: late-surviving neural crest boundary cap cells at dorsal root entry sites may regulate sensory afferent ingrowth and entry zone morphogenesis. Mol Cell Neurosci 9: 381-396.

Graham A, Begbie J (2000) Neurogenic placodes: a common front. Trends Neurosci 23: 313-316.

Graham A, Begbie J, McGonnell I (2004) Significance of the cranial neural crest. Dev Dyn 229: 5-13.

Henry MA, Rzasa RS, Beeler JJ, Levinson SR (2005) Caspr reveals an aggregation of nodes and flanking node free zones at the rat trigeminal sensory root and dorsal root entry zones. Glia 49: 445-450.

Hjerling-Leffler J, Marmigere F, Heglind M, Cederberg A, Koltzenburg M, Enerback S, Ernfors P (2005) The boundary cap: a source of neural crest stem cells that generate multiple sensory neuron subtypes. Development 132: 2623-2632.

Ishibashi T, Dakin KA, Stevens B, Lee PR, Kozlov SV, Stewart CL, Fields RD (2006) Astrocytes promote myelination in response to electrical impulses. Neuron 49: 823-832.

Jackman A, Fitzgerald M (2000) Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. J Comp Neurol 418: 281-298.

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

Jessen KR, Mirsky R (2005) The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6: 671-682.

Kirchhoff F, Dringen R, Giaume C (2001) Pathways of neuron-astrocyte interactions and their possible role in neuroprotection. Eur Arch Psychiatry Clin Neurosci 251: 159-169.

Kleber M, Lee HY, Wurdak H, Buchstaller J, Riccomagno MM, Ittner LM, Suter U, Epstein DJ, Sommer L (2005) Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. J Cell Biol 169: 309-320.

Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17: 5820-5829.

Le Douarin NM, Renaud D, Teillet MA, Le Douarin GH (1975) Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantations. Proc Natl Acad Sci U S A 72: 728-732.

Lee HY, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L (2004) Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303: 1020-1023.

Leimeroth R, Lobsiger C, Lussi A, Taylor V, Suter U, Sommer L (2002) Membrane-bound neuregulin1 type III actively promotes Schwann cell differentiation of multipotent Progenitor cells. Dev Biol 246: 245-258.

Liem KF, Jr., Tremml G, Roelink H, Jessell TM (1995) Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82: 969-979.

Lin SC, Bergles DE (2004) Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. Nat Neurosci 7: 24-32.

Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P (2004) Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7: 930-938.

Masuda T, Shiga T (2005) Chemorepulsion and cell adhesion molecules in patterning initial trajectories of sensory axons. Neurosci Res 51: 337-347.

McDermott KW, Barry DS, McMahon SS (2005) Role of radial glia in cytogenesis, patterning and boundary formation in the developing spinal cord. J Anat 207: 241-250.

Merkle FT, Tramontin AD, Garcia-Verdugo JM, Alvarez-Buylla A (2004) Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci U S A 101: 17528-17532.

Mirnics K, Koerber HR (1995) Prenatal development of rat primary afferent fibers: II. Central projections. J Comp Neurol 355: 601-614.

Morrison SJ (2001) Neuronal potential and lineage determination by neural stem cells. Curr Opin Cell Biol 13: 666-672.

Murphy P, Topilko P, Schneider-Maunoury S, Seitanidou T, Baron-Van Evercooren A, Charnay P (1996) The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. Development 122: 2847-2857.

Nilsson R, I, Berthold CH, Carlstedt T (1998) Redistribution of Schwann cells at the developing PNS-CNS borderline. An ultrastructural and autoradiographic study on the S1 dorsal root of the cat. J Neurocytol 27: 85-97.

O'Brien D, Dockery P, McDermott K, Fraher JP (1998) The ventral motoneurone axon bundle in the CNS--a cordone system? J Neurocytol 27: 247-258.

O'Brien D, Dockery P, McDermott K, Fraher J (2001) Early development of rat ventral root transitional zone: an immunohistochemical and morphometric study. J Neurocytol 30: 11-20.

Ozaki S, Snider WD (1997) Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J Comp Neurol 380: 215-229.

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399: 366-370.

Polito A, Reynolds R (2005) NG2-expressing cells as oligodendrocyte progenitors in the normal and demyelinated adult central nervous system. J Anat 207: 707-716.

Rakic P (2003) Elusive radial glial cells: historical and evolutionary perspective. Glia 43: 19-32.

Reissmann E, Ernsberger U, Francis-West PH, Rueger D, Brickell PM, Rohrer H (1996) Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. Development 122: 2079-2088.

Rossiter JP, Fraher JP (1990) Intermingling of central and peripheral nervous tissues in rat dorsolateral vagal rootlet transitional zones. J Neurocytol 19: 385-407.

Rowitch DH (2004) Glial specification in the vertebrate neural tube. Nat Rev Neurosci 5: 409-419.

Sanes DH, Reh TA, Harris WA (2006) Development of the Nervous System. Elsevier Academic Press.

Sepp KJ, Schulte J, Auld VJ (2001) Peripheral glia direct axon guidance across the CNS/PNS transition zone. Dev Biol 238: 47-63.

Shah NM, Groves AK, Anderson DJ (1996) Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85: 331-343.

Sherman DL, Brophy PJ (2005) Mechanisms of axon ensheathment and myelin growth. Nat Rev Neurosci 6: 683-690.

Taveggia C, Zanazzi G, Petrylak A, Yano H, Rosenbluth J, Einheber S, Xu X, Esper RM, Loeb JA, Shrager P, Chao MV, Falls DL, Role L, Salzer JL (2005) Neuregulin-1 type III determines the ensheathment fate of axons. Neuron 47: 681-694.

Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. Nature 371: 796-799.

Trajkovic K, Dhaunchak AS, Goncalves JT, Wenzel D, Schneider A, Bunt G, Nave KA, Simons M (2006) Neuron to glia signaling triggers myelin membrane exocytosis from endosomal storage sites. J Cell Biol 172: 937-948.

Tsai HH, Macklin WB, Miller RH (2006) Netrin-1 is required for the normal development of spinal cord oligodendrocytes. J Neurosci 26: 1913-1922.

Vermeren M, Maro GS, Bron R, McGonnell IM, Charnay P, Topilko P, Cohen J (2003) Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. Neuron 37: 403-415.

Vesce S, Bezzi P, Volterra A (1999) The active role of astrocytes in synaptic transmission. Cell Mol Life Sci 56: 991-1000.

Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289: 74-88.

Wanner IB, Guerra NK, Mahoney J, Kumar A, Wood PM, Mirsky R, Jessen KR (2006) Role of N-cadherin in Schwann cell precursors of growing nerves. Glia 54: 439-459.

Chapter 2

COMPARATIVE POSTNATAL DEVELOPMENT OF SPINAL, TRIGEMINAL, AND VAGAL SENSORY ROOT ENTRY ZONES[1]

Introduction

During embryonic development in the rodent, sensory axons extend centrally from their peripheral ganglia into the central nervous system (CNS) and form functional connections with higher order neurons (Ozaki and Snider, 1997). In the adult rodent, the glial environment of the sensory axons consists of Schwann cells in the peripheral nervous system (PNS) and astrocytes and oligodendrocytes in the CNS. Sensory axons span an abutment of peripheral and central nervous tissue along nerve root entry zones that anatomically connect the PNS with the CNS. These PNS-CNS boundaries are located in both spinal and cranial sensory nerve root entry zones.

While the growth of sensory axons into the CNS occurs embryonically, the development of the glial elements of the root entry zones is largely a postnatal process. At the dorsal root entry zone (DREZ), this development has been characterized morphometrically as a progressive extension of central tissue (mainly astrocytes) distally into the dorsal root during the first two postnatal weeks, after which a solid glial dome forms at the distal end of the projection (Fraher and Sheehan, 1987). Cranial root entry zone morphology in the adult rodent has been described in detail by Fraher (1992). For example, the trigeminal root entry zone (TREZ) (cranial nerve V) is very similar in

[1] A version of this chapter has been published. Toma JS, McPhail LT, Ramer MS. (2006) Comparative postnatal development of spinal, trigeminal, and vagal sensory root entry zones. International Journal of Developmental Neuroscience. 24: 373-388.

structure to the DREZ. The PNS-CNS boundary of the TREZ also contains a high nodal density that is comparable to that of the DREZ (Henry et al., 2005). In contrast, the structural components of the adult vagal dorsal root entry zone (vDREZ) (cranial nerve X) have been shown to be more complex than those of the spinal DREZ (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991). Whereas the PNS-CNS boundary of the DREZ is very abrupt and occurs over one node of Ranvier, in the vDREZ this boundary is less marked due to the existence of peripheral tissue insertions (PTIs) that penetrate several hundred microns into the brainstem (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991). This results in a more complex glial environment in the vDREZ, as one axon may span multiple PNS-CNS boundaries.

While the development of central tissue projections in the DREZ has been studied (Fraher and Sheehan, 1987; Nilsson et al., 1998), the postnatal development of cranial root entry zones such as the vDREZ and TREZ has yet to be described. The aim of this study was to establish, immunohistochemically, a comparative timecourse of postnatal development of the glial elements of the cranial vDREZ and TREZ and spinal DREZ.

Materials and Methods

Long Evans rats were killed on postnatal day (P)0, P3, P7, P14, and in adulthood. Rats were deeply anaesthetized with 3g/ml chloral hydrate in water before transcardial perfusion with 0.1 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M PB. The pons with attached trigeminal root, brainstems with attached vagal roots, and cervical spinal cords with attached dorsal roots were removed, cryoprotected in 20% sucrose in 0.1M PB, and frozen over liquid nitrogen (Fig. 2-1). The spinal cord and brainstem were cryosectioned transversely at 16 μm; the pons was cryosectioned (at 16 μm) longitudinally, and all sections were thaw-mounted onto Superfrost Plus glass slides (VWR). Features of the dorsal root entry zone (DREZ), the vagal dorsal root entry zone (vDREZ), and the trigeminal root entry zone (TREZ) were visualized immunohistochemically. After blocking with 10% normal donkey serum in PBS for 30 minutes, slides were incubated with primary antibody solutions in order to visualize the following: astrocytes [mouse anti-glial fibrillary acidic protein, (GFAP), 1:1000, Sigma, St. Louis, MO and rabbit anti-GFAP, 1:1000, Dako, Denmark, used in double-labelling study with anti-3CB2 antibody]; Schwann cells and neurons [rabbit anti-p75 (1:1000, Chemicon, Temecula, CA)]; oligodendrocyte precursor cells [rabbit anti-NG2 (1:500, Chemicon)]; oligodendrocytes [mouse anti-oligodendrocytes (RIP),1:2000, Chemicon]; basal laminae (rabbit anti-laminin, 1:1000, Sigma); radial glial cells (mouse anti-3CB2, 1:1000, developed by EJ de la Rosa, obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, IA); and voltage-gated potassium channels (rabbit anti-Kv1.2, 1:200, Alomone Labs, Jerusalem, Israel). Primary antibody solutions were left on slides overnight. Slides were then washed with PBS for 30 minutes

before secondary antibody solutions were applied. The following secondary antibodies were used at 1:300 and left on slides for 2 hr: donkey anti-rabbit Alexa Fluor 488, Molecular Probes, Eugene, OR and donkey anti-mouse Cy3, Jackson ImmunoResearch, West Grove, PA. All antibodies were prepared in solution with PBS with 0.1% sodium azide and 0.2% Triton X-100. A Q-Imaging (Burnaby, British Columbia, Canada) CCD camera mounted on a Zeiss (Oberkochen, Germany) Axioplan 2 fluorescent light microscope with a motorized z-drive, along with Northern Eclipse (Empix Imaging, Mississauga, Ontario, Canada) imaging software were used to construct digital images. Relative expression levels of GFAP, 3CB2, RIP antigen, p75, and NG2 were tabulated based on qualitative inspection of the immunoreactivity levels present in the images shown in the figures; for example, a score of "highest" was assigned to the highest levels of immunoreactivity present both across timepoints and across entry zones and similarly, a score of "lowest" was assigned to the lowest amount of immunoreactivity present. Scores between highest and lowest were arbitrarily assigned but based on the immunoreactivity present. The contrast and brightness of the images presented in the following figures were adjusted using Adobe Photoshop 7.0 to allow for the clearest possible demonstration of immunoreactive tissue. In total, 12 P0, 7 P3, 7 P7, 8 P14, and 5 adult rats were used in this study. The experimental procedures undertaken conform to the rules and regulations governing animal care at the University of British Columbia and follow the guidelines of the Canadian Council on Animal Care.

Results

GFAP expression in the developing DREZ, TREZ and vDREZ

The glial components of the adult DREZ have been extensively characterized (reviewed in Cafferty and Ramer, 2002). Astrocytes expressing GFAP (a glial filament protein highly expressed in astrocytes) form a characteristic glial dome of central tissue at the DREZ (Cafferty and Ramer, 2002) and the TREZ. We sought to characterize the developmental expression of GFAP from P0 to adult in the DREZ, vDREZ, and the TREZ (Fig. 2-2, Table 2-1). Astrocytic processes were present in the TREZ and vDREZ at P0, while distal extension of GFAP-positive astrocytes into the DREZ did not occur until P3 (Fig. 2-2, Table 2-1). Progressive extension of astrocytic processes outward from the surface of the CNS occurred from P0 to P14 in all three root entry zones (arrows, Fig. 2-2). By P14, the morphology of the three entry zones, based on the presence of GFAP expression, resembled that of the adult.

RIP immunoreactivity in the developing DREZ, TREZ and vDREZ

To examine oligodendrocytes and the development of central myelination in each of the three root entry zones, we examined RIP immunoreactivity. The RIP monoclonal antibody labels oligodendrocyte cell bodies and their myelinating processes (Friedman et al.,1989; Jhaveri et al., 1992; Berger and Frotscher, 1994; Butt et al., 1995; Butt et al., 1998; Hwang et al., 2006). Initial extension of oligodendrocytes and their processes into the central portion of the root occurred between P0 and P3 at the TREZ and vDREZ and

later (between P3 and P7) at the DREZ (Fig. 2-3, Table 2-2). In the developing vDREZ, the source of the central tissue projections was the developing inferior cerebellar peduncle (ICP). The ICP first became apparent at P3 and was labelled with RIP immunoreactivity (arrows, Fig. 2-3). There was a delay in oligodendrocyte extension into the three root entry zones as compared to astrocyte (GFAP) extension (Fig. 2-3). However, a progressive distal expansion of oligodendrocyte processes up to P14 occurred in all three root entry zones, which paralleled the extension of GFAP-positive astrocytes. Consistent with developing GFAP-positive astrocytic expression, the adult morphology of the central oligodendroglial components of the three root entry zones became apparent by P14.

Unexpectedly, high levels of RIP immunoreactivity were located throughout the PNS portions of the three root entry zones (Fig. 2-3, Table 2-2). RIP immunoreactivity appeared as a punctate staining pattern that was especially evident at the P14 TREZ (arrowheads, Fig. 2-3), suggesting that the RIP antibody binds to an antigen present in peripheral as well as central myelin. This RIP immunoreactivity continually declined from P0 until the adult in the peripheral components of the three root entry zones but remained at paranodal regions in the PNS (Fig. 2-4). Paranodal RIP immunoreactivity was found adjacent to Kv1.2 immunoreactivity as revealed through double-labelling with the anti-Kv1.2 antibody, which recognizes potassium channels localized to the juxtaparanodal region of myelinated axons (Wang et al., 1993; Rasband et al., 1998; Fig. 2-4).

Laminin expression in the developing DREZ, TREZ and vDREZ

Laminin is produced by Schwann cells, endothelial cells lining blood vessels, and is a component of the basal lamina of the PNS but is present only around blood vessels in the postnatal CNS (Cornbrooks et al., 1983; Sosale et al., 1988; Cafferty and Ramer, 2002); therefore, it is ideal for identification of the PNS components of the three root entry zones. Laminin expression levels in the developing root entry zones clearly demonstrated a gradual retraction of PNS tissue distally along the roots throughout postnatal development (Fig. 2-5). This retraction was most evident in the DREZ and TREZ. Withdrawal of laminin-expressing peripheral tissue into the peripheral portion of the root was already underway by P0 at the TREZ and vDREZ, and began between P3 and P7 at the DREZ. This retraction of PNS tissue was correlated with the extension of central glial tissue distally into the root of the three root entry zones (Fig. 2-6). As the PNS-CNS interface developed, it was displaced distally along the roots of all three root entry zones (Fig. 2-6). PTIs extending from the vDREZ deep to the brainstem surface (Ramer 2003; Rossiter and Fraher, 1990; Fraher and Rossiter, 1991) were present at P0, but increased in length throughout development, concomitant with the thickening of the ICP (Fig. 2-5, arrowheads). Blood vessels or possibly remnants of developing peripheral tissue were labelled with laminin in the brainstem and TREZ (especially at P0, Fig. 2-5, arrows) throughout development.

While the overall structure of the adult TREZ and DREZ are similar, the pattern of laminin immunoreactivity differed between the two entry zones. In the DREZ, laminin immunoreactivity was uniformly distributed throughout the PNS compartment.

In the trigeminal root and ganglion, laminin was consistently patchy (Fig. 2-7), with reduced staining toward the core of the root. In the ganglion, patchy laminin immunoreactivity also occurred, but in no consistent pattern. This was not an artefact of the staining procedure, since the geniculate ganglion and root, often present in the same sections, had uniformly intense laminin immunoreactivity.

p75 expression in the developing DREZ, TREZ and vDREZ

The p75 neurotrophin receptor is expressed in neurons and in immature-myelinating and non-myelinating Schwann cells (Taniuchi et al., 1986a; Taniuchi et al., 1986b; Jessen et al., 1990; Mikol et al., 2002). We examined p75 expression in all three root entry zones. Substantial p75 expression was present in both the central and peripheral components of all entry zones from P0 until P7, followed by a gradual decrease in expression from P7 to adulthood that was particularly apparent in the DREZ and TREZ (Fig. 2-8, Table 2-3). As expected, expression levels remained higher in the PNS than the CNS component of the three root entry zones from P14 to adulthood, as p75 decreases in the developing CNS but remains in non-myelinating Schwann cells in the PNS (Luque and Wintzer, 1999; Crockett et al., 1999; Jessen et al., 1990; reviewed in Jessen and Mirsky, 1999). An interesting pattern of p75 expression became apparent at the PNS-CNS interface of the P7 TREZ, the P14 vDREZ, and the adult DREZ. Dense, punctate areas of p75 immunoreactivity were aligned along the PNS-CNS boundary in the TREZ (P7 to adult), in the vDREZ from (P14 to adult) and the adult DREZ (arrowheads, Fig. 2-8). p75 expression levels appeared higher in the adult vDREZ than

the adult DREZ and TREZ. This was, in part, attributed to p75 immunoreactivity in the PTIs of the vDREZ (arrow, Fig. 2-8), which paralleled laminin expression.

NG2 expression in the developing DREZ, TREZ and vDREZ

The chondroitin sulphate proteoglycan NG2 is expressed by oligodendrocyte precursor cells (Levine et al., 1993; Nishiyama et al., 1996; Reynolds and Hardy, 1997; reviewed in Polito and Reynolds, 2005) as well as by pericytes associated with developing microvasculature (Ozerdem et al., 2001). We reasoned that a decrease in NG2 expression would indicate maturation of the three root entry zones, or more specifically, a transition of oligodendrocyte precursors to mature oligodendrocytes. NG2 expression decreased throughout postnatal development in both the PNS and CNS portions of all three root entry zones (Fig. 2-9, Table 2-3), and the developmental decline of NG2 expression corresponded with the increase of RIP-immunoreactive oligodendrocytes (Fig. 2-3). Expression levels remained high from P0 to P7 followed by a continual decrease in expression into adulthood in the DREZ. In the TREZ, NG2 levels were very high in early neonatal development prior to progressive decreases in expression after P3 into adulthood. NG2 expression levels in the vDREZ were high from P0 to P14 with decreased expression present in the adult. NG2 appeared most prominent in the neonatal (P0-P3) TREZ compared to the DREZ and vDREZ, although decline in NG2 expression throughout postnatal development occurred in the TREZ prior to the initiation of developmental decreases in NG2 expression at the DREZ and vDREZ. Diffuse, punctate depositions of NG2 were present in the PNS component of mature root

entry zones. They were most evident in the TREZ and vDREZ (arrowheads, Fig. 2-9). These depositions may be localized to nodes of Ranvier in the PNS (Martin et al., 2001).

3CB2 expression in the developing DREZ, TREZ and vDREZ

3CB2, a glial filament protein, is an established marker for the identification of radial glial cells in vivo (Barry and McDermott, 2005; Shibuya et al., 2003; and Prada et al., 1995). Because radial glial cells are known to differentiate into astrocytes in the spinal cord and brain of the developing CNS in vivo (Barry and McDermott, 2005; Levitt and Rakic, 1980; Edwards et al., 1990), we reasoned that by 3CB2 and GFAP double-labelling, we could establish a postnatal timecourse for this differentiation in each root entry zone. In each case, 3CB2 was present in a subset of GFAP-positive astrocytes, which was expected, as 3CB2 expression has been demonstrated in astrocytes in the developing brain (Prada et al., 1995). In the DREZ, 3CB2 expression remained low from P0 to P7, after which there was an increase in expression in a subset of GFAP-positive astrocytes (Fig. 2-10, Table 2-1). A decline in 3CB2 expression was present at the TREZ between P0 and P7 and was followed by an increase in expression in GFAP-positive astrocytes by P14 (Fig. 2-11, Table 2-1). In the vDREZ, co-labelling of GFAP and 3CB2 in astrocytes increased between P7 and P14 (Fig. 2-12, Table 2-1). Parallel to expression changes in the TREZ, 3CB2 levels decreased from P0 to P7. Early neonates (between P0 and P7) demonstrated a low level of 3CB2 expression in all three root entry zones that was rarely co-localized with GFAP expression. However, increases of 3CB2 occurred only in GFAP-positive astrocytes after P7.

Discussion

Formation of the DREZ, TREZ, and vDREZ: postnatal development of glial elements

Substantial rearrangement of PNS and CNS tissue occurs throughout postnatal development which precedes the formation of the mature PNS-CNS interface at sensory root entry zones. This rearrangement involves the extension of central tissue distally along the root, paralleled by a distal retraction of peripheral tissue from the surface of the CNS proper. The extent to which this retraction occurred and the developmental timecourse of concomitant central extension differed amongst the three root entry zones examined. The results of this study show that formation of the cranial nerve root entry zones investigated (the TREZ and vDREZ) begins earlier in development than that of the spinal dorsal root entry zone (DREZ). This is the first comprehensive and comparative immunohistochemical study of the postnatal development of the DREZ, TREZ, and vDREZ.

Postnatal development of the DREZ

The mature adult DREZ is characterized by a glial dome of central tissue that is composed of CNS glia (Berthold and Carlstedt, 1977; Fraher and Sheehan, 1987; Fraher, 1992; Fraher, 2000; Cafferty and Ramer, 2002). The distal edge of the glial dome is surrounded by the glia limitans, which itself is coated in basal lamina. The boundary between the glial dome and Schwann cells on the PNS side of the DREZ is very abrupt, occurring over one node of Ranvier (Cafferty and Ramer, 2002). During postnatal

development, distal extension of central glia into the dorsal root occurs. Fraher and Sheehan noted that this extension into the C7 dorsal root of the rat was complete by the second postnatal week (Fraher and Sheehan, 1987; reviewed in Fraher, 1992). In the present study, the extension of astrocytic processes into the DREZ was first evident at P3, similar to the time noted by Fraher and Sheehan (1987). This was followed shortly thereafter (between P3 and P7) by a progressive extension of oligodendrocytes into the dorsal root which then paralleled astrocytic extension until P14. Our immunohistochemical results indicate that the mature phenotype of the central tissue projections in the root was achieved by P14. Both GFAP-positive astrocytes and RIP-positive oligodendrocytes were present in a prominent dome at the distal end of the central tissue projection by the end of the second postnatal week. A displacement of peripheral tissue from the surface of the CNS occurred simultaneously with extension of central tissue, resulting in a distally-migrating PNS-CNS boundary into the dorsal root from P0 to P14. Not only did the boundary migrate distally over this time, but it also became denser, as there was an increase in central glial fibres (astrocytic and oligodendrocytic) from P0 to P14 that culminated in a glial dome.

Postnatal development of the TREZ

The glial structure of the adult rodent TREZ is similar to that of the DREZ, as both contain an abrupt PNS-CNS interface characterized by dense central tissue projections. In both the TREZ and DREZ, central tissue is present as a conical projection into the trigeminal and spinal dorsal roots, respectively. However, differences exist in the shape of the central projections between the TREZ and the DREZ. The TREZ contains a

central tissue projection that terminates in a smooth, convex fashion that is unlike the distally-tapering, more conically-shaped dome of central tissue in the DREZ (Fraher, 1992). In addition, the mature PNS-CNS interface exists further distally along the root from the CNS proper in the TREZ than in the DREZ. We found that the formation of the PNS-CNS boundary during postnatal development of the TREZ involved progressive extension and increased density of central tissue projections and concomitant retraction of peripheral tissue until P14, which is similar to DREZ development. The timeline of these events in the TREZ differed from that of the DREZ, as initiation of the extension of central tissue occurred earlier in the TREZ (Fig. 2-13). By P0, astrocytic processes were present in the TREZ, whereas astrocytes were not present in the DREZ until P3. Oligodendrocytes appeared in the TREZ between P0 and P3 while they were not present in the DREZ until between P3 and P7.

Postnatal development of the vDREZ

The adult structure of the rodent vDREZ is unique amongst sensory root entry zones. The PNS-CNS interface of the vDREZ is a jagged and uneven boundary, while the interfaces of the DREZ and TREZ are much more uniform. The PNS-CNS interface of the vDREZ is complicated by PTIs that lie deep within the brainstem in addition to central tissue projections lying distally within the dorsolateral sensory root of the vagus (Rossiter and Fraher, 1990; Fraher and Rossiter, 1991). Early development of the vDREZ is not well understood, but it may involve the invasion of Schwann cells along blood vessels that lead deep into the brainstem, forming PTIs and islands of peripheral tissue (Rossiter and Fraher, 1990). Alternatively, PTI Schwann cells could have derived

from the neural tube, or resulted from overgrowth of rootlet tissue from a nearby developing region of the brainstem, such as the ICP (Rossiter and Fraher, 1990). While the function of PTIs is unclear, they have been shown to support spontaneous and functional regeneration of injured vagal afferents into the brainstem (Ramer, 2003). Here we have demonstrated immunohistochemically (with laminin immunoreactivity) that PTIs are present at birth, suggesting that their early formation occurs embryonically. We found that the sequence of postnatal developmental processes involved in the formation of the mature vDREZ was similar to that of the other two root entry zones investigated, however the timeline of events most closely resembled that of the TREZ (Fig. 2-13). At birth, astrocytic processes had begun to extend into the root, however not to the same extent as that present in the TREZ. By P3, progressive extension of oligodendrocytes was underway, and increasing density of central glia (both astrocytes and oligodendrocytes) occurred until P14. These glia were apparently derived from the neighbouring ICP, which developed concomitantly with the vDREZ (Fig. 2-13). One aspect of vDREZ development differed substantially from that of the other root entry zones investigated. Progressive retraction of peripheral tissue from the CNS proper was not as apparent throughout the postnatal development of the vDREZ as it was in the DREZ and TREZ due to the relatively complex glial environment of the former, which is characterized by a jagged and uneven PNS-CNS boundary. Part of this complexity is attributable to the variable presence of PTIs within the vDREZ, as a series of crossections through the vDREZ reveals PTIs in only a portion of sections (Rossiter and Fraher, 1990, Fraher and Rossiter, 1991). Therefore, the structure of the PNS-CNS interface is much more complex within the vDREZ as compared to the TREZ and DREZ.

Changes in p75 expression throughout the postnatal development of the DREZ, TREZ, and vDREZ

p75, also known as the low-affinity neurotrophin receptor, is expressed in neurons and in premyelinating and non-myelinating Schwann cells (Taniuchi et al., 1986a; Taniuchi et al., 1986b; Jessen et al., 1990; Mikol et al., 2002). During postnatal development, p75 expression is downregulated in neurons in the CNS (Luque and Wintzer, 1999; Crockett et al., 1999) and myelinating Schwann cells but is maintained in non-myelinating Schwann cells (Jessen et al., 1990; reviewed in Jessen and Mirsky, 1999). In the present study, we demonstrated that p75 expression decreased in all three root entry zones after the first postnatal week. In the adult, p75 expression remained at low levels in Schwann cells, however p75 levels were much lower by comparison in mature sensory axons present in the CNS. In the P7 to adult TREZ, the P14 to adult vDREZ and the adult DREZ, punctate p75-positive staining was present along the PNS-CNS interface. It is likely that this is indicative of nonmyelinating Schwann cells of Remak fibres that have been aligned along the PNS-CNS boundary and which interact with unmyelinated sensory afferent fibres. Their presence at the transitional node (the node of Ranvier that spans the PNS-CNS interface) suggests that these Schwann cells support the high density of unmyelinated fibres which cross as bundles at this junction (Cafferty and Ramer, 2002). Nilsson et al. (1998) generated similar findings in a study of Schwann cell reorganization at the PNS-CNS interface of the S1 DREZ in the cat. They demonstrated that the numbers of short Schwann cells with non-functional myelin that associated with unmyelinated fibres at the PNS-CNS interface increased during postnatal development. The presence of Remak fibres at the PNS-CNS interface of the TREZ and

vDREZ prior to that of the spinal DREZ seems to suggest an earlier or accelerated development of the glial interface in cranial root entry zones.

Changes in RIP immunoreactivity throughout the postnatal development of the DREZ, TREZ, and vDREZ

The RIP antibody binds to an antigen present in oligodendrocyte cell bodies and myelinating processes (Friedman et al.,1989; Jhaveri et al., 1992; Berger and Frotscher, 1994; Butt et al., 1995; Butt et al., 1998; Hwang et al., 2006). RIP immunoreactivity was present in the central portions of all three root entry zones between P3 and P7, and increased over the first two postnatal weeks. However, RIP immunoreactivity was also located in what is very likely to be peripheral myelin and Schwann cell cytoplasm. RIP immunoreactivity was ubiquitously distributed throughout the PNS portion of all three root entry zones from P0 to P7. There was a marked decline in RIP immunoreactivity in the PNS portion of all three root entry zones throughout the first two postnatal weeks. Following this decline, RIP immunoreactivity was localized to paranodal regions along axons, as was especially evident in the P14 TREZ. This is the first example of RIP immunoreactivity in the PNS, as it is routinely purported to be an oligodendrocyte-specific marker. It may prove to be a useful marker for developing or immature PNS myelin.

Changes in 3CB2 expression throughout the postnatal development of the DREZ, TREZ, and vDREZ

3CB2 is expressed in radial glial cells of the CNS during embryonic and early postnatal development and in astrocytes later on in the development of the brain (Barry and McDermott, 2005; Prada et al., 1995). Our results support previously published observations, as 3CB2 expression was mainly localized to radial glial cells in the first week of postnatal development of the three root entry zones examined. There was little co-localization of this glial filament protein with GFAP throughout this period of development. Later on (between P7 and P14), 3CB2 was upregulated in a subset of GFAP-positive astrocytes in all three root entry zones. Increased co-localization of 3CB2 and GFAP occurred in astrocytes between the first and second postnatal weeks in the three root entry zones, reaching adult levels by P14. It is not clear from our results whether or not the GFAP-positive astrocytes that upregulated 3CB2 were exclusively derived from 3CB2-positive radial glial cells or rather that 3CB2 was being upregulated in a general population of GFAP-positive astrocytes between P7 and P14 in the three root entry zones. The latter explanation seems more likely, as 3CB2 expression was very low in the three root entry zones throughout the first postnatal week, followed by a dramatic increase by P14.

Changes in NG2 expression throughout the postnatal development of the DREZ, TREZ, and vDREZ

The chondroitin sulphate proteoglycan NG2 is present in developing pericytes surrounding blood vessels and oligodendrocyte precursor cells of the developing CNS

(Ozerdem et al., 2001; Levine et al., 1993; Nishiyama et al., 1996; Reynolds and Hardy, 1997; reviewed in Polito and Reynolds, 2005). NG2 expression declined throughout postnatal development as expected in all three root entry zones investigated. The decrease in NG2 expression throughout development correlated loosely with increased RIP immunoreactivity in the central portions of all three root entry zones, which is expected as oligodendrocytes present in the central tissue projections are maturing throughout postnatal development. NG2 expression peaked earlier in the TREZ than in the vDREZ or the DREZ, and the subsequent decreases of NG2 were evident in the TREZ prior to that of the other two root entry zones. This may reflect the earlier maturation of the glial elements of the TREZ compared to the other root entry zones. While the timeframe for decreases in NG2 differed slightly between root entry zones, the developmental trend and the end results were similar: high levels of NG2 were present in the first postnatal week, followed by a progressive decrease into adulthood. In the adult, punctate NG2 reactivity was present in all three root entry zones and may be localized to nodes of Ranvier in the PNS, as has been demonstrated by Martin et al. (2001). In the adult CNS, one population of NG2-positive cells (synantocytes) have been demonstrated to interact with nodes of Ranvier and these may partly account for the low level of NG2-expressing cells in the CNS (Butt et al., 1999; reviewed in Butt et al., 2002). In addition, other studies suggest that a population of NG2-expressing cells in the adult CNS are actively dividing and may give rise to oligodendrocytes (Horner et al., 2000; Dawson et al., 2003; Polito and Reynolds, 2005).

Concluding remarks

The initial stages of cranial nerve root entry zone development occur prior to those of the spinal dorsal root entry zone (Fig. 2-13). The reasons for this are not clear, however this may reflect the functional requirements of the neonatal animal. A neonatal rat must have fully-developed facial innervation which is necessary for suckling behaviour (mediated by the trigeminal nerve), and fully-developed visceral innervation for maintaining internal homeostatic mechanisms (mediated by the vagus), whereas trunk somatosensation (mediated by the dorsal root) is arguably less critical to the survival of the altricial neonate.

By P14, the mature phenotype had been reached in all three root entry zones studied, suggesting that the first two postnatal weeks are the most critical for root entry zone development. A study in support of our developmental findings was conducted by Carlstedt (1988). It was found that early on in postnatal development (P0-P2), axonal regeneration is possible through the DREZ into the spinal cord following a crush injury of the dorsal root, whereas axons are unable to cross the PNS-CNS interface of the DREZ following root injury after the first postnatal week, demonstrating that the immature DREZ is capable of supporting regeneration, whereas the more mature DREZ is not (Carlstedt, 1988). The glial elements of the mature DREZ, in particular astrocytes, are known to inhibit axons from regenerating into the spinal cord following root injury (Carlstedt, 1985; Liuzzi and Lasek, 1987; Liu et al., 1998; Zhang et al., 2001; McPhail et al., 2005; reviewed in Cafferty and Ramer, 2002). Given that the glial elements of the

TREZ mature earlier than those of the DREZ, the TREZ may be impermeable to regenerating axons following trigeminal rhizotomy as early as P0.

An interesting developmental feature that became apparent in all three root entry zones was the delay in oligodendrocyte migration compared to astrocyte invasion of the root. Perhaps extension of astrocytic processes is needed to signal to peripheral tissue in order to initiate retraction, and oligodendrocyte extension into the root occurs after peripheral tissue has retracted. Although the molecular mechanisms involved in the developmental processes illustrated here remain unknown, this study has provided a comprehensive summary of the developmental events involved in forming the glial elements of the DREZ, TREZ, and vDREZ.

Table 2-1. Relative expression levels of GFAP and 3CB2 throughout the postnatal development of the DREZ, TREZ, and vDREZ.

Age	GFAP			3CB2		
	DREZ	TREZ	vDREZ	DREZ	TREZ	vDREZ
P0	-	++	+	++	+++	+++
P3	++	++	++	+	+	+
P7	+++	+++	+++	+	+	+
P14	++++	++++	++++	++++	++++	++++
Adult	++++	++++	++++	++++	++++	++++

Relative expression levels: "-" = absent, "+" = lowest, "++++" = highest.

Table 2-2. Relative expression levels of RIP in the CNS and PNS compartments of the DREZ, TREZ, and vDREZ throughout postnatal development.

Age	RIP					
	DREZ		TREZ		vDREZ	
	CNS	PNS	CNS	PNS	CNS	PNS
P0	-	++++	-	++++	-	++++
P3	-	+++	++	+++	++	++
P7	+++	+++	+++	+++	+++	+++
P14	++++	++	++++	+++	++++	+++
Adult	++++	++	++++	++	++++	++

Relative expression levels: "-" = absent, "+" = lowest, "++++" = highest.

Table 2-3. Relative expression levels of p75 and NG2 throughout the postnatal development of the DREZ, TREZ, and vDREZ.

Age	p75			NG2		
	DREZ	TREZ	vDREZ	DREZ	TREZ	vDREZ
P0	+++	++++	+++	+++	++++	+++
P3	++++	++++	+++	+++	++++	+++
P7	++++	+++	+++	++++	+++	+++
P14	++	++	++	++	++	++
Adult	+	+	++	++	+	+

Relative expression levels: "-" = absent, "+" = lowest, "++++" = highest.

Figure 2-1. A schematic representation of the anatomical locations of the dorsal root entry zone (DREZ), trigeminal root entry zone (TREZ), and vagal dorsal root entry zone (vDREZ) in the Long Evans rat. **a**, Transections of cervical spinal cord were prepared for immunohistochemical analysis of the developing DREZ. **b**, Sagittal sections through the pons, where trigeminal sensory afferents project to central nuclei, were prepared for immunohistochemical analysis of the developing TREZ. Vagal afferents that project through the vDREZ terminate at the paratrigeminal nucleus (Pa5) and the nucleus of the solitary tract (nts) (Ramer, 2003). **c**, Transverse sections through the brainstem were prepared for immunohistochemical analysis of the developing vDREZ. Images were adapted from Paxinos and Watson (1997).

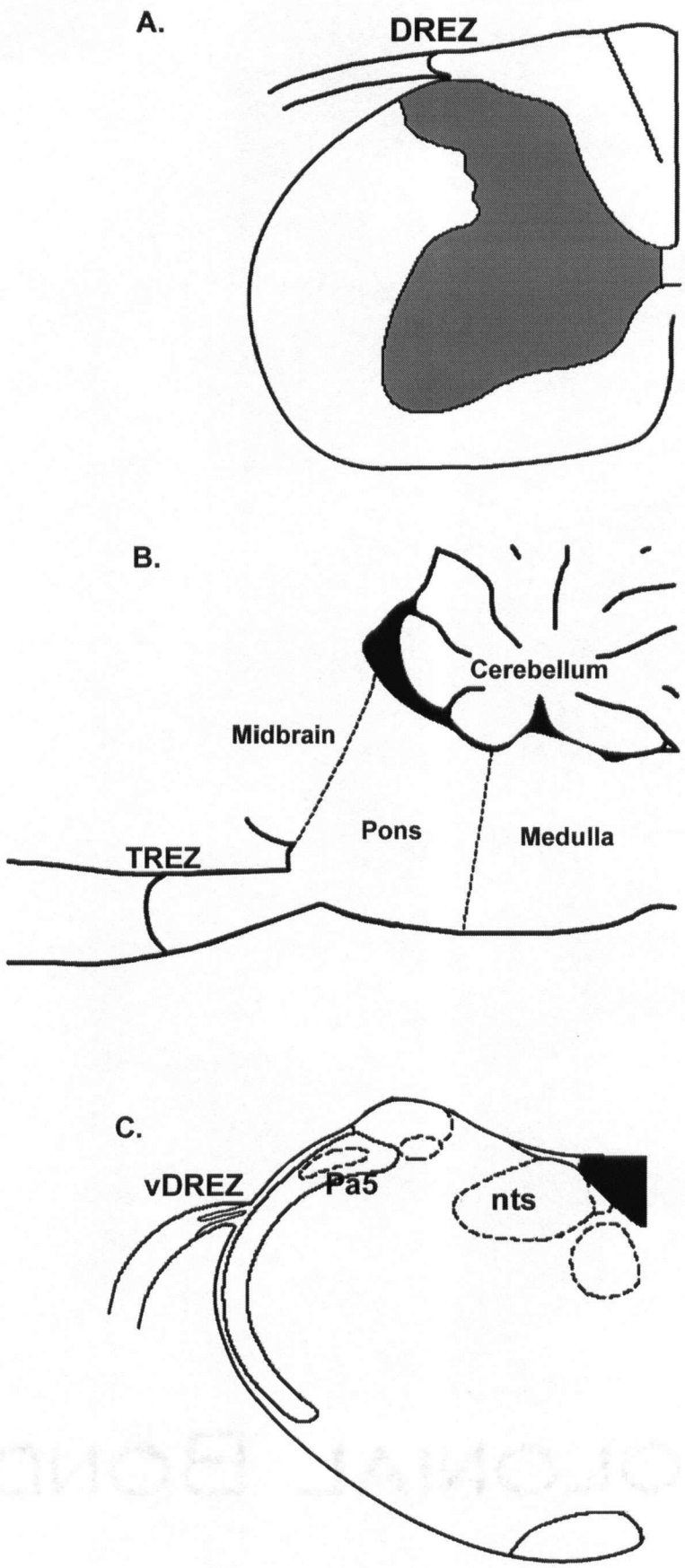

A.
DREZ
B.
Cerebellum
Midbrain
Pons
Medulla
TREZ
C.
vDREZ
Pa5
nts

Figure 2-2. GFAP expression in the DREZ, TREZ, and vDREZ of the neonatal (P0, P3, P7, and P14) and adult Long Evans rat. GFAP-positive astrocytes were present in the trigeminal sensory root and vagal root by P0, prior to their presence in the dorsal root. Distal extension of GFAP-positive astrocytes and their processes (arrows) into the root from the CNS occurred progressively from P0-P14 in the TREZ and vDREZ and P3-P14 in the DREZ. Dotted lines indicate the root boundary. Scale = 100 μm.

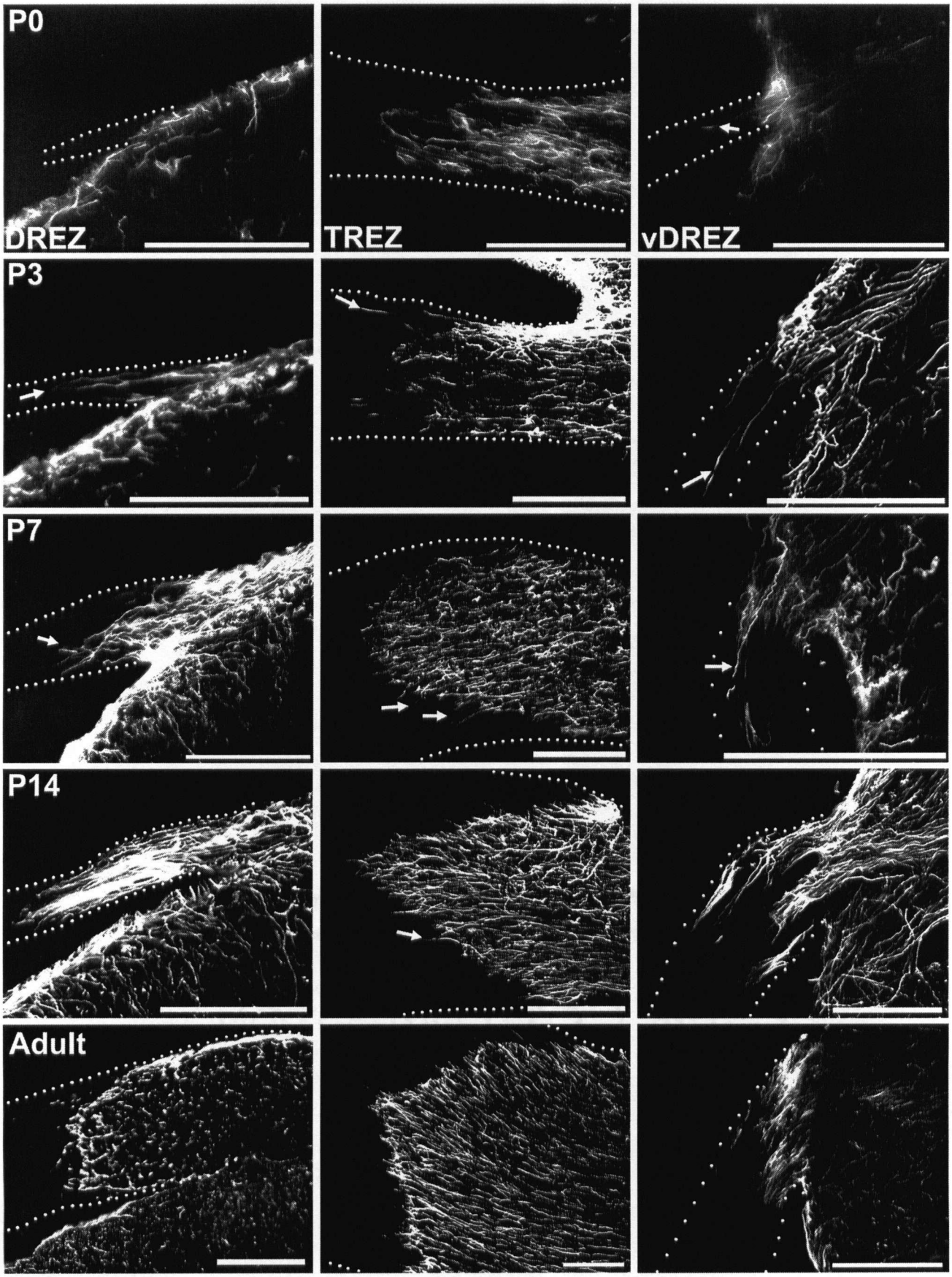

P0
DREZ
TREZ
vDREZ
P3
P7
P14
Adult

Figure 2-3. RIP immunoreactivity in the DREZ, TREZ, and vDREZ of the neonatal (P0, P3, P7, and P14) and adult Long Evans rat. Extensive RIP immunoreactivity was present in peripheral nervous tissue of the roots of all three root entry zones at early timepoints in neonatal development (P0-P7). By P14, punctate RIP staining was present in the peripheral component of the root in all three root entry zones, but was especially apparent in the TREZ (arrowheads). This punctate staining remained in the adult. Progressive distal expansion of RIP immunoreactive central tissue (oligodendrocytes) into the root occurred from P3 to P14 in the TREZ and vDREZ, and from sometime between P3 and P7 to P14 in the DREZ. Note the development of the inferior cerebellar peduncle (ICP) at the vDREZ, which was first evident at P3 (arrows). Scale = 100 μm.

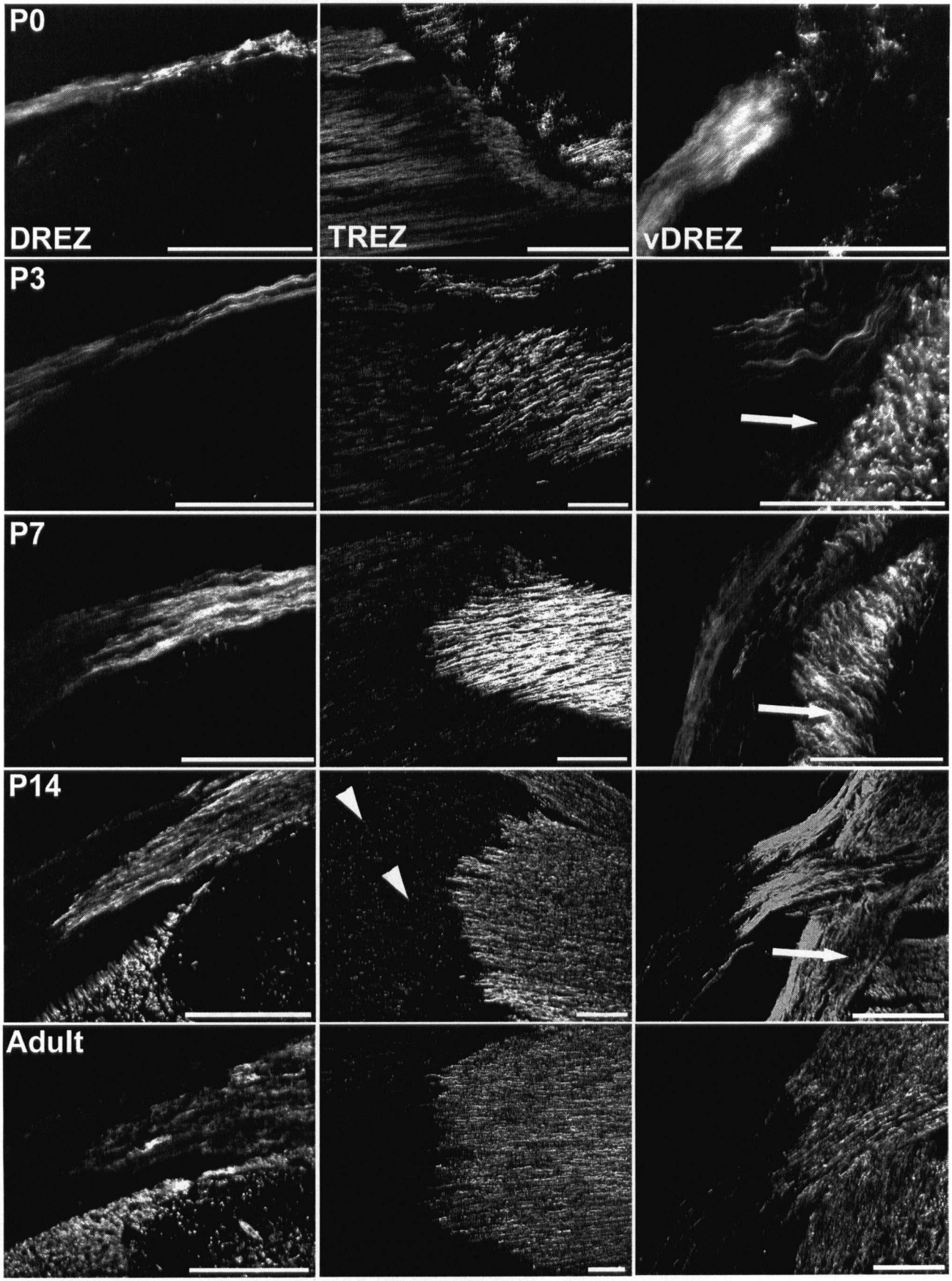

P0
DREZ
TREZ
vDREZ
P3
P7
P14
Adult

Figure 2-4. RIP stains paranodal regions surrounding peripheral nodes of Ranvier. Double-staining with RIP (red) and anti-Kv1.2 (green), which labels voltage-gated potassium channels known to be localized to the juxtaparanodal region of myelinated axons (Wang et al., 1993; Rasband et al., 1998), demonstrated RIP immunoreactivity in paranodal regions of axons in the peripheral component of the P14 TREZ (inset). The arrowhead indicates the origin of the enlarged nodal region in the inset. Scale of large image = 100 μm, scale of inset = 16 μm.

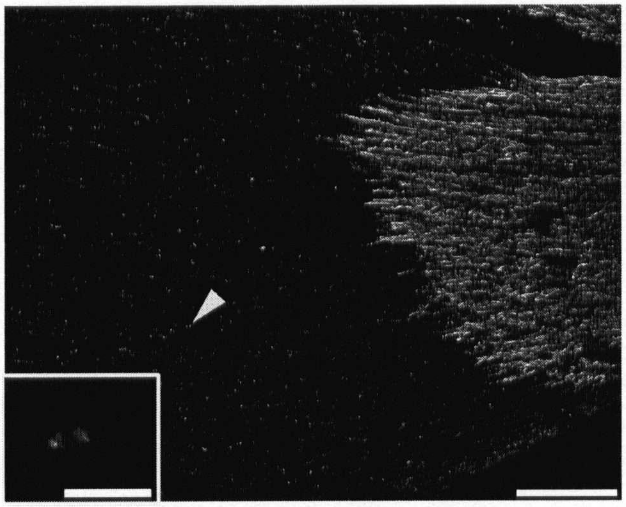


Figure 2-5. Laminin expression in the DREZ, TREZ, and vDREZ of the neonatal (P0, P3, P7, and P14) and adult Long Evans rat. Laminin immunoreactivity was present in the peripheral components of all three root entry zones throughout development. Retraction of peripheral tissue in all three root entry zones from the CNS proper was evident throughout postnatal development, occurring throughout P3-P14 in the DREZ and P0-P14 in the TREZ and vDREZ. Note the laminin-positive peripheral tissue insertions (PTIs) deep into the brainstem in the P0, P14 and adult vDREZ (arrowheads). Laminin-positive blood vessels or remnants of developing peripheral tissue were clearly present in the central component of the P0 TREZ and in the brainstem throughout development (arrows). Scale = 100 μm.

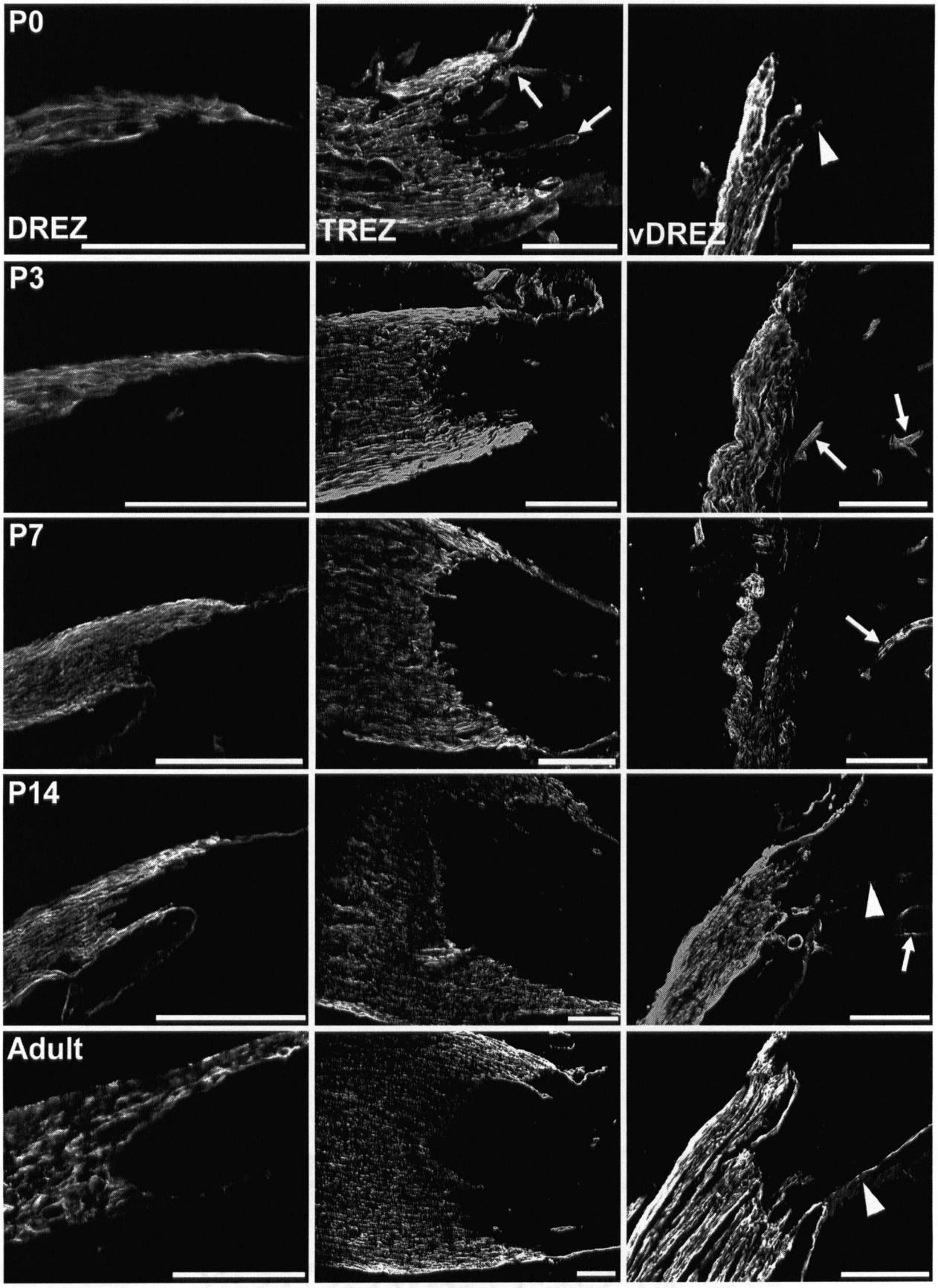
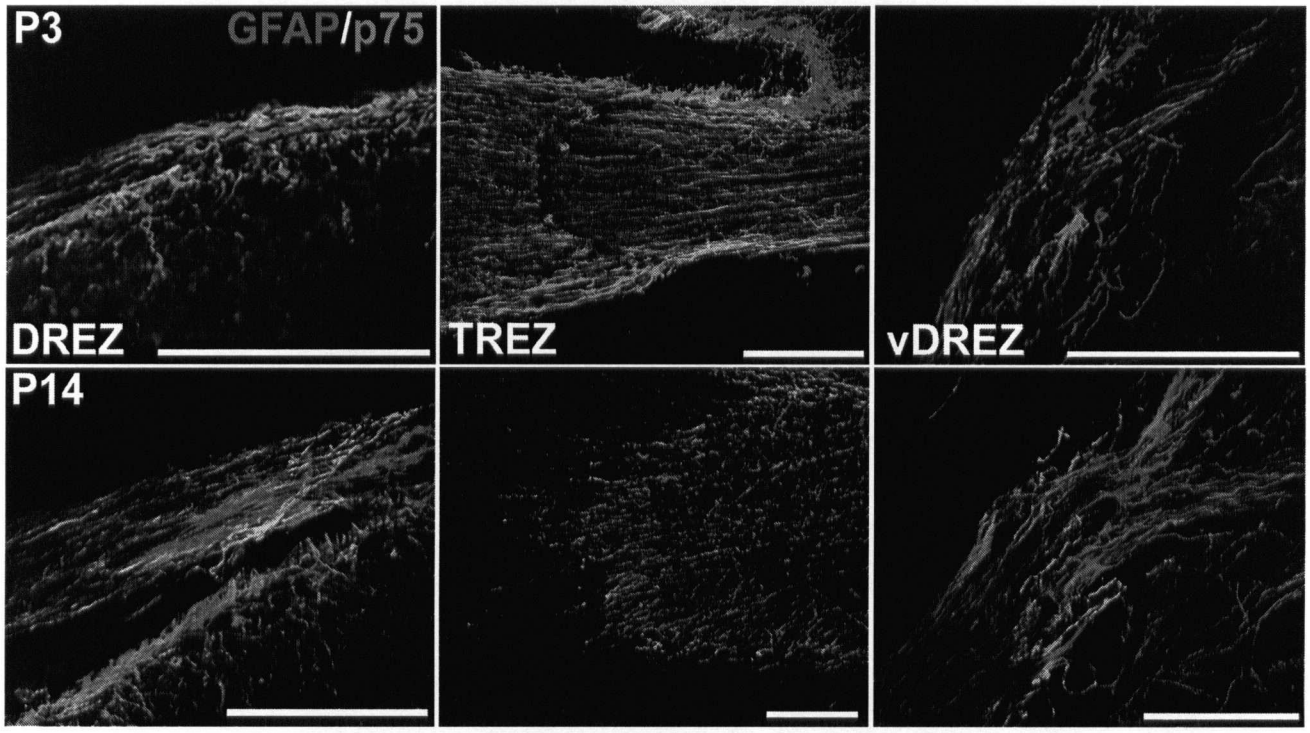

P0
DREZ
TREZ
vDREZ
P3
P7
P14
Adult

Figure 2-6. Merged images illustrating the development of the DREZ, TREZ, and vDREZ. GFAP-p75 and RIP-laminin double-labelling at the P3 and P14 root entry zones. Distal displacement of the PNS-CNS interface along the root of each entry zone occurred throughout postnatal development. The density of central glia (GFAP and RIP immunoreactivity) increased throughout postnatal development in each sensory root entry zone. Scale = 100 μm.


P3
GFAP/p75
DREZ
TREZ
vDREZ
P14

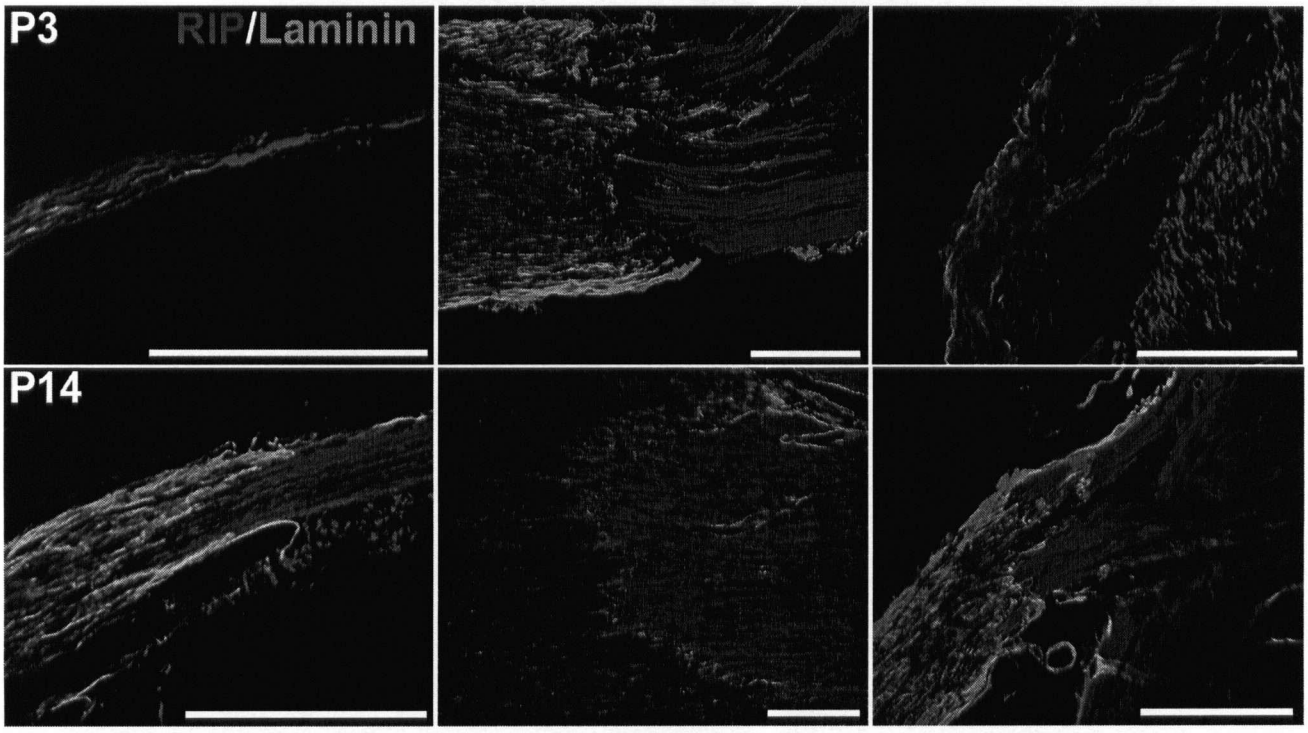

P3
RIP/Laminin
P14

Figure 2-7. Laminin immunoreactivity is patchy in the adult trigeminal ganglion and root. A laminin-stained parasaggital section which included the trigeminal ganglion, root, pons and geniculate root and ganglion. The interface between CNS and PNS is indicated by arrows. Laminin immunoreactivity in the peripheral portion of the trigeminal root was more intense around the root periphery, and less intense in the core region. Insets show patchy laminin immunoreactivity in the trigeminal ganglion and uniformly bright laminin immunoreactivity in the geniculate. Scale = 400 μm (or 200 μm in the insets).

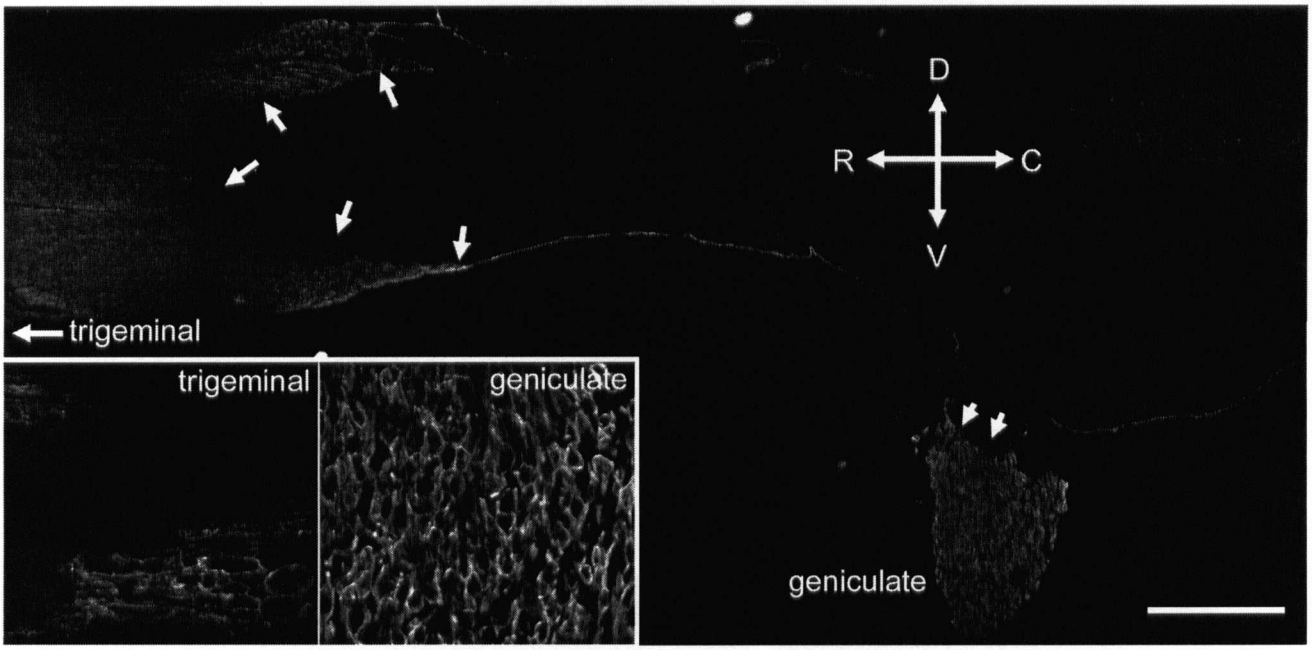

D
R
C
V
trigeminal
trigeminal
geniculate
geniculate

Figure 2-8. p75 expression in the DREZ, TREZ, and vDREZ of the neonatal (P0, P3, P7, and P14) and adult Long Evans rat. p75 expression levels remained high in the three root entry zones throughout early neonatal development (P0-P7) but declined from P7 to adult. By P7, dense regions of p75 immunoreactivity were located in the PNS tissue along the PNS-CNS interface of the TREZ and remained in the adult. A similar staining pattern was present in the P14 and adult vDREZ and in the adult DREZ (arrowheads). p75-positive PTIs were evident in the adult vDREZ (arrow). Scale = 100 μm.

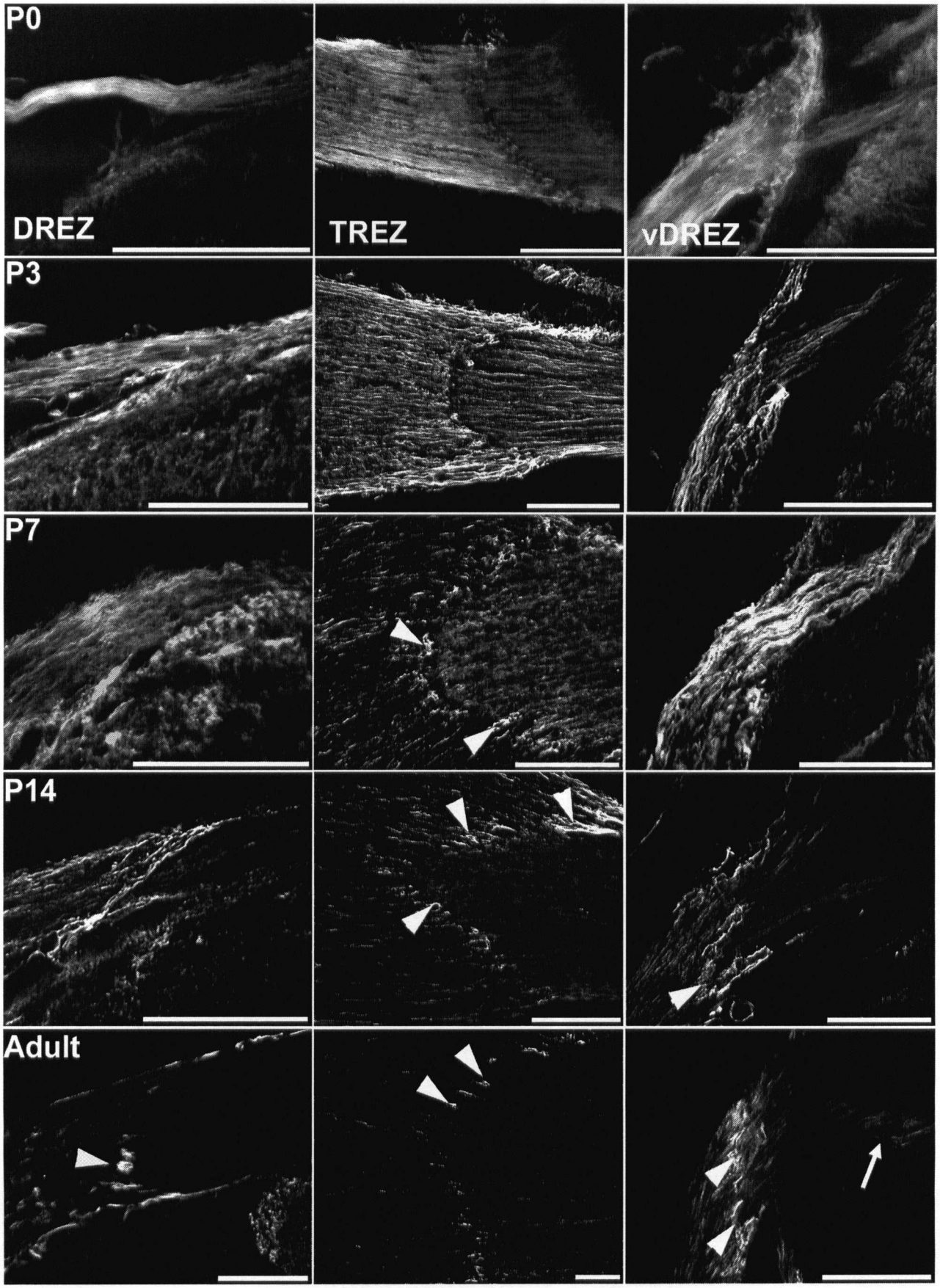

P0
DREZ
TREZ
vDREZ
P3
P7
P14
Adult

Figure 2-9. NG2 expression in the DREZ, TREZ, and vDREZ of the neonatal (P0, P3, P7, and P14) and adult Long Evans rat. NG2 was present in both the peripheral and central tissue components of all three root entry zones. In the TREZ, NG2 expression was highest early on in neonatal development (P0-P3), then progressively decreased into adulthood. Expression levels in the DREZ and vDREZ remained high from P0-P14, but decreased into adulthood. NG2 expression levels peaked at P7 in the DREZ and P0-P7 in the vDREZ. Note the punctate NG2 staining in the adult TREZ and vDREZ (arrowheads). Scale = 100 μm.

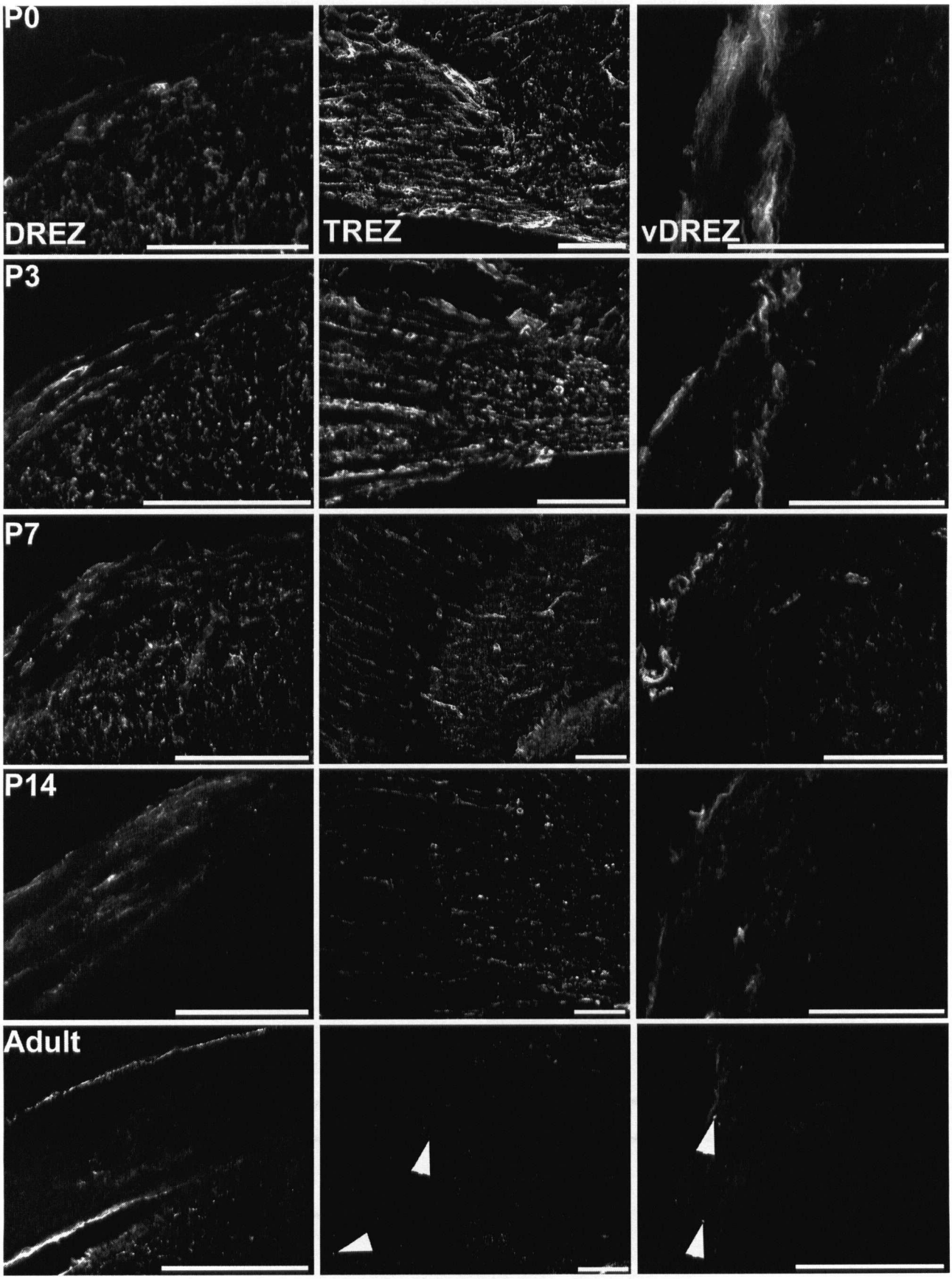

P0
DREZ
TREZ
vDREZ
P3
P7
P14
Adult

Figure 2-10. 3CB2-GFAP double-labelling in the neonatal (P0-P14) and adult DREZ of the Long Evans rat. 3CB2 expression increased after P7 in GFAP-positive astrocytes. There were no 3CB2-GFAP co-labelled processes evident at P0, but by P3, co-labelling was present. A greater proportion of GFAP-positive astrocytes were also 3CB2-positive in the DREZ of older neonatal (P14) and adult animals than in the P0-P7 DREZ. Scale = 100 μm.

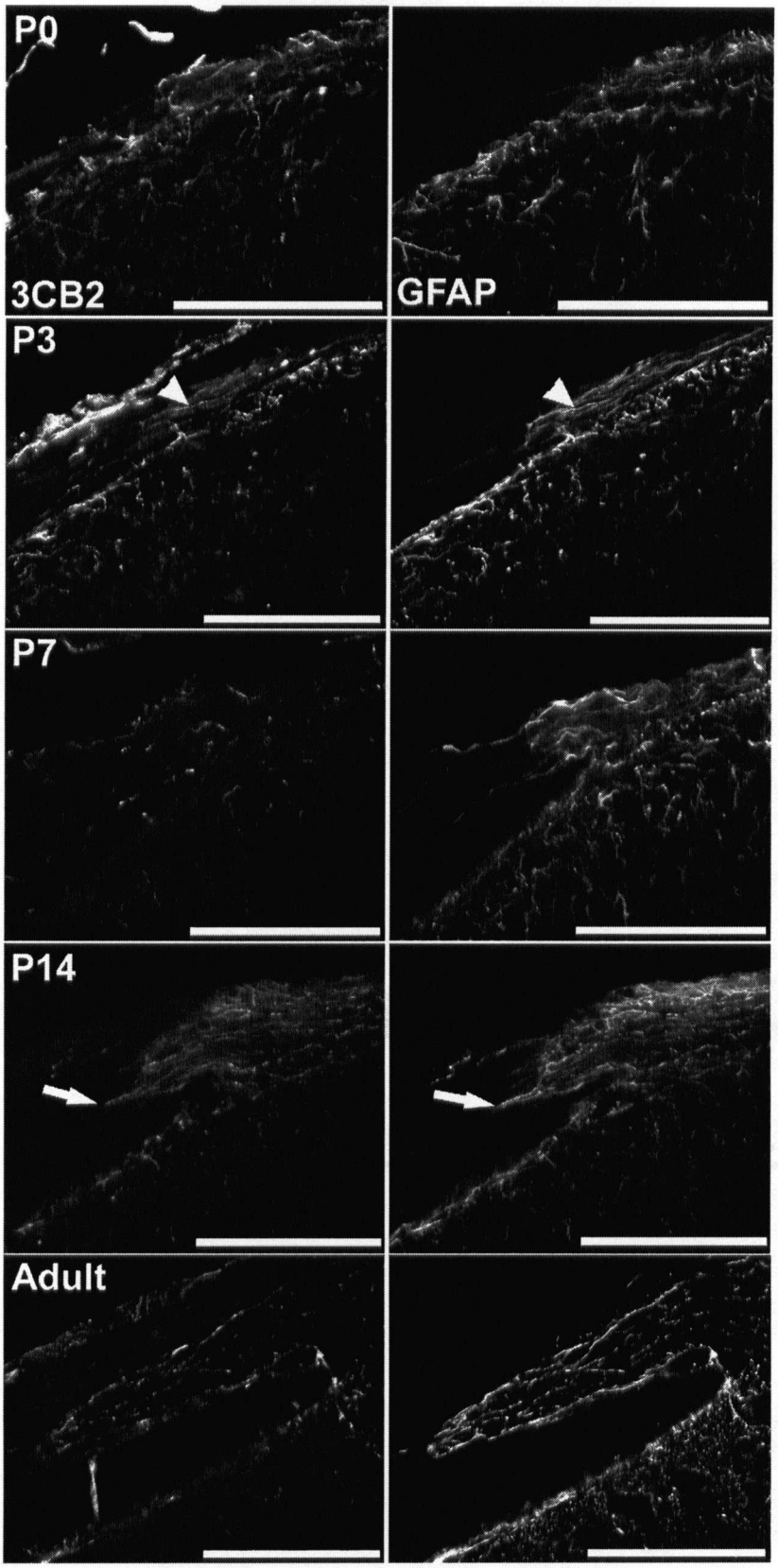

P0
3CB2
GFAP
P3
P7
P14
Adult

Figure 2-11. 3CB2-GFAP double-labelling in the neonatal (P0-P14) and adult TREZ of the Long Evans rat. 3CB2 expression levels decreased in the TREZ between P0 and P3, with an increase by P7. By P14 and into adulthood, there was an increase in 3CB2 expression in many GFAP-positive astrocytes. Dotted lines indicate root boundaries. Scale = 100 μm.

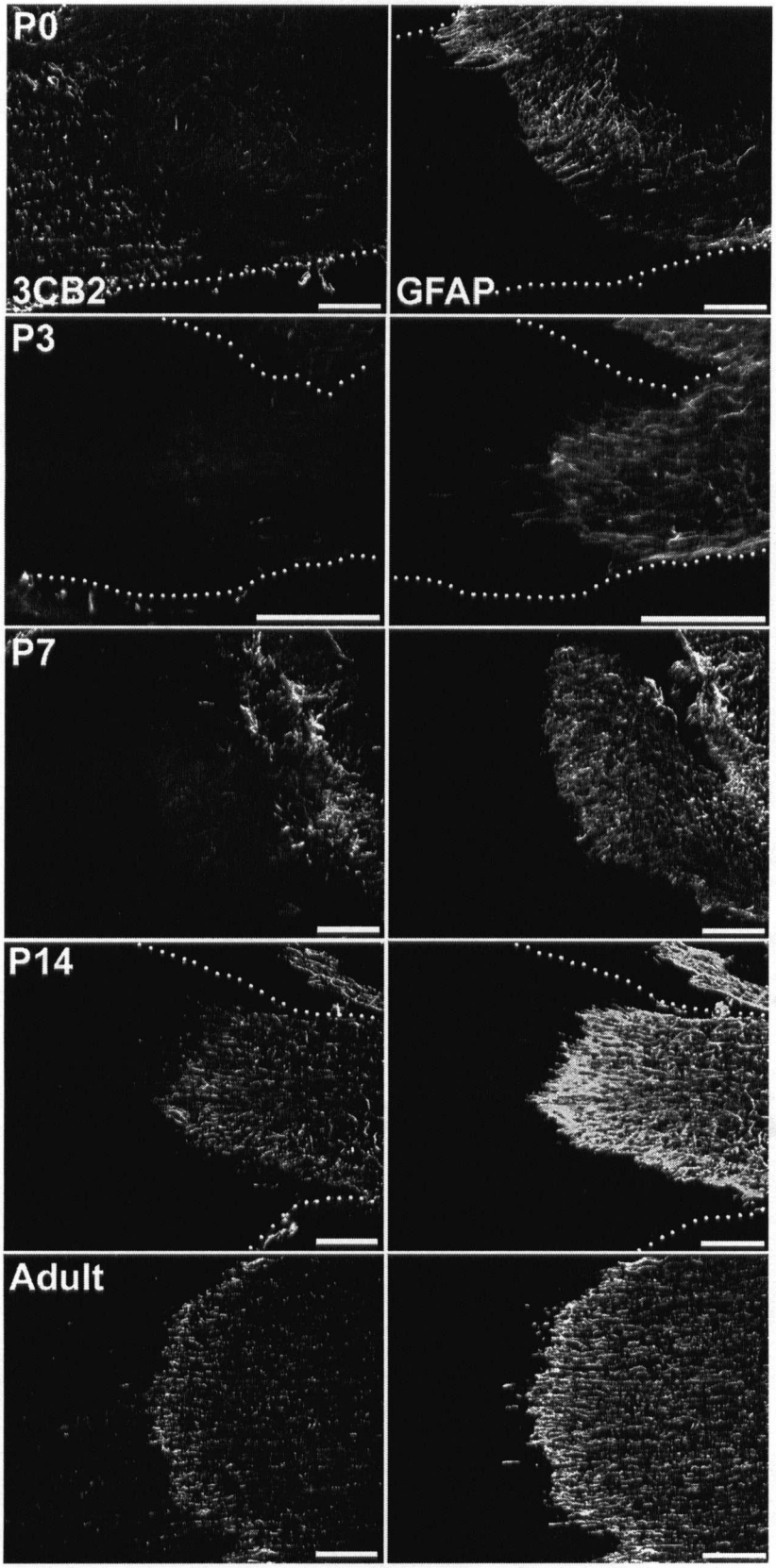

P0
3CB2
GFAP
P3
P7
P14
Adult

Figure 2-12. 3CB2-GFAP double-labelling in the neonatal (P0-P14) and adult vDREZ of the Long Evans rat. 3CB2 expression decreased in the vDREZ after P0 until P7, and was upregulated in GFAP-positive astrocytes by P14 and in the adult. 3CB2-GFAP co-labelled astrocytic processes in the brainstem were first apparent by P3. Scale = 100 μm.

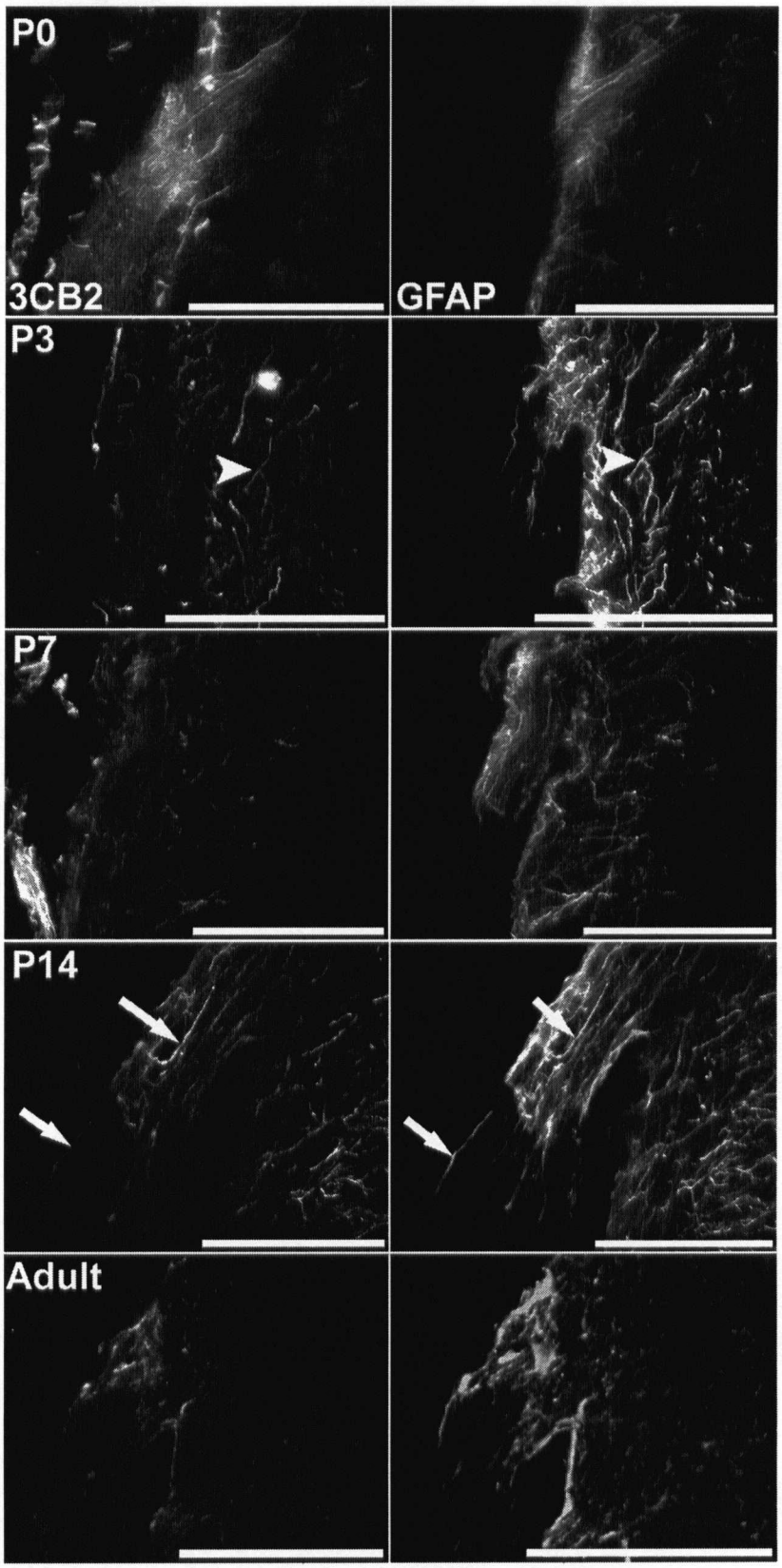
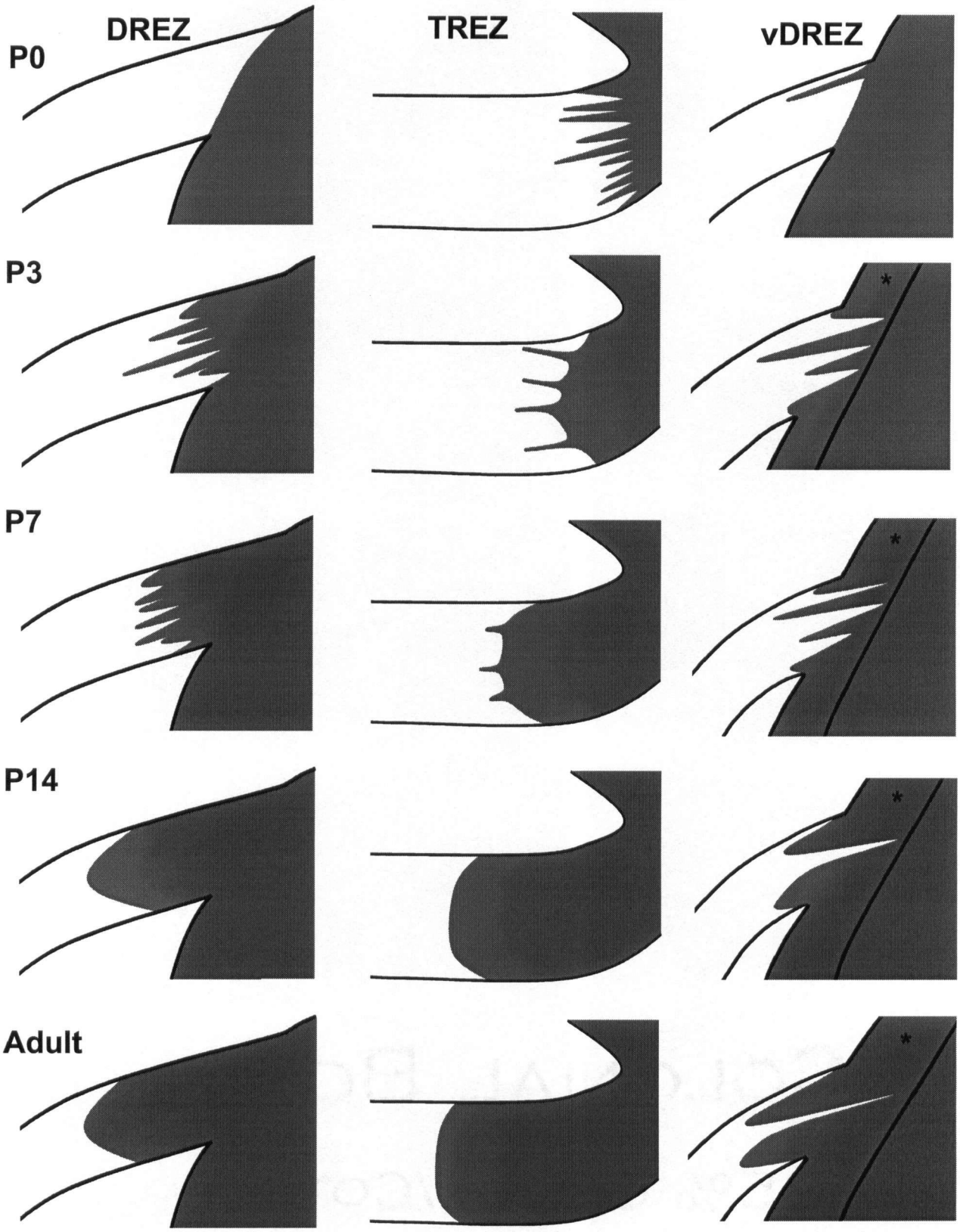

P0
3CB2
GFAP
P3
P7
P14
Adult

Figure 2-13. Schematic illustrating the postnatal development of the PNS-CNS interface of the DREZ, TREZ, and vDREZ. The central glia present in the early neonatal (P0-P3) root entry zones mainly consisted of astrocytes, as oligodendrocyte extension did not occur until after P3. Numerous astrocyte extensions were present in the TREZ at P0, while few were located in the vDREZ at this time. Astrocytic extension was not present in the DREZ until P3. In each root entry zone, the density of central tissue projections increased throughout postnatal development until the mature, adult phenotype was reached at P14. After P14, only modest extension of central glia into the three root entry zones occurred. Note the development of the ICP adjacent to the developing vDREZ (*). As the amount of central tissue derived from the ICP increased within the vDREZ throughout postnatal development, the PTIs present in the vDREZ were elongated but reduced in overall size.


DREZ
TREZ
vDREZ
P0
P3
P7
P14
Adult
*
*
*
*

Bibliography

Barry D, McDermott K (2005) Differentiation of radial glia from radial precursor cells and transformation into astrocytes in the developing rat spinal cord. Glia 50: 187-197.

Berger T, Frotscher M (1994) Distribution and morphological characteristics of oligodendrocytes in the rat hippocampus in situ and in vitro: an immunocytochemical study with the monoclonal Rip antibody. J Neurocytol 23: 61-74.

Berthold CH, Carlstedt T (1977) Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. II. General organization of the transitional region in S1 dorsal rootlets. Acta Physiol Scand Suppl 446: 23-42.

Butt AM, Ibrahim M, Ruge FM, Berry M (1995) Biochemical subtypes of oligodendrocyte in the anterior medullary velum of the rat as revealed by the monoclonal antibody Rip. Glia 14: 185-197.

Butt AM, Ibrahim M, Berry M (1998) Axon-myelin sheath relations of oligodendrocyte unit phenotypes in the adult rat anterior medullary velum. J Neurocytol 27: 259-269.

Butt AM, Duncan A, Hornby MF, Kirvell SL, Hunter A, Levine JM, Berry M (1999) Cells expressing the NG2 antigen contact nodes of Ranvier in adult CNS white matter. Glia 26: 84-91.

Butt AM, Kiff J, Hubbard P, Berry M (2002) Synantocytes: new functions for novel NG2 expressing glia. J Neurocytol 31: 551-565.

Cafferty WBJ, Ramer MS (2002) Promoting Sensory Axon Regeneration Across the PNS-CNS Interface. In: Glial Interfaces in the Nervous System: Role in Repair and Plasticity (Aldskogius H, Fraher JP, eds), pp 23-40. Amsterdam: IOS Press.

Carlstedt T (1985) Regenerating axons form nerve terminals at astrocytes. Brain Res 347: 188-191.

Carlstedt T (1988) Reinnervation of the mammalian spinal cord after neonatal dorsal root crush. J Neurocytol 17: 335-350.

Cornbrooks CJ, Carey DJ, McDonald JA, Timpl R, Bunge RP (1983) In vivo and in vitro observations on laminin production by Schwann cells. Proc Natl Acad Sci U S A 80: 3850-3854.

Crockett DP, Wang L, Zhang RX, Egger MD (1999) Distribution of the low-affinity neurotrophin receptor (p75) in the developing trigeminal brainstem complex in the rat. Anat Rec 254: 549-565.

Dawson MR, Polito A, Levine JM, Reynolds R (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 24: 476-488.

Edwards MA, Yamamoto M, Caviness VS, Jr. (1990) Organization of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. Neuroscience 36: 121-144.

Fraher JP, Sheehan MM (1987) The CNS-PNS transitional zone of rat cervical dorsal roots during development and at maturity. A morphological and morphometric study. J Anat 152: 189-203.

Fraher JP, Rossiter JP (1991) Myelin-axon relationships established by rat vagal Schwann cells deep to the brainstem surface. J Comp Neurol 304: 253-260.

Fraher JP (1992) The CNS-PNS transitional zone of the rat. Morphometric studies at cranial and spinal levels. Prog Neurobiol 38: 261-316.

Fraher JP (2000) The transitional zone and CNS regeneration. J Anat 196 (Pt 1): 137-158.

Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG (1989) In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. Glia 2: 380-390.

Henry MA, Rzasa RS, Beeler JJ, Levinson SR (2005) Caspr reveals an aggregation of nodes and flanking node free zones at the rat trigeminal sensory root and dorsal root entry zones. Glia 49: 445-450.

Horner PJ, Power AE, Kempermann G, Kuhn HG, Palmer TD, Winkler J, Thal LJ, Gage FH (2000) Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J Neurosci 20: 2218-2228.

Hwang IK, Yoo KY, Kim DS, Kang TC, Lee BH, Kim YS, Won MH (2006) Chronological distribution of rip immunoreactivity in the gerbil hippocampus during normal aging. Neurochem Res 31: 1119-1125.

Jessen KR, Morgan L, Stewart HJ, Mirsky R (1990) Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. Development 109: 91-103.

Jessen KR, Mirsky R (1999) Schwann cells and their precursors emerge as major regulators of nerve development. Trends Neurosci 22: 402-410.

Jhaveri S, Erzurumlu RS, Friedman B, Schneider GE (1992) Oligodendrocytes and myelin formation along the optic tract of the developing hamster: an immunohistochemical study using the Rip antibody. Glia 6: 138-148.

Levine JM, Stincone F, Lee YS (1993) Development and differentiation of glial precursor cells in the rat cerebellum. Glia 7: 307-321.

Levitt P, Rakic P (1980) Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J Comp Neurol 193: 815-840.

Liu L, Persson JK, Svensson M, Aldskogius H (1998) Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transection. Glia 23: 221-238.

Liuzzi FJ, Lasek RJ (1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. Science 237: 642-645.

Luque JM, Wintzer ME (1999) Detection of p75 mRNA in developing marsupial CNS by cross-hybridization with rat oligonucleotide probes. Brain Res Mol Brain Res 65: 129-134.

Martin S, Levine AK, Chen ZJ, Ughrin Y, Levine JM (2001) Deposition of the NG2 proteoglycan at nodes of Ranvier in the peripheral nervous system. J Neurosci 21: 8119-8128.

McPhail LT, Plunet WT, Das P, Ramer MS (2005) The astrocytic barrier to axonal regeneration at the dorsal root entry zone is induced by rhizotomy. Eur J Neurosci 21: 267-270.

Mikol DD, Scherer SS, Duckett SJ, Hong HL, Feldman EL (2002) Schwann cell caveolin-1 expression increases during myelination and decreases after axotomy. Glia 38: 191-199.

Nilsson R, I, Berthold CH, Carlstedt T (1998) Redistribution of Schwann cells at the developing PNS-CNS borderline. An ultrastructural and autoradiographic study on the S1 dorsal root of the cat. J Neurocytol 27: 85-97.

Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB (1996) Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J Neurosci Res 43: 299-314.

Ozaki S, Snider WD (1997) Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J Comp Neurol 380: 215-229.

Ozerdem U, Grako KA, Dahlin-Huppe K, Monosov E, Stallcup WB (2001) NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev Dyn 222: 218-227.

Paxinos G., Watson C. (1997) The rat brain in stereotaxic coordinates. San Diego: Academic.

Polito A, Reynolds R (2005) NG2-expressing cells as oligodendrocyte progenitors in the normal and demyelinated adult central nervous system. J Anat 207: 707-716.

Prada FA, Dorado ME, Quesada A, Prada C, Schwarz U, de la Rosa EJ (1995) Early expression of a novel radial glia antigen in the chick embryo. Glia 15: 389-400.

Ramer MS (2003) Spontaneous functional viscerosensory regeneration into the adult brainstem. J Neurosci 23: 9770-9775.

Rasband MN, Trimmer JS, Schwarz TL, Levinson SR, Ellisman MH, Schachner M, Shrager P (1998) Potassium channel distribution, clustering, and function in remyelinating rat axons. J Neurosci 18: 36-47.

Reynolds R, Hardy R (1997) Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo. J Neurosci Res 47: 455-470.

Rossiter JP, Fraher JP (1990) Intermingling of central and peripheral nervous tissues in rat dorsolateral vagal rootlet transitional zones. J Neurocytol 19: 385-407.

Shibuya S, Miyamoto O, Itano T, Mori S, Norimatsu H (2003) Temporal progressive antigen expression in radial glia after contusive spinal cord injury in adult rats. Glia 42: 172-183.

Sosale A, Robson JA, Stelzner DJ (1988) Laminin distribution during corticospinal tract development and after spinal cord injury. Exp Neurol 102: 14-22.

Taniuchi M, Clark HB, Johnson EM, Jr. (1986a) Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc Natl Acad Sci U S A 83: 4094-4098.

Taniuchi M, Schweitzer JB, Johnson EM, Jr. (1986b) Nerve growth factor receptor molecules in rat brain. Proc Natl Acad Sci U S A 83: 1950-1954.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric K+ channels in terminal and juxtaparanodal regions of neurons. Nature 365: 75-79.

Zhang Y, Tohyama K, Winterbottom JK, Haque NS, Schachner M, Lieberman AR, Anderson PN (2001) Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. Mol Cell Neurosci 17: 444-459.

Chapter 3

DIFFERENTIAL RIP ANTIGEN (CNPase) EXPRESSION IN PERIPHERAL ENSHEATHING GLIA[2]

Introduction

The RIP monoclonal antibody was generated by Friedman et al. (1989) for the immunohistochemical detection of mature oligodendrocytes of the central nervous system (CNS). The antibody was directed toward antigens present in mature oligodendrocyte cytoplasm for the purpose of developing a reliable marker of somata and myelinating processes, rather than a marker of myelin sheath components. Since 1989, numerous immunocytochemical and immunohistochemical studies of the CNS have used the RIP antigen as a marker of mature oligodendrocytes (Friedman et al., 1989; Valverde and Lopez-Mascaraque, 1991; Jhaveri et al., 1992; Berger and Frotscher, 1994; Philpot et al., 1995; Butt et al., 1995; Butt et al., 1998; Hwang et al., 2006a, b); however, the identity of the antigen recognized by the RIP antibody remained unknown until very recently when Watanabe et al. (2006) identified the RIP antigen in oligodendrocytes as 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), a known non-compact myelin protein.

The functions of CNPase in myelinating glia are not entirely clear, but recent studies have elucidated some key roles for CNPase in myelination and axonal support in

[2] A version of this chapter has been accepted for publication. Toma JS, McPhail LT, Ramer MS. (2006) Differential RIP antigen (CNPase) expression in peripheral ensheathing glia. Brain Research. Accepted December 14, 2006.

the CNS. CNPase is involved in cytoskeletal rearrangement necessary for process expansion in oligodendrocytes (Lee et al., 2005). In addition, CNPase function appears crucial in maintaining axonal integrity, as CNPase-deficient mice demonstrate axonal degeneration throughout maturity (Lappe-Siefke et al., 2003; Rasband et al., 2005). However, no defects are evident in the myelin structure of these mice, suggesting that CNPase activity is not critical for sustaining myelin composition.

We have recently shown that RIP immunoreactivity, in addition to its previously characterized staining pattern in mature oligodendrocytes of the CNS, is also expressed at surprisingly high levels in the early postnatal peripheral nervous system (PNS) (Toma et al., 2006). RIP immunoreactivity is ubiquitous throughout the peripheral nerve compartment of the developing sensory root entry zones of the spinal dorsal nerve, the vagus nerve, and the trigeminal nerve. Upon maturation of the root entry zones, RIP immunoreactivity becomes localized to paranodal regions of myelinated axons. These findings suggest that the RIP antibody is recognizing an antigen present in Schwann cell cytoplasm, as it is in the paranodal regions that loops containing non-compact myelin contact the axolemma. Some interesting questions were raised in light of these observations: 1) Does the RIP monoclonal antibody recognize a myelin-specific protein in both the myelinating cells of the CNS and the PNS, namely oligodendrocytes and Schwann cells, respectively? 2) Is RIP immunoreactivity altered in the PNS following injury, and does the expression pattern of the RIP antigen in the regenerating peripheral nerve recapitulate that seen in the developing peripheral nerve? 3) Is RIP

immunoreactivity present in any other ensheathing glia in the PNS, or is its expression exclusively localized to myelinating Schwann cells?

In the present study we characterize the cellular localization of RIP immunoreactivity within a variety of glial populations of the adult rat PNS. We show that RIP immunoreactivity is differentially-expressed in discrete populations of peripheral glia, and that within the Schwann cell lineage but not in olfactory ensheathing cells, its relative intensity is correlated with the amount of axoglial contact. These results suggest a "supporting" role for glial CNPase in the PNS akin to that previously demonstrated in the CNS (Lappe-Siefke et al., 2003).

Materials and Methods

Sciatic nerve surgery and tissue preparation

Adult male Sprague Dawley rats (250-300g) were anaesthetized with a solution of 75 mg/kg ketamine hydrochloride (Bimeda-MTC, Cambridge, ON) and 0.5 mg/kg medetomidine hydrochloride (Orion Corporation, Espoo, Finland). In the study investigating RIP immunoreactivity following sciatic nerve crush injury, rats received a unilateral sciatic nerve crush injury using fine forceps, followed by an injection of 0.02 mg/kg buprenorphine (Schering-Plough Ltd., Hertfordshire, UK). To investigate RIP immunoreactivity in satellite cells surrounding injured neurons in the lumbar (L) level 4 dorsal root ganglia (DRG) and lumbar sympathetic ganglia, a 5% Fluorogold (Flurochrome Inc, Englewood, CO) solution in distilled water was delivered via GelFoam (Upjohn, Kalamazoo, MI) and placed at the site of nerve injury immediately following unilateral nerve transection (n = 3). To investigate sympathetic sprouting and RIP immunoreactivity in the L5 DRG, the L5 spinal nerve was ligated 2 mm distal to the DRG (Chung et al., 1993); both ipsilateral and uninjured contralateral DRGs were harvested 2 weeks following injury (n = 6). In addition, thoracic level 5 DRGs of uninjured animals (n = 3) were used to study RIP immunoreactivity in uninjured DRGs.

Both uninjured and injured rats (at 7, 14 or 28 days post injury, n = 4/timepoint for sciatic nerve crush injury study) were deeply anaesthetized with 900 mg/kg chloral hydrate in distilled water followed by transcardial perfusion with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 M PB. A 20 mm section of

sciatic nerve was removed 3-5 mm distal to the site of injury from each injured nerve. A segment of approximately the same length was harvested from both the contralateral nerve of injured animals and from sciatic nerves of uninjured animals (n = 5). Sciatic nerves, DRGs, sympathetic ganglia, olfactory epithelium and bulbs, and mylohyoid muscles were removed, cryoprotected in 20% sucrose in 0.1 M PB, and frozen at -80^0C. Sciatic nerves were cryosectioned longitudinally at 16 μm and at 10 μm in cross-section. DRGs, olfactory epithelium (n = 2) and bulb (n = 3), mylohyoid muscle (n = 3), and sympathetic ganglia (n = 3) were cryosectioned at 16 μm. The olfactory bulbs were cryosectioned coronally; the mylohyoid muscle longitudinally; and DRGs, sympathetic ganglia, and olfactory epithelium were cut in cross-section. All sections were thaw-mounted onto Superfrost Plus glass slides (VWR, West Chester, PA). In total, 37 adult rats were used for this study. All surgical and other experimental procedures undertaken conform to the rules and regulations governing animal care at the University of British Columbia and follow the guidelines of the Canadian Council on Animal Care.

Immunohistochemical analysis

After blocking with 10% normal donkey serum (or 10% normal goat serum in studies involving the goat-anti chicken Alexa Fluor 488-conjugated antibody) in PBS for 30 minutes, slides were incubated with the following primary antibody solutions: mouse anti-oligodendrocytes (RIP, 1:2000, Chemicon, Temecula, CA; rabbit anti-p75 (1:1000, Chemicon, Temecula, CA); chicken anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, 1:100, Neuromics, Edina, MN); rabbit anti-calcitonin gene-related peptide (CGRP, 1:4000, Sigma, St. Louis, MO); rabbit anti-Kv1.1 (1:200, Alomone Labs,

Jerusalem, Israel); rabbit anti-protein gene product 9.5 (PGP9.5, 1:3000, Affiniti, Mamhead, UK); and sheep anti-tyrosine hydroxylase (TH, 1:100, Chemicon). Primary antibody solutions were left on the slides overnight. Slides were then washed with PBS for 30 min before application of secondary antibody solutions. The following secondary antibodies were used at a concentration of 1:300 and left on slides for 2 hr: donkey anti-rabbit, donkey anti-sheep and goat anti-chicken Alexa Fluor 488 (Molecular Probes, Eugene, OR) and donkey anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA). Primary and secondary antibodies were prepared in solution with PBS with 0.1% sodium azide and 0.2% Triton X-100. Sections of DRGs were stained with bisBenzimide (Sigma) at 1 μg/ml for 2 min and washed in PBS for 2-5 min to identify satellite cell nuclei. Sections taken from the ipsilateral and contralateral DRGs following L5 sciatic nerve ligation and those taken from the ipsilateral and contralateral sciatic nerves following sciatic nerve crush were stained simultaneously to control for variability in staining. Images were acquired using a Q-Imaging (Burnaby, British Columbia, Canada) CCD camera mounted on an Axioplan 2 fluorescent light microscope with a motorized z-drive (Zeiss, Jena, Germany). Deconvolution steps were carried out on series of superimposed images taken that were not clearly focussed at any single plane of focus; however, images that were entirely in focus on any one plane did not undergo deconvolution as it was deemed unnecessary. Therefore, no standardized protocol for image deconvolution was employed and only obvious qualitative differences were included in any comparative immunohistochemical analyses used in this study. All images presented in this study are representative of the typical staining observed. Northern Eclipse (Empix Imaging, Mississauga, Ontario, Canada) imaging software was

used to capture digital images. The contrast and brightness of the images presented in this study were adjusted using Adobe Photoshop 7.0 to allow for the clearest possible demonstration of immunoreactive tissue.

Quantification of RIP staining intensity

Axon diameter and surrounding myelin in 10 μm cross-sections of uninjured sciatic nerves were measured using SigmaScan Pro 5.0 (SSPS, Chicago, IL) image analysis software. The profiles of PGP9.5-immunoreactive axons were traced on-screen and their diameters were calculated. The corresponding average red (RIP) intensity (0-255 redscale) was measured within (and including the myelinated area immediately surrounding) each axon (n = 750 axons, 250 randomly-selected axons/rat, 3 rats).

Results

RIP immunoreactivity in the intact sciatic nerve

The following immunohistochemical analyses were conducted in order to ascertain the distribution of RIP immunoreactivity in the intact sciatic nerve. RIP immunoreactivity had a similar localization pattern as CNPase in the sciatic nerve, demonstrating that the RIP antibody likely recognizes CNPase in Schwann cells of the PNS in addition to oligodendrocytes of the CNS (arrowheads in Fig. 3-1a, b). However, differences did exist in the immunoreactivity of the two antibodies. RIP immunoreactivity resulted in much more robust cytoplasmic staining than did the anti-CNPase antibody. RIP immunoreactivity was clearly evident in the cytoplasm of myelinating Schwann cell processes, Schmidt-Lantermann incisures (SLIs; arrowhead, Fig. 3-1c), and paranodal regions of myelinated axons (inset, Fig. 3-1c). Double-staining for RIP and Kv1.1 voltage-gated potassium channels, known to be localized to the juxtaparanodal region of myelinated axons (Fig. 3-1c, asterisks in d; Wang et al., 1993; Mi et al., 1995; Rasband et al., 1998), confirmed the presence of RIP immunoreactivity at paranodal regions. Intense RIP immunoreactivity was also evident in Remak fibres, as was determined by colocalization of RIP with p75, a neurotrophin receptor expressed by non-myelinating Schwann cells and neurons in the mature PNS (Fig. 3-1g, h; Taniuchi et al., 1986a, b; Jessen et al., 1990; Mikol et al., 2002). In order to demonstrate that RIP immunoreactivity was indeed present in non-myelinating Schwann cells of Remak fibres and not in p75-positive axons, we colabelled sciatic nerve sections with RIP and PGP9.5, a pan-neuronal marker (Fig. 3-1e, f). RIP staining was found to be absent in PGP9.5-

positive axons, suggesting that the RIP antigen is not expressed in axons. The absence of RIP immunoreactivity from neurons was further demonstrated in dorsal root ganglia (DRGs) (see below). In addition, RIP immunoreactivity did occur in the sheaths surrounding CGRP-positive axons, which further suggests the presence of RIP immunoreactivity in Remak fibres, as CGRP is a marker of unmyelinated, small-diameter C-fibres (Fig. 3-1i, j).

Using cross-sections through sciatic nerves, the RIP antigen was identified in the cytoplasm of myelinating Schwann cells surrounding axons (which were labelled with PGP 9.5) (Fig. 3-2a). The intensity of RIP immunoreactivity was generally greater in the cytoplasm of myelinating Schwann cells surrounding axons of a smaller diameter than those of a larger diameter (Fig. 3-2b) (n = 750 axons, 250 randomly-selected axons/rat, 3 rats). However, because there were populations of small-diameter axons that contained low levels of RIP immunoreactivity, it is likely that high levels of RIP staining occurred only in the cytoplasm of myelinating Schwann cells associated with a sub-population of small-diameter axons. RIP immunoreactivity was always less intense in the Schwann cell cytoplasm surrounding large-calibre axons (Fig. 3-2b).

RIP immunoreactivity in the injured sciatic nerve

We have previously shown that RIP immunoreactivity is distributed throughout the peripheral compartment of certain sensory root entry zones during early postnatal development but becomes localized to paranodes in the young adult rat (Toma et al., 2006). To determine if these changes that occur throughout neonatal development were

recapitulated following injury, RIP immunoreactivity was assayed in the distal stump of crush injured sciatic nerves at 7, 14, and 28 days post injury (dpi) (Fig. 3-3, n = 4/timepoint).

RIP immunoreactivity was very diffuse and ubiquitously distributed throughout the sciatic nerve 7 dpi, resembling the appearance of RIP distribution at root entry zones early in postnatal development (Fig. 3-3b). By 14 dpi, RIP immunoreactivity was once again localized to paranodes and SLIs, however, some diffuse immunoreactivity remained (Fig. 3-3c). Axonal thickness increased between 14 dpi and 28 dpi and by 28 dpi, the morphology of myelinated axons approached that of the uninjured nerve (Fig. 3-3a, d). This increase in axonal thickness correlated with an increase in the number of SLIs.

RIP immunoreactivity in peripheral ganglia

We next sought to determine if other non-myelinating glial populations aside from the non-myelinating Schwann cells of Remak fibres expressed the RIP antigen. RIP immunoreactivity was investigated in satellite cells, the non-myelinating glial cells associated with the neurons of peripheral ganglia. We assessed RIP immunoreactivity in peripheral ganglia (DRGs and sympathetic ganglia) with and without prior (seven days earlier) peripheral axotomy. RIP immunoreactivity in the uninjured DRG was abundant in the fibre layer but only diffuse in satellite cells (Fig. 3-4a). Following injury, RIP immunoreactivity in the L4 DRG increased in satellite cells, many of which surrounded large-diameter neurons that had axons projecting into the sciatic nerve (retrogradely-

filled with Fluorogold) (Fig. 3-4b). Increases in RIP immunoreactivity were also evident in satellite cells surrounding injured L5 DRG neurons following L5 spinal nerve ligation (see below for description) (Fig. 3-5b, arrowhead in inset; Fig. 3-6a, arrowheads).

However, heavy RIP immunoreactivity was also apparent around cells which did not contain Fluorogold and were therefore not likely axotomized. No RIP immunoreactivity was present in any satellite cells located in intact or axotomized sympathetic ganglion neurons (arrow, Fig. 3-4c). However, RIP staining was clearly evident in Remak fibres containing unmyelinated sympathetic axons of the grey rami exiting sympathetic ganglia (inset, Fig. 3-4c) and entering the spinal nerve at the distal pole of the DRG (arrow, Fig. 3-6a).

High RIP antigen expression in Remak bundles prompted us to ask whether other glia which normally express little RIP antigen upregulate the antigen upon contact with unmyelinated axons. A well-established consequence of peripheral nerve injury is sympathetic sprouting in the DRG: tyrosine hydroxylase (TH)-expressing sympathetic axons (which are unmyelinated) form pericellular baskets surrounding large-diameter axons following peripheral nerve injury (McLachlan et al., 1993; Ramer et al., 1999; Shinder et al., 1999). Two weeks following L5 spinal nerve ligation, TH-positive sympathetic baskets had formed as described previously (Chung et al., 1996; Fig. 3-6c, f), and RIP immunoreactivity was intense in reactive and proliferating satellite cells surrounding only a subset of large-diameter neurons, as not all satellite cells surrounding injured large-diamer neurons displayed high levels of RIP immunoreactivity (Fig. 3-6a,

arrowheads and asterisk). L5 spinal nerve injuries were used here because the injury is more severe than a sciatic nerve transection or crush (not all neurons in either the L4 or L5 DRG are injured following sciatic nerve injury) and we were not attempting to distinguish between injured and uninjured neurons of the L5 DRG in this case; we were attempting to maximize injury to the DRG and therefore maximize sprouting of sympathetic axons. In addition, all neurons in the L5 DRG are injured following L5 spinal nerve ligation, so the use of a retrogradely-transported tracer (e.g. Fluorogold) is unnecessary for the identification of injured neurons. All TH-positive baskets were associated with high RIP immunoreactivity in reactive satellite cell sheaths (Fig. 3-6d, g; ~ 100 baskets investigated). While the converse was not true, several populations of axons sprout in the DRG following axotomy, not just sympathetic axons (McLachlan and Hu, 1998). Because L5 spinal nerve ligation axotomizes all L5 DRG neurons but only a sub-population become wrapped with sprouting axons, the absence of RIP immunoreactivity from some satellite cell sheaths strongly suggests that upregulation is stimulated by sprouting axons rather than by injured neuronal somata.

RIP immunoreactivity in terminal neuromuscular glia and olfactory ensheathing cells

RIP immunoreactivity was assayed in two other types of non-myelinating glial cells: terminal Schwann cells of the mylohyoid neuromuscular junction and olfactory ensheathing cells (OECs) of the olfactory mucosa and bulb. Levels of RIP immunoreactivity were assayed in these types of peripheral glia in order to determine if RIP immunoreactivity was high in other types of non-myelinating glia in addition to the

non-myelinating Schwann cells of Remak fibres. Terminal Schwann cells of the neuromuscular junction were weakly RIP-positive (Fig. 3-7a, b). Surprisingly, RIP immunoreactivity was found surrounding axons in only some nerve bundles present within the olfactory lamina propria (Fig. 3-7c). One explanation for this variability in RIP immunoreactivity is that the RIP antigen may be in the cytoplasm of Schwann cells surrounding trigeminal sensory axons while being absent in OECs surrounding olfactory sensory axons. This explanation is supported by the fact that the RIP antigen was absent from OECs of the olfactory nerve layer that surrounds the olfactory bulb (Fig. 3-7d). In the olfactory bulb, the RIP antigen was present in the interstitial spaces separating glomeruli and was highly expressed in glia of the granular layer (Fig. 3-7d; Valverde and Lopez-Mascaraque, 1991; Philpot et al., 1995). A small population of RIP-positive oligodendrocyte-like cells was scattered throughout the olfactory nerve layer (inset, Fig. 3-7d).

Discussion

RIP immunoreactivity in peripheral glia

Here we have shown that RIP immunoreactivity is differentially-distributed in sub-populations of peripheral glia. RIP demarcates paranodal regions of myelinated axons in the mature PNS (Toma et al., 2006), and clearly defines SLIs in myelinating Schwann cells. Even more dramatic was the robust RIP immunoreactivity in Remak bundles in mixed nerves and in sympathetic ganglia and grey rami. RIP immunoreactivity was also detectable, although at a much lower level, in satellite cells surrounding DRG neurons in uninjured animals and in terminal Schwann cells at neuromuscular junctions (NMJs). Peripheral nerve injury resulted in increases in RIP immunoreactivity in satellite cells surrounding large-diameter DRG neurons. Sprouting sympathetic baskets surrounding neurons in axotomized DRGs always associated with RIP-immunoreactive satellite cells. OECs were conspicuously RIP-negative.

Linking RIP immunoreactivity to CNPase expression and function

Watanabe et al. (2006) revealed that the non-compact myelin protein CNPase is recognized by the RIP monoclonal antibody in the CNS. In the present study we demonstrated similar localization of RIP and CNPase immunoreactivity in the PNS, suggesting that the antigen recognized by the RIP antibody in the PNS is likely CNPase. Gomes et al. (2003) demonstrated CNPase immunoreactivity in the glomerular and granular layers of the developing olfactory bulb in much the same pattern as RIP immunoreactivity demonstrated here and by others (Valverde and Lopez-Mascaraque,

1991; Philpot et al., 1995). Taken together, these results indicate that RIP also recognizes CNPase in the olfactory bulb.

CNPase is expressed in the cytoplasm of non-compact myelin loops, such as those located at paranodes and SLIs (Braun et al., 1988; Trapp et al., 1988). CNPase is synthesized in the cytoplasm where it is translated by free polysomes (Gillespie et al., 1990; Kim and Pfeiffer, 1999). In oligodendrocytes, its hydrophobic domains allow its association with the plasmalemma where it anchors tubulin and mediates the cytoskeletal reorganization underlying process extension and branching (Bifulco et al., 2002; Lee et al., 2005). However, the lack of any gross or ultrastructural myelin abnormalities in CNPase-null mice (Lappe-Siefke et al., 2003) argues against its involvement in myelin formation in the CNS. The most striking phenotypic feature of CNPase-null mice is overt axonal degeneration, implicating CNPase in the traditional "supporting" function of ensheathing glia. Degeneration of CNS axons in the absence of CNPase is likely to be mediated by the progressive loss of axoglial interactions, as Na^+-channels and the paranodal adhesion protein Caspr become mislocalized from the nodal and paranodal regions respectively (Rasband et al., 2005). Whether CNPase deficiency leads to similar degenerative effects in the PNS has yet to be investigated.

RIP immunoreactivity surrounding myelinated axons

Although RIP immunoreactivity was present surrounding all myelinated axons, the intensity of immunoreactivity varied with axonal diameter. Specifically, Schwann cell cytoplasm surrounding a sub-population of small-diameter (2-3 μm) axons was

strongly immunoreactive while that associated with larger diameter axons expressed the RIP antigen at lower levels (except at paranodes and SLIs). On the one hand it might be argued that since intensity measurements were made on cross sections of the myelin sheath, thicker compact (and relatively RIP-negative) myelin surrounding larger axons may have artificially reduced intensity. However, such signal dilution would lead us to predict a tight negative correlation between axon diameter and the RIP immunoreactivity of its sheath. This was not the case: RIP intensity varied broadly among the sheaths of small, thinly-myelinated axons. Furthermore, simple inspection of the abaxonal and adaxonal cytoplasmic leaflets consistently verified low RIP immunoreactivity in the sheaths of large axons. Since RIP intensity is highest in non-compact myelin, its differential distribution amongst small-calibre sheaths may indicate different degrees of compaction. More plausibly, given the lack of evidence for varying compactness amongst axonal subsets in peripheral nerve, separate sub-populations of thinly-myelinated axons may dictate the level of CNPase expression in their associated glia.

RIP immunoreactivity following peripheral nerve injury: implications for CNPase activity

Seven days following sciatic nerve injury, RIP immunoreactivity was mislocalized from paranodes and SLIs and was redistributed throughout Schwann cells. By 28 dpi, RIP immunoreactivity redistributed to the paranodes and SLIs. Changes in the pattern of RIP antigen expression following axonal injury recapitulated those observed during development (Toma et al., 2006): in developing nerve root entry zones, RIP immunoreactivity was distributed diffusely throughout the peripheral compartment

during the first 2 weeks of postnatal development. By the end of the second week, RIP immunoreactivity was localized to paranodes.

Between 14 dpi and 28 dpi, the amount of SLIs increased in the regenerating sciatic nerve. In adult peripheral nerve, the number of SLIs per internode is positively correlated with internodal width, a relationship which becomes even tighter in regenerating nerve, supporting the hypothesis that SLIs play a vital role in metabolic activities necessary for myelin sheath development and maintenance (Hiscoe, 1947; Sotnikov, 1965; Ghabriel and Allt, 1980; Berger and Gupta, 2006).

The effect of nerve injury on satellite cells associated with injured neurons of the L4 DRG was also investigated. RIP immunoreactivity increased in satellite cells associated with some, particularly large-diameter neurons, but following sciatic nerve injury was not specific to satellite cell surrounding Fluorogold-filled (axotomized) neurons. This was not entirely unexpected since both axotomized DRG neurons and their spared neighbours, along with their respective satellite cell sheaths, undergo changes in gene expression following peripheral nerve injury (Zhou et al., 1996; Ma and Bisby, 1998; Tsuzuki et al., 2001; Obata et al., 2003). Spinal nerve injury also resulted in robust RIP antigen upregulation in proliferating satellite cells surrounding large neurons. However, even though all DRG neurons in the L5 ganglion are axotomized by this manipulation, increases in RIP immunoreactivity did not occur in all satellite cell sheaths. These data represent the sole report of an injury-induced increase in RIP

immunoreactivity, and demonstrate that neuron-glial signalling does indeed influence RIP antigen expression, at least in the DRG.

RIP immunoreactivity in peripheral glia may vary with axonal contact

Peripheral glia of the Schwann cell lineage (perisynaptic glia at the NMJ, myelinating and non-myelinating Schwann cells, satellite cells) varied greatly in their levels of RIP immunoreactivity. Given the apparent requirement for CNPase in maintaining axonal integrity in the CNS (Lappe-Siefke et al., 2003), it is tempting to speculate that CNPase performs a similar function in the periphery. If this is indeed the case, it stands to reason that levels of RIP immunoreactivity are correlated with the amount of contact between neurons and their associated glia in the PNS. This notion is substantiated by intense RIP immunoreactivity in the non-myelinating Schwann cells of Remak fibres, which ensheathe multiple axons in sympathetic rami communicantes and mixed nerves such as the sciatic, and lower immunoreactivity in the sheaths surrounding large-calibre axons. RIP immunoreactivity was faint in terminal Schwann cells, corroborating Georgiou and Charlton's (1999) finding that CNPase is weakly expressed in these cells. The relative absence of RIP immunoreactivity from terminal Schwann cells at the NMJ and satellite cells in intact peripheral ganglia, both of which contact relatively small amounts of neuronal membrane, also supports the hypothesis stated above. The injury-induced upregulation of the RIP antigen in satellite glia in the DRG is particularly interesting since these cells may support sympathetic axons which sprout within the ganglion following peripheral nerve injury (Ramer et al., 1999). These form pericellular baskets investing the satellite cell sheath (Shinder et al., 1999) surrounding

primarily large-diameter DRG neurons (McLachlan et al., 1993) which may be either intact or axotomized (Ma and Bisby, 1999). The pattern of RIP antigen upregulation in the DRG following peripheral nerve injury thus bore a striking resemblance to that of sympathetic pericellular basket formation. Indeed, all sympathetic baskets which formed following L5 spinal nerve lesion were ensheathed by RIP-positive satellite cells. The fact that many DRG neurons had RIP-positive sheaths but no TH-positive baskets is not unexpected since peptidergic sensory axons also form baskets around axotomized DRG neurons (McLachlan and Hu, 1998). Whether sprouting axons or neuronal somata are responsible for RIP antigen upregulation in satellite glia remains to be determined. However, the former scenario is more likely given the absence of RIP-positive sheaths from many injured DRG neurons following L5 spinal nerve ligation.

An exception to the correlation between the amount of axon-glial contact and RIP immunoreactivity is apparent in OECs, which also ensheathe multiple axons in the olfactory lamina propria and olfactory bulb (Doucette, 1991), yet are not RIP immunoreactive. These results support those of an earlier study by Pixley (1996) demonstrating that in cell cultures of rat olfactory mucosa, CNPase immunoreactivity was absent from OECs but did label "oligodendrocyte-like" cells analogous to those we found thinly scattered throughout the olfactory nerve layer. The difference in RIP immunoreactivity between subsets of ensheathing glia may be attributed simply to the fact that OECs are of placodal origin, rather than being derived from neural crest which gives rise to the Schwann cell lineage (Doucette, 1991). The olfactory system is unique in that primary afferent neurons in the olfactory epithelium undergo death and

replacement throughout life (Calof et al., 1996), and it may therefore be that the CNPase-mediated axonal "support" role is irrelevant and therefore inoperative.

OEC transplantation is a popular, albeit controversial, experimental approach to the treatment of spinal cord injury since these cells have been reported to support axonal regeneration and to myelinate denuded or re-growing axons (reviewed in: Boyd et al., 2005; Ibrahim et al., 2006; Ruitenberg et al., 2006). The absence of CNPase from OECs may on the one hand implicate their unsuitability in the long-term maintenance of CNS axons. However, it has become apparent that one of the primary effects of OEC transplantation is the recruitment of endogenous (CNPase-expressing) Schwann cells (Ramer et al., 2004), and that much of the myelination occurring following OEC transplantation is in fact mediated by endogenous Schwann cells or those contaminating "purified" preparations (Boyd et al., 2004; Rizek and Kawaja, 2006). It is also possible that like satellite cells in the DRG, OECs upregulate CNPase in response to contact with non-olfactory axons, although this has yet to be tested.

RIP as a glial marker: uses and misuses

RIP immunoreactivity is commonly used to identify oligodendrocyte cytoplasm (Friedman et al., 1989; Valverde and Lopez-Mascaraque, 1991; Jhaveri et al., 1992; Berger and Frotscher, 1994; Philpot et al., 1995; Butt et al., 1995; Butt et al., 1998; Hwang et al., 2006a, b). The RIP antibody has been applied for this purpose in studies ranging from characterizing the relationship between axons and oligodendrocyte myelinating units in the anterior medullary velum to investigating oligodendroglial

development in the hippocampus (Butt et al., 1998; Hwang et al., 2006, b). In the present study, we have established the RIP antigen as a useful marker of Schwann cell cytoplasm, specifically in SLIs and paranodes in the myelin surrounding axons as well as in the non-myelinating Schwann cells of Remak fibres. As RIP immunoreactivity is present in both oligodendrocytes and Schwann cells, there are caveats inherent in the use of this antibody in distinguishing between endogenous and invading myelinating glia following CNS injury (Tzeng et al., 2001; Bacia et al., 2004; Mey et al., 2005; Labombarda et al., 2006).

Figure 3-1. RIP immunoreactivity in longitudinal sections of the uninjured sciatic nerve of the adult rat. **a**, **b**, RIP immunoreactivity localized with CNPase immunoreactivity (arrowheads). The image presented in **b** represents the best quality of staining available for the anti-CNPase antibody. **c**, RIP immunoreactivity was present in the paranodal regions of myelinated axons (inset, **c**) and in Schmidt-Lantermann incisures (arrowhead), as well as in Remak fibres (arrows in **c** and **g**). Note the RIP immunoreactivity along the outermost boundaries of the myelinated axon internodes. **d**, RIP and Kv1.1 co-labelling further indicated paranodal localization of the RIP antigen (asterisks). **e**, **f**, RIP immunoreactivity was absent in axons, as the RIP antigen was not present in PGP9.5-positive fibres. **g**, **h**, RIP immunoreactivity colocalized with p75, suggesting the presence of the RIP antigen in Remak fibres, as p75 is expressed by both neurons and non-myelinating Schwann cells. **i**, **j**, RIP immunoreactivity associated with sheaths of small-diameter, CGRP-positive fibres, providing further evidence for the presence of RIP immunoreactivity in Remak fibres (arrowhead). Scales: **a** and **b** = 16 μm; **c** and **d** = 34 μm, inset in **c** = 8.5 μm; **e** and **f** = 25 μm; **g** and **h** = 100 μm, inset in **h** = 25 μm; **i** and **j** = 50 μm.

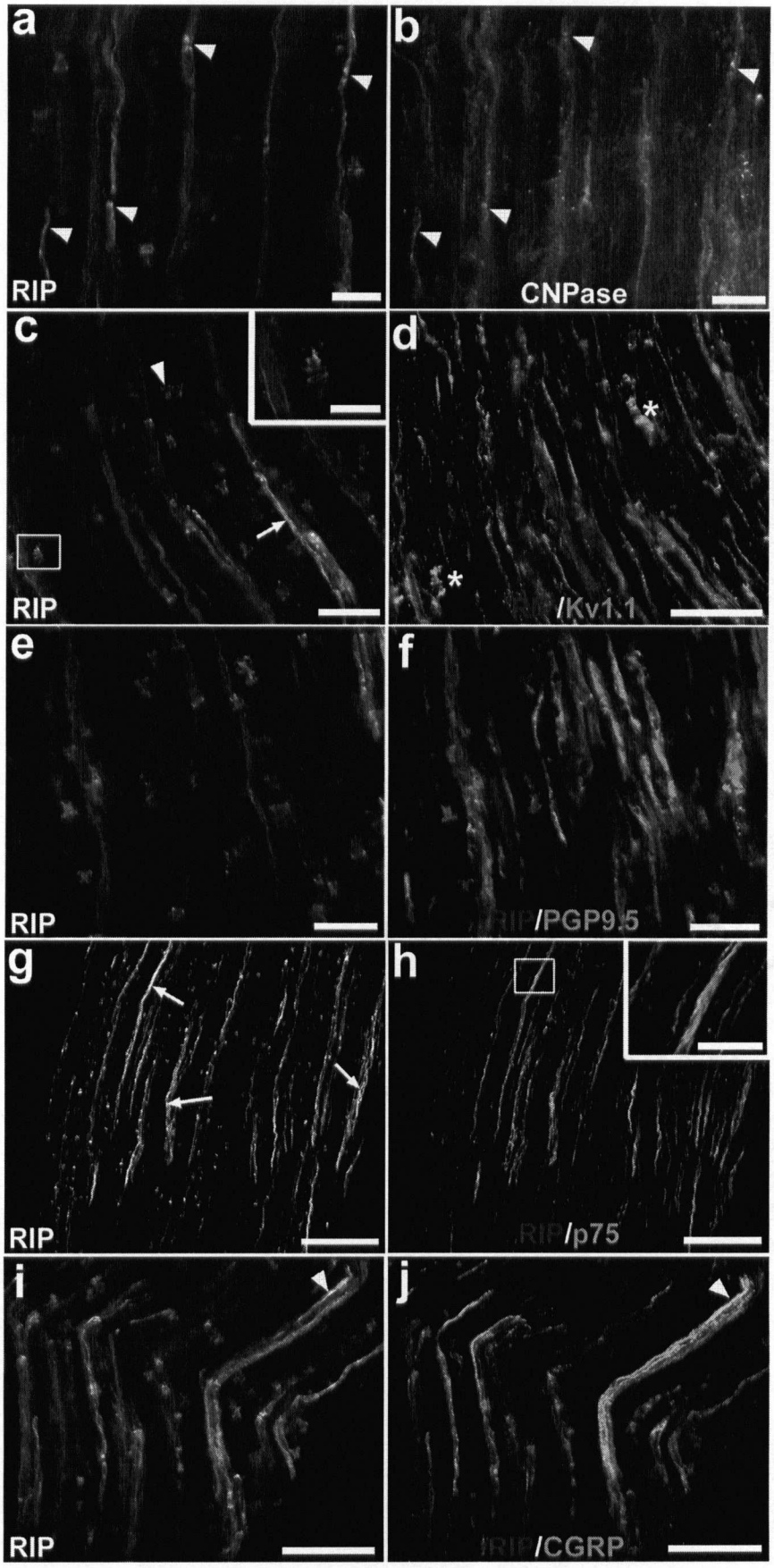
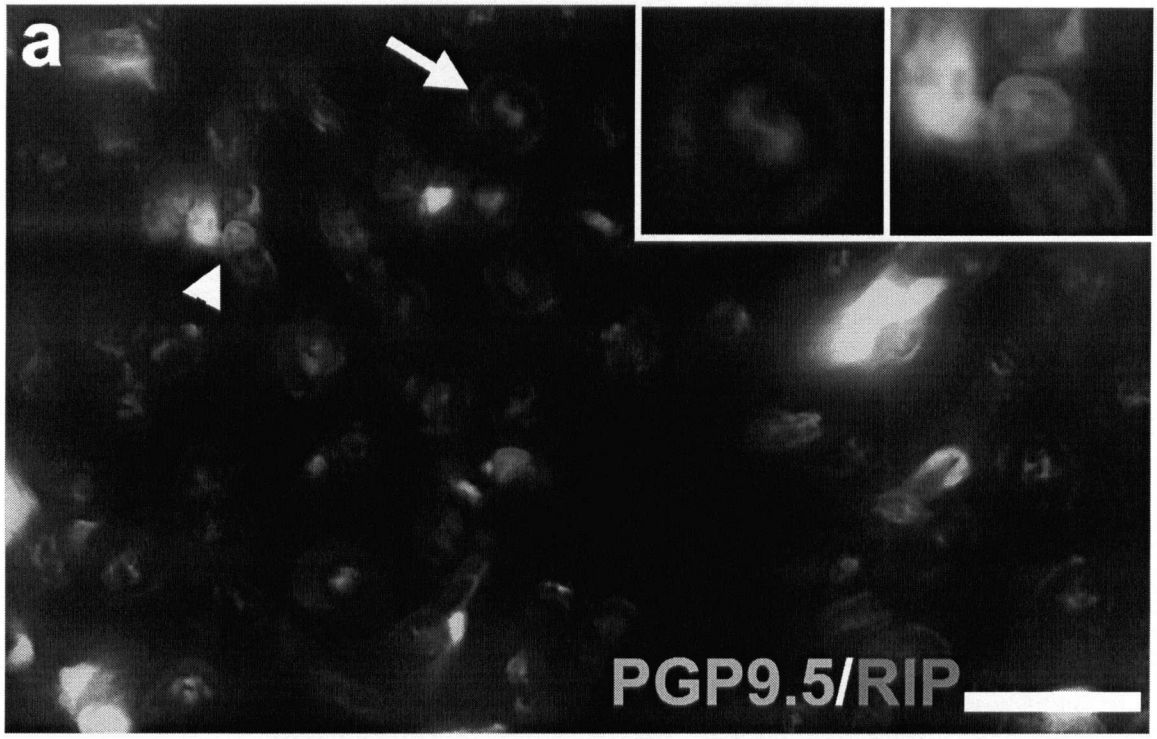

a
RIP
b
CNPase
c
RIP
d
/Kv1.1
e
RIP
f
/PGP9.5
g
RIP
h
/p75
i
RIP
j
/CGRP

Figure 3-2. RIP immunoreactivity in cross-sections of sciatic nerve. **a**, Intense RIP immunoreactivity was present in the cytoplasm of myelinating Schwann cells surrounding a population of small-diameter axons (arrowhead and right inset), whereas larger diameter axons were associated with consistently low levels of RIP immunoreactivity in the cytoplasm of their surrounding myelinating Schwann cells (arrow and left inset). Red = RIP, Green = PGP9.5. **b**, Scatter plot analysis of axon diameter and RIP staining intensity revealed high levels of RIP immunoreactivity surrounding only a sub-population of small-diameter axons (cross-sections of sciatic nerves taken from 3 rats, n = 750 axons). Scale = 16 μm.


a
PGP9.5/RIP

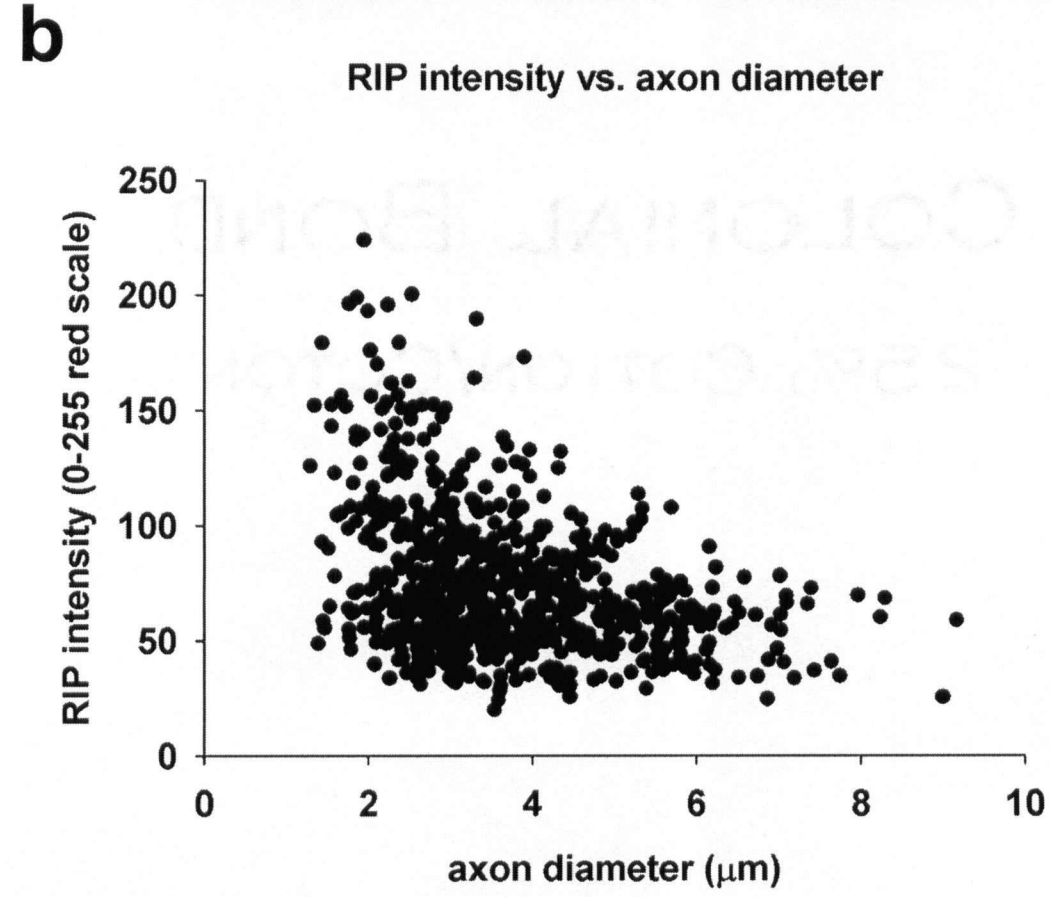

b
RIP intensity vs. axon diameter
RIP intensity (0-255 red scale)
250
200
150
100
50
0
0
2
4
6
8
10
axon diameter (μm)

Figure 3-3. RIP immunoreactivity following sciatic nerve crush lesion in the adult rat at 7, 14, and 28 days post injury (dpi). **b**, RIP immunoreactivity was ubiquitous and diffuse 7 dpi; it was no longer localized to Schmidt-Lantermann incisures and paranodal regions of myelinated axons. **c**, By 14 dpi, RIP immunoreactivity was re-distributed to paranodes and Schmidt-Lantermann incisures of newly-ensheathed regenerated axons. **d**, At 28 dpi, paranodal and incisure morphology more closely resembled that of the contralateral uninjured nerve, **a**. There was an increased incidence of Schmidt-Lantermann incisures at 28 dpi than at 14 dpi. Scale = 50 μm.

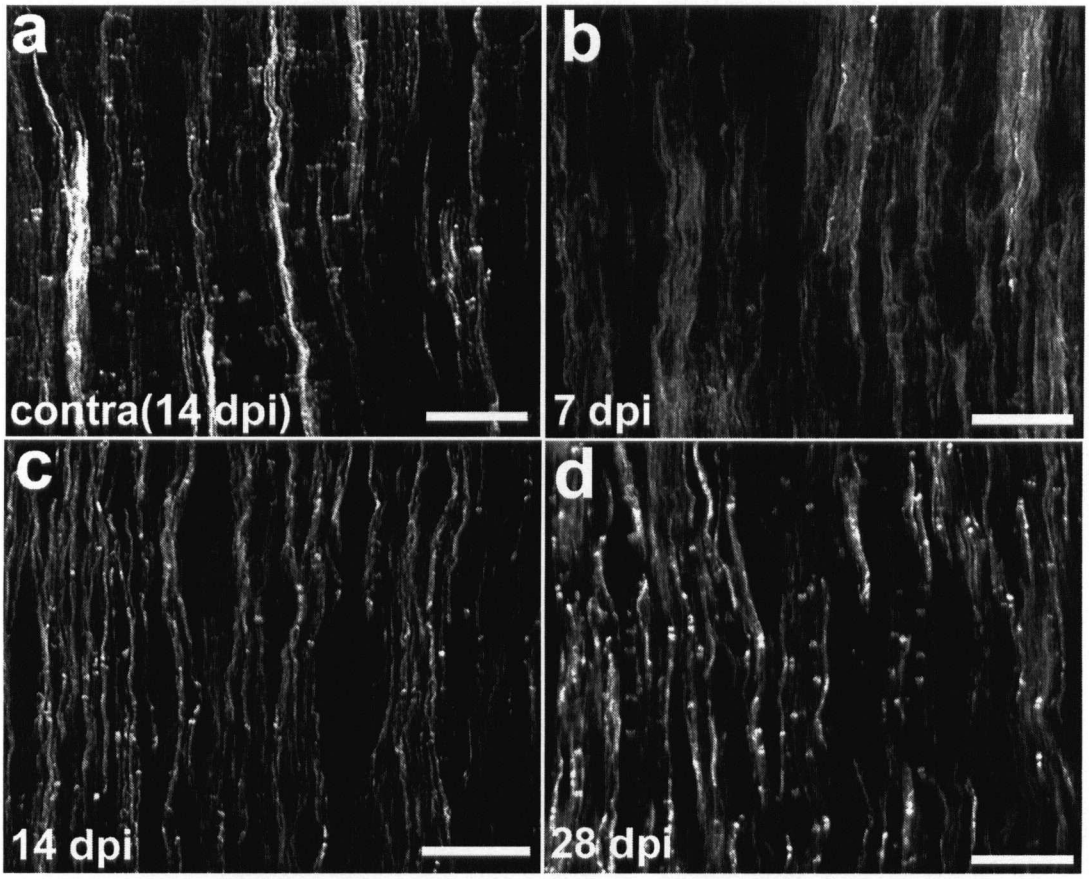

a
contra(14 dpi)
b
7 dpi
c
14 dpi
d
28 dpi

Figure 3-4. RIP immunoreactivity in peripheral ganglia. **a**, RIP immunoreactivity (red) was low in satellite cells (nuclei in green, stained with bisBenzimide) surrounding neurons (in blue, stained with PGP9.5) in the uninjured DRG (arrowheads in inset). RIP staining was prominent surrounding myelinated axons of the fibre layer (arrow). **b**, The satellite cells (green nuclei) surrounding injured (Fluorogold-positive) neurons (green cytoplasm) of the DRG 7 days following sciatic nerve cut injury had increased RIP immunoreactivity (red) (arrowheads in inset). **c**, Satellite cells surrounding neurons of sympathetic chain ganglia that had projections into the injured sciatic nerve were not RIP-immunoreactive (arrow). However, RIP immunoreactivity was clearly present in the sympathetic grey rami (inset, **c**) and in the Remak fibres present in the sympathetic ganglia (arrowhead, **c**). Scales: **a** = 100 μm, inset in **a** = 32 μm; **b** = 100 μm, inset in **b** = 16 μm; **c** = 100 μm, inset in **c** = 50 μm.

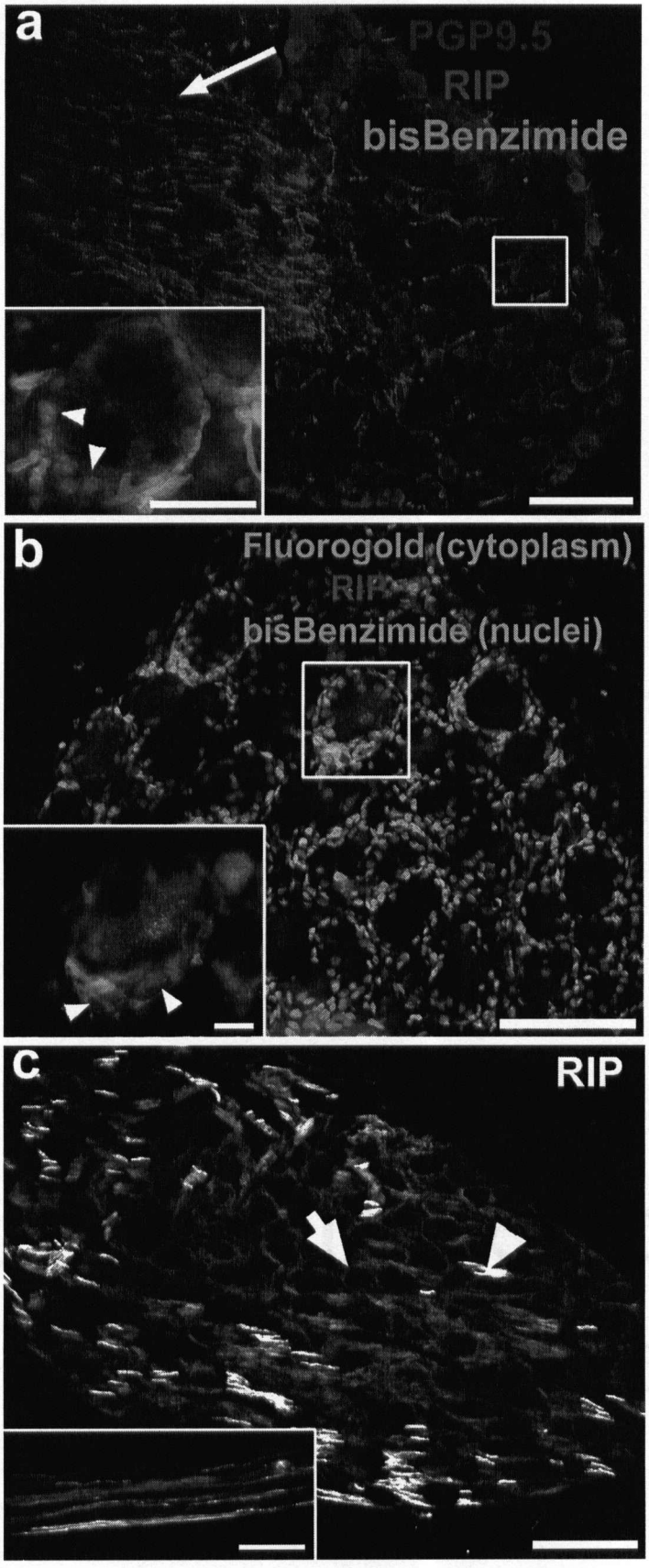

a
PGP9.5
RIP
bisBenzimide
b
Fluorogold (cytoplasm)
RIP
bisBenzimide (nuclei)
c
RIP

Figure 3-5. RIP immunoreactivity increases in the cell layer of the injured (ipsilateral) L5 DRG following L5 spinal nerve ligation. **a**, RIP immunoreactivity in the contralateral L5 DRG. Faint RIP immunoreactivity is evident in satellite cells surrounding DRG neurons (arrowhead in inset, **a**). The majority of RIP immunoreactivity situated around the neurons is present in myelinating Schwann cells and localized to Schmidt-Lantermann incisures and paranodal regions (asterisks in inset, **a**). **b**, High levels of RIP immunoreactivity are present surrounding injured neurons of the ipsilateral L5 DRG. Levels of RIP immunoreactivity are higher in satellite cells surrounding injured neurons than those present in the contralateral DRG (compare arrowhead in inset, **b** with arrowhead in inset, **a**). RIP immunoreactivity is comparable in the fibre layer of both the ipsilateral and contralateral L5 DRGs (arrows in **a** and **b**). Notice the low RIP immunoreactivity in the ventral root lying directly adjacent to the ipsilateral L5 DRG (arrowhead and surrounding area directly above cell layer in **b**). Scale = 100 μm.

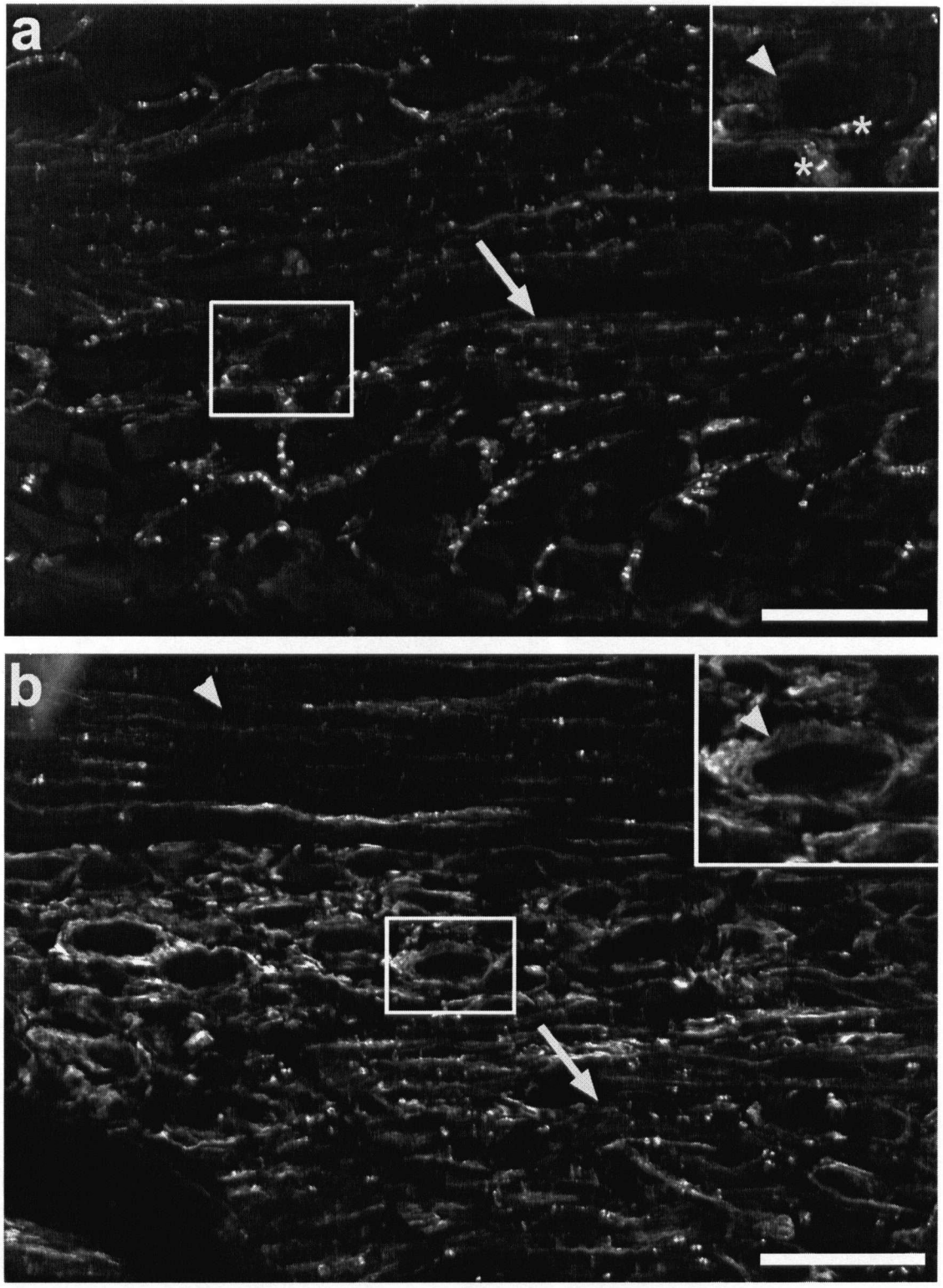

a
b

Figure 3-6. RIP immunoreactivity and sympathetic sprouting in the L5 DRG. **a**, High RIP immunoreactivity (red) was present in many reactive satellite cells (arrowheads) surrounding neurons (PGP9.5, blue) of the injured DRG; however, the RIP antigen was not highly expressed in all satellite cells surrounding injured neurons (asterisk). Post-ganglionic sympathetic axons of the grey rami (TH, green) were evident at the distal pole of the DRG (arrow). Sprouting TH-positive fibres formed pericellular baskets that invariably associated with RIP-immunoreactivity in satellite cells (RIP immunoreactivity in **b**, **e**; TH immunoreactivity in **c**, **f**; and merges shown in **d**, **g**). Scale = 25 μm.

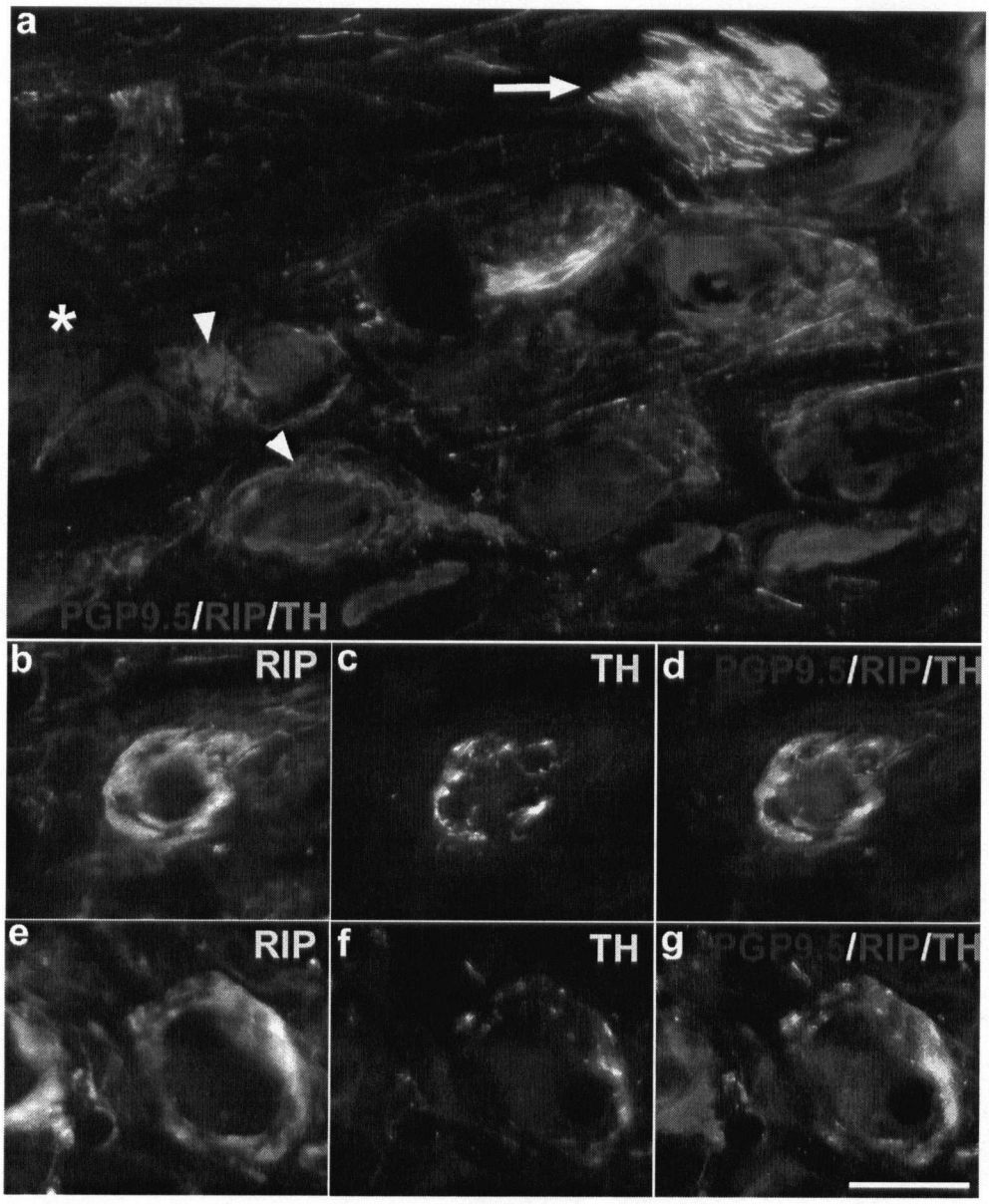
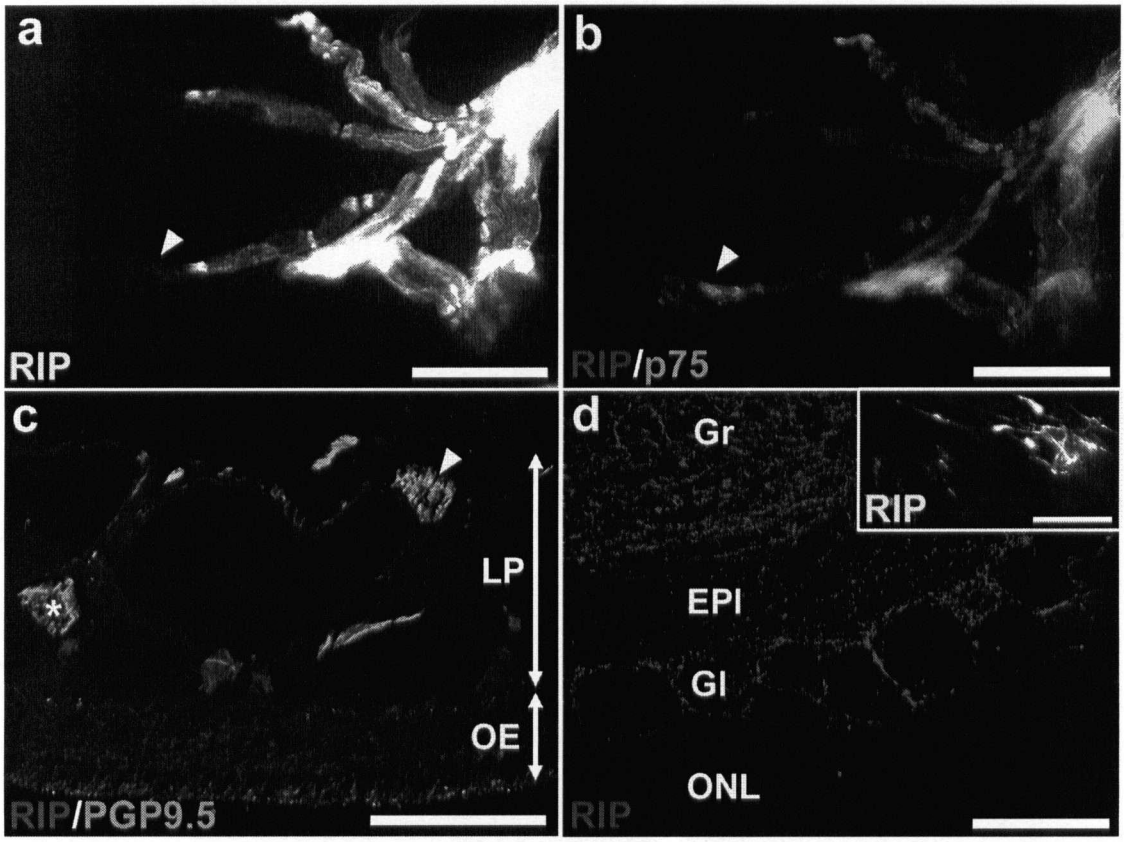

a
PGP9.5/RIP/TH
b
RIP
c
TH
d
PGP9.5/RIP/TH
e
RIP
f
TH
g
PGP9.5/RIP/TH

Figure 3-7. RIP immunoreactivity in two types of non-myelinating glia: the terminal Schwann cells at the mylohyoid neuromuscular junction and olfactory ensheathing cells (OECs) of the olfactory mucosa and bulb. **a**, Faint RIP immunoreactivity was present in terminal Schwann cells (arrowhead), as demonstrated by colocalization with p75 in **b**. (red = RIP, green = p75). **c**, In the lamina propria (LP) beneath the olfactory epithelium (OE), RIP immunoreactivity was variably present in nerve bundles (red = RIP, green = PGP9.5, arrowhead indicates RIP-positive bundle, asterisk indicates RIP-negative bundle). **d**, RIP immunoreactivity was absent from OECs of the olfactory nerve layer (ONL) yet abundant in glia throughout other layers of the olfactory bulb (Gl = glomerular layer, EPl = external plexus layer, Gr = granular layer). A sparse population of RIP-positive oligodendrocyte-like cells was located in the ONL (**d**, inset). Scales: **a** and **b** = 32 μm; **c** = 100 μm; **d** = 200 μm; inset in **d** = 32 μm.


a
RIP
b
RIP/p75
c
LP
OE
RIP/PGP9.5
d
Gr
RIP
EPI
GI
ONL
RIP

Bibliography

Bacia A, Wollmann R, Soliven B (2004) K+ channel blockade impairs remyelination in the cuprizone model. Glia 48: 156-165.

Berger BL, Gupta R (2006) Demyelination secondary to chronic nerve compression injury alters Schmidt-Lantermann incisures. J Anat 209: 111-118.

Berger T, Frotscher M (1994) Distribution and morphological characteristics of oligodendrocytes in the rat hippocampus in situ and in vitro: an immunocytochemical study with the monoclonal Rip antibody. J Neurocytol 23: 61-74.

Bifulco M, Laezza C, Stingo S, Wolff J (2002) 2',3'-Cyclic nucleotide 3'-phosphodiesterase: a membrane-bound, microtubule-associated protein and membrane anchor for tubulin. Proc Natl Acad Sci U S A 99: 1807-1812.

Boyd JG, Lee J, Skihar V, Doucette R, Kawaja MD (2004) LacZ-expressing olfactory ensheathing cells do not associate with myelinated axons after implantation into the compressed spinal cord. Proc Natl Acad Sci U S A 101: 2162-2166.

Boyd JG, Doucette R, Kawaja MD (2005) Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord. FASEB J 19: 694-703.

Braun PE, Sandillon F, Edwards A, Matthieu JM, Privat A (1988) Immunocytochemical localization by electron microscopy of 2'3'-cyclic nucleotide 3'-phosphodiesterase in developing oligodendrocytes of normal and mutant brain. J Neurosci 8: 3057-3066.

Butt AM, Ibrahim M, Ruge FM, Berry M (1995) Biochemical subtypes of oligodendrocyte in the anterior medullary velum of the rat as revealed by the monoclonal antibody Rip. Glia 14: 185-197.

Butt AM, Ibrahim M, Berry M (1998) Axon-myelin sheath relations of oligodendrocyte unit phenotypes in the adult rat anterior medullary velum. J Neurocytol 27: 259-269.

Calof AL, Hagiwara N, Holcomb JD, Mumm JS, Shou J (1996) Neurogenesis and cell death in olfactory epithelium. J Neurobiol 30: 67-81.

Chung K, Kim HJ, Na HS, Park MJ, Chung JM (1993) Abnormalities of sympathetic innervation in the area of an injured peripheral nerve in a rat model of neuropathic pain. Neurosci Lett 162: 85-88.

Chung K, Lee BH, Yoon YW, Chung JM (1996) Sympathetic sprouting in the dorsal root ganglia of the injured peripheral nerve in a rat neuropathic pain model. J Comp Neurol 376: 241-252.

Doucette R (1991) PNS-CNS transitional zone of the first cranial nerve. J Comp Neurol 312: 451-466.

Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG (1989) In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. Glia 2: 380-390.

Georgiou J, Charlton MP (1999) Non-myelin-forming perisynaptic schwann cells express protein zero and myelin-associated glycoprotein. Glia 27: 101-109.

Ghabriel MN, Allt G (1980) Schmidt-Lantermann Incisures. I. A quantitative teased fibre study of remyelinating peripheral nerve fibres. Acta Neuropathol (Berl) 52: 85-95.

Gillespie CS, Bernier L, Brophy PJ, Colman DR (1990) Biosynthesis of the myelin 2',3'-cyclic nucleotide 3'-phosphodiesterases. J Neurochem 54: 656-661.

Gomes SS, Carvalho SL, Santiago MF, Lopez LB, Barradas PC, Cavalcante LA (2003) Expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in the developing olfactory bulb and subventricular zone rostral extension. J Neurosci Res 73: 471-480.

Hiscoe H (1947) Distribution of nodes and incisures in normal and regenerated nerve fibers. Anat Rec 99: 447-475.

Hwang IK, Yoo KY, Kim DS, Kang TC, Lee BH, Kim YS, Won MH (2006a) Chronological distribution of rip immunoreactivity in the gerbil hippocampus during normal aging. Neurochem Res 31: 1119-1125.

Hwang IK, Lee HY, Yoo KY, Kim JC, Kim JH, Kim CH, Kang TC, Kim JD, Won MH (2006b) Rip immunoreactivity significantly decreases in the stratum oriens of hippocampal CA1 region after transient forebrain ischemia in gerbils. Brain Res 1073-1074: 491-496.

Ibrahim A, Li Y, Li D, Raisman G, El Masry WS (2006) Olfactory ensheathing cells: ripples of an incoming tide? Lancet Neurol 5: 453-457.

Jessen KR, Morgan L, Stewart HJ, Mirsky R (1990) Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. Development 109: 91-103.

Jhaveri S, Erzurumlu RS, Friedman B, Schneider GE (1992) Oligodendrocytes and myelin formation along the optic tract of the developing hamster: an immunohistochemical study using the Rip antibody. Glia 6: 138-148.

Kim T, Pfeiffer SE (1999) Myelin glycosphingolipid/cholesterol-enriched microdomains selectively sequester the non-compact myelin proteins CNP and MOG. J Neurocytol 28: 281-293.

Labombarda F, Gonzalez S, Gonzalez Deniselle MC, Garay L, Guennoun R, Schumacher M, De Nicola AF (2006) Progesterone increases the expression of myelin basic protein and the number of cells showing NG2 immunostaining in the lesioned spinal cord. J Neurotrauma 23: 181-192.

Lappe-Siefke C, Goebbels S, Gravel M, Nicksch E, Lee J, Braun PE, Griffiths IR, Nave KA (2003) Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nat Genet 33: 366-374.

Lee J, Gravel M, Zhang R, Thibault P, Braun PE (2005) Process outgrowth in oligodendrocytes is mediated by CNP, a novel microtubule assembly myelin protein. J Cell Biol 170: 661-673.

Ma W, Bisby MA (1998) Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. Eur J Neurosci 10: 2388-2399.

Ma W, Bisby MA (1999) Partial sciatic nerve transection induced tyrosine hydroxidase immunoreactive axon sprouting around both injured and spared dorsal root ganglion neurons which project to the gracile nucleus in middle-aged rats. Neurosci Lett 275: 117-120.

McLachlan EM, Janig W, Devor M, Michaelis M (1993) Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. Nature 363: 543-546.

McLachlan EM, Hu P (1998) Axonal sprouts containing calcitonin gene-related peptide and substance P form pericellular baskets around large diameter neurons after sciatic nerve transection in the rat. Neuroscience 84: 961-965.

Mey J, Morassutti J, Brook G, Liu RH, Zhang YP, Koopmans G, McCaffery P (2005) Retinoic acid synthesis by a population of NG2-positive cells in the injured spinal cord. Eur J Neurosci 21: 1555-1568.

Mi H, Deerinck TJ, Ellisman MH, Schwarz TL (1995) Differential distribution of closely related potassium channels in rat Schwann cells. J Neurosci 15: 3761-3774.

Mikol DD, Scherer SS, Duckett SJ, Hong HL, Feldman EL (2002) Schwann cell caveolin-1 expression increases during myelination and decreases after axotomy. Glia 38: 191-199.

Obata K, Yamanaka H, Fukuoka T, Yi D, Tokunaga A, Hashimoto N, Yoshikawa H, Noguchi K (2003) Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. Pain 101: 65-77.

Philpot BD, Klintsova AY, Brunjes PC (1995) Oligodendrocyte/myelin-immunoreactivity in the developing olfactory system. Neuroscience 67: 1009-1019.

Pixley SK (1996) Characterization of olfactory receptor neurons and other cell types in dissociated rat olfactory cell cultures. Int J Dev Neurosci 14: 823-839.

Ramer LM, Au E, Richter MW, Liu J, Tetzlaff W, Roskams AJ (2004) Peripheral olfactory ensheathing cells reduce scar and cavity formation and promote regeneration after spinal cord injury. J Comp Neurol 473: 1-15.

Ramer MS, Thompson SW, McMahon SB (1999) Causes and consequences of sympathetic basket formation in dorsal root ganglia. Pain Suppl 6: S111-S120.

Rasband MN, Trimmer JS, Schwarz TL, Levinson SR, Ellisman MH, Schachner M, Shrager P (1998) Potassium channel distribution, clustering, and function in remyelinating rat axons. J Neurosci 18: 36-47.

Rasband MN, Tayler J, Kaga Y, Yang Y, Lappe-Siefke C, Nave KA, Bansal R (2005) CNP is required for maintenance of axon-glia interactions at nodes of Ranvier in the CNS. Glia 50: 86-90.

Rizek PN, Kawaja MD (2006) Cultures of rat olfactory ensheathing cells are contaminated with Schwann cells. Neuroreport 17: 459-462.

Ruitenberg MJ, Vukovic J, Sarich J, Busfield SJ, Plant GW (2006) Olfactory ensheathing cells: characteristics, genetic engineering, and therapeutic potential. J Neurotrauma 23: 468-478.

Shinder V, Govrin-Lippmann R, Cohen S, Belenky M, Ilin P, Fried K, Wilkinson HA, Devor M (1999) Structural basis of sympathetic-sensory coupling in rat and human dorsal root ganglia following peripheral nerve injury. J Neurocytol 28: 743-761.

Sotnikov O (1965) Structure of Schmidt-Lantermann incisures. Arkh Anat Gistol Embriol 43: 31-42 (English translation in Fed. Am Soc Exp Biol (1966) 25, T204-210).

Taniuchi M, Clark HB, Johnson EM, Jr. (1986a) Induction of nerve growth factor receptor in Schwann cells after axotomy. Proc Natl Acad Sci U S A 83: 4094-4098.

Taniuchi M, Schweitzer JB, Johnson EM, Jr. (1986b) Nerve growth factor receptor molecules in rat brain. Proc Natl Acad Sci U S A 83: 1950-1954.

Toma JS, McPhail LT, Ramer MS (2006) Comparative postnatal development of spinal, trigeminal and vagal sensory root entry zones. Int J Dev Neurosci 24: 373-388.

Trapp BD, Bernier L, Andrews SB, Colman DR (1988) Cellular and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. J Neurochem 51: 859-868.

Tsuzuki K, Kondo E, Fukuoka T, Yi D, Tsujino H, Sakagami M, Noguchi K (2001) Differential regulation of P2X(3) mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia. Pain 91: 351-360.

Tzeng SF, Bresnahan JC, Beattie MS, de Vellis J (2001) Upregulation of the HLH Id gene family in neural progenitors and glial cells of the rat spinal cord following contusion injury. J Neurosci Res 66: 1161-1172.

Valverde F, Lopez-Mascaraque L (1991) Neuroglial arrangements in the olfactory glomeruli of the hedgehog. J Comp Neurol 307: 658-674.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric K+ channels in terminal and juxtaparanodal regions of neurons. Nature 365: 75-79.

Watanabe M, Sakurai Y, Ichinose T, Aikawa Y, Kotani M, Itoh K (2006) Monoclonal antibody Rip specifically recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase in oligodendrocytes. J Neurosci Res 84: 525-533.

Zhou XF, Rush RA, McLachlan EM (1996) Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. J Neurosci 16: 2901-2911.

Chapter 4

Discussion and concluding remarks

With respect to conceptual design, differences exist between the two studies presented in this thesis: the first study (Chapter 2) is concerned with glial development at a very defined anatomical region – the sensory root entry zone – while the second study (Chapter 3) is an investigation of RIP immunoreactivity among populations of peripheral ensheathing glia. The study presented in Chapter 3 is a direct follow-up of immunohistochemical results obtained in Chapter 2. However, these differences are rather superficial as common thematic threads do tie together the studies of this thesis. These themes fall into three very general categories of neuroscience: 1) developmental origin; 2) neuron-glial relationships; and 3) axonal regeneration. This final chapter examines how these three broad topics relate to the work presented in both experimental chapters and concludes with some final thoughts on the immunohistochemical techniques that were critical for both studies.

Developmental origin

In both studies, the developmental origins of the nervous tissue studied may have had an effect on the results presented. For example, in Chapter 2 it was demonstrated that the mature structures of the cranial TREZ and the vDREZ develop earlier than those of the spinal DREZ. While the mechanisms for this developmental trend are unknown, it may be possible that the neurons of placodal origin signal to the surrounding glia that form the structures of the cranial root entry zones to initiate root formation prior to the

instigation of the analogous formation of the DREZ by the neural crest-derived DRG neurons. This would suggest that specific neuronal populations of separate developmental origin play a role in the timing of signals necessary for formation of root entry zones; neurons of a placodal origin may reach maturity and subsequently generate the necessary signals earlier than those of a neural crest origin. If developmental origin is an important factor in determining the onset and completion of entry zone development, then it seems unlikely that the signals responsible for initiating these processes in the sensory root entry zones examined are derived from the associated glia, as the glia that form both cranial and spinal nerve root entry zones (i.e. astrocytes, oligodendrocytes and Schwann cells) are of a similar developmental origin. The above explanation provides a developmental sequence of events that would support the hypothesis that cranial root entry zones develop earlier in order to facilitate neuronal functions that are immediately necessary at birth in altricial mammals.

In the study presented in Chapter 3, OECs (which are of placodal origin; Doucette, 1991) were found to be negative for the RIP antigen while peripheral ensheathing glia of neural crest origin (i.e. myelinating and non-myelinating Schwann cells, satellite cells of the DRG and terminal Schwann cells) all expressed the RIP antigen to varying degrees. The reason for this differential expression is unknown, but perhaps glia of placodal orgin do not require RIP antigen expression in order to function as ensheathing glia whereas this antigen may be functionally important for maintaining axoglial contacts between axons and glia of neural crest origin (see below for a more detailed discussion of the RIP antigen and axoglial interactions). In both studies, we saw

that the developmental origin of the nervous tissue may influence the specific interactions between neurons and glia, possibly through regulating gene expression patterns that prime certain neural populations to transiently express developmentally-important genes at certain times, thus regulating developmental processes that determine the arrangement of the mature nervous system.

Neuron-glial relationships

Nervous system function is dependent upon the interactions between neurons and glia. One example of a neuron-glial interaction that exists in the CNS is between astrocytes and axons, as astrocytic end-feet provide metabolic support for axons at synapses and uptake excess glutamate as well as actively participate in neurotransmission via glutamate release (reviewed in: Kirchhoff et al., 2001; Vesce et al., 1999). Another example involves astrocytes that interact with the nodes of Ranvier (i.e. perinodal astrocytes) that may aid in structural development of the nodes as well as buffer ions at these sites (reviewed in Black and Waxman, 1988). In one study of the adult axolotl (salamander) spinal cord, Sims et al. (1991) demonstrated that radial glia (which are abundant in the mature CNS of this animal) interact with axons at the nodes of Ranvier in much the same manner as the perinodal astrocytes of mammals. This suggests a potentially similar role for astrocytes and radial glia at nodes of the vertebrate CNS. In the PNS, Schwann cells perform functions integral to nodal formation; however, the majority of axon-Schwann cell interactions occur along other internodal domains of the axon (discussed in detail below).

In the present studies, many examples of neuron-glial associations are illustrated. In Chapter 2, for example, the development of axoglial interactions at sensory root entry zones was investigated. The mature sensory root entry zone is an area of transition for specific neuron-glial interactions. In the PNS compartment, myelinated axons are associated with Schwann cells and unmyelinated axons are associated with non-myelinating Schwann cells of Remak fibres, while in the CNS, there are interactions between axons and astrocytes and oligodendrocytes. The astrocyte-axon interaction is highlighted at the sensory root entry zone. At the DREZ, there is a sudden shift across the PNS-CNS interface in the major axoglial interaction from Schwann cell-axon in the PNS to astrocyte-axon in the CNS (reviewed in Fraher, 2002).

In the developing rodent DREZ, the axons are directed towards and synapse onto their targets within the spinal cord prior to their ensheathment by glia (Mirnics and Koerber, 1995; Ozaki and Snider, 1997; reviewed in Cafferty and Ramer, 2002). The mature axoglial interactions that occur along the spinal nerve root up to the developing DREZ and into the spinal cord are largely established postnatally; however, transient interactions between axons and Schwann cell precursor cells occur embryonically. A recent study by Wanner et al. (2006) demonstrated that the cell adhesion molecule N-cadherin is heavily expressed in Schwann cell precursors and in BC cells at the rat DREZ at E14-16; during this time, primary afferent axons are reaching their central targets in rats (Mirnics and Koerber, 1995). Furthermore, N-cadherin-expressing Schwann cell cultures were shown to promote growth of sensory axons in vitro. These results suggest that contacts between Schwann cell precursors and axons are integral for sensory axon

growth from the DRG, through the immature DREZ, and toward central targets in the spinal cord. Treating cultures of Schwann cell precursors with cadherin blocking conditions (i.e. low Ca^{2+} culture media) resulted in the loss of contact between precursor cells, demonstrating the importance of N-cadherin in mediating this glioglial contact. The tight association between Schwann cell precursors is likely to be conducive to axonal growth through the dorsal root of the spinal nerve by aiding in nerve compaction and axonal fasciculation in the embryonic nerve – this is important as fasciculation increases the growth capacity of axons. The expression of N-cadherin progressively declines as Schwann cell precursors differentiate into mature Schwann cells, and this may occur in order to loosen contact between the Schwann cells and permit endoneurial space to form. This space is necessary for allowing axoglial contacts to form between mature Schwann cells and axons and also allows connective tissue to interact with both Schwann cells and axons (Wanner et al., 2006). The decrease in N-cadherin at the DREZ parallels the decrease of neural cell adhesion molecules in the cluster cells of the developing VRTZ (O'Brien et al., 2001), and the reason for these decreases may in fact be the same: to allow mature axoglial and glioglial interactions to develop in and around the TZs present in both the DREZ and the VRTZ.

In Chapter 2, I demonstrated that the glial elements characteristic of the mature sensory root entry zones were not in place until about P14, suggesting that mature axoglial interactions were not present until this time as well. The changes in the expression pattern of the RIP antigen (putatively CNPase in the PNS) throughout postnatal root entry zone development seem to indicate that this is likely the case, as RIP

immunoreactivity was not localized to the paranodes until P14, suggesting that this antigen may trace the development of axoglial (i.e. axon-Schwann cell) interactions in the PNS (see below for discussion of the expression of CNPase in paranodes).

Many axoglial interactions are mediated by certain families of adhesion proteins present on both the axolemma and the surface of the cell membrane of the interacting cell, such as a type of ensheathing glia. Examples of these proteins are present in interactions between axons and myelin in both the PNS and CNS. At the paranodal regions of myelinated axons, which lie adjacent to the nodes of Ranvier, high amounts of axoglial contact exist between the cytoplasm-filled terminal loops of myelin (that are situated at the edges of the internode of a myelinated axon) and the axolemma. This area of contact is referred to as the "paranodal septate-like junction" in the vertebrate, as it resembles the septate junctions present in *Drosophila* that are important for ensheathing axons (Brophy, 2001; Charles et al., 2002; Faivre-Sarrailh et al., 2004; Bellen et al., 1998). While the molecular interactions that are critical for maintaining the structural integrity of this region are not entirely understood, studies have suggested potential candidates. One complex of proteins known as the paranodin/contactin-associated protein (Caspr)-contactin complex is present on the axolemma and may interact with the neurofascin (NF155) glial receptor expressed on paranodal myelin (Charles et al., 2002). However, a later study by Gollan et al. (2003) demonstrated that Caspr actually inhibits contactin binding to NF155, thereby casting doubt on the relative importance of the interaction of this complex with NF155 in paranodal adhesion. On the other hand, studies have demonstrated that Caspr, contactin, and NF155 are all critical for

maintaining paranodal integrity (Bhat et al., 2001; Boyle et al., 2001; Sherman et al., 2005). Caspr-null mice contain irregular ion channel arrangements such as the presence of voltage-gated potassium channels, which are normally localized to the juxtaparanodal region, in the paranodes (Rios et al., 2003; Bhat et al., 2001; Rasband et al., 1998; Wang et al., 1993). In addition, nodal components such as voltage-gated sodium channels, which are necessary for maintaining saltatory conduction along the length of the axon, are abnormally distributed within the axon in these mice (Rios et al., 2003). These results illustrate the importance of the paranode in maintaining ion channel localization along the axolemma. Caspr-null mice also exhibit tremors that may result from altered conduction properties along axons, such as loss of current due to the separation of the paranodal myelin from the axolemma (Bhat et al., 2001). Some recent work has demonstrated how these key paranodal molecules interact with the axonal cytoskeleton. Ogawa et al. (2006) isolated the adaptor protein ankyrinB and members of the actin-associated spectrin family (αII spectrin and βII spectrin) from the cytoskeleton at the paranode. These proteins were found to interact with the paranodal cytoskeleton binding protein 4.1B in both the CNS and the PNS. Protein 4.1B is known to bind to the cytoplasmic region of Caspr and retains the Caspr-contactin cell adhesion complex to the paranodal axolemma (Gollan et al., 2002). In Caspr-null mice, ankyrinB expression is not localized to paranodes, thus providing molecular clues as to how the cytoskeleton of the paranodes is comprised (Ogawa et al., 2006). Taken together, these studies demonstrate that complicated multi-protein interactions at the paranodal region are important for sustaining axoglial interactions at this junction.

Axoglial interactions involving very similar proteins to those present at the paranode maintain the structure of nodes of Ranvier in both the CNS and PNS. Another ankyrin protein, ankyrinG, is present in high amounts in developing nodes of the optic nerve and its expression precedes that of mature nodal components such as voltage-gated sodium channels (Jenkins and Bennett, 2002). A later study by Koticha et al. (2006) demonstrated, through binding of neurofascin fusion proteins, that the axonal neurofascin (NF186) is important for recruiting sodium channels, ankyrinG, and the nodal spectrin βIV to nodal regions. This process is initiated by signalling through the glial ligand gliomedin in Schwann cells which binds to NF186 on the axolemma (Eshed et al., 2005). Furthermore, the absence of NF186 results in voltage-gated sodium channel diffusion throughout the axon (Sherman et al., 2005). These results indicate that it is the glial cell that initiates signalling necessary for nodal formation, at least in the PNS. A similar function is likely provided by oligodendrocytes in the CNS, as the absence of these cells prevents the normal clustering of voltage-gated sodium channels and ankyrinG at nodes (Mathis et al., 2001). However, astrocytes and NG2-positive cells that contact nodes may play a role in nodal maintenance in the CNS as well (Black and Waxman, 1988; Butt et al., 1999).

Recently, the previously unknown antigen bound by the RIP monoclonal antibody in oligodendrocytes was identified as CNPase (Watanabe et al., 2006). In chapter 3, I demonstrated that RIP likely recognized CNPase in the PNS as well. RIP immunoreactivity is robust in the cytoplasm of the paranodal loops in the PNS, suggesting that CNPase may play a role in establishing and maintaining axoglial contact

at the paranodal junction in the PNS. Studies have confirmed the importance of CNPase in this capacity in the CNS. Lappe-Siefke et al. (2003) demonstrated gradual axonal degeneration in CNPase-null mice that is at least partly the result of aberrant paranodal junctions, as Rasband et al. (2005) found that Caspr and voltage-gated sodium channels were not properly localized to the paranode and node respectively in these mice. The exact function of CNPase in maintaining axoglial junctions is unknown, but due to the role of CNPase in cytoskeletal rearrangement necessary for process expansion in oligodendrocytes (Lee et al., 2005; Zhang et al., 2005), it is reasonable to hypothesize that CNPase might be involved in establishing cytoskeletal interactions with proteins (such as gliomedin and NF155) within Schwann cells that mediate axoglial interactions.

Other examples of axoglial contact include those which occur in Remak fibres, where non-myelinating Schwann cells ensheathe small-diameter unmyelinated axons. Protein 4.1B, the cytoskeletal-binding protein that is involved in paranodal adhesion, is also located on the plasma membrane of unmyelinated axons, suggesting a role for this protein in maintaining axoglial interactions in Remak fibres (Terada et al., 2004). The high levels of RIP immunoreactivity present in the non-myelinating Schwann cells of Remak fibres suggest that CNPase is likely to be involved in axoglial interactions here in much the same fashion as posited in myelinating Schwann cells: through interaction with the cytoskeleton. The high levels of RIP immunoreactivity present may reflect the relatively large amount of axoglial interactions along the length of unmyelinated axons. These interactions are initially established postnatally in the developing nervous system and increase progressively until maturity. In chapter 2, I demonstrated that there was an

increase in punctate p75 immunoreactivity on the PNS side of the PNS-CNS interface in developing root entry zones. This accumulation of puncta was indicative of developing interactions between non-myelinating Schwann cells and unmyelinated fibres that enter the CNS through sensory root entry zones (Nilsson et al., 1998).

Still other neuron-glial associations were investigated in the experiments presented in Chapter 3. In both terminal Schwann cells of the NMJ and satellite cells surrounding neuronal somata in the DRG, only faint RIP immunoreactivity was identified. This may be due to the relatively small amount of neuron-glial interaction that occurs in these areas. In the olfactory entry zone, OECs are the major glial type that interacts with olfactory neurons (Doucette, 1991). RIP immunoreactivity was absent in these cells, suggesting that the axoglial interactions that occur between olfactory neurons and OECs do not involve CNPase. These results indicate that CNPase expression and its possible role in axoglial interactions may vary according to developmental origin as discussed above. While RIP immunoreactivity was low in satellite cells in the uninjured DRG, substantial increases in immunoreactivity were observed in satellite cells surrounding primarily large-diameter neurons in injured DRGs. Sympathetic sprouting in the DRG was assessed following peripheral nerve injury in order to test the hypothesis that RIP immunoreactivity and amount of axoglial contact were related. Increases in RIP immunoreactivity were associated with perineuronal sympathetic baskets, suggesting that expression of the RIP antigen (CNPase) is induced by axoglial contact.

Axonal regeneration: implications from development and RIP antigen expression

In the studies presented in this thesis, implications for axonal regeneration during development were addressed and regeneration of axons following peripheral nerve injuries was studied. In Chapter 2, I demonstrated that the developmental timeline of root entry zones was correlated to rostrocaudal localization; rostrally located cranial nerve root entry zones (the TREZ and the vDREZ) matured earlier than the more caudally located spinal DREZ. The expression of inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs), which prevents axonal regeneration through the DREZ, does not begin in earnest until after a "critical period" (that occurs in the first postnatal week) where axonal regeneration is possible through the DREZ (Pindzola et al., 1993; Carlstedt, 1988). CSPGs are extracellular matrix molecules that are composed of a core protein which is attached to various sulphated sugar chains composed of glycosaminoglycan; these chains inhibit axonal growth (reviewed in Properzi et al., 2003). CSPGs have been found to be expressed at the DREZ as early as postnatal day 3 in the rat along with cyotactin/tenascin (another extracellular matrix molecule) and CSPGs have been implicated in axonal guidance and growth inhibition elsewhere in developing CNS boundaries, including the retina (Pindzola et al., 1993; reviewed in Silver, 1994). If a similar "critical period" for axonal regeneration exists at the TREZ, it stands to reason, based on our results, that this may be present prenatally and that by birth, regeneration may no longer be possible through the TREZ. The developmental study presented in Chapter 2 illustrated that the expression patterns of proteins used to identify certain tissue types changed in similar fashion longitudinally in all three root entry zones investigated. It did not seem that there was a difference in the overall rate of

development of the three root entry zones, but rather that the cranial nerve root entry zones began to develop earlier than the spinal DREZ and completed development earlier as a result, suggesting that if there is a critical period for regeneration in the TREZ and vDREZ, it should last roughly as long as that present in the DREZ. It is possible that a similar critical period is present at the vDREZ, as regenerating axons are unable to cross the glial dome comprised of reactive astrocytes following injury in the adult, however this remains to be tested. Vagal afferent axons are capable of spontaneous regeneration into the brainstem, although this requires the presence of peripheral tissue insertions (Ramer, 2003). It is interesting to note that these tissue insertions decrease in relative size throughout vDREZ development, suggesting that their ability to promote axonal regeneration may decline as postnatal development proceeds.

The change in expression patterns of antigens throughout development of the root entry zones examined was no more evident than that of the RIP antigen. There was progressive decline along the length of the nerve and localization of RIP immunoreactivity at paranodal regions throughout the peripheral component of the DREZ, TREZ and vDREZ. The recapitulation of this expression pattern was present in the regenerating sciatic nerve, as the RIP antigen was redistributed throughout the cytoplasm of dedifferentiated Schwann cells (i.e. Schwann cells that convert from a myelinating to a non-myelinating state) that are present at the distal portion of an injured nerve (i.e. the portion of the nerve on the other side of the injury from the cell body of the neuron). Following peripheral nerve injury, these Schwann cells phagocytose the axonal debris present at the distal nerve, and proceed to form the bands of Büngner which

produce a support structure that provide a conduit for regenerating axons (reviewed in Fenrich and Gordon, 2004). Subsequently, the Schwann cells redifferentiate into a myelinating state. This progression of dedifferentiation of Schwann cells and consequent redifferentiation following injury to the sciatic nerve was evident in the results presented in Chapter 3. At 7 dpi, the Schwann cells appeared dedifferentiated but by 14 dpi, redifferentiation into a myelinating state was obvious as paranodes and Schmidt-Lantermann incisures were again visible with RIP staining. By 28 dpi, the morphology of the injured nerves closely resembled that of the uninjured nerve.

Final thoughts on immunohistochemical technique

The results of the studies presented were entirely dependent on the technique of immunohistochemistry. While this technique is generally considered useful and normally quite reliable for histological analysis, its validity is not always assured. In immunohistochemical studies, specificity issues are always a concern, as is the blind faith exercised by some investigators that certain antibodies are binding exclusively to the cell types that they were designed to identify. Even supposing the primary antibody used is binding to the correct antigen (ie. assuming that there is no cross-reactivity with undesired antigens), there is no guarantee that this antigen is specific to the cell type of interest. Unfortunately, the fact that certain antibodies are commercially available does not ensure their specificity with regards to cell type. As an example, the RIP monoclonal antibody has been marketed as a "specific oligodendrocyte marker", and I have clearly demonstrated that this is not the case. As it turns out, the RIP antigen (recently identified as CNPase in oligodendrocytes; Watanabe et al., 2006) is not solely expressed by

oligodendrocytes, as my studies show that it is present in discrete populations of both myelinating and non-myelinating peripheral glia as well. The story of the RIP monoclonal antibody exemplifies how dangerous conclusions can be drawn from immunohistochemical results, as it was used for 17 years in studies of the CNS before the identity of its antigen was revealed. Much evidence was presented in Chapter 3 to suggest that RIP recognizes CNPase in many peripheral glial populations (both myelinating and non-myelinating) in addition to oligodendrocytes; however, this remains unproven. The necessary biochemical experiments needed to verify this (i.e. isolating and identifying the protein complex recognized by RIP in the PNS) would be worthwhile future studies. CNPase expression, therefore, is not likely to be confined to the myelinating glia of both the PNS and CNS and it should not be used as a specific marker for such cells. A very recent study demonstrated transient expression of CNPase in microglia of the developing CNS (Wu et al., 2006), further indicating that this protein is not specific to myelinating glial cells. These results illustrate just one of many examples of using a purported "specific" antigen to mark a cell type when in fact the antigen in question is actually expressed in multiple cell populations that occupy similar environments or are of a similar class.

In immunohistochemical studies, care should always be taken to disclose as much as is reasonably possible about an antigen used to mark a particular cell type so that the most accurate interpretation of the results is undertaken and any confounding immunoreactivity is able to be taken into account. Because this is not always done, the results of immunohistochemical studies should be taken with caution, especially if the

antibodies used in such studies have yet to be fully characterized. Problems with substandard immunohistochemical technique have drawn the ire of some editors of scientific journals, such as those of the Journal of Comparative Neurology (see editorial by Saper and Sawchenko, 2003). Despite the inherent pitfalls associated with this technique, the tool of immunohistochemistry can be powerful for visualizing the cellular architecture of the nervous system. If adequate microscopy and photography accompany proper tissue processing techniques and co-operative antibodies, the contrast between immunoreactive and non-immunoreactive tissue in immunohistochemical data is usually striking enough to provide clear demonstrations of immunopositive tissue. The studies presented in this thesis demonstrate the versatility of this technique, as it is useful for examining the dynamic processes of neural development and regeneration. If used properly, immunohistochemistry can answer very fundamental and important questions concerning nervous system development and structure.

Bibliography

Bellen HJ, Lu Y, Beckstead R, Bhat MA (1998) Neurexin IV, caspr and paranodin--novel members of the neurexin family: encounters of axons and glia. Trends Neurosci 21: 444-449.

Bhat MA, Rios JC, Lu Y, Garcia-Fresco GP, Ching W, St Martin M, Li J, Einheber S, Chesler M, Rosenbluth J, Salzer JL, Bellen HJ (2001) Axon-glia interactions and the domain organization of myelinated axons requires neurexin IV/Caspr/Paranodin. Neuron 30: 369-383.

Black JA, Waxman SG (1988) The perinodal astrocyte. Glia 1: 169-183.

Boyle ME, Berglund EO, Murai KK, Weber L, Peles E, Ranscht B (2001) Contactin orchestrates assembly of the septate-like junctions at the paranode in myelinated peripheral nerve. Neuron 30: 385-397.

Brophy PJ (2001) Axoglial junctions: separate the channels or scramble the message. Curr Biol 11: R555-R557.

Butt AM, Duncan A, Hornby MF, Kirvell SL, Hunter A, Levine JM, Berry M (1999) Cells expressing the NG2 antigen contact nodes of Ranvier in adult CNS white matter. Glia 26: 84-91.

Cafferty WBJ, Ramer MS (2002) Promoting Sensory Axon Regeneration Across the PNS-CNS Interface. In: Glial Interfaces in the Nervous System: Role in Repair and Plasticity (Aldskogius H, Fraher JP, eds), pp 23-40. Amsterdam: IOS Press.

Carlstedt T (1988) Reinnervation of the mammalian spinal cord after neonatal dorsal root crush. J Neurocytol 17: 335-350.

Charles P, Tait S, Faivre-Sarrailh C, Barbin G, Gunn-Moore F, Denisenko-Nehrbass N, Guennoc AM, Girault JA, Brophy PJ, Lubetzki C (2002) Neurofascin is a glial receptor for the paranodin/Caspr-contactin axonal complex at the axoglial junction. Curr Biol 12: 217-220.

Doucette R (1991) PNS-CNS transitional zone of the first cranial nerve. J Comp Neurol 312: 451-466.

Eshed Y, Feinberg K, Poliak S, Sabanay H, Sarig-Nadir O, Spiegel I, Bermingham JR, Jr., Peles E (2005) Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. Neuron 47: 215-229.

Faivre-Sarrailh C, Banerjee S, Li J, Hortsch M, Laval M, Bhat MA (2004) Drosophila contactin, a homolog of vertebrate contactin, is required for septate junction organization and paracellular barrier function. Development 131: 4931-4942.

Fenrich K, Gordon T (2004) Canadian Association of Neuroscience review: axonal regeneration in the peripheral and central nervous systems--current issues and advances. Can J Neurol Sci 31: 142-156.

Fraher J (2002) Axons and glial interfaces: ultrastructural studies. J Anat 200: 415-430.

Gollan L, Sabanay H, Poliak S, Berglund EO, Ranscht B, Peles E (2002) Retention of a cell adhesion complex at the paranodal junction requires the cytoplasmic region of Caspr. J Cell Biol 157: 1247-1256.

Gollan L, Salomon D, Salzer JL, Peles E (2003) Caspr regulates the processing of contactin and inhibits its binding to neurofascin. J Cell Biol 163: 1213-1218.

Jenkins SM, Bennett V (2002) Developing nodes of Ranvier are defined by ankyrin-G clustering and are independent of paranodal axoglial adhesion. Proc Natl Acad Sci U S A 99: 2303-2308.

Kirchhoff F, Dringen R, Giaume C (2001) Pathways of neuron-astrocyte interactions and their possible role in neuroprotection. Eur Arch Psychiatry Clin Neurosci 251: 159-169.

Koticha D, Maurel P, Zanazzi G, Kane-Goldsmith N, Basak S, Babiarz J, Salzer J, Grumet M (2006) Neurofascin interactions play a critical role in clustering sodium channels, ankyrin G and beta IV spectrin at peripheral nodes of Ranvier. Dev Biol 293: 1-12.

Lappe-Siefke C, Goebbels S, Gravel M, Nicksch E, Lee J, Braun PE, Griffiths IR, Nave KA (2003) Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nat Genet 33: 366-374.

Lee J, Gravel M, Zhang R, Thibault P, Braun PE (2005) Process outgrowth in oligodendrocytes is mediated by CNP, a novel microtubule assembly myelin protein. J Cell Biol 170: 661-673.

Mathis C, Denisenko-Nehrbass N, Girault JA, Borrelli E (2001) Essential role of oligodendrocytes in the formation and maintenance of central nervous system nodal regions. Development 128: 4881-4890.

Mirnics K, Koerber HR (1995) Prenatal development of rat primary afferent fibers: II. Central projections. J Comp Neurol 355: 601-614.

Nilsson R, I, Berthold CH, Carlstedt T (1998) Redistribution of Schwann cells at the developing PNS-CNS borderline. An ultrastructural and autoradiographic study on the S1 dorsal root of the cat. J Neurocytol 27: 85-97.

O'Brien D, Dockery P, McDermott K, Fraher J (2001) Early development of rat ventral root transitional zone: an immunohistochemical and morphometric study. J Neurocytol 30: 11-20.

Ogawa Y, Schafer DP, Horresh I, Bar V, Hales K, Yang Y, Susuki K, Peles E, Stankewich MC, Rasband MN (2006) Spectrins and ankyrinB constitute a specialized paranodal cytoskeleton. J Neurosci 26: 5230-5239.

Ozaki S, Snider WD (1997) Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J Comp Neurol 380: 215-229.

Pindzola RR, Doller C, Silver J (1993) Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. Dev Biol 156: 34-48.

Properzi F, Asher RA, Fawcett JW (2003) Chondroitin sulphate proteoglycans in the central nervous system: changes and synthesis after injury. Biochem Soc Trans 31: 335-336.

Ramer MS (2003) Spontaneous functional viscerosensory regeneration into the adult brainstem. J Neurosci 23: 9770-9775.

Rasband MN, Trimmer JS, Schwarz TL, Levinson SR, Ellisman MH, Schachner M, Shrager P (1998) Potassium channel distribution, clustering, and function in remyelinating rat axons. J Neurosci 18: 36-47.

Rasband MN, Tayler J, Kaga Y, Yang Y, Lappe-Siefke C, Nave KA, Bansal R (2005) CNP is required for maintenance of axon-glia interactions at nodes of Ranvier in the CNS. Glia 50: 86-90.

Rios JC, Rubin M, St Martin M, Downey RT, Einheber S, Rosenbluth J, Levinson SR, Bhat M, Salzer JL (2003) Paranodal interactions regulate expression of sodium channel subtypes and provide a diffusion barrier for the node of Ranvier. J Neurosci 23: 7001-7011.

Saper CB, Sawchenko PE (2003) Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry. J Comp Neurol 465: 161-163.

Sherman DL, Tait S, Melrose S, Johnson R, Zonta B, Court FA, Macklin WB, Meek S, Smith AJ, Cottrell DF, Brophy PJ (2005) Neurofascins are required to establish axonal domains for saltatory conduction. Neuron 48: 737-742.

Silver J (1994) Inhibitory molecules in development and regeneration. J Neurol 242: S22-S24.

Sims TJ, Gilmore SA, Waxman SG (1991) Radial glia give rise to perinodal processes. Brain Res 549: 25-35.

Terada N, Ohno N, Yamakawa H, Baba T, Fujii Y, Ohara O, Ohno S (2004) Protein 4.1B localizes on unmyelinated axonal membranes in the mouse enteric nervous system. Neurosci Lett 366: 15-17.

Vesce S, Bezzi P, Volterra A (1999) The active role of astrocytes in synaptic transmission. Cell Mol Life Sci 56: 991-1000.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric K+ channels in terminal and juxtaparanodal regions of neurons. Nature 365: 75-79.

Wanner IB, Guerra NK, Mahoney J, Kumar A, Wood PM, Mirsky R, Jessen KR (2006) Role of N-cadherin in Schwann cell precursors of growing nerves. Glia 54: 439-459.

Watanabe M, Sakurai Y, Ichinose T, Aikawa Y, Kotani M, Itoh K (2006) Monoclonal antibody Rip specifically recognizes 2',3'-cyclic nucleotide 3'-phosphodiesterase in oligodendrocytes. J Neurosci Res 84: 525-533.

Wu CY, Lu J, Cao Q, Guo CH, Gao Q, Ling EA (2006) Expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase in the amoeboid microglial cells in the developing rat brain. Neuroscience 142: 333-341.

Zhang B, Cao Q, Guo A, Chu H, Chan YG, Buschdorf JP, Low BC, Ling EA, Liang F (2005) Juxtanodin: an oligodendroglial protein that promotes cellular arborization and 2',3'-cyclic nucleotide-3'-phosphodiesterase trafficking. Proc Natl Acad Sci U S A 102: 11527-11532.