ROLE OF GALECTIN-1 IN SENSORY NEURON DEVELOPMENT AND PERIPHERAL NERVE REPAIR

by

ANDREW DAVID GAUDET

B.Sc., The University of British Columbia, 2003

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

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2010

© Andrew David Gaudet, 2010
Abstract

In comparison to adult central nervous system (CNS) axons, peripheral nervous system (PNS) axons have a high propensity for regrowth following injury. The PNS axon’s capacity to regenerate depends on an effective response within the neuron itself, combined with a supportive environment maintained by cells surrounding the axon. Unfortunately, successful reconnection of peripheral axons with appropriate targets is hampered by discontinuities associated with injury and by a decreased growth response over time. Studying strategies that improve peripheral nerve repair could enhance functional outcomes following peripheral nerve injury (PNI), and might provide insight for CNS repair.

The small protein galectin-1 (Gal1) is required for developmental targeting of specific olfactory axons and promotes peripheral axon regeneration. Despite this, Gal1’s role in sensory axon development and regeneration is not well-defined. In this dissertation, I explore how Gal1 affects developmental and regenerative axon growth.

Using mice lacking Gal1 (Lgals1−/−), I show that Gal1 is required for proper targeting of central axons of small-diameter, nociceptive dorsal root ganglion (DRG) neurons. Interestingly, Lgals1−/− mice had corresponding deficits in behavioural responses to noxious stimuli.

Next, I characterize the regulation of Gal1 in DRG neurons and their environment following PNI and dorsal root injury (DRI). DRG neurons mount a robust regenerative response following injury of their peripheral, but not central branch. Neuronal Gal1 was
upregulated after PNI, but not DRI. In addition, Gal1 expression in the regrowing axon’s environment correlated with the permissiveness of that environment.

I then examine whether Gal1 promotes axonal regeneration through mechanisms intrinsic and/or extrinsic to the injured neuron. Gal1 did not affect DRG neurons’ intrinsic growth state: Lgals1−/− neurons did not display abnormal neurite outgrowth, and exogenous oxidized Gal1 (Gal1/Ox) did not affect neurite outgrowth.

Gal1 does affect the response of non-neuronal cells. I show that Gal1 promotes accumulation of immune cells called macrophages following PNI. Injection of Gal1-specific antibodies attenuated typical PNI-induced accumulation of macrophages; conversely, Gal1/Ox injection into uninjured nerves facilitated macrophage accumulation in wild-type mice. My data suggest that Gal1 does not elicit axon growth directly; rather, Gal1 likely promotes axon regeneration indirectly by enhancing PNI-induced macrophage accumulation.
# Table of contents

Abstract............................................................................................................................ ii

Table of contents ........................................................................................................... iv

List of tables ................................................................................................................. ix

List of figures ................................................................................................................ x

List of abbreviations ..................................................................................................... xiii

Acknowledgements ....................................................................................................... xiv

Co-authorship statement............................................................................................... xvi

1 Introduction: gaining perspective from the periphery......................................... 1

1.1 Overview .......................................................................................................... 1

1.2 Nerve trauma .................................................................................................. 3

1.2.1 Peripheral nerve injury: causes and implications ................................................. 3

1.2.2 Central nervous system injury: causes and implications .................................... 5

1.3 Importance of studying peripheral axon regeneration .............................. 7

1.3.1 Peripheral axon regeneration: challenges and current treatment ................. 7

1.3.2 Central nervous system injury: challenges and current treatment ..................... 9

1.4 The dorsal root ganglion: home to a large heterogeneous population of sensory neurons ........................................................................................................... 10

1.4.1 DRG neuron development: a new sensation ...................................................... 10

1.4.2 DRG neuron neurochemistry ........................................................................... 11

1.4.3 Anatomy and functions of DRG neurons ............................................................ 15

1.5 Cellular responses to peripheral nerve injury ............................................ 19

1.5.1 Intrinsic response of the neuron to injury ........................................................... 22

1.5.2 Responses extrinsic to the neuron after nerve injury ....................................... 28

1.6 Galectin-1: the little protein that could......................................................... 45

1.6.1 Galectins: discovery and classification ............................................................... 45

1.6.2 Galectin-1: a ubiquitous protein with diverse functions ................................. 46

1.6.3 Roles of galectin-1 in the immune system .......................................................... 48

1.6.4 Roles of galectin-1 in the nervous system .......................................................... 49

1.7 Experimental hypotheses............................................................................. 55

1.8 References ..................................................................................................... 58
2 Altered primary afferent anatomy and reduced thermal sensitivity in mice lacking galectin-1 ........................................................................................................ 84

2.1 Introduction ................................................................................................... 84
2.2 Materials and methods ................................................................................. 87
  2.2.1 Animals ................................................................................................... 87
  2.2.2 Behavioral testing .................................................................................. 87
  2.2.3 Fos activation ....................................................................................... 88
  2.2.4 Tissue processing / immunohistochemistry .......................................... 88
  2.2.5 Image analysis ...................................................................................... 89
  2.2.6 Thin sectioning and electron microscopy ............................................. 91
  2.2.7 In situ hybridization ............................................................................ 92
  2.2.8 Imaging and Statistics .......................................................................... 93
2.3 Results ......................................................................................................... 94
  2.3.1 Lgals1−/− mice have increased thermal nociceptive thresholds ........ 94
  2.3.2 Gal1 is expressed mainly in small-diameter DRG neurons .................. 94
  2.3.3 Altered neuron subtype distribution in the Lgals1−/− DRG .................. 100
  2.3.5 Fewer Lgals1−/− dorsal horn neurons express Fos after noxious stimulation 105
2.4 Discussion ................................................................................................... 110
  2.4.1 Overview .............................................................................................. 110
  2.4.2 Gal1 distribution .................................................................................. 111
  2.4.3 Role of IB4-binding neurons in thermal nociception ......................... 113
  2.4.4 Conclusions ......................................................................................... 115
2.5 References .................................................................................................. 116

3 Regulation of neuronal and glial galectin-1 expression by peripheral and central axotomy of rat primary afferent neurons ............................................. 120

3.1 Introduction ................................................................................................. 120
3.2 Materials and methods ............................................................................... 123
  3.2.1 Animals and surgery .......................................................................... 123
  3.2.2 Tissue processing ............................................................................... 123
  3.2.3 In situ hybridization (ISH) ................................................................ 124
  3.2.4 Immunohistochemistry ...................................................................... 124
  3.2.5 Image Analysis ................................................................................... 125
  3.2.6 Statistics ............................................................................................. 127
3.3 Results ....................................................................................................... 128
3.3.1 Altered Gal1 mRNA and protein expression in the DRG following peripheral, but not central axotomy ................................................................. 128
3.3.2 Novel Gal1 expression patterns in the dorsal horn following dorsal rhizotomy and peripheral axotomy ................................................................ 129
3.3.3 Dorsal rhizotomy-induced Gal1 mRNA increase is restricted to the peripheral portion of the DREZ ............................................................. 130

3.4 Discussion ......................................................................................... 149
3.4.1 Overview ....................................................................................... 149
3.4.2 Gal1 expression in the naïve DRG .................................................. 149
3.4.3 Gal1 expression after injury ............................................................. 150
3.4.4 Conclusions ................................................................................... 152

3.5 References .......................................................................................... 154

4 The effect of galectin-1 on neurite outgrowth from cultured adult dorsal root ganglion neurons ................................................................. 158

4.1 Introduction ....................................................................................... 158
4.2 Materials and methods .................................................................... 161
4.2.1 Animals ......................................................................................... 161
4.2.2 Dissociated dorsal root ganglion neuron culture .............................. 161
4.2.3 Immunocytochemistry .................................................................. 162
4.2.4 Image analysis .............................................................................. 163

4.3 Results ............................................................................................... 165
4.3.1 Neurite outgrowth in the absence of Gal1..................................... 165
4.3.2 The effects of Gal1/Ox treatment on neurite outgrowth .................. 165

4.4 Discussion ......................................................................................... 175
4.4.1 Overview ....................................................................................... 175
4.4.2 Potential links between intracellular Gal1 and the neuronal switch to growth mode ................................................................. 175
4.4.3 Potential effects of extracellular Gal1 on axon growth ................. 177
4.4.4 Conclusions ................................................................................... 180

4.5 References .......................................................................................... 181

5 A role for galectin-1 in the immune response to peripheral nerve injury ..... 184

5.1 Introduction ....................................................................................... 184
5.2 Materials and methods .................................................................... 186
5.2.1 Preparation of oxidized galectin-1 ............................................... 186
5.2.2 Animals and surgery ..................................................................... 186
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.3 Tissue processing / immunohistochemistry</td>
<td>188</td>
</tr>
<tr>
<td>5.2.4 Image analysis</td>
<td>189</td>
</tr>
<tr>
<td>5.3 Results</td>
<td>190</td>
</tr>
<tr>
<td>5.3.1 The density of Gal1-immunoreactive cells is increased in distal nerve following sciatic nerve injury</td>
<td>190</td>
</tr>
<tr>
<td>5.3.2 Endogenous Gal1 is necessary for the typical injury-induced macrophage response during Wallerian degeneration</td>
<td>190</td>
</tr>
<tr>
<td>5.3.3 Mice lacking Gal1 exhibit impaired macrophage responses following peripheral axotomy</td>
<td>194</td>
</tr>
<tr>
<td>5.3.4 Exogenous Gal1/Ox facilitates macrophage accumulation in uninjured Lgals1⁺/⁻, but not Lgals1⁻⁻ sciatic nerve</td>
<td>197</td>
</tr>
<tr>
<td>5.3.5 Nerve transplantation in Lgals1⁻⁻ mice: compensation and (lack of) responsiveness to Gal1</td>
<td>200</td>
</tr>
<tr>
<td>5.4 Discussion</td>
<td>209</td>
</tr>
<tr>
<td>5.4.1 Overview</td>
<td>209</td>
</tr>
<tr>
<td>5.4.2 Gal1 and the immune response</td>
<td>209</td>
</tr>
<tr>
<td>5.4.3 Role of Gal1 following peripheral nerve injury</td>
<td>210</td>
</tr>
<tr>
<td>5.4.4 Conclusions</td>
<td>213</td>
</tr>
<tr>
<td>5.5 References</td>
<td>214</td>
</tr>
<tr>
<td>6 Discussion: exploring galectin-1’s impact on axon growth</td>
<td>218</td>
</tr>
<tr>
<td>6.1 Overview</td>
<td>218</td>
</tr>
<tr>
<td>6.2 Fateful moments: galectin-1 and DRG neuron development</td>
<td>223</td>
</tr>
<tr>
<td>6.2.1 Galectin-1 is required for the development of specific DRG neuron subpopulations</td>
<td>223</td>
</tr>
<tr>
<td>6.2.2 Wiring before firing: galectin-1’s role in developmental axon targeting</td>
<td>225</td>
</tr>
<tr>
<td>6.3 Good as new? Enhancing axon growth with galectin-1</td>
<td>229</td>
</tr>
<tr>
<td>6.3.1 Gal1 expression is correlated with the regenerative capacity of injured DRG neurons</td>
<td>229</td>
</tr>
<tr>
<td>6.3.2 Assessing intrinsic value: galectin-1 as a potential neuronal regeneration-associated gene</td>
<td>230</td>
</tr>
<tr>
<td>6.3.3 Galectin-1 is good for the environment</td>
<td>231</td>
</tr>
<tr>
<td>6.3.4 Branching out: galectin-1-mediated regeneration placed into context</td>
<td>234</td>
</tr>
<tr>
<td>6.4 Summary</td>
<td>241</td>
</tr>
<tr>
<td>6.5 References</td>
<td>243</td>
</tr>
<tr>
<td>Appendix</td>
<td>250</td>
</tr>
</tbody>
</table>
Animal care certificates

250
List of tables

Table 1.1. Comparison of properties of C, Aβ and Aδ primary afferent fibres ............... 16
List of figures

Figure 1.1. Neurochemical phenotypes and anatomy of dorsal root ganglion (DRG) neurons and their projections ................................................................. 14

Figure 1.2. The progression of Wallerian degeneration and axon regeneration following peripheral nerve injury ................................................................. 21

Figure 2.1. Lgals1−/− mice have reduced nocifensive thermal responses compared to Lgals1+/+ mice ................................................................. 96

Figure 2.2. Distribution of Gal1 protein in the C7 and C8 DRGs of Lgals1+/+ mice .... 98

Figure 2.3. Gal1 in situ hybridization signal in Lgals1+/+ and Lgals1+/− DRGs ............. 99

Figure 2.4. Differences in distribution of CGRP-IR, IB4-binding, and NF200-IR neurons in the DRG between Lgals1+/+ and Lgals1+/− mice .......................... 102

Figure 2.5. Thin plastic sections and high power electron micrographs demonstrating axonal morphology dorsal roots of Lgals1+/+ and Lgals1+/− mice ................. 104

Figure 2.6. Differences in distribution of nociceptive fibre terminals within the dorsal horn of Lgals1+/+ and Lgals1+/− mice .......................................................... 107

Figure 2.7. Fos activation induced by noxious thermal forepaw stimulation is reduced in the Lgals1−/− cervical dorsal horn ................................................. 108

Figure 2.8. The reduced number of Fos-positive neurons in Lgals1−/− mice is not a result of a difference in the number of second order neurons .............................. 109

Figure 3.1. DRG neuron Gal1 mRNA expression is regulated by axotomy of their peripheral, but not central projections ..................................................... 132

Figure 3.2. Gal1 autoradiographic signal (silver grain density) quantification of uninjured, axotomized, and rhizotomized DRGs ........................................ 134
Figure 3.3. Gal1- and CGRP-IR in cervical DRGs after peripheral axotomy and dorsal rhizotomy .......................................................... 136

Figure 3.4. Gal1-IR and IB4-binding in cervical DRG neurons following peripheral axotomy and dorsal rhizotomy .......................................................... 138

Figure 3.5. Gal1- and NF200-IR in cervical DRGs following peripheral and central axotomy ........................................................................................................ 140

Figure 3.6. Summary of Gal1-IR cell size distribution compared to the size distribution of the entire neuronal population in uninjured and injured DRGs .......................................................... 142

Figure 3.7. Differential regulation of Gal1-IR distribution in the C7 dorsal horn by central and peripheral axotomy ........................................................................................................ 144

Figure 3.8. Gal1 mRNA increased significantly in degenerating sensory tracts in the spinal cord following dorsal rhizotomy .......................................................... 146

Figure 3.9. Gal1 mRNA increased significantly in the peripheral, but not central, compartment of the dorsal root entry zone at 7 and 14 days following dorsal rhizotomy .......................................................... 148

Figure 4.1. Inverted fluorescent photomicrographs of dissociated \textit{Lgals1}^{+/+} and \textit{Lgals1}^{-/-} DRG neurons cultured in the absence or presence of trophic factors .......................................................... 167

Figure 4.2. Neurite outgrowth from dissociated \textit{Lgals1}^{-/-} DRG neurons is not significantly different from outgrowth from \textit{Lgals1}^{+/+} neurons ........................................................................................................ 169

Figure 4.3. Inverted fluorescent photomicrographs of dissociated rat DRG neurons cultured in the absence or presence of Gal1/Ox and/or trophic factors ........................................................................................................ 172

Figure 4.4. Gal1/Ox treatment has minimal effects on neuritic outgrowth from dissociated rat DRG neurons ........................................................................................................ 174

Figure 5.1. Gal1-IR cell density is increased in the distal nerve following peripheral axotomy ........................................................................................................ 191

Figure 5.2. The axotomy-induced increase in macrophage density in the \textit{Lgals1}^{+/+} sciatic nerve distal to ligation was attenuated by injection of Gal-1-specific function-blocking antibody (Gal1-Ab) at 3 days post-injury ........................................................................................................ 193
Figure 5.3. Axotomy-induced accumulation of macrophages was delayed and diminished in sciatic nerves of $Lgals1^{-/-}$ mice, as visualized by F4/80-IR ........................ 196

Figure 5.4. Injury-induced macrophage accumulation was delayed in L5 DRGs of $Lgals1^{+/+}$ mice, as visualized by F4/80-IR .......................................................... 199

Figure 5.5. Oxidized Gal1 (Gal1/Ox) injection into uninjured sciatic nerve was sufficient to facilitate macrophage accumulation in $Lgals1^{+/+}$ (but not $Lgals1^{-/-}$) mice ................. 203

Figure 5.6. Macrophage accumulation in segments of sciatic nerve transplanted to $Lgals1^{+/+}$ mice or $Lgals1^{-/-}$ mice three and seven days post-transplantation ................ 205

Figure 5.7. Dorsal rhizotomy-induced microglial/macrophage accumulation in the dorsal horn was reduced at 21 dpi in $Lgals1^{+/+}$ mice ............................................... 207

Figure 5.8. Comparison of macrophage/microglial accumulation in the dorsal horn of $Lgals1^{+/+}$ mice after sciatic nerve ligation or dorsal rhizotomy ..................................... 208

Figure 6.1. Schematic representing mechanisms that underlie Gal1-mediated peripheral axon regeneration ........................................................................................................ 222
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AraC</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C-</td>
<td>cervical spinal cord segment</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CST</td>
<td>corticospinal tract</td>
</tr>
<tr>
<td>DH</td>
<td>dorsal horn</td>
</tr>
<tr>
<td>dh2O</td>
<td>distilled water</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-injury</td>
</tr>
<tr>
<td>DREZ</td>
<td>dorsal root entry zone</td>
</tr>
<tr>
<td>DRI</td>
<td>dorsal root injury (dorsal rhizotomy)</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>E-</td>
<td>embryonic day (e.g. E8)</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>Gal1</td>
<td>galectin-1</td>
</tr>
<tr>
<td>Gal1-Ab</td>
<td>function-blocking antibody against galectin-1</td>
</tr>
<tr>
<td>Gal1/Ox</td>
<td>oxidized galectin-1</td>
</tr>
<tr>
<td>Gal1/Red</td>
<td>reduced galectin-1</td>
</tr>
<tr>
<td>GAP-43</td>
<td>43kDa growth-associated protein</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GF</td>
<td>gracile fasciculus</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H-Ras-GTP</td>
<td>GTP-bound (activated) H-Ras</td>
</tr>
<tr>
<td>IB4</td>
<td>isolectin B4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin (e.g. IL-6)</td>
</tr>
<tr>
<td>-IR</td>
<td>-immunoreactivity (or -immunoreactive)</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>K-Ras-GTP</td>
<td>GTP-bound (activated) K-Ras</td>
</tr>
<tr>
<td>L-</td>
<td>lumbar spinal cord segment</td>
</tr>
<tr>
<td>Lgals1+/-</td>
<td>galectin-1 wild-type</td>
</tr>
<tr>
<td>Lgals1-/-</td>
<td>galectin-1 null mutant</td>
</tr>
<tr>
<td>lamina II</td>
<td>inner part of lamina II</td>
</tr>
<tr>
<td>lamina IIo</td>
<td>outer part of lamina II</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1 (a.k.a. CCL-2)</td>
</tr>
<tr>
<td>NF200</td>
<td>heavy neurofilament (200 kDa)</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NT-</td>
<td>neurotrophin (e.g. NT-3)</td>
</tr>
<tr>
<td>OMgp</td>
<td>oligodendrocyte-myelin glycoprotein</td>
</tr>
<tr>
<td>P-</td>
<td>postnatal day</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>pheochromocytoma cell line</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PNI</td>
<td>peripheral nerve injury</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RAG</td>
<td>regeneration-associated gene</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>T-</td>
<td>thoracic spinal cord segment</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin-related kinase</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>W</td>
<td>Watts</td>
</tr>
<tr>
<td>Wld-s</td>
<td>slow Wallerian degeneration mouse</td>
</tr>
</tbody>
</table>
Acknowledgements

Damn right I like the life I live
‘Cause I went from negative to positive
And it’s all…
(It’s all good)
- Notorious B.I.G., “Juicy”

Rather than just a list of names, I picture the wonderful friends and scientists described herein as contributors to a nebulous of positive energy that has helped me immensely on my road to success as a graduate student, whether they know it or not. Of course, the difficulty comes in describing the effects that these people have had in this small space.

My biggest scientific influence over the past few (?) years has been my supervisor, Dr Matt Ramer. Matt’s passion for discovery is infectious, and he is driven to complete rigorous, high-quality science. He always comes through in the clutch for his students. I also appreciate Matt’s flexibility: he allowed me to explore many avenues during my degree, such as volunteer work, teaching, and traveling. I learned that I love to teach, and to write, thanks to these opportunities. Matt sent me away to fantastic places for conferences and collaborations, which allowed me to experience science — and life — from new perspectives. In addition to being my supervisor, Matt makes a great teaching colleague: we had loads of fun leading Funky Shirt Fridays in class and sharing the odd pint in the Gallery. Matt leads by example.

During my “formative” research years, Drs John McGraw and Lowell McPhail had important roles. John provided guidance with the galectin-1 project and eased me into its takeover, and also taught me the infamous and everlasting Three Life Rules. Lowell gave me a figurative kick in the butt and encouraged me to adopt a more positive perspective in the lab.

Other graduate students (listed alphabetically here) made the lab a wonderful place to be over the years. Jess Inskip was a late recruit to our office in the NCE Building, and made tasty contributions to Lunch Club. Her creative and adventurous spirit is inspiring. Leanne Ramer is my teaching buddy — we taught our first (85 person!) class together — and is now my pedagogy study colleague. She makes a great ski partner, is straightforward, and always adds spice to conversations or nights out on the town. Dr Angela Scott was my grad student buddy from the start. She was another officemate and Lunch Clubber. We shared many memorable moments, from riding bikes and go-karts near the Golden Gate Bridge to eating smoked meat in Montreal, not to mention routine Friday night drinks. She is a great travel partner: together, we attended conferences in Asilomar, Australia/Fiji, Montreal, and Washington. Lesley Soril, another former officemate, was a student in a class I TAed years ago. It turns out that she is a superb listener who has good taste in music and fashion. She’s also fun to hang out with, and organizes epic birthday parties every March. We enjoyed bula dancing and racing hermit crabs together on a remote Fijian island in 2007. Jeremy Toma is a unique character. He brought a different type of fashion to the lab with his Funky Shirt Friday attire, which was exemplified by his paisley/pink plaid two-shirt combination Hallowe’en costume.

Employees in the lab also had important influences. Jacquelyn Cragg brings a wonderful positive attitude to everything she approaches, and her passion for statistics
also benefited everyone in the lab. Mario Cruz also has a sunny demeanour, and is always willing to help out in any way possible. The 2009 hike to a foggy Dog Mountain with Jac, Mario, and Jess was a particularly fun day with these guys (not to mention Asilomar shenanigans). Emily Lipinski was another excellent lab manager who brightened up everyone’s day. She is also a karaoke expert.

Professor Wolfram Tetzlaff provided guidance on the galectin-1 project. More importantly, his (sometimes long-winded) opining in journal club was instrumental in students’ development of critical and scientific thinking. Incidentally, the weekly journal club was an invaluable venue for discussing recent findings and for establishing what constitutes good and bad science. Tetzlaffians that made especially notable contributions during my stay include Jason Plemel, Dr Ward Plunet, Joe Sparling, and Dr Femke Streijger.

I would also like to thank my collaborators. Dr Natalie Gardiner was an extremely hospitable host during my visit to the University of Manchester, where I learned how to culture DRG neurons. In addition, she taught me invaluable general cell culture skills. UBC’s Prof. Fabio Rossi allowed me to use his lab for my macrophage work, where I collaborated with Dr Jami Bennett and Sherie Duncan. Dr Hidenori Horie, my Japanese collaborator, provided input on the galectin-1 project and played generous host when I visited Tokyo.

Drs Victoria and Thomas Claydon have also been enormously supportive over the years. Back in our office days, Vic always had a good album ready for a Friday afternoon – Robbie Williams, The Darkness, Ben Folds – that would lighten the mood before lab meeting and/or beers, and was a key Lunch Club member. (It must be said that time spent in the office – whether working hard, listening to music, discussing a random article on napping, or actually napping – was some of the best that I experienced during my degree.) Vic and Tom’s generosity, and their ability to have both frank and funny conversations, is remarkable.

My friends outside of the lab have also been incredible. Jamey Trewartha has been a great running, hiking, and triathlon buddy, and together we’ve garnered scars from many a jagged mountain including Black Tusk, Weart, Saxifrage, and Gardiner. Cameron Gross was an excellent roommate, and makes watching hockey (and playing Mario Kart) that much more entertaining. Playing euchre into the wee hours with Jamey, Cam, Paul Huntley, and Brooks Young will never get old (BJPAC!). When I’m up for watching cheesy television shows (that I refuse to name here), I know that Denise Jesudason and Cathy Harrison will be ready to meet up. Outdoor adventures with Karine Duval are always tons of fun too.

Finally, I’d like to thank my family, starting with the West Coast Family (Sherril, Nanny, Phil, Elaine), who gave me gentle nudges at first by asking when I would finish my degree, before recognizing that it’s best not to ask (just in time for me to actually finish – my brother will reap the benefits from that). But really, they always ensure that I am well-fed and are very welcoming. My parents, Pat and Norm, have been incredibly supportive throughout my 25 (!) years of schooling, and have provided valuable advice (and $$) along the way. My brother Michael also deserves mention, although he broke my bicycle in 2007. Living in the same city as him is fantastic. My most vivid recent family memories include trips to Montreal (for F1), to Quebec City, and to frigid and blustery Buffalo (to see the Bills).
Co-authorship statement

The work presented in Chapters 2, 3, and 5, as well as portions of section 1.6, has been previously published. Colleagues at the International Collaboration of Repair Discoveries made especially notable contributions to these studies.

Section 1.6:

A version of this section has been published:


I wrote the entire section that was included in this dissertation. The section was edited by my co-authors.

Chapter 2:


* JM and ADG contributed equally to this manuscript.

I wrote the manuscript, provided intellectual input, and performed some of the experiments (some immunohistochemistry, microscopy, analysis, and behaviour). Drs McGraw and Ramer provided much of the intellectual input to the paper, and Dr McGraw performed and directed most of the experiments. Dr Oschipok performed in situ hybridization, while Professors Tetzlaff and Steeves provided intellectual input. Dr Poirier provided the Lgals1−/− mice.

Chapter 3:

I contributed intellectual input, completed some experiments (microscopy and image analysis), and wrote the manuscript. Dr McGraw was the primary author for this manuscript. He and Dr Ramer contributed most of the intellectual input, and Dr McGraw completed much of the experimentation along with me. Dr Oschipok performed in situ hybridization. Professors Tetzlaff, Steeves, and Horie provided intellectual input. Drs Horie and Kadoya have expertise with galectin-1, and provided reagents.

Chapter 4:


This chapter is being prepared for publication. I was the primary researcher for this project and was involved with every aspect of the research, including experiment design, research, analysis, and writing. Dr Ramer provided intellectual input. I was trained in dissociated dorsal root ganglion neuron culture and analysis by the convivial Dr Natalie Gardiner at the University of Manchester. The amiable undergraduate researchers Ms Rebecca Rowen and Ms Griselle León assisted with cell culture and analysis under my supervision.

Chapter 5:


I was the primary researcher on this manuscript. I had the major role in experimental design, research, analysis, and manuscript preparation. Dr Ramer provided intellectual input and assisted with surgeries. Dedicated undergraduate researcher Ms Margaret Leung performed high-quality microscopy and analysis under my supervision. Drs Horie and Kadoya provided reagents and minor input.
1 Introduction: gaining perspective from the periphery

If it were permissible to apply such a metaphor where only physical and chemical forces are at work, we might say that, among all the organs, the nervous system, leader and protector of the hive, feels profoundly its responsibility. Docile to the mandates of the latter, it occupies the post of honour and danger, facing bravely the struggle with cosmic forces and pathogenic agents.

- Ramon y Cajal, 1991, p. xix

1.1 Overview

Given an appropriate environment, injured mammalian peripheral nervous system (PNS) axons have the remarkable ability to regenerate. Unfortunately, the growth potential of these axons declines with time, and is often hampered by discontinuity with the distal nerve or gaps introduced by injury. Current surgical treatments for peripheral nerve injury (PNI) are limited in their capacity to promote functional recovery, especially when axons must grow over long distances or traverse large gaps (Hood et al., 2009). Studying mechanisms that underlie axon growth and targeting in the developing and adult rodent PNS will hopefully lead to the development of new therapies that aid nerve repair after PNI. Moreover, studying models of PNS axon growth may lead to the discovery of new approaches for the treatment of injured central nervous system (CNS) axons, which fail to regenerate.

In this dissertation, I explore the role of the protein galectin-1 (Gal1) in peripheral axon growth and regeneration. Gal1 is required for the appropriate pathfinding of olfactory axon populations during development (Puche et al., 1996), and it promotes axonal regeneration and functional recovery in various rodent PNI models (Horie et al., 1999; 1 A portion of this chapter has been published. Gaudet AD, Steeves JD, Tetzlaff W, Ramer MS (2005) Expression and functions of galectin-1 in sensory and motoneurons. Curr Drug Targets 6: 419-25.
McGraw et al., 2004a; Horie et al., 2004). Gal1 is therefore a candidate therapeutic molecule for improving nerve repair in humans. Here, I show that proper development of dorsal root ganglion (DRG) neurons and their axons as well as normal sensitivity to thermal stimuli require developmental Gal1 expression; that Gal1 expression within DRG neurons and their axons’ environment is correlated with axons’ regenerative capacity; that Gal1 does not elicit axon growth directly when applied to cultured neurons; and that Gal1 likely enhances peripheral axon regeneration by promoting macrophage accumulation after injury.

In chapter one, I begin with a general introduction to the causes and treatments of PNS and CNS injuries, and a discussion of the development, anatomy and phenotypes of dorsal root ganglion (DRG) neurons. I then examine characteristics that define whether a regenerative response is successful (as in the PNS) or unsuccessful (as in the CNS). Finally, I consider the roles of Gal1 in the body generally, and in the immune and nervous systems, before outlining the hypotheses that laid the foundation for the experiments described in subsequent data chapters.
1.2 Nerve trauma

In this section, I will discuss the causes and implications of both PNI and SCI. There are clear differences in the potential for functional recovery following each type of injury.

1.2.1 Peripheral nerve injury: causes and implications

In contrast with CNS axons, which are generally unable to regrow, injured adult mammalian PNS axons can regenerate and re-innervate appropriate targets. The PNS axon’s ability to regrow results from the synergy of two favourable factors: first, the injured neuron itself switches to a “growth mode”; and second, the cellular environment surrounding the growing axon mounts an efficient response that removes debris and supports axonal regeneration. This differs from the CNS, where regeneration fails because the neuronal response is insufficient and its axon’s environment is inhibitory to growth (for review, see Fenrich and Gordon, 2004; Chen et al., 2007; Vargas and Barres, 2007). In Canada (excluding Quebec), there were 1,788 hospital admissions due to nerve injuries in 2008, which represented 1.15 percent of all injury-related admissions that year (Canadian Institute for Health Information, 2010).

PNIs are often caused by direct mechanical insult to the nerve. These injuries are especially prevalent in combat situations, where 14 to 18 percent of extremity injuries involve the peripheral nerves (Jobe and Martinez, 2008). Interestingly, much of the foundation for the clinical treatment of PNIs was established by physicians in the first and second World Wars, who encountered these types of injuries frequently (Langley and Hashimoto, 1917; Seddon, 1947; Woodhall and Beebe, 1956; Jobe and Martinez, 2007). In addition, PNI is one of the most common afflictions suffered by survivors of
severe earthquakes. After Iran’s 2003 earthquake, radial and sciatic nerve injuries were the most prevalent PNIs (Ahrari et al., 2006).

Obviously combat-related injuries and severe earthquakes are not the only causes of PNI: the general population is more likely to experience PNIs that result from aging, overuse, or accidents. Lumbar spinal stenosis – an abnormal narrowing of the canal containing the lumbar spinal nerve – is a major cause of pain and disability. This condition affects three percent of the general U.S. population, and 14 percent of the U.S. population over 60 years of age (Kalichman et al., 2009). PNIs are also commonly associated with broken long bones. The PNS is involved in 74 percent of upper, and 20 percent of lower body extremity long bone breaks (Jobe and Martinez, 2008). In addition, 28 percent of patients with rotator cuff tears present with a PNI (7/25 patients; Vad et al., 2003).

One final cause of nerve trauma, the brachial plexus lesion, is particularly relevant to this dissertation. Brachial plexus lesion can occur during childbirth when excess traction is placed upon the baby’s brachial plexus during labour. Obstetric brachial plexus lesions occur in two out of 1000 births, and deficits persist in 20 to 30 percent of these cases (Malessy and Pondaag, 2009). The high rate of recovery is likely due to the enhanced regenerative capacity of neonatal peripheral and central axons. In adults, brachial plexus injury is often caused by motorcycle accidents (Flores, 2006).

Brachial plexus injury is any injury to nerves associated with the plexus that exits the spinal cord around the neck and projects to the arm. Thus, although the plexus itself consists of peripheral sensory, motor, and autonomic axons, brachial plexus injury can
also involve sensory axons that project centrally via the dorsal root. These sensory axons are derived from neurons that reside in the dorsal root ganglion (DRG).

DRG neurons are pseudo-unipolar: one axon projects to peripheral targets (e.g. muscle); the other projects to central targets (e.g. spinal dorsal horn) (for more information on DRG neurons, see Section 1.4). Interestingly, injured peripherally-projecting sensory axons can reconnect with targets, whereas injured dorsal root axons fail to regrow into the CNS (as described by Ramon y Cajal, 1991). Since both PNS and CNS injury can be modeled in this single type of neuron, manipulation of DRG neuron projections provides immense insight into how axon regeneration succeeds or fails.

1.2.2 Central nervous system injury: causes and implications

Injury to the CNS is devastating. The adult mammalian CNS – which consists of the brain and the spinal cord – does not have an efficient repair process, meaning that deficiencies caused by CNS injury are usually permanent. In the case of spinal cord injury (SCI), loss of axonal input and conduction in areas caudal to injury results in deficits in critical functions, including basic bodily functions (e.g. excretion, sexual function), movement, and sensation. In Canada, approximately 36,000 people are afflicted with chronic disabilities related to SCI (Canadian Paraplegic Association, 2009). There are over 1000 new cases every year, 38% of which involved patients under 40 years of age in 2007 and 2008 (Rick Hansen Registry, 2009). Therefore, designing new therapies that improve SCI patients’ treatment, care, or recovery are of paramount importance.
The most common causes of SCI in Canada (excluding Quebec) in 2007-2008 were unintentional falls (46%) and motor vehicle accidents (34%) (Canadian Institute for Health Information, 2010).
1.3 Importance of studying peripheral axon regeneration

The study of peripheral axon regeneration addresses two major aims:

1. To improve peripheral axon regeneration, and, by extension, enhance functional recovery after PNI; and

2. To improve axonal regeneration and functional recovery after SCI.

1.3.1 Peripheral axon regeneration: challenges and current treatment

Despite having the ability to regrow, peripheral axons often do not reconnect with appropriate targets and functional recovery after PNS injury is not always achieved. Thus, deficits induced by PNI may resolve in a matter of weeks or can be permanent. The severity and duration of deficit depends on a number of factors, including the severity, location, and type of insult. In general, more distance between the injury site and an axon’s target means that the axon will take more time to reconnect with its target. A simple calculation illustrates this point well: a fingertip denervated after injury nearby might be reinnervated within days or weeks, whereas reinnervation of that same fingertip after nerve injury around the shoulder would take more than six months (assuming no initial delay of growth and an unusually high axon growth rate of three millimetres per day over a distance of 60 centimetres; see Sunderland, 1947). The net effect of this large distance is that axons will not regenerate as precisely, and that fewer axons will regenerate successfully to targets due to the long period of time required (addressed in Section 1.5).
The current gold standard for treating a PNI is to surgically reattach severed nerve stumps. The situation is complicated if the PNI is not a clean cut, if scar tissue has formed, or if nerve stumps are separated. In these cases, stretching of nerves to facilitate simple reattachment produces tension that is inhibitory to axonal regrowth (Millesi et al., 1972; Lundborg and Rydevik, 1973; Millesi et al., 1976; Miyamoto et al., 1979; Radek, 1986). Therefore, the surgeon resects the nerve (if necessary) and bridges the nerve gap, using an autograft or an allograft. The autograft, which is the gold standard for the treatment of segmental peripheral nerve loss, involves the transplant of “non-critical” autologous nerve into space between proximal and distal stumps of injured nerve (Seddon, 1947; Dellon and Mackinnon, 1988; Millesi, 2007). Limitations of autografts include the creation of a novel neurological deficit and the potential formation of painful neuromas (Hood et al., 2009). Peripheral nerve allografts can allow for the transplant of a more appropriate nerve; however, allografts require a donor (usually deceased) and continued host immunosuppression, and they support mediocre axon regeneration when compared with autografts (Mackinnon et al., 1984; reviewed by Song et al., 2009).

Traumatic human PNIs are highly varied, and functional recovery following PNI is often inadequate. Current treatments are focused on the axon’s basic requirement for a growth-permissive physical substrate, and are often not effective. The study of mechanisms underlying peripheral axon regeneration may allow us to elucidate molecular and cellular interactions that elicit and enhance axonal regrowth, and, ultimately, improve functional recovery after PNI.
1.3.2 Central nervous system injury: challenges and current treatment

Promoting CNS axonal regeneration is a fundamental challenge that has been recognized for over a century. Indeed, contemporary researchers’ inability to promote CNS axon regrowth has led some to emphasize the importance of limiting destructive cascades facilitated by initial SCI (secondary damage), rather than focusing on the promotion of axon regeneration after acute injury (Blight, 2002; Bethea and Dietrich, 2002; Kwon et al., 2005; Donnelly and Popovich, 2008).

There are currently no universally-accepted treatments for acute SCI. In some cases, vertebrae are fused surgically in order to stabilize the spine after injury, though the surgery’s effectiveness has never been tested using controlled trials (Bagnall et al., 2008). Methylprednisolone, a steroid reported to dampen secondary damage in rat models of SCI, has been administered to humans with SCI in at least seven clinical trials (Hawryluk et al., 2008); however, it is no longer a standard of care due to its questionable efficacy and safety: the steroid must be delivered within eight hours of injury and is associated with an increased chance of infection (Sayer et al., 2006; Hurlbert and Hamilton, 2008).

Studying the composition of environments permissive to axon growth and the response of neurons that mount a robust regenerative response will be instructive for enhancing CNS repair. As mentioned earlier, manipulation of the DRG neuron provides a unique opportunity to study nerve injuries; developing treatments that encourage axon regrowth into the CNS and enhance the responses of endogenous CNS cells after dorsal root injury (DRI; dorsal rhizotomy) may be instructive for other types of CNS injury. In the next section, I will examine the properties of neurons contained within the DRG.
1.4 The dorsal root ganglion: home to a large heterogeneous population of sensory neurons

Although peripheral nerves contain sensory, sympathetic, and motor axons, I will focus on sensory neurons and their axons, whose cell bodies reside in DRGs at the spinal level. The adult DRG contains thousands of neurons that transduce a wide variety of sensory information from the periphery into the CNS (see Devor (1999) for a unique perspective on DRG neuron function). Although individual DRG neurons are relatively specialized and respond only to a limited repertoire of stimuli (e.g. mechanical or noxious), the population of neurons within the DRG comprises an array of primary afferent subtypes such that the animal can sense itself and its environment in a meaningful way. In this section, I will consider the development, neurochemistry, anatomy, and functions of DRG neurons, with particular focus on the subset of DRG neurons that respond to noxious stimuli, nociceptors.

1.4.1 DRG neuron development: a new sensation

During development, glial cells and neurons of the DRG arise from neural crest cells that migrate laterally from the dorsal edge of the closing neural tube (prospective spinal cord and brain) (Basch et al., 2004). The formation of a DRG and its spinal nerve is guided by mesodermal somites at each segment of the animal (Krull, 2001; Roffers-Agarwal and Gammill, 2009; Schwarz et al., 2009). While cells of the developing somite migrate and differentiate to form tissues such as skin, muscles, and vertebrae, the peripheral branches of developing DRG neurons grow distally in parallel, using extracellular matrix (ECM) substrates for directional guidance. Peripheral projections of DRG neurons have characteristic somatotopic patterns of termination in the skin and muscle, called dermatomes and myotomes, respectively.
Development and elongation of the central process of DRG neurons occurs in waves and is incredibly precise (Altman and Bayer, 1984). Larger fibres from prospective Aβ and Aδ neurons enter the spinal cord first, through the medial division of the dorsal root. In rats, large diameter primary afferent fibres initially penetrate the spinal cord at embryonic day (E) 13 (Altman and Bayer, 1984), equivalent to eight weeks of gestational age in humans (Konstantinidou et al., 1995). In the second wave of axonal growth, fine afferents derived from small DRG neurons (including prospective C nociceptors) penetrate the spinal cord through the lateral division of the dorsal root. The directed outgrowth of the central projections may be mediated by specific interactions between ECM carbohydrates and proteins on the surface of the axon (Dodd and Jessell, 1985; Regan et al., 1986) and by sensitivity to attractive and repulsive target-derived chemotropic factors such as nerve growth factor (NGF) and semaphorin-3A, respectively (Zhang et al., 1994; Redmond et al., 1997). Semaphorin-3A, which is expressed in the ventral spinal cord during pathfinding of DRG neurons’ central axons at E14, restricts the termination of axons expressing its receptor (those that are small-diameter) to the superficial layers of the dorsal horn (Messersmith et al., 1995; Masuda and Shiga, 2005). In contrast, large-diameter primary afferents, which do not express semaphorin-3A receptors, grow readily in its presence and terminate in deeper laminae of the spinal cord.

1.4.2 DRG neuron neurochemistry

Expression of certain high-affinity receptors for neurotrophic factors is among the earliest defining features of DRG neuron subclasses (Figure 1.1; see Ernsberger, 2008; Ernsberger, 2009 for review). Neurotrophic factors are secreted proteins that support
DRG neuron survival and axon growth during embryonic and early postnatal development (Levi-Montalcini and Hamburger, 1951). Post-mitotic DRG neurons begin to express neurotrophic factor receptors soon after differentiating, and the expression of specific receptors in this population changes throughout development.

In the adult, most nociceptive neurons express either tropomyosin-related kinase (Trk)A, the high-affinity receptor for the prototypical neurotrophin NGF (41 to 47 percent of all DRG neurons) (Mu et al., 1993; McMahon et al., 1994; Orozco et al., 2001; Kobayashi et al., 2005b), or Ret, a co-receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family (60 to 70 percent of all DRG neurons) (Molliver et al., 1997; Bennett et al., 1998; Orozco et al., 2001; Kobayashi et al., 2005b; Chen et al., 2006). There is a limited population of DRG neurons that express both TrkA and Ret (three to nine percent in lumbar DRGs) (Molliver et al., 1997; Kashiba et al., 2003). TrkA expression is required for the expression of various ion channels, receptors and trophic factors implicated in nociception (Pezet and McMahon, 2006), and Ret-expressing neurons are required for normal thresholds to thermal and mechanical stimuli and for neuropathic pain development (Tarpley et al., 2004; Joseph and Levine, 2010; but see Golden et al., 2010). Interestingly, these nociceptive subpopulations can also be defined by their expression of specific antigens. TrkA-positive neurons express the neuropeptides substance P (Risling et al., 1994; Kashiba et al., 1996) and calcitonin gene-related peptide (CGRP) (Lindsay and Harmar, 1989; Averill et al., 1995; Bennett et al., 1996), and are therefore peptidergic. Ret-positive neurons, which are non-peptidergic, bind the isolectin IB4 (Molliver et al., 1997).
**Figure 1.1.** Neurochemical phenotypes and anatomy of dorsal root ganglion (DRG) neurons and their projections.  

a) The approximate proportions of cells expressing specific trophic factor receptors in the adult rat. Some cells are sensitive to multiple growth factors: there is a significant number that express both Ret and TrkA. Various neurochemicals are expressed in distinct DRG neuron subpopulations; these can be used to identify the subtypes immunohistochemically (represented by the lines that encircle the pie chart).  

b) The three major classes of DRG neurons have distinct physical and neurochemical properties: large-diameter Aβ neurons generally transmit mechanical or proprioceptive information, whereas small- to medium-diameter Aδ neurons and small-diameter C-type neurons often transmit nociceptive and temperature information. Neurons that express TrkA and/or Ret are generally of the C or Aδ variety. TrkC- and TrkB-expressing neurons are generally Aβ neurons. Neurons’ colours in panel b) correspond to the colours of trophic factor receptors shown in panel a). The neurons have two colours not because they co-express the receptor necessarily; rather, that type of neuron generally expresses one of the two receptors (e.g. an Aβ neuron generally expresses TrkB or TrkC). The central and peripheral terminations of these neurons’ axons also differ. Major termination zones in the dorsal horn for each different neuron class are shown for clarity. Identities of dorsal horn laminae are represented by the Roman numerals to the right of the figure. CGRP, calcitonin gene-related peptide; SP, substance P; IB4, isolectin B4; NF200, neurofilament (200 kDa). After Boucher and McMahon (2001); see text for additional references.
a

b

peripheral target (e.g. skin)

DRG

spinal cord

I
II
III, IV
V

Aβ
Aδ
C
Most large-diameter mechanoreceptive and proprioceptive primary sensory neurons express TrkB (the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-4/5) and the high-affinity NT-3 receptor TrkC, respectively (Klein, 1994). In the adult rat, five to 25 percent of all DRG neurons express \textit{trkB} mRNA and 15 to 30 percent express \textit{trkC} mRNA (Wetmore and Olson, 1995; Kashiba et al., 1996; Kobayashi et al., 2005a). Large-diameter DRG neurons can be identified using antibodies against the 200 kDa heavy neurofilament NF200.

1.4.3 Anatomy and functions of DRG neurons

In addition to categorizing these neurons by neurochemistry, the heterogeneous population of sensory neurons within each DRG can be classified based on the type of sensory information transduced (noxious or innocuous mechanical, thermal, and/or chemical) and the amount of myelination (none, light, or heavy) (Table 1.1). Rat DRG neurons are approximately spherical and have diameters in the range of 10-60 µm (McDougal, Jr. et al., 1985; Swett et al., 1991). Generally, both branches of a given neuron are either myelinated or unmyelinated and there is positive correlation between soma size and axon diameter (Lieberman, 1976). There are very few axons that branch within the peripheral nerve or dorsal root (Devor et al., 1984), although considerable branching is noted in both the peripheral and central terminal fields.

The central branches of DRG neurons project through the dorsal root and either terminate in the dorsal horn of the spinal cord (nociceptive and temperature, and some mechanoreceptive information) (Figure 1.1), or project directly to the brainstem via funiculi in the dorsal columns (proprioceptive and some mechanoreceptive information; reviewed by (Beck, 1976)). Most primary afferent fibres terminate in the dorsal horn.
Table 1.1. Comparison of properties of C, Aδ and Aβ primary afferent fibres. Various physical, phenotypic, and anatomical characteristics distinguish the various classes (reviewed by Wilson and Kitchener, 1996; Millan, 1999).

<table>
<thead>
<tr>
<th>Class of fibre</th>
<th>Cell size; myelination</th>
<th>Conduction velocity</th>
<th>Information transmitted*</th>
<th>Terminal dorsal horn laminae*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Small; unmyelinated</td>
<td>Slow (0.5-2 m/s)</td>
<td>Noxious thermal and/or mechanical (some polymodal)</td>
<td>I, IIo Some V (and X)</td>
</tr>
</tbody>
</table>
| Aδ            | Medium; light          | Intermediate (12-30 m/s) | Type I: pinch, low noxious thermal  
Type II: pinch, high noxious thermal  
Type III: noxious thermal only | I Some II, V (X) |
| Aβ            | Large; heavy           | Fast (30-100 m/s)   | Innocuous mechanical and proprioceptive | III, IV Some IIi |

*Note that although these categories include the majority of fibres within a given class, there are exceptions (Lawson, 2002)
ipsilaterally (although some fibres project to the contralateral dorsal horn) (Culberson et al., 1979; Light and Perl, 1979; Dumoulin et al., 1996; Millan, 1999). The dorsal horn consists of the six most superficial layers of the grey matter, which are named sequentially beginning with lamina I as the most dorsal layer (Rexed, 1952; Molander et al., 1984; Molander et al., 1989). Small-diameter fibres (Aδ- and C-types), which generally transmit nociceptive information, terminate in laminae I, II, and V of the dorsal horn, although some Aδ fibres arborize on either side of the lamina II-III border. Large-calibre Aβ cutaneous afferents typically arborize in laminae III-IV and subserve low-threshold mechanoreception. The sensory fibres synapse on neurons within the dorsal horn, where afferent and efferent input is integrated and information is transmitted to supraspinal centres (Millan, 1999; Craig, 2003). About 70 percent of cutaneous innervation is contributed by C-fibres, 10 percent by Aδ-fibres, and 20 percent by Aβ-fibres (Millan, 1999).

1.4.3.1 The nociceptor

Primary afferent neurons that are selectively sensitive to noxious stimuli are called nociceptors. Nociceptors transmit information from all areas of the body and have small-calibre fibres that terminate centrally in laminae I, II, and V of the dorsal horn (some also terminate in area X, around the central canal) (reviewed by Willis, Jr., 1988; Craig, 2003). Whereas laminae I and V receive nociceptive input from a variety of tissues, lamina II receives primarily cutaneous nociceptive input. Most nociceptive information is transmitted via two subtypes of DRG neurons, Aδ and C nociceptors (although small subpopulations of large-diameter Aβ fibres transmit nociceptive information as well (Lawson, 2002)).
A\textgreek{\delta} nociceptors are small-diameter, thinly-myelinated neurons that are mainly exteroceptive (provide information about the external environment) and are sensitive to noxious mechanical or thermal stimulation. The central branches of these neurons terminate mainly in lamina I, and some project sparsely to laminae II and V (and X) (Craig and Mense, 1983; Cervero and Connell, 1984; Kuo and de Groat, 1985; Millan, 1999). In mammals, temperatures above about 43°C and those below 15°C evoke pain-like responses (Tillman et al., 1995; Tominaga and Caterina, 2004).

C-type nociceptors are small-diameter, unmyelinated neurons that can be exteroceptive and/or interoceptive (detect changes in tissues that threaten homeostasis). Most C-fibres respond to various types of noxious stimuli (i.e. they are polymodal), and these neurons innervate all types of tissue, including visceral and deep tissues. Some C-fibres are only activated by inflammation or tissue damage that may result from injury (so-called “silent nociceptors” (Schmelz et al., 1997; Schmidt et al., 2002; Schmelz et al., 2003)). The central projections of C nociceptors terminate mainly in laminae I and II (outer) (IIO) of the dorsal horn, although some provide weak input to lamina V and perhaps X (Light and Perl, 1979; Sugiura et al., 1986; Millan, 1999).

Here, I have described the phenotypes and functions of DRG neuron subpopulations. The link between neuron function and the expression of specific antigens is invaluable for establishing the relevance of phenotypic alterations in DRG neuron neurochemistry induced during development or after nerve injury. In the next section, I will discuss the effects of PNI on neurons and their axons' environments.
1.5 Cellular responses to peripheral nerve injury

Injury-induced cell death is the first obstacle to successful regeneration. Approximately 10 to 30 percent of DRG neurons die after peripheral axotomy, most of which are small diameter (Arvidsson et al., 1986; Ygge, 1989; Vestergaard et al., 1997; Tandrup et al., 2000; Navarro et al., 2007). The amount of apoptosis induced by PNI depends on the animal’s age, and on injury severity and location: immature neurons are more likely to succumb after injury than are adult neurons (Snider et al., 1992; Lowrie et al., 1994), and injuries more proximal to the cell body facilitate more cell death than do distal lesions (Ygge, 1989). The extent of neuronal death is augmented if target reinnervation is delayed (Tornqvist and Aldskogius, 1994, but see McPhail et al., 2004).

Neurons that survive PNI have the ability to regenerate their axons (Figure 1.2). The injured axon in the proximal nerve stump – the part of the axon still connected to its cell body – degenerates to the first intact node of Ranvier (Stoll et al., 1993). (This proximal degeneration, called dieback, also occurs after SCI (Stirling et al., 2004; Kerschensteiner et al., 2005), although the process is more extensive and permanent there.) Regenerative axon sprouts then begin to grow from this first node of Ranvier (Hopkins and Slack, 1981; McQuarrie, 1985; Ramon y Cajal, 1991). Detached axonal and myelin debris are removed by phagocytic Schwann cells and macrophages in the distal stump, which creates a more favourable environment for regrowing axons (Vargas and Barres, 2007). The axons grow along Schwann cell tubes, called bands of Büngner, that guide axons towards targets. Although the axon may successfully reconnect with its proper target, its regenerated portion is usually thinner and internodal length is smaller than in the uninjured portion of nerve (Navarro et al., 2007).
Figure 1.2. The progression of Wallerian degeneration and axon regeneration following peripheral nerve injury. A single axon with associated myelinating Schwann cells is shown for clarity. 1. The endoneurium of an uninjured nerve consists of axons, associated Schwann cells (myelinating and nonmyelinating), and resident, inactivated macrophages. 2. Soon after nerve injury, denervated myelinating Schwann cells shed their myelin. These Schwann cells then proliferate, produce cytokines/trophic factors, and phagocytose detached debris. In addition, the reaction within the neuron cell body begins: this is characterized by cell soma hypertrophy, displacement of the nucleus to an eccentric position, and dissolution of Nissl bodies. 3. Wallerian degeneration is well underway within a week of injury. Soluble factors produced by Schwann cells and injured axons activate resident macrophages and lead to recruitment of hematogenous macrophages. The activated macrophages clear myelin and axon debris efficiently, and produce factors that facilitate Schwann cell migration and axon regeneration. 4. After a lag period, injured axons form a growth cone and begin to regenerate along bands of Büngner formed by Schwann cells. These tubes provide a permissive growth environment and guide extending axons towards potential peripheral targets. Schwann cells that have been chronically denervated (e.g. for a few months) are less supportive of regrowth and are more likely to undergo apoptosis. 5. If the axon is able to traverse the injury site, and its environment supports its growth along the entire distal stump, then the axon can connect with peripheral targets. Although myelinating Schwann cells do remyelinate the regenerated portion of axon, the myelin is thinner and the nodal length is shorter than in the uninjured portion of axon. For review, see Fu and Gordon (1997); Fenrich and Gordon (2004); Chen et al. (2007); and Vargas and Barres (2007). Please see text for additional references.
1. Uninjured nerve

2. Initial reaction to injury
   (~24 hours)

3. Macrophage recruitment;
   Wallerian degeneration
   (one week)

4. Schwann cell alignment;
   axon regeneration
   (weeks to months)

5. Successful target
   reinnervation
   (weeks to years)

LEGEND
- peripheral neuron
- resident macrophage
- injury site
- activated macrophage
- myelinating Schwann cell
- cytokines / growth factors
- nonmyelinating Schwann cell
- myelin debris
- apoptotic Schwann cell
Successful axon regeneration therefore depends on initiation and maintenance of concerted injury-induced cellular responses. These responses have classically been divided into two categories (Fu and Gordon, 1997; Fenrich and Gordon, 2004):

1. Intrinsic responses of the neuron to injury; and
2. Responses extrinsic to the neuron.

1.5.1 Intrinsic response of the neuron to injury

As mentioned above, the adult peripheral neuron generally has the capacity to mount an effective cell body response that supports its axon’s regeneration after PNI. A robust neuronal response involves a switch from “transmission” to “growth” mode that requires timely integration of three processes: first, the neuron must sense the injury; second, the neuron must coordinate appropriate changes in gene expression within the nucleus; and third, the neuron must initiate axon regrowth through the injury site.

1.5.1.1 Sound the alarm: neuron detection of axonal injury

Injured neurons detect injury through intense depolarizations and altered retrograde transport (Ambron and Walters, 1996). Rapid ion influx caused by trauma likely initiates vigorous action potentials immediately after injury that travel retrogradely to the cell body. PNI-induced electrical activity is required for typical peripheral axon regeneration: when axotomy is performed in the presence of the sodium channel blocker tetrodotoxin, the repair process is delayed (Mandolesi et al., 2004). Interestingly, periodic electrical stimulation of peripheral nerves post-axotomy increases intracellular cyclic AMP (cAMP) levels and expedites regeneration of injured DRG neuron central projections in rat dorsal columns (Udina et al., 2008). However, electrical stimulation of CNS axons with
less growth potential does not enhance their regeneration, even when presented with a more permissive peripheral nerve graft (rubrospinal tract axons; Harvey et al., 2005).

In addition to sustained depolarization immediately after PNI, slower, more permanent alterations in retrograde transport from the injured axon provide cues that induce changes in the cell body (Hanz and Fainzilber, 2006; Abe and Cavalli, 2008). For instance, an injured axon might no longer retrogradely transport a target-derived trophic factor, or it might begin transport of novel or altered proteins found at the lesion site. Injury-induced changes in extracellular signal-related kinase (Erk) localization and activation provide excellent examples of how axotomy can alter retrograde transport of important proteins. Following rodent sciatic nerve injury, soluble fragments of the intermediate filament vimentin accumulate in the axon (through local translation and calpain-dependent cleavage) (Perlson et al., 2005). Meanwhile, Erk1 and Erk2, important signaling molecules implicated in neuronal survival and growth, are phosphorylated and are thereby activated. Phospho-Erk binds to vimentin, which both prevents Erk dephosphorylation and interacts with retrograde transport machinery (importins and dynein) (Hanz et al., 2003; Perlson et al., 2005). Once in the cell body, activated Erk translocates into the nucleus and regulates gene expression. c-Jun N-terminal kinase (JNK), ATF3, and JAK are other signaling molecules that, like Erk1/2, may provide injury-induced retrograde signals (Tsujino et al., 2000; Lee et al., 2004; Cavalli et al., 2005; Lindwall and Kanje, 2005).

1.5.1.2 Injury-induced changes in the cell body

The most conspicuous change initiated by PNI-induced retrograde signaling is chromatolysis, which is the set of morphological changes observed in the axotomized neuronal soma (Fenrich and Gordon, 2004). Hallmarks of chromatolysis include
dispersal of organized arrays of rough endoplasmic reticulum, displacement of the nucleus from the cell body’s centre, and somatic and nuclear hypertrophy. Axotomy-induced structural changes within the cell body – particularly the dissolution of Nissl-staining ribosome clusters from the endoplasmic reticulum – reflect an overall increase in mRNA and protein production.

PNI modulates expression or activation of a plethora of transcription factors (reviewed in (Abe and Cavalli, 2008). Peripheral axotomy leads to elevated expression of transcription factors implicated in the immediate-early response to injury (e.g. c-Jun and JunD), and of those that are constitutively expressed (e.g. cAMP response element binding protein (CREB), STAT3, Sox11, ATF3). These transcription factors have pivotal roles in integrating axonal injury detection and directing an appropriate regenerative response. For instance, c-Jun activation (by JNK-mediated phosphorylation) is crucial for initiation of transcriptional programs associated with an effective facial motoneuron regenerative response: recovery of function after facial nerve injury is never fully achieved in mice lacking neuronal c-Jun (Raivich et al., 2004). c-Jun deletion in injured neurons was associated with reduced immunoreactivity for regeneration-associated proteins normally upregulated by injury, including the cell adhesion receptor α7β1 integrin and the neuropeptide galanin. Raivich et al. (2004) therefore established that injury-induced expression of a single immediate-early gene can have a profound impact on initiation and success of the regenerative program. The fact that c-Jun, ATF3, and STAT3 are all activated in response to injury of DRG neurons’ peripheral, but not central branch implies that these proteins may facilitate the neuronal switch to growth mode.

The role of the cAMP-CREB pathway in axon regeneration has garnered recent attention, as cAMP elevation in neuronal soma enhances axonal regrowth (Spencer and
Filbin, 2004). cAMP signaling must activate the transcription factor CREB to function (Gao et al., 2004). cAMP signaling induces expression of arginase I (Cai et al., 2002), an enzyme implicated in polyamine synthesis and the regenerative response, as well as cytokines (e.g. interleukin (IL-) 6) (Wu et al., 2007). The conditioning lesion effect – enhanced DRG neuron central branch regeneration facilitated by prior lesion of the peripheral DRG branch that “conditions” the neuron (Richardson and Issa, 1984; Neumann and Woolf, 1999) – requires upregulation of cAMP, and cAMP administration to the DRG prior to spinal dorsal column injury mimics this effect (Qiu et al., 2002; Neumann et al., 2002). Elevating cAMP levels also improves anatomical and functional recovery following SCI (Nikulina et al., 2004; Pearse et al., 2004; Hannila and Filbin, 2008). However, elevating cAMP levels in cultured postnatal retinal ganglion cells is not sufficient to induce axonal extension comparable to extension from embryonic cells (Goldberg et al., 2002), suggesting that multiple pathways are required to overcome challenges confronted by mature CNS neurons.

1.5.1.3 Regeneration-associated genes: expression in injured peripheral neurons

The regenerative program is characterized by altered expression of a battery of proteins that involve cytoskeletal dynamics, cell adhesion, or cell communication. As their name implies, regeneration-associated genes (RAGs) comprise a diverse set of genes implicated in growth cone elongation and axon sprouting or pathfinding. The first RAGs identified included the 43 kDa growth-associated protein (GAP-43), CAP-23, the neurotransmitter calcitonin gene-related peptide (CGRP), and cytoskeletal subunits (Tetzlaff et al., 1988; Haas et al., 1990; Tetzlaff et al., 1991; Fu and Gordon, 1997). Researchers have since identified countless other potential RAGs, reinforcing the view that axonal regeneration is a complex and multi-faceted process.
GAP-43 and CAP-23 modulate cytoskeletal dynamics following PNI. These functionally-related (but structurally-distinct) proteins are found in the growth cone’s subplasmalemmal region, where they promote actin assembly and affect neurite extension (Frey et al., 2000; Laux et al., 2000). GAP-43 and CAP-23 are expressed highly during development and after injury of the DRG’s peripheral, but not central branch (Skene and Willard, 1981; Basi et al., 1987; Skene, 1989; Mason et al., 2002). Bomze and colleagues (2001) found that cultured DRG neurons overexpressing both GAP-43 and CAP-23 were more likely to extend neurites, and that these neurites had an extending (versus branching) phenotype that was comparable to that induced by conditioning lesion. Furthermore, CAP-23 and GAP-43 overexpression enhanced DRG neuron central axonal growth into peripheral nerve grafts within the dorsal column, although this growth was not as robust as was that induced by conditioning lesion.

In addition to identifying GAP-43 as a potential RAG, Skene and Willard (1981) were the first to show that the cytoskeletal components actin and tubulin are upregulated during regeneration. Actin and tubulin subunits travel anterogradely via slow component b transport. Several factors related to injury-induced cytoskeletal changes strongly suggest that axon regeneration recapitulates developmental axon growth: first, the apparent requirement for actin and tubulin subunits; second, downregulation of less dynamic intermediate filament components; and third, the tight correlation between rates of regeneration and slow component b transport (Wujek and Lasek, 1983; Tetzlaff et al., 1988; Hoffman and Cleveland, 1988; McQuarrie and Lasek, 1989; Fu and Gordon, 1997).
More recently, the protein SPRR1A was identified as a RAG. SPRR1A is not expressed in DRG neurons until after injury, at which point it has a key role in promoting axonal growth (Bonilla et al., 2002). As with DRG neurons co-transfected with GAP-43 and CAP-23, cultured adult DRG neurons transfected with SPRR1A displayed an extending neuritic phenotype that was similar to that induced by conditioning lesion. Using Sprr1A mRNA knockdown, Bonilla et al. (2002) showed that SPRR1A is required for enhanced neurite growth elicited by conditioning lesion. Moreover, SPRR1A overexpression in E13 DRG neurons increased neurite extension on inhibitory CNS myelin substrates. Collectively, these results imply that SPRR1A may promote CNS axon regeneration; however, there is no published evidence that shows that the protein enhances regeneration in vivo.

I have provided a brief overview of the peripheral neuron’s response to axon injury. Promoting a robust regenerative response in CNS neurons presents a particularly difficult challenge, as activation of individual signaling pathways or proteins associated with regeneration effect relatively little CNS axon regrowth (e.g. cAMP elevation, GAP-43/CAP-23 overexpression). These studies demonstrate that eliciting a robust neuronal response to injury requires the complex interplay of multiple signaling pathways, and development of treatments aimed at augmenting the cell body response must take this into account. With advances in our understanding of the limited potential of promoting neural repair through a robust regenerative neuronal response alone, the importance of studying the milieu surrounding the injured axon has become more apparent. In the next section, I will shift focus to the impact of the axon’s environment on axon regeneration.
1.5.2 Responses extrinsic to the neuron after nerve injury

Injury elicits a vigorous response from non-neuronal cells in the peripheral nerve, especially in the portion distal to injury. This degenerative process is called Wallerian degeneration after Augustus Volney Waller, who first characterized morphological changes in sectioned frog glosopharyngeal and hypoglossal nerves 160 years ago (Waller, 1850; cf. Stoll et al., 2002). More recent studies have identified the intrinsic degeneration of detached distal axons as the key event in Wallerian degeneration: this triggers a cascade of non-neuronal cellular responses that leads to clearing of inhibitory debris in the peripheral nerve and to the production of an environment that can support axon regrowth for months after injury (Griffin et al., 1995; Vargas and Barres, 2007; Griffin and Thompson, 2008). Here, I will discuss injury-induced axon degeneration, the roles of glial and immune cells after injury, and alterations in the extracellular environment of the pathological nerve.

1.5.2.1 Axonal degeneration: the spark for widespread changes in the distal nerve

Axon degeneration in the distal nerve is the critical event that instigates subsequent degenerative processes after PNI; however, axon degeneration does not begin immediately after injury. The detached axons remain intact for a period after PNI, and can still transmit action potentials when stimulated (Luttges et al., 1976; Tsao et al., 1999). The lag period between injury and axon degeneration varies by species: the period lasts 24 to 48 hours in young rats (Miledi and Slater, 1970; Lubinska, 1977), whereas it takes several days for primate (including human) axons to degenerate (Gilliatt and Hjorth, 1972; Chaudhry and Cornblath, 1992). After this lag period, axons begin to bead and swell before catastrophic granular disintegration of the cytoskeleton occurs (George et al., 1995; Sievers et al., 2003). Granular disintegration of the cytoskeleton –
the sudden destruction of cytoskeletal elements into fine debris – is completed in less than an hour (Kerschensteiner et al., 2005; Beirowski et al., 2005).

Interestingly, mechanisms intrinsic to the detached axon underlie injury-induced degeneration. For instance, axotomized neurites from rat sympathetic ganglion explants degenerate within 8-24 hours (Zhai et al., 2003). Influx of extracellular calcium into detached axons has a key role in axon degeneration: application of calcium ionophores induces degeneration of intact cultured DRG neurites, and factors that prevent extracellular calcium entry delay degeneration of injured DRG neurites \textit{in vitro} (George et al., 1995). This calcium influx activates the calcium-dependent protease calpain, which is essential for cytoskeletal degradation and axon degeneration. Activation of the axon’s ubiquitin-proteasome system has also been implicated in these degenerative processes (Zhai et al., 2003).

The most compelling evidence that supports a pivotal position for axon degeneration in Wallerian degeneration comes from studies that involve the slow Wallerian degeneration (Wld$^S$) mouse. The Wld$^S$ mouse, a C57BL substrain, has nerves that degenerate exceptionally slower than wild-type nerves (Lunn et al., 1989). This difference is exemplified by studying the ability of distal nerves to conduct action potentials after injury: whereas normal distal nerves transmit compound action potentials for two or three days after injury, Wld$^S$ nerves do so for up to three weeks post-injury (Lunn et al., 1989; Perry et al., 1992; Tsao et al., 1999). Although axonal granular disintegration of cytoskeleton is significantly delayed in Wld$^S$ mice, the process still precedes later degenerative events such as myelin sheath breakdown and macrophage accumulation, as well as axonal regeneration (Bisby and Chen, 1990). In addition, transplant experiments using Wld$^S$ and wild-type nerves have shown that intrinsic properties of the
WldS axons are responsible for the delayed progression of Wallerian degeneration in WldS nerves (Glass et al., 1993). Therefore, the timing of granular disintegration of the cytoskeleton and axon degeneration defines when subsequent, more extensive degenerative processes will begin.

A variety of changes occur in the nerve soon after PNI-induced axon degeneration, including blood-nerve barrier compromise and initiation of cellular changes associated with degeneration. The blood-nerve barrier (and the blood-brain barrier) comprises non-fenestrated endothelial cells connected by tight junctions, and it restricts the movement of proteins, hormones, ions, and toxic substances from blood into neural tissue (Olsson, 1966; Olsson, 1968; Poduslo et al., 1994; Smith et al., 2001). Although the blood-nerve barrier is often breached at the lesion site during injury, it is not compromised elsewhere along the nerve until axon degeneration begins. At that point, the barrier opens for the length of the nerve distal to injury for at least four weeks post-injury (Gray et al., 2007). Blood-nerve barrier breakdown allows blood-borne factors and cells to enter the nerve more readily, thereby promoting an efficient response to damage that will facilitate tissue repair.

1.5.2.2 The Schwann cell response to peripheral nerve injury

During the three or four weeks following the section, nearly all the nerve sprouts present in the peripheral stump are devoid of nuclei and myelin sheath. But from the fourth week on and sometimes before, fusiform cells with an elongated nucleus apply themselves around the fibres. These cells are produced by the proliferation of the old cell of Schwann.

- Ramon y Cajal, 1991, p. 244

Schwann cells, the ensheathing glial cells of the PNS, are crucial for normal nerve function and for nerve repair. Schwann cells constitute 90 percent of nucleated cells
within peripheral nerves (Campana, 2007). They provide trophic support for growing, mature, and regenerating axons. In addition, basal lamina produced by these cells surrounds the Schwann cell and their associated axon(s), and its components support axonal growth (discussed in Section 1.4.2.4). There are two types of Schwann cells in adult peripheral nerve: the myelinating Schwann cell and the ensheathing (non-myelinating) Schwann cell (Griffin and Thompson, 2008). The myelinating Schwann cells form a multi-layered, membranous myelin sheath around large-calibre axons (motor or sensory). These cells associate with one segment of a single axon. Myelinating Schwann cells are evenly spaced along the length of large-diameter axons; their myelin provides insulation that allows these axons to conduct action potentials much more rapidly than they otherwise would. In contrast, the ensheathing Schwann cells, or Remak cells, loosely ensheathe multiple small-diameter unmyelinated axons. The ensheathing cell’s cytoplasmic processes segregate and surround the axons.

PNI induces massive changes in the differentiation state of Schwann cells in the distal nerve beginning soon after injury. Myelinating Schwann cells associated with detached axons respond to injury even before axon degeneration occurs by altering gene expression (Liu et al., 1995a; Murinson et al., 2005; Guertin et al., 2005). Within 48 hours of injury, these Schwann cells cease myelin protein production (Trapp et al., 1988; White et al., 1989), upregulate RAGs (GAP-43, specific neurotrophic factors and their receptors, neuregulin and its receptors), and begin to proliferate (Pellegrino et al., 1986; Liu et al., 1995a; Murinson et al., 2005). Both myelinating and ensheathing Schwann cells undergo cell division, which peaks at four days post-injury (dpi). The proliferating Schwann cells are confined to their basal lamina tubes where they align to form bands of Büngner, which provide a supportive substrate and growth factors for regenerating axons (Stoll et al., 1989; Griffin and Thompson, 2008).
Schwann cells also play an important early role in removing detached myelin debris, which acts as a molecular and physical barrier to regrowing axons in the distal nerve. After the axons degenerate and disappear, Schwann cell myelin sheath divides longitudinally to form ovoids that shorten over time (Stoll et al., 1989). The myelin debris, which is shed by the Schwann cell, contains molecules that are inhibitory to axonal growth, including myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) (Shen et al., 1998; Huang et al., 2005). These Schwann cells then have an active role in removing myelin debris: they can degrade their own myelin, phagocytose extracellular debris, and offer myelin to other phagocytic cells (e.g. macrophages) (Holtzman and Novikoff, 1965; Hirata et al., 1999). Denervated Schwann cells are the major phagocytic cells at early timepoints after injury, and they secrete cytokines and chemokines that cause massive immune cell influx into the nerve (Tofaris et al., 2002). The recruited immune cells include the phagocytic neutrophils and macrophages, which take over the bulk of debris removal and produce growth factors in the next stage of Wallerian degeneration.

The loss of Schwann cell support in the distal nerve over time is one factor that limits successful long-distance peripheral axon regeneration. Many denervated Schwann cells die by apoptosis within months after injury and leave behind their basal lamina, which is eventually degraded and removed (Pellegrino et al., 1986; Sulaiman and Gordon, 2002; Vargas and Barres, 2007; Ebenezer et al., 2007; Griffin and Thompson, 2008). Moreover, those Schwann cells that persist in the chronically injured nerve atrophy and do not sustain their supportive phenotype (Li et al., 1997; Sulaiman and Gordon, 2002; Dedkov et al., 2002; Vargas and Barres, 2007). Interestingly, chronically denervated Schwann cells can be reactivated by treatment with the cytokine TGF-β, which is released by proliferating Schwann cells and macrophages. These reactivated cells...
strongly support axon regeneration (Sulaiman and Gordon, 2002). In summary, denervated Schwann cells promote nerve repair by proliferating, secreting trophic factors and cytokines, and phagocytosing myelin debris; however, this growth-supportive Schwann cell response declines with time.

1.5.2.3 The inflammatory response to peripheral nerve injury

The typical inflammatory response to tissue injury and infection is largely conserved in the pathological PNS: many phagocytic neutrophils and macrophages arrive within hours or days, whereas major lymphocyte accumulation occurs a week or more after injury in the distal nerve.

Neutrophils (polymorphonuclear granulocytes), the first cells to invade tissue from the circulation during an inflammatory event, phagocytose debris and modulate recruitment and activation of other immune cells during Wallerian degeneration (Nathan, 2006). Although neutrophils are very sparsely distributed in the uninjured rat nerve, their density in the area immediately around the injury site increases massively within eight hours, and peaks at 24 hours after partial sciatic nerve transection (only a few neutrophils infilitrate more distal areas of the distal stump) (Perkins and Tracey, 2000). These cells have a high rate of turnover; after entering tissue, neutrophils briefly phagocytose debris before undergoing apoptosis (Kennedy and DeLeo, 2009). It is not known whether this neutrophil response is beneficial for peripheral axon regeneration.

Macrophages constitute another major immune cell population that respond to PNI, and they remove myelin debris during later phases of Wallerian degeneration. Endoneurial macrophages account for two to nine percent of nucleated cells within the uninjured peripheral nerve (Perry et al., 1987; Vass et al., 1993; Mueller et al., 2003). These
resident cells are thought to perform a surveillance role in the PNS, where they likely act as antigen-presenting cells – they express major histocompatibility complex proteins and complement receptor 3 (Bruck and Friede, 1991; Mueller et al., 2003; Griffin and Thompson, 2008). Using sciatic nerve explants, it has been shown that resident macrophages respond to PNI by proliferating and by phagocytosing myelin (Bonnekoh et al., 1989; Shen et al., 2000). Endoneurial macrophage proliferation, activation, and phagocytic activity commences within two days post-axotomy, before the wave of hematogenous macrophage infiltration begins (Mueller et al., 2001).

Although proliferating endoneurial macrophages contribute significantly to the early stages of Wallerian degeneration, a massive population of monocytes recruited from the circulation accumulates by four days after injury (Mueller et al., 2003). These monocytes, which differentiate into macrophages following extravasation, are thought to be recruited from the blood by Schwann cell- and macrophage-derived cytokines and chemokines, such as leukemia inhibitory factor (LIF), MCP-1 and TNF-α (Siebert et al., 2000; Tofaris et al., 2002). Local cues direct macrophages to distinct areas of damage: after injury to a subset of axons within a nerve, macrophages accumulate preferentially around the injured fibres (Stoll et al., 1989). Hematogenous macrophage accumulation in the injured nerve is also enhanced by serum components such as antibodies and complement, as macrophage recruitment is delayed in B cell-deficient mice (which lack antibodies) (Vargas and Barres, 2007) and in C5-deficient mice (C5 is a key complement component) (Liu et al., 1999). Breakdown of the distal nerve’s blood-nerve barrier within 48 hours of injury allows influx of these serum components (Bouldin et al., 1991), which then facilitate macrophage recruitment and label debris for phagocytosis (Bruck and Friede, 1990; Bruck, 1997; Smith et al., 1998).
Hematogenous macrophages are essential for effective myelin phagocytosis (Beuche and Friede, 1984; Bruck et al., 1996), and produce cytokines that activate Schwann cells (e.g. IL-1; La Fleur et al., 1996) and trophic factors that aid axon regeneration (e.g. nerve growth factor, NGF; (Perry et al., 1987; Heumann et al., 1987; Hikawa and Takenaka, 1996). Neurites from DRG explants, which normally grow minimally on uninjured nerve cryosections, grow in a more stable and robust fashion when nerve sections are treated with macrophage-conditioned medium or are derived from pre-degenerated nerves (Luk et al., 2003), supporting a role for macrophages in PNS axon regrowth. Macrophages also re-model the distal nerve’s ECM in preparation for regrowing axons (La Fleur et al., 1996). Excess macrophages remain in the nerve for days to months, after which they either emigrate to lymphatic organs via the circulation or die by apoptosis (Kuhlmann et al., 2001).

The final major immune cell type to arrive in the nerve is the T lymphocyte. These cells begin infiltrating the injured sciatic nerve at three days after chronic constriction injury, and their numbers peak between 14 and 28 days after injury (Moalem et al., 2004). T lymphocytes shape the later phase of the immune response by producing pro- or anti-inflammatory cytokines that support cellular and humoral immunity, respectively (Mosmann et al., 1986). Whereas pro-inflammatory cytokines (e.g. TNF-α, IFN-γ) secreted by Type 1 helper T cells activate nearby macrophages, neutrophils and natural killer cells, anti-inflammatory cytokines (e.g. IL-4, IL-10) released by Type 2 helper T cells inhibit various macrophage functions and prevent a pro-inflammatory response (London et al., 1998). Although T lymphocytes have been implicated in neuropathic pain after PNI (Moalem et al., 2004; Cao and DeLeo, 2008; Costigan et al., 2009), their role in peripheral axon regeneration remains undefined.
PNI also causes inflammatory and glial reactions in areas proximal to injury, including the DRG (Lu and Richardson, 1993; Fenzi et al., 2001; Hu and McLachlan, 2002; Hu and McLachlan, 2003; Hu et al., 2007), the ventral horn of the spinal cord (Hu et al., 2007), and the spinal dorsal horn (Eriksson et al., 1993; Liu et al., 1998; Colburn et al., 1999; Zhang and De Koninck, 2006; Hu et al., 2007). In DRGs associated with PNI, satellite cells abutted to injured neurons upregulate glial fibrillary acidic protein (GFAP) and the low affinity neurotrophin receptor p75NTR within seven days of sciatic nerve transection, indicating their “activation” (Hu et al., 2007). Neutrophils – the first immune cells to invade distal nerve – are extremely scarce in DRGs, even after PNI (McLachlan et al., 2007). In contrast, a considerable number of macrophages accumulate in regions near the injured neurons within three dpi and remain present and activated for months (Hu and McLachlan, 2003). The density of immunoreactivity of a marker for resident macrophages (ED2) is not altered significantly by injury, suggesting that the high number of macrophages is most likely due to recruitment from circulation rather than resident cell proliferation (Hu et al., 2007). This is feasible since DRGs have no barrier to the blood (Olsson, 1968; Jacobs et al., 1976). Finally, a small number of T lymphocytes accumulate around the injured sensory neurons concomitantly with the invading macrophages (Hu and McLachlan, 2003; Hu et al., 2007).

Within the spinal cord, the dorsal horn, which includes the central terminals of axotomized primary afferents, and ventral horn, which includes injured motor neuron somata, are both affected similarly by injury. Sciatic nerve transection provokes widespread activation of astrocytes (a CNS glial cell) and microglia (the resident CNS immune cell) in these areas; however, this injury does not facilitate T lymphocyte recruitment to the spinal cord (Hu et al., 2007). Although little is known about the role of proximal PNI-induced inflammatory reactions in peripheral axon regeneration, these
reactions have been associated with neuropathic pain states (Scholz and Woolf, 2007; Dublin and Hanani, 2007; Hu et al., 2007; Cao and DeLeo, 2008; Costigan et al., 2009).

Therefore, PNI initiates an inflammatory response that is widespread, involves multiple cell types, and lasts for months. Whereas Schwann cells mediate myelin clearance in early stages of Wallerian degeneration, resident endoneurial and hematogenous macrophages play a crucial role in debris removal within a week of PNI. Peripheral axon regeneration is thought to be dependent on this efficient macrophage-mediated degenerative response within the peripheral nerve. Finally, PNI elicits inflammatory reactions in proximal regions associated with injury, including the DRG, dorsal horn, and ventral horn.

1.5.2.4 Peripheral axotomy-induced changes to the extracellular environment

The composition of the extracellular milieu in the distal peripheral nerve is another feature that contributes to successful (if transient) axon regrowth. Here, I will briefly consider expression of ECM components and their receptors, exposure and removal of myelin-associated inhibitory proteins, and expression of growth-promoting molecules.

PNS ECM contains three major matrix protein classes: laminins, fibronectins, and collagens. After collagens, laminins are the most prevalent glycoproteins in PNS basal lamina, where they can promote cell adhesion, migration, or proliferation. Laminins are large cross-shaped heterotrimers that include one $\alpha$ chain, one $\beta$ chain, and one $\gamma$ chain. There are 15 laminin variants; laminins 2 ($\alpha2\beta1\gamma1$) and 8 ($\alpha4\beta1\gamma1$) are found in intact peripheral nerve endoneurium, and their expression increases after PNI (Wallquist et al., 2002). Laminins play important roles in neurite extension in culture. For instance, Agius
and Cochard (1998) found that addition of a laminin 2-specific antibody decreased neurite outgrowth on pre-degenerated, but not intact, nerve sections. More recently, Chen and Strickland (2003) showed that conditional knock-out of the laminin γ1 chain gene in Schwann cells resulted in diminished axon regeneration in the sciatic nerve and abnormal Schwann cell function (e.g. errors in differentiation and axon ensheathment). These results suggest that increased laminin expression and deposition after injury is required for normal Schwann cell function and to create a supportive environment for regrowing axons. Laminins directly promote neurite extension, and are therefore commonly used as a substrate for cultured neurons (e.g. Colognato et al., 2005; Gardiner et al., 2005).

Laminin receptors include the integrins and dystroglycan. Integrins are heterodimeric receptors that comprise one α and one β chain, and are expressed highly in sensory neurons during development and in growth cones during regeneration. Integrin chains that have been implicated in a successful regenerative response include β1 (Tomaselli et al., 1993; Condic and Letourneau, 1997; Agius and Cochard, 1998; Ekstrom et al., 2003), α7 (Werner et al., 2000; Hammarberg et al., 2000; Gardiner et al., 2005), and α9 (Andrews et al., 2009).

Fibronectin, another growth-promoting ECM protein, is expressed widely during development in the PNS and CNS. Fibronectin is expressed in the adult PNS, and its deposition increases after injury. Although fibronectin can support axon regeneration – fibronectin guidance channels facilitate axon growth – it is not as effective as laminin at promoting regrowth (Gardiner et al., 2007). Finally, collagens, including the fibril-forming collagens (I-III, V) and basal lamina collagen (IV), are ubiquitous in peripheral nerves.
The collagens regulate ECM structure and Schwann cell function. Following PNI, collagen expression seems to increase beyond ideal levels, thereby hindering axon regeneration (Koopmans et al., 2009).

Another factor that affects regenerative success is the liberation of myelin-associated inhibitors in the distal stump following injury. Degenerating myelin shed by denervated Schwann cells contains newly-exposed inhibitors, including myelin-associated glycoprotein (MAG) (Shen et al., 1998) and oligodendrocyte-myelin glycoprotein (OMgp) (Huang et al., 2005), that robustly inhibit axon growth by acting on specific receptor complexes on axons’ membranes. These inhibitory factors are cleared relatively efficiently in the PNS. Whereas axon regeneration into injured Wld<sup>S</sup> nerves is severely impaired (Bisby and Chen, 1990; Brown et al., 1991; Chen and Bisby, 1993), regrowth is significantly improved in Wld<sup>S</sup> mice that lack MAG (Schafer et al., 1996). These results suggest that the persistence of myelin-associated inhibitors in their nerves is one factor that limits axon regeneration in Wld<sup>S</sup> mice, and that the effective clearance in wild-type nerves allows regeneration to proceed.

Nerve injury also induces significant changes in the release of growth factors and cytokines (for review, see Chen et al., 2007). Some of these factors promote axonal growth directly. For instance, IL-6, which is elevated in both neurons and non-neuronal cells by PNI (Murphy et al., 1995; Bourde et al., 1996), signals via its receptor to increase neuronal RAG expression and promote neurite growth (Cafferty et al., 2004; Cao et al., 2006). Neurotrophic factors such as NGF are elevated by nerve injury, although their roles in regenerative axon growth are not well-defined (Heumann et al., 1987; Fu and Gordon, 1997; Snider et al., 2002; Markus et al., 2002a). Other factors
promote nerve repair indirectly: for example, neuron-derived neuregulin promotes Schwann cell migration and aids in recovery (Mahanthappa et al., 1996).

Therefore, efficient regulation of the extracellular environment’s composition after PNI supports axon regrowth. Increased expression of permissive ECM molecules (and their receptors on axons), rapid removal of myelin-associated inhibitory factors in the distal stump, and increased secretion of trophic factors and cytokines all contribute to successful PNS axon regeneration in the weeks following injury.

1.5.2.5 Responses extrinsic to the neuron after CNS injury

In contrast with the efficient response coordinated within injured peripheral nerves, the non-neuronal response to CNS injury contributes to regenerative failure. CNS injury elicits changes in blood-brain barrier permeability, in cells’ activation states, in the cellular composition of the CNS, and in the extracellular milieu.

Whereas PNI-induced blood-nerve breakdown is extensive, the blood-brain barrier does not fully break down following CNS injury. After spinal cord contusion, the permeability of the blood-brain barrier increases at and around the injury site for about three weeks (Popovich et al., 1996; Whetstone et al., 2003; Pan et al., 2006). Even though granular disintegration of the axon cytoskeleton occurs relatively soon after CNS injury, as in the PNS, the blood-brain barrier is not fully compromised in areas associated with degenerating CNS tracts. Therefore, the maintenance of the blood-brain barrier that covers disconnected CNS tracts could be a factor that underlies protracted Wallerian degeneration – and poor axon regeneration – in the pathological spinal cord and brain.
The growth-supportive phenotype of denervated Schwann cells contrasts starkly with the response of endogenous CNS glia to injury (Fawcett, 2006; Yiu and He, 2006). The myelinating cells of the CNS, oligodendrocytes, respond to denervation by either undergoing apoptosis or entering a quiescent state (Barres et al., 1993; Crowe et al., 1997; Casha et al., 2001). Compared to Schwann cells, oligodendrocytes are more sensitive to denervation, provide minimal growth support, and have little phagocytic activity (Vaughn and Pease, 1970; Ludwin, 1990; Dougherty et al., 2000). The astrocyte, which is the most numerous resident CNS glial cell type, responds to injury by forming an impenetrable glial scar around the injury site and by producing factors that inhibit axon growth (Davies et al., 1999; Hermanns et al., 2001).

Compared to that in the PNS, the CNS immune response to injury is muted and is much less efficient at clearing inhibitory debris. For instance, myelin debris can be found in the degenerating human corticospinal tract years after injury (Becerra et al., 1995; Buss et al., 2004). Although resident microglia react to injury by proliferating, becoming activated, and phagocytosing debris, their response is delayed by two or three days and is not nearly as effective as is the PNS macrophage response (Lawson et al., 1994). Few hematogenous macrophages infiltrate the injured CNS; this is likely due to the preservation of the blood-brain barrier along degenerating tracts (Vargas and Barres, 2007). Therefore, the limited phagocytic and growth-promoting response of microglia and oligodendrocytes, combined with the dearth of hematogenous macrophages, may underlie the delayed removal of inhibitory debris during CNS Wallerian degeneration.

The composition of CNS ECM is also very different from that of the PNS. CNS ECM includes the massive glycosaminoglycan hyaluronan and the glycoproteins tenascin-C and thrombospondin (Gladson, 1999). CNS ECM lacks significant amounts of collagen,
laminin, and fibronectin, which contribute to the structure and strength of other tissues (Bellail et al., 2004). In contrast with its expression pattern in the PNS, laminin is confined to the pial and vascular basal lamina in the CNS (Gladson, 1999). In addition, oligodendrocytes do not have an associated basal lamina (Colognato et al., 2005), which is a key contributor to PNS axon regeneration. Finally, after CNS injury, the dense ECM network that forms at the injury site acts as a physical and molecular barrier to axon regeneration (McKeon et al., 1991).

Due to protracted Wallerian degeneration, myelin-associated inhibitory factors have a profound impact on injured CNS axons. In addition to MAG and OMgp, degenerating CNS myelin contains the potent outgrowth inhibitor NogoA (GrandPre et al., 2000; Chen et al., 2000). These inhibitors linger at and around the injury site, where they contribute to regenerative failure.

### 1.5.2.6 Responses extrinsic to the neuron after dorsal root injury

The effects of DRI on the extracellular environment are unique, but they are also instructive for other injury models. Although axons regenerate through the peripheral portion of the dorsal root, they halt at the PNS-CNS interface (the dorsal root entry zone (DREZ)) and fail to reach their denervated dorsal horn and/or brainstem targets (Bradbury et al., 2000; Fraher et al., 2002; Tessler, 2004). The striking change in axons’ growth capacity precisely at the DREZ can be attributed to differential reactions extrinsic to the axons within the PNS and CNS segments of the root.

Although their axons can regrow to the DREZ, DRG neurons mount a relatively muted regenerative response to injury of their central process. Whereas almost all peripherally-injured DRG neurons robustly upregulate RAGs such as GAP-43, few neurons induce
GAP-43 expression after DRI (Mason et al., 2002). This difference is manifested in regeneration rates: rat dorsal root axon regeneration proceeds at about 2.5 mm per day, compared to 4.5 mm per day in peripheral nerves (Wujek and Lasek, 1983; Oblinger and Lasek, 1984).

Once at the DREZ, dorsal root axons form large end bulbs as they encounter the inhibitory environment of the CNS (Carlstedt, 1985; Ramon y Cajal, 1991). The inhibitory nature of the central portion of the DREZ is induced by DRI, as untreated axons from transplanted DRG neurons can grow across an uninjured, but not an injured, DREZ (McPhail et al., 2005). Within the CNS, DRI induces astrocyte hypertrophy, proliferation, and reactivity (including increased GFAP immunoreactivity (-IR)) (Liu et al., 2000). DRI also causes an inflammatory response associated with delayed degeneration of disconnected axon and myelin debris. Hematogenous leukocytes invade in small numbers within a week of DRI (Ling, 1979; George and Griffin, 1994; Rutkowski et al., 2002); however, microglia are the predominant phagocytic cells after DRI: they proliferate and accumulate in the dorsal horn and dorsal column nuclei, as well as in the central part of the DREZ and the dorsal columns (George and Griffin, 1994; Liu et al., 1995b; Liu et al., 2000).

As mentioned in Section 1.5.1.2, prior conditioning lesion of the DRG neuron’s peripheral branch enhances growth of the central branch into the CNS compartment of the DREZ. Interestingly, treatment with specific neurotrophic factors also promotes axon regeneration across the DREZ and functional reconnection with central targets (Ramer et al., 2000), suggesting that the growth state of DRG neurons can be augmented pharmacologically. Ramer and colleagues (2001) extended these findings by showing that NT-3 treatment enhanced central regeneration when delivered immediately, but not
when delayed one week. They established that NT-3 treatment was sufficient for axons to surmount the first barrier to regeneration formed by astrocytes within two dpi, but NT-3-treated axons could not overcome a second barrier that develops within a week of injury and consists of activated microglia/macrophages and associated degenerating myelin (these axons remained at the DREZ). NT-3 remains one of the most effective treatments for promoting axon regeneration and functional recovery following DRI (cf. Ramer et al., 2002).

In summary, changes in the environment of injured CNS neurons and axons effectively prohibit axon regeneration. Endogenous CNS cells contribute to the hostile environment: astrocytes proliferate and release inhibitory factors, oligodendrocytes atrophy and release myelin-associated inhibitors, and microglia coordinate a dismal degenerative response. DRI proves to be an instructive model for regeneration after CNS injury, since cellular responses to injury are similar, while complicating factors such as glial scar formation are limited.
1.6 Galectin-1: the little protein that could

Galectins are classified based on their binding specificity for carbohydrates containing galactosides; however, not all of their varied effects are dependent on this lectin activity.

1.6.1 Galectins: discovery and classification

The first vertebrate $\beta$-galactoside-binding lectin to be characterized was isolated from the electric organ of the electric eel (Teichberg et al., 1975). This protein was called electrolectin, and its discovery led to a widespread search for orthologues in other vertebrates. Identification of a variety of lactose-sensitive agglutinins from rat and chick tissues followed, and soon it was clear that these proteins were ubiquitously expressed in vertebrate tissues (Harrison, 1991; Kilpatrick, 2002). Proteins in this family are defined by the presence of a highly-conserved carbohydrate-recognition domain specific for $\beta$-galactosides, and are now known as galectins (Barondes et al., 1994).

There are 16 known vertebrate galectins that are divided into three subtypes based on subunit structure. Galectins of the prototype subtype, including galectin-1 (Gal1), contain a single galectin domain, those of the repeat subtype have two homologous domains, and chimera subtype galectins comprise one galectin domain and an unrelated domain. Galectins are expressed in a variety of tissues, and galectin-like proteins are present in highly divergent species, including slime mould, *C. elegans*, and humans (Rosen et al., 1973; Gitt and Barondes, 1986; Hirabayashi et al., 1992; Cooper and Barondes, 1999; Dodd and Drickamer, 2001). It is therefore not surprising that galectins have important roles in key cellular processes, such as cell adhesion, proliferation, differentiation, growth, and cell death.
1.6.2 Galectin-1: a ubiquitous protein with diverse functions

The structure and function of Gal1 depend on its oxidation state. In solution, Gal1 most often exists as a homodimer with two active carbohydrate recognition domains (Lopez-Lucendo et al., 2004). When Gal1 is present at low concentrations, the 14.5 kDa monomers can dissociate to form two independent lactose-binding units (Cho and Cummings, 1995). Under oxidizing conditions, Gal1 is a monomer that lacks lectin activity (Gal1/Ox, as opposed to the reduced form, Gal1/Red) (Inagaki et al., 2000); its structure is maintained by the formation of three intramolecular disulfide bonds between cysteine residues under these conditions. Most of Gal1’s extracellular interactions require its lectin activity, whereas its intracellular protein-protein interactions are generally lactose-independent (Camby et al., 2006).

In the extracellular space, Gal1 interacts with a multitude of glycoproteins in a carbohydrate-dependent manner. Gal1 binds ECM glycoproteins laminin and fibronectin, and is implicated in ECM assembly and remodelling (Moiseeva et al., 1999; Moiseeva et al., 2003). In addition, Gal1 binding partially activates β1 integrin (Moiseeva et al., 2003), and Gal1 modulates α7β1 integrin interaction with laminin during skeletal muscle differentiation (Gu et al., 1994). Gal1 also associates with αMβ2 integrin (complement receptor 3) on mouse macrophages (Avni et al., 1998). Other major Gal1 binding partners include GM1 ganglioside and various T-cell glycoproteins (see Camby et al., 2006).

Gal1 has been implicated in fewer protein-protein interactions. Intracellular Gal1 interacts with a version of the monomeric G protein Ras, GTP-bound H-Ras (H-Ras-GTP) (Paz et al., 2001; Rotblat et al., 2004). Gal1 enhances H-Ras activity by
strengthening its membrane association. Interestingly, Gal1-mediated Ras activation could affect neurite outgrowth (see Section 1.6.4.2). In addition, Gal1 interacts with the protein Gemin4 (Park et al., 2001); these proteins are involved in pre-mRNA splicing in the nucleus (Vyakarnam et al., 1997; Voss et al., 2008). Finally, Horie et al. (2004) showed that extracellular Gal1/Ox binds to an unidentified cell surface receptor on macrophages that activates intracellular signaling cascades. Since Gal1/Ox lacks lectin activity, this is likely a protein-protein interaction.

Gal1 has countless, often contradictory, effects on major cellular processes. In some cases, Gal1’s effects depend critically on its concentration: whereas high Gal1 levels inhibit proliferation of human cells (and may initiate apoptosis), low levels facilitate the process (Adams et al., 1996; Vas et al., 2005). Gal1 signaling induces proliferation in various mammalian tissues, such as spleen (Symons et al., 2000), vascular (Sanford and Harris-Hooker, 1990), and hepatic stellate cells (Maeda et al., 2003); however, it represses growth of other cell types, including neuroblastoma (Kopitz et al., 2001) and stromal bone marrow cells (Andersen et al., 2003). Gal1 modulates cell adhesion to the ECM by cross-linking cell surface glycoproteins (e.g. integrins) with extracellular glycoproteins (e.g. laminin) (Zhou and Cummings, 1993; van den Brule et al., 1995; Moiseeva et al., 1999; van den et al., 2003). Finally, Gal1 regulates cell motility: Gal1 is associated with increased mobility of glioma cells (Camby et al., 2002), has roles in immune cell transmigration (Rabinovich et al., 1999; Delbrouck et al., 2002), and is implicated in Schwann cell migration after PNI (Fukaya et al., 2003). Before discussing the role of Gal1 in the nervous system in more detail, I will briefly consider Gal1’s functions in the immune system.
1.6.3 Roles of galectin-1 in the immune system

Galectin-1 (Gal1) has wide-ranging effects on cells of the immune system that can be either pro- or anti-inflammatory (reviewed by Liu and Rabinovich, 2010). Gal1-treated neutrophils cultured on endothelial monolayers have diminished ability to transmigrate, and exhibit less capture, rolling and adhesion on the cell layer compared to cells in the control group (La et al., 2003; Cooper et al., 2008). In contrast, Gal1 can also have a pro-inflammatory effect on neutrophils: Gal1 treatment of cultured neutrophils causes increased superoxide release (Almkvist et al., 2002). Gal1 also modulates the macrophage response during inflammation. Gal1 suppresses arachidonic acid release, prevents synthesis of nitric oxide, and increases arginase activity (Rabinovich et al., 2000; Correa et al., 2003), supporting an anti-inflammatory role for Gal1. However, the bi-functional nature of Gal1 is also evident in the regulation of the macrophage response. Whereas Gal1 treatment of naïve macrophages induced increased FcγRI expression and phagocytosis, Gal1 treatment of IFN-γ-activated macrophages decreased both of these parameters (Barrionuevo et al., 2007). Finally, Gal1 regulates T cell survival and favours a Th2 T cell response. Gal1 can induce apoptosis of specific T cell subtypes (Perillo et al., 1995; Rabinovich et al., 1998). In addition, Gal1 limits T cell transmigration across endothelial cell layers (Rabinovich et al., 1999), and promotes expression of Th2 cytokines (Motran et al., 2008; Stowell et al., 2008). Therefore, Gal1 has dual roles in the modulation of the cellular response to inflammation. Given that the majority of the data suggest that Gal1 tempers immune cell migration and cytokine production, Gal1 might ultimately have an anti-inflammatory effect on immune cells.
1.6.4 Roles of galectin-1 in the nervous system

Gal1 has key functions in the growth/targeting of subpopulations of olfactory and DRG axons. I will consider Gal1’s roles in developmental axon growth before discussing its effects on peripheral axon regeneration.

1.6.4.1 Galectin-1 in primary sensory neuron development

Many lectins are developmentally regulated, and lectins are often found to be at highest concentrations in differentiating tissue (Barondes et al., 1984). This generalization holds true for the regulation of Gal1 expression during sensory neuron development. Gal1 mRNA and protein are first detected in rat DRGs during early neurogenesis, at E13 (Dodd and Jessell, 1986; Regan et al., 1986; Hynes et al., 1990). At this stage, Gal1-IR is localized to sensory neuron somata within the DRG and in their central projections, some of which have grown up to the DREZ (Dodd and Jessell, 1986; Regan et al., 1986). At E14, Gal1-IR is observed in growing intrasegmental dorsal root collateral axons in the dorsal root bifurcation zone (Regan et al., 1986), and is also found in afferents penetrating the dorsal horn at E16 (Dodd and Jessell, 1986). By E18, Gal1 mRNA and protein are expressed in almost all sensory neurons, although small- and intermediate-diameter neurons, which were produced on E15 (Altman and Bayer, 1984), have the highest Gal1-IR (Regan et al., 1986; Hynes et al., 1990). At post-natal day (P) 0, Gal1 mRNA is detected at much higher levels in the DRG and spinal cord than in the brain, a pattern that is maintained in the adult animal (Hynes et al., 1990). Approximately 30-40% of DRG neurons contained high Gal1 levels at P0 (Regan et al., 1986). Within the spinal dorsal horn, sensory terminals in laminae I and II are intensely Gal1-IR, while those in laminae III-IV contain lower levels of Gal1. Other areas of the
spinal cord that display Gal1-IR at P0 include sensory collaterals in the dorsal columns and motoneurons in the ventral horn (Regan et al., 1986).

Gal1 is localized to the nucleus and cytoplasm of DRG neurons, and is externalized by sensory neurons and Schwann cells via a non-classical pathway into the extracellular milieu (Barondes et al., 1984; Cooper and Barondes, 1990; Sango et al., 2004). Interestingly, early studies showed that neurons expressing Gal1 often co-express its specific lactoseries glycoconjugate (Dodd and Jessell, 1985; Regan et al., 1986), and that embryonic DRG neurons have the ability to bind Gal1 in dissociated cell culture (Dodd and Jessell, 1986). Gal1-expressing neurons that also express its specific carbohydrate ligands are generally small-diameter and have central projections that terminate in the superficial laminae of the dorsal horn (Dodd and Jessell, 1985; Regan et al., 1986; Hynes et al., 1990). Although Gal1 is expressed by nearly all DRG neurons, differential expression of its lactoseries epitope on these neurons suggests that this interaction could mediate the cross-linking of specific axonal subsets, possibly assisting in the formation discrete nerve fascicles and terminal fields (Dodd and Jessell, 1985; Hynes et al., 1990). Thus, the pattern of regulation of Gal1 in sensory neurons during neurogenesis suggests that the reduced form of this protein may act in concert with its complementary carbohydrate ligands to promote changes in cellular proliferation within the cell, or mediate axonal adhesion or targeting on the cell surface.

More recent studies have used the Gal1 null mutant (Lgals1−/−) mouse (Poirier and Robertson, 1993) to elucidate whether Gal1 has a role in the development of primary sensory neurons and their axonal connections. Puche et al. (1996) studied axonal connectivity in the olfactory system of Lgals1−/− mice, and found that a subset of primary sensory olfactory neurons failed to project to their correct targets in the caudal olfactory
bulb in these mice. They also showed that Gal1 had neurite outgrowth-promoting activity when used as a substrate \textit{in vitro}. This study was the first to implicate a lectin in the promotion of neurite outgrowth and in axonal pathfinding. Subsequent studies have demonstrated that the pattern of expression of Gal1 in the olfactory system is consistent with its proposed role in the targeting and maintenance of sensory axon connections in the mouse, rat and human (Tenne-Brown et al., 1998; St John and Key, 1999; Heilmann et al., 2000; Crandall et al., 2000). Given that Gal1 is important for development of the typical connectivity of the olfactory system and is expressed highly in and around growing DRG neurons, Gal1 may be involved in the proper development of DRG neurons and their central projections. I address this issue in chapter two.

1.6.4.2 Expression and functions of galectin-1 in the adult nervous system

In the adult rat, Gal1 expression in the nervous system becomes localized mainly to neurons that project peripherally, although Gal1 is also found in some central tissues. Distribution of Gal1 mRNA and protein expression in sub-populations of DRG neurons during development is essentially maintained in the adult. While Gal1 is expressed in most adult DRG neurons, a sub-population of small diameter DRG neurons contains the highest levels of Gal1 mRNA and protein (Dodd and Jessell, 1986; Regan et al., 1986; Imbe et al., 2003; Fukaya et al., 2003; Sango et al., 2004). I examine the injury-induced regulation of Gal1 expression within DRG neurons and their environment in chapter three.

Gal1 expression is regulated by PNI. Cameron and colleagues (1997) were the first to demonstrate that density of Gal1-IR is increased in the central terminals of injured DRG neurons in the spinal dorsal horn in a peripheral neuropathy model. In the uninjured rat spinal cord, Gal1-positive terminals are found mainly within lamina II, with diffuse
staining in deeper laminae. Following chronic constriction injury, Gal1-IR is more dense in laminae I-III by 14 dpi and levels remain elevated for at least 70 dpi; the augmented Gal1 levels were abolished after DRI, suggesting that the central primary afferent terminals had more Gal1 after PNI (Cameron et al., 1997). Similarly, Imbe et al. (2003) found that Gal1-IR is increased in the medial portion of the superficial dorsal horn following sciatic nerve transection.

Gal1 promotes peripheral axon regeneration. Horie et al. (1999) delivered Gal1 or Gal1 function-blocking antibody using a tubulization model in the rat sciatic nerve, and established that Gal1 promotes Schwann cell migration and axon regeneration. Subsequent studies showed that Gal1/Ox, but not Gal1/Red, effects axon regeneration in a variety of models (Inagaki et al., 2000; Horie et al., 2004; Okada et al., 2005). Early studies on Gal1’s role in axon regeneration did not investigate the underlying mechanisms satisfactorily: it is conceivable, of course, that Gal1 could promote regeneration by acting within the neuron itself, and/or through actions in regrowing axons’ environment.

Gal1 may act as a RAG within injured peripheral neurons. Gal1 mRNA is upregulated within hours of facial nerve injury (Akazawa et al., 2004), remains elevated for at least seven dpi, and returns to uninjured levels by 14 dpi (McGraw et al., 2004a), a time at which many axons have re-connected with peripheral targets. Recovery of whisking function, a behavioural measure of regenerative success, was delayed significantly in Lgals1−/− mice, supporting a role for Gal1 in facial nerve repair. In another study, McGraw and colleagues (2004b) compared the injury-induced regulation of Gal1 in spinal motoneurons and rubrospinal neurons. PNI caused a significant increase in the expression of Gal1 mRNA and protein in cervical spinal motoneurons at seven and 14
dpi, whereas injury to CNS rubrospinal tract axons (C4 dorsolateral funiculus transection) induced a significant downregulation of Gal1 mRNA in their somata within the red nucleus at seven and 14 dpi. BDNF application to the red nucleus, which increases RAG expression and regenerative capacity of rubrospinal neurons (Kobayashi et al., 1997; Kwon et al., 2002), increased Gal1 mRNA expression in these neurons at seven dpi relative to vehicle-treated animals (McGraw et al., 2004b). Interestingly, cultured PC12 cells extend longer neurites when they are transfected with Gal1 (Rotblat et al., 2004). This effect is mediated through its interaction with Ras, since a point mutation in Gal1 that prevents its interaction with Ras restricts neurite outgrowth (Ras activation has been implicated in neuron survival and axon extension (Markus et al., 2002b; Hall and Lalli, 2010)). Thus, Lgals1 deletion results in delayed functional recovery following PNI, the expression of Gal1 in rubrospinal and motoneurons is correlated with their axons’ regenerative potential, and intracellular Gal1 overexpression is associated with enhanced neuritic growth. I discuss a potential role for Gal1 within neurons in chapter four.

There is also evidence that Gal1-mediated regeneration is dependent on non-neuronal cells. Using DRG explants, Horie et al. (2004) found that conditioned medium from Gal1/Ox-treated macrophages enhanced neurite outgrowth and Schwann cell migration. Gal1/Ox also elicited enhanced neuritic outgrowth, although this growth was likely less than that from explants treated with the conditioned medium. Gal1/Ox binding to an unidentified receptor on macrophages induced changes in tyrosine phosphorylation of unidentified intracellular proteins, and ultimately may have led to the secretion of a protein that mediates the aforementioned effects on regeneration. Interestingly, Gal1/Ox has been shown to promote axon regeneration in a model of CNS injury (Okada et al., 2005). Following intravitreal injection of Gal1/Ox (one hour post-injury), retinal ganglion
cell axon regeneration was enhanced to a level comparable to that promoted by 
zymosan or forskolin injection. The authors assumed, but never confirmed that these 
effects were due to Gal1/Ox-induced macrophage activation. Gal1 also potentiates 
injury-induced axon degeneration (Plachta et al., 2007); it is possible that this effect 
could lead to more rapid Wallerian degeneration and enhanced axon regeneration. I 
study the relationship between Gal1 and macrophages in the nerve in chapter five.

There is growing evidence that Gal1 has an important role in axonal regeneration. Gal1 
upregulation is positively correlated with the switch to growth mode in injured neurons. 
In addition, Gal1/Ox-treated macrophages produce factors that enhance axon 
regeneration. Therefore, Gal1 may act both within the injured neuron and/or within the 
neuron’s external milieu to promote axonal regeneration following nerve injury.
 Experimental hypotheses 

Although Gal1 has been implicated in developmental axon targeting and in peripheral axon growth, several details remain to be brought to light. I address some of these unresolved topics in the following four chapters.

During development, Gal1 is involved in olfactory axon targeting and is expressed highly during extension of DRG neurons’ central projections into the spinal dorsal horn. In chapter two, I clarify whether Gal1 has a role in the development of DRG neurons and their central projections. I compare the distribution of DRG neuron subpopulations in $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mice, as well as the terminal fields of their central projections in the dorsal horn. I also explore whether the formation of functional connections between primary afferents and projection neurons in the dorsal horn is Gal1-dependent. In addition, I assessed sensory thresholds in $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mice to determine whether observed differences in anatomy are correlated with a measureable behavioural affect. I hypothesized that Gal1 would be required for targeting of central branches of DRG neurons to correct dorsal horn laminae during development, and that anatomical differences revealed after development in the absence of Gal1 would be manifested in altered sensitivity to various stimuli.

Regulation of neuronal Gal1 expression by injury has been correlated with regenerative potential in various systems. In chapter three, I characterize the regulation of Gal1 expression by injury to the peripheral or central processes of DRG neurons. I examine Gal1 protein and mRNA levels in DRG neurons that were uninjured, as well as those subjected to PNI or DRI. I also describe Gal1 regulation in the peripheral and central portion of the dorsal root to elucidate whether Gal1 regulation in the root is related to the
growth capacity of extending axons. Finally, I study the regulation of Gal1 mRNA expression in the spinal cord after DRI. I hypothesized that Gal1 expression would be correlated with regenerative capacity both within the neuron and in its axons environment: specifically, I predicted that Gal1 expression within DRG neurons would be upregulated after injury to their peripheral, but not central, processes; and that Gal1 would be upregulated in the peripheral, but not central, compartment of the DREZ after DRI.

Although many studies have implicated Gal1 in peripheral axon regeneration, none have established whether its effects are intrinsic or extrinsic to the neuron. In chapter four, I elucidate whether Gal1 promotes neurite outgrowth from isolated adult DRG neurons. I dissociated and cultured DRG neurons from adult rats and mice overnight in the absence or presence of Gal1 and/or neurotrophic factors. In order to establish whether intrinsic or intracellular Gal1 affected neurite outgrowth, I cultured mouse DRG neurons from \( Lgals1^{+/+} \) and \( Lgals1^{-/-} \) mice. I also characterized neurite outgrowth from rat DRG neurons exposed to exogenous Gal1/Ox, in order to determine whether this protein can effect neurite outgrowth directly. I assessed neurite outgrowth by comparing neurite length and branching, as well as the proportion of neurons extending neurites. I hypothesized that neurites from \( Lgals1^{-/-} \) neurons would not grow as extensively as would those from \( Lgal1^{+/+} \) neurons, and I predicted that Gal1/Ox would enhance neuritic elaboration compared to control cultures.

In chapter five, I study a potential role for Gal1 extrinsic to injured neurons, and demonstrate a role for Gal1 in PNI-induced macrophage accumulation. I used immunohistochemistry to study macrophage density in peripheral nerve, and in areas proximal to PNI, in \( Lgals1^{+/+} \) and \( Lgals1^{-/-} \) mice at various stages after axotomy. I also
injected Gal1/Ox or a function-blocking Gal1 antibody into the nerve to test whether Gal1 is sufficient or necessary, respectively, for macrophage accumulation in the nerve. Finally, I performed nerve transplant experiments to elucidate mechanisms underlying differences in macrophage accumulation in the two mouse strains. I hypothesized that Gal1 would promote macrophage accumulation following PNI.
1.8 References


Canadian Institute for Health Information. National Trauma Registry. 2010. Ottawa, ON. Ref Type: Report


in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. J Neurosci 29: 14415-14422.


Kashiba H, Ueda Y, Senba E (1996) Coexpression of preprotachykinin-A, alpha-
calcitonin gene-related peptide, somatostatin, and neurotrophin receptor family


Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld T (2005) In vivo imaging of


Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent
neurons with adelta/c-fibers and colocalization with trk receptors. J Comp Neurol 493:
596-606.

Differential expression patterns of mRNAs for P2X receptor subunits in neurochemically

and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate
GAP-43 and Talph1-tubulin mRNA expression, and promote axonal regeneration. J
Neurosci 17: 9583-9595.


HJ (2001) Negative regulation of neuroblastoma cell growth by carbohydrate-dependent
surface binding of galectin-1 and functional divergence from galectin-3. J Biol Chem 276:
35917-35923.


Kuhlmann T, Bitsch A, Stadelmann C, Siebert H, Bruck W (2001) Macrophages are
eliminated from the injured peripheral nerve via local apoptosis and circulation to

Kuo DC, de Groat WC (1985) Primary afferent projections of the major splanchnic nerve


Lawson SN (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 87: 239-244.


Ref Type: Electronic Citation


Tornqvist E, Aldskogius H (1994) Motoneuron survival is not affected by the proximo-distal level of axotomy but by the possibility of regenerating axons to gain access to the distal nerve stump. J Neurosci Res 39: 159-165.


van den BF, Califice S, Garnier F, Fernandez PL, Berchuck A, Castronovo V (2003) Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary
carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin. Lab Invest 83: 377-386.


2 Altered primary afferent anatomy and reduced thermal sensitivity in mice lacking galectin-1

2.1 Introduction

Small diameter dorsal root ganglion (DRG) neurons transmit nociceptive information from the periphery to the central nervous system (CNS). They have thinly-myelinated and unmyelinated small diameter axons and constitute approximately 70% of all neurons within the DRG (Snider and McMahon, 1998). Nociceptive neurons can be further subdivided based on neurochemistry and termination pattern within the spinal cord: those expressing the neuropeptide calcitonin gene-related peptide (CGRP) terminate in laminae I and II outer (IIo) (Averill et al., 1995), while non-peptidergic C afferents that express the ATP purinoceptor receptor P2X3 (non-peptidergic neurons) and bind the lectin Bandeiraea simplicifolia (IB4) terminate in lamina II inner (IIi) of the spinal cord (Chen et al., 1995; Molliver et al., 1997; Bradbury et al., 1998; Vulchanova et al., 1998). Large-calibre axons carrying proprio- and mechanoceptive information terminate in deeper laminae (III-X) and are identifiable within the DRG by their expression of the large molecular weight neurofilament NF200 (Lawson et al., 1984).

During development, different neurotrophic factors regulate distinct functional classes of sensory neurons. For example, the nerve growth factor (NGF)-specific receptor TrkA localizes to 70-80% of all DRG neurons early in development (Molliver et al., 1995; Molliver and Snider, 1997) and is required for their survival (Crowley et al., 1994; Smeyne et al., 1994; Silos-Santiago et al., 1995). However, as development proceeds, half of the NGF-dependent neurons lose their TrkA expression and begin to express Ret.

* JM and ADG contributed equally to this manuscript.
the signaling receptor for the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors (Molliver and Snider 1997; Molliver et al., 1997), leading to an adult DRG population that comprises 40% TrkA- and CGRP-immunoreactive (IR) neurons and 30% Ret-expressing and IB4-binding neurons.

During development and into adulthood in the rat, these small-diameter neurons also express the carbohydrate-binding protein galectin-1 (Gal1) (Regan et al., 1986). Using antibodies against a Gal1 epitope, Reagan et al. (1986) reported Gal1 (then called RL-14.5) protein expression in 63% of primary sensory somata as well as expression in spinal motor neurons. The initial expression in sensory neurons began at E13–14 as they finished their final mitotic division and began their growth towards their targets within the dorsal horn of the spinal cord. When Gal1-expressing axons reached their targets, Gal1 expression remained elevated, albeit at lower levels (Regan et al., 1986; Hynes et al., 1990; John and Key, 1999; Sango et al., 2004). Furthermore, exogenous Gal1 protein promotes DRG axonal growth in vitro and in vivo (Horie et al., 1999; Horie and Kadoya, 2000; Horie et al., 2004). These data strongly suggest that Gal1 plays a role in sensory axon outgrowth and maintenance; however, to date the role of Gal1 in sensory neuron development remains unknown.

Gal1 homozygous null mutant (Lgals1−/−) mice are viable without obvious phenotypic abnormalities (Poirier and Robertson, 1993). Interestingly, in these mice, a subpopulation of olfactory bulb axons that normally express Gal1 do not reach appropriate targets in olfactory glomeruli (Puche et al., 1996). These data suggest that Gal1 may be involved in axonal growth or pathfinding more generally. Here, we examine Gal1 expression in the DRG and spinal cord of wild-type (Lgals1+/+) mice, study changes
in neuronal populations and primary afferent terminations in $Lgals1^{−/−}$ mice, and correlate these changes with nociceptive behavioural responses.
2.2 Materials and methods

2.2.1 Animals

A total of 26 adult age-matched 129P3/J (Lgals1+/+, Jackson Labs, Maine) and 26 adult 129P3/J Lgals1+/− (Poirier and Robertson, 1993) were used for these experiments. All experiments were performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee.

2.2.2 Behavioral testing

A total of 7 Lgals1+/+ and 7 Lgals1−/− mice were used for behavioral testing. Two tests (punctate pressure and either radiant heat or cold plate, see below) were carried out three times per day with at least two hours between each trial. To avoid sensitization, the tests were repeated on four different days with three days between each testing day. Student’s t-tests were used to determine differences between groups with statistical significance attained when \( p < 0.05 \).

**Progressive plantar punctate force test**: A mouse was placed on a metal grate and allowed to adjust to the surroundings for 10 minutes. A dynamic plantar aesthesiometer (model # 37400 UGO Basile Biological Research, Comerio VA) with a dull metal wire was maneuvered under a paw. The ramp was set to 7 seconds (s), increasing to a maximum force of 20 grams (g). Upon paw withdrawal (involving some or all of: sustained elevation, biting, licking, or shaking of the paw), the instantaneous force applied to the plantar surface eliciting the withdrawal was recorded automatically. Random paw movements were not recorded.
Radiant heat: A mouse was placed on a Plexiglas surface and allowed to habituate to the surroundings for 20-30 minutes. When the mouse was still, an infrared beam connected to a timer was shone on the palmar or plantar surface of mouse paw (model #7371 UGO Basile Biological Research, Comerio VA). Latency to withdrawal was recorded. Only nocifensive movements were counted.

Cold plate test: A mouse was placed on a 1°C cold plate (model # 0134-002l Columbus Instruments, OH) and a timer was immediately started. The latency to the initial nocifensive response was recorded.

2.2.3 Fos activation

Fos protein expression is used as a marker for neuronal activation in the spinal cord (Hunt et al., 1987). We used a total of 8 Lgals1+/+ and 8 Lgals1−/− mice to determine if there were differences in second order neuron activation after noxious temperature stimulation. Under light anesthesia, the mouse’s left front paw was carefully submerged in a water bath (1 or 52°C) three times for 10 s with a 30 s delay between each submersion. Animals were killed 2 hours after thermal stimulation, when Fos activation in the dorsal horn peaks (Hunt et al., 1987; Dai et al., 2001).

2.2.4 Tissue processing / immunohistochemistry

At the appropriate time, animals were injected with a lethal dose of chloral hydrate (1.75 g/kg body weight) and monitored. Upon the loss of nociceptive reflexes, animals were perfused transcardially with 0.1 M phosphate buffered saline (PBS), pH 7.4 followed by cold 4% paraformaldehyde in 0.1 M PBS. The spinal cord and attached DRGs were removed and the tissue was post-fixed for 24 hours in 4% paraformaldehyde at 4°C,
then cryoprotected overnight in a 24% sucrose solution in PBS. Cryoprotected tissue was rapidly frozen in supercooled 2-methylbutane and, later, 14 µm cryosections were cut, thaw-mounted onto glass slides (Superfrost plus) and stored at -80°C.

Standard immunohistochemical techniques and controls for indirect immunofluorescence were used in order to visualize specific antigens on cryosectioned tissue. Slides were washed in 0.1M PBS for 20 minutes, blocked for 20 minutes in 10% normal goat serum and then goat anti-Gal1 (1:500 R&D systems, MN), rabbit anti-NeuN (1:100, Chemicon, Temecula CA) (McPhail et al., 2004), rabbit anti-CGRP (1:2000, Sigma, Oakville, ON), biotin-conjugated IB4 (1:50, Vector Labs), mouse anti-βIII tubulin (1:500, Sigma), mouse anti-NF200 (clone N52, 1:500, Sigma) or rabbit anti-Fos (1:5000, Oncogene, Cambridge, MA), in 0.1M PBS (in 0.2% Triton X-100, and 0.1% sodium azide) was applied to the slides overnight. After washing, secondary antibodies raised in donkey and conjugated to Cy3, Alexa 488, AMCA (1:300, Jaskson Immunological Research, West Grove, PA) or extravidin conjugated Cy3 or FITC (1:500, Sigma) was applied for 1 hour at room temperature. After a final wash, slides were coverslipped with a 3:1 solution of glycerol:PBS. A fluorescent microscope (Carl Zeiss, Axioskop, Toronto, ON) was used to visualize chromophore-labeled tissue and then greyscale images were captured using a digital camera (Carl Zeiss, Axioskop, Toronto, ON) in combination with Northern Eclipse software (Empix Inc, Mississauga ON). All images for an individual antigen were taken at the same time and under the same light intensities.

### 2.2.5 Image analysis

Analysis of DRG neuron markers was carried out as described by Ramer et al. (2001b). Briefly, triple-labeled images of Gal1, CGRP and IB4 were imported into SigmaScan Pro.
5.0 (SPSS Inc., Chicago, IL). The DRG cell bodies were outlined creating an image overlay. The average intensity and Feret diameter of each object identified by the overlay was automatically measured. The threshold for immunopositivity for Gal1, IB4 and CGRP was determined by averaging three cell bodies in each section that were judged to be minimally positive. We used recursive translation, a stereological counting method which reconstructs cell populations based on size-distribution of profiles (Rose and Rohrlich, 1988), to determine actual soma size and the proportion of cells expressing an antigen. Proportions of labeled neurons were compared using Student’s t-tests.

Dorsal horn analysis was carried out as described previously (Ramer et al., 2001a; Ramer et al., 2003; Gaudet et al., 2004). Briefly, for each mouse cervical spinal cord level, three images of CGRP- and IB4-stained tissue were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL) where a threshold was applied. Staining was measured along three non-overlapping 50 µm-wide strips starting from uppermost border of grey matter and extending 450 µm ventrally. Measurements at each depth were averaged across all sections per mouse and mean ± SEM axonal density was plotted as a function of depth. For every 10 µm in depth, the average axon density was determined. Using Student’s t-test, differences between Lgals1+/+ and Lgals1−/− mice were determined at 10 µm intervals.

For quantification of noxious stimulus-induced Fos immunoreactivity, three images were captured for each cervical section of the left dorsal horn for each animal. These images were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL), a threshold was applied to discount background staining, and Fos-positive cells were counted automatically.
We compared the number of NeuN-positive neurons in $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mice. For each animal, three images were captured for each cervical section (C7-C8) of the left dorsal horn. These images were then imported into Photoshop (Adobe Systems, San Jose, CA) and only laminae I–II were selected. Using the Image Processing toolkit 3.0 (Reindeer Graphics, Asheville, NC) a threshold was used to separate the individual NeuN positive cells. The cells were then automatically counted.

2.2.6 Thin sectioning and electron microscopy

To compare total numbers of primary afferent neurons, dorsal roots from four $Lgals1^{+/+}$ and four $Lgals1^{-/-}$ adult mice were examined at the electron microscope level. A 2-3 mm length of dorsal root just central to the DRG was harvested from each mouse. Deeply anesthetized mice were transcardially perfused with a mixture of 4% paraformaldehyde and 1% glutaraldehyde. Tissue processing was performed using the Pelco 3450 laboratory microwave with a Pelco coldspot (Ted Pella, Inc., Redding, CA) in place. We fixed tissue in 2.5% gluteraldehyde, with a cycle of 2 minutes on, 2 minutes off, 2 minutes on, under vacuum at 100W. The fixative was washed out with two cycles of 40 s at 100W, using fresh buffer with each wash. Tissue was then placed in 1% osmium tetroxide in 0.1% potassium ferrocyanide for membrane enhancement and microwaved for 2 minutes on, 2 minutes off, 2 minutes on at 100W (repeated once without changing solution). After two distilled water washes (40 s, 100W), the tissue was dehydrated through an ethanol series (50, 70, 90, 100, 100, 100%, each at 40 s, 250W). Microwave infiltration of the 1:1 Spurr’s:Epon resin (1:1 acetone:resin, followed with three 100% resin changes) was completed with 3 minutes, 300W, under vacuum cycles. Resin polymerization was completed at 65°C for 16 hours and thin sections were cut using a Leica Ultracut T (Leica Microsystems AG, Wetzlar, Germany) on a 45 degree Diatome
diamond knife. Sections were picked up on copper grids and stained in 2% UA (12 minutes) and Reynolds’s lead citrate (6 minutes). Sections were viewed in a Hitachi H-7600 transmission electron microscope (Hitachi, Ltd, Tokyo, Japan) and photos were taken using a built-in AMT digital camera (American Technologies, Corp., San Francisco, CA). Counting of myelinated axons was performed on thin plastic sections using micrographs taken using bright field microscopy. In electron micrographs the number of unmyelinated axons in a randomly sampled area was counted, from which the total number per root was extrapolated.

2.2.7 In situ hybridization

The mouse Gal1 probe was a 51-mer oligonucleotide complementary to the 3’-untranslated sequence of Gal1 and 5’-TCA CTC AAA GGC CAC GCA CTT AAT CTT GAA GTC TCC ATC CGC CGC CAT GTA -3’ (GenBank accession number BC002063). The Gal1 probe was complementary to bases 424-474. The mouse probes were end-labeled with 33P-dATP (Perkin-Eimer, Woodbridge, ON) by using deoxynucleotide terminal transferase according to a standard protocol (Kobayashi et al., 1996). Perfusion-fixed sections were hybridized to 1.2 x 106 cpm of probe for 16-18 h at 44°C. The slides were dipped in Kodak NTB-2 emulsion and exposed for 3 days. Slides were then dehydrated in a series of alcohols and stored at room temperature. Spinal cord sections were later re-hydrated in dH2O for 1 hour and then the fluorescent Nissl stain; Neurotrace (1:200, Molecular Probes Inc. Eugene, OR) was added to the slides. Slides were then dehydrated in a series of alcohols and coverslipped with Entallen (Fisher Scientific, Nepean, ON).
2.2.8 Imaging and Statistics

All images were imported into Photoshop (7.0, Adobe, Ottawa, ON) and adjustments were made to brightness and contrast to the whole image. Some images were false-coloured in Photoshop to provide clarity. Quantification was performed blind with respect to the treatment groups. All results were analyzed using SigmaStat 3.0 (SPSS Inc., Chicago, IL) and the criterion for significance was $p<0.05$. Unless otherwise stated, Student's t-test was used to determine significance. All results are stated as mean ± standard error of the mean (SEM).
2.3 Results

2.3.1 *Lgals1*−/− mice have increased thermal nociceptive thresholds

The responses to both noxious and non-noxious stimuli of transgenic mice carrying the homozygous null mutation of the Gal1 gene (*Lgals1*−/−) were compared to those of the inbred *Lgals1*+/+ mice. When placed on a 1°C cold plate, null mutant mice displayed significantly longer latencies before exhibiting a nociceptive withdrawal response (involving some or all of sustained elevation, biting, licking or shaking of the paw) when compared to *Lgals1*+/+(75.4 ± 6.8 s for *Lgals1*−/−, mean ± SEM, compared to 18.4 ± 1.3 s for *Lgals1*+/+; ANOVA, p<0.001, Fig. 2.1). In addition, *Lgals1*−/− mice had increased withdrawal latency from radiant heat (8.3 ± 0.3 s, front paw; 8.7 ± 2.6 s, hind paw) compared to *Lgals1*+/+ mice (5.3 ± 0.3 s, front paw; 5.4 ± 2.6 s, hind paw, ANOVA, p<0.012).

A dynamic plantar punctate pressure test was used to assess the amount of force (grams) at which a mouse would withdraw. Here, no significant difference was observed between the *Lgals1*−/− (front: 6.7 ± 0.6 hind paw: 7.5 ± 2.6 grams of force) compared to *Lgals1*+/+ groups (front: 5.7 ± 1.3 hind paw: 6.7 ± 0.6 grams of force, Fig. 2.1).

2.3.2 Gal1 is expressed mainly in small-diameter DRG neurons

Next, we examined Gal1 protein and mRNA expression in *Lgals1*+/+ DRGs. Gal1 protein was expressed in 68 ± 8.3 % of somata (of all sizes) within the C7 and C8 DRGs of *Lgals1*−/− mice (Fig. 2.2, histogram). 32 ± 4 % of Gal1-IR neurons expressed CGRP, while 37 ± 2 % of Gal1-positive neurons were IB4-binding (Fig. 2.2, histogram inset). Using *in situ* hybridization and autoradiography, we found Gal1 mRNA in DRG somata (Fig. 2.3). Silver grains were predominantly localized within neurons. Silver grain
Figure 2.1. *Lgals1*−/− mice have reduced nocifensive thermal responses compared to *Lgals1*+/+ mice (n=7 for each group). Nocifensive withdrawal involves some or all of: sustained elevation, biting, licking or shaking the paw during stimulation.

**Cold Plate**, top graph: *Lgals1*−/− mice (grey bar) could remain on a 1°C cold plate for a significantly longer period of time than *Lgals1*+/+. *Lgals1*−/− mice remain on the cold plate for 75.4 ± 6.8 seconds (s) (mean ± SEM) before exhibiting a nociceptive cold response, while *Lgals1*+/+ mice lasted for 18.4 ± 1.3 s.

**Radiant heat**, middle graph: *Lgals1*−/− mice (grey bars) withdraw from radiant heat after 8.3 ± 0.3 s for the front paw and 8.7 ± 2.6 s for the hind paw, which is significantly longer than the withdrawal latencies of *Lgals1*+/+ mice (black bars, 5.3 ± 0.3 s, front paw; 5.4 ± 0.3 s, hind paw).

**Progressive punctate force**, bottom graph: There is no significant difference in the amount of non-noxious force required to elicit a response between *Lgals1*−/− mice (grey bars) and *Lgals1*+/+ mice (black bars). *Lgals1*−/− mice required 6.8 ± 0.42 grams of force for the front paw and 7.5 ± 2.6 grams of force for the hind paw compared to *Lgals1*+/+ mice that required 5.7 ± 1.3 grams of force for the front paw and 6.7 ± 0.6 grams of force for the hind paw.

Asterisks indicate significant difference compared to *Lgals1*+/+ mice.
Figure 2.2. Distribution of Gal1 protein in the C7 and C8 DRGs of Lgals1+/+ mice.

Representative photomicrographs of a C8 mouse DRG triple-labeled for IB4 (red), Gal1 (green), CGRP (blue) and the merged picture of all three images (colour). 68 ± 8.3% of all somata express Gal1, which is distributed evenly across somata size (grey bars, large histogram). Of the Gal1-expressing population, 37 ± 2.4 % also bind the lectin IB4, 32 ± 3.8% express CGRP, 18 ± 0.6% both bind IB4 and express CGRP and 23 ± 4.1% do not bind IB4 nor express CGRP (histogram bottom inset). Scale bar, 50 µm.
Figure 2.3. Gal1 in situ hybridization signal in \textit{Lgals1}\textsuperscript{+/+} and \textit{Lgals1}\textsuperscript{-/-} dorsal root ganglia (DRGs). Gal1 in situ hybridization signal (red) can clearly be seen in the \textit{Lgals1}\textsuperscript{+/+} DRG (top middle panel) and is localized to neuronal somata (top right panel). Sections have been counter stained with the fluorescent Nissl stain (green). \textit{Lgals1}\textsuperscript{-/-} mouse DRGs (middle panel) have no ISH signal. Scale bar, 100 µm.
density was at background levels in $Lgals1^{-/-}$ tissue sections (even when exposed for 2
days longer than $Lgals1^{+/+}$ sections), confirming the probe’s specificity (Fig. 2.3 middle
panel).

2.3.3 Altered neuron subtype distribution in the $Lgals1^{-/-}$ DRG

We examined differences in the proportions of large-diameter mechanoreceptive
(NF200-IR) and nociceptive (peptidergic, CGRP-expressing; and non-peptidergic, IB4- 
binding) neurons within $Lgals1^{-/-}$ and $Lgals1^{+/+}$ mouse DRGs. There was no significant
difference in CGRP-IR between groups, with $56 \pm 4 \%$ CGRP-positive somata in
$Lgals1^{+/+}$ mice and $50 \pm 4 \%$ CGRP positive somata in $Lgals1^{-/-}$ mice (Fig. 2.4).
Interestingly, there was a significant reduction in the proportion of cells binding IB4 from
$59 \pm 3 \%$ in $Lgals1^{+/+}$ to $38 \pm 5 \%$ in $Lgals1^{-/-}$ mice ($p < 0.05$, Fig. 2.4). In addition, the
proportion of neurons that showed co-localization of both IB4-binding and CGRP-IR was
also significantly decreased from $38 \pm 3 \%$ in $Lgals1^{+/+}$ mice to $21 \pm 3 \%$ in $Lgals1^{-/-}$ mice
($p < 0.05$, data not shown). In contrast with the decreased proportion of IB4-binding
neurons, there was a significant increase in the proportion of NF200-IR DRG neurons in
$Lgals1^{-/-}$ (29 \pm 3 \%) compared to $Lgals1^{+/+}$ (20 \pm 3 \%) mice ($p < 0.05$, Fig. 2.4).

The morphology and number of axons in the L5 dorsal root of $Lgals1^{-/-}$ mice were
quantified (Fig. 2.5). We found that there was no significant difference in the total
number of axons projecting centrally from the DRG. However, there was a significant
increase in the number of myelinated axons in $Lgals1^{-/-}$ mice when compared with
$Lgals1^{+/+}$ mice ($Lgals1^{+/+}$: 1490 ± 90, $Lgals1^{-/-}$: 1980 ± 170 axons). No significant
difference between $Lgals1^{-/-}$ and $Lgals1^{+/+}$ mice in the axonal-Remak cell (ensheathing,
non-myelinating Schwann cell) relationship, axonal size distribution, nor in the g-ratio
(ratio of axon diameter to axon plus myelin diameter) was observed (data not shown).
Figure 2.4. Differences in distribution of CGRP-immunoreactive (IR) (green), IB4-binding (red), and neurofilament (NF)200-IR (blue) neurons in the DRG between $Lgals1^{-/-}$ and $Lgals1^{+/+}$ mice (n=4 for both groups). There was no difference in the proportion of CGRP-IR DRG neurons in $Lgals1^{+/+}$ and $Lgals1^{-/-}$ C7-8 DRGs (around 50-55% of neurons). $Lgals1^{-/-}$ DRGs had a significantly smaller proportion of IB4-binding neurons than did $Lgals1^{+/+}$ DRGs (~40% versus ~60%, respectively). In contrast, $Lgals1^{-/-}$ DRGs had a significantly higher proportion of NF200-IR neurons compared to $Lgals1^{+/+}$ DRGs (~29% versus ~20%, respectively). Asterisk indicates significant difference compared to $Lgals1^{+/+}$. Scale bar, 100 µm.
Figure 2.5. Thin plastic sections (top) and high power electron micrographs (middle) demonstrating axonal morphology dorsal roots of $Lgals1^{-/-}$ and $Lgals1^{+/+}$ mice. There was no difference in morphology of myelinated axons or unmyelinated axons in the dorsal root of $Lgals1^{-/-}$ mice compared to $Lgals1^{+/+}$ mice. There were significantly more myelinated axons (white bars) in $Lgals1^{-/-}$ compared with $Lgals1^{+/+}$ mice. There was no significant difference in the number of unmyelinated axons (grey portion of bars) or the total number of axons (myelinated plus unmyelinated) between groups. Asterisk indicates significant difference compared to $Lgals1^{+/+}$. Scale bar, 25 $\mu$m (top panels); 500 nm (bottom panels).
Comparison of axon density in Lgals1+/+ and Lgals1-/- conditions. The images above show a higher density of axons in Lgals1+/+ mice compared to Lgals1-/- mice. The bar graph below quantifies the number of axons, with significantly lower counts in Lgals1-/- mice indicated by the asterisk.
2.3.4 Altered central distribution of Lgals1\(^{-/-}\) nociceptive axons

Afferents that bind IB4 normally terminate in lamina II. In Lgals1\(^{+/+}\) mice, these axons extended deeper into the dorsal horn than in Lgals1\(^{+/+}\) mice (middle panel Fig. 2.6). In Lgals1\(^{-/-}\) mice, the peak IB4 binding density occurred between 70-80 \(\mu\)m ventral to the top of lamina I, whereas in Lgals1\(^{+/+}\) mice, the maximum binding intensity occurred 30-40 \(\mu\)m deep in the dorsal horn (Fig. 2.6, bottom right graph). CGRP-positive axons also terminated deeper in the dorsal horn of Lgals1\(^{-/-}\) mice than Lgals1\(^{+/+}\) mice (Fig. 2.6, bottom left graph).

2.3.5 Fewer Lgals1\(^{-/-}\) dorsal horn neurons express Fos after noxious stimulation

Using Fos expression as a marker for neuronal primary afferent-elicited activity in second-order neurons in the dorsal horn (Dai et al., 2001; Hunt et al., 1987), we tested whether Lgals1\(^{-/-}\) and Lgals1\(^{+/+}\) mice differed in their ability to transmit nociceptive information after thermal stimulation. Two hours after exposure of the front paw to noxious thermal stimuli, the dorsal horns of spinal segments C7 and C8 were examined. These two segments were examined since these two spinal levels receive the majority of sensory input from the forepaw. After exposure to 1\(^\circ\)C water, Lgals1\(^{+/+}\) mice had 21.1 ± 1.9 Fos-positive nuclei per section at C7 and 24.1 ± 2.7 Fos-positive nuclei per section at C8 (Fig. 2.7, left panel). Lgals1\(^{-/-}\) mice had 14.7 ± 0.9 Fos-positive nuclei per section C7 and 13.1 ± 0.6 Fos-positive nuclei at C8 (\(p < 0.05\) compared at the same level between strains, Fig. 2.7).

After exposure to 52\(^\circ\)C water, Lgals1\(^{+/+}\) mice had 27.5 ± 2.9 Fos-positive nuclei at per section C7 and 34.5 ± 1.5 Fos positive nuclei per section at C8, whereas Lgals1\(^{-/-}\) mice
had 18.7 ± 2.1 Fos-positive nuclei per section at C7 and 28.0 ± 1.9 Fos-positive nuclei per section at C8 (p < 0.05 compared at the same level between strains, Fig. 2.7). The neuronal-specific antibody NeuN was used to determine if the differences in Fos expression could be attributed to differences in the number of second order neurons. There was no significant difference in the number of neuronal cell bodies at either the C7 or C8 spinal levels between Lgals1+/+ and Lgals1−/− mice (Fig. 2.8).
Figure 2.6. Differences in distribution of nociceptive fibre terminals within the dorsal horn of \textit{Lgals1}^{+/+} and \textit{Lgals1}^{-/-} mice (n=4 for both groups). CGRP-positive axons terminated deeper within the \textit{Lgals1}^{-/-} dorsal horn (top left) than the \textit{Lgals1}^{+/+} dorsal horn (top right panel, bottom left graph). IB4-binding axons within the dorsal horn terminate deeper in \textit{Lgals1}^{-/-} mice (middle right panel, bottom right graph) than in \textit{Lgals1}^{+/+} mice (middle left panel, bottom right graph). Red arrows (middle panel) demarcate the increased area of the termination field of IB4 binding afferents in \textit{Lgals1}^{-/-} mice; white arrows indicate the increased depth of IB4-positive axons in these mice. Asterisks indicate significant differences. Scale bar, 100 $\mu$m.
Figure 2.7. Fos activation induced by noxious thermal forepaw stimulation is reduced in the $Lgals1^{-/-}$ cervical dorsal horn. Two hours after exposure to 1°C water (left panels), $Lgals1^{-/-}$ C7 and C8 dorsal horns had significantly fewer Fos-positive nuclei per section compared to the $Lgals1^{+/+}$ dorsal horn. Likewise, two hours after exposure to 52°C water (right panels), $Lgals1^{-/-}$ C7 and C8 dorsal horns had significantly fewer Fos-IR nuclei per section than did $Lgals1^{+/+}$ dorsal horns. Asterisk indicates significant difference between $Lgals1^{-/-}$ and $Lgals1^{+/+}$ animals; n=4 for both $Lgals1^{-/-}$ and $Lgals1^{+/+}$ mice.
Figure 2.8. The reduced number of Fos-positive neurons in *Lgals1*−/− mice is not a result of a difference in the number of second order neurons. NeuN immunohistochemistry was performed on *Lgals1*+/+ and *Lgals1*−/− spinal cords at C7 and C8 to quantify the number of neuronal cell bodies within the dorsal horn (n=4 for both groups). There was no significant difference in the number of dorsal horn neurons in *Lgals1*+/+ and *Lgals1*−/− mice at C7 or C8. Scale bar, 100 µm.
2.4 Discussion

2.4.1 Overview

The principal findings of this study are (1) that $Lgals1^{-/-}$ mice have reduced sensitivity to noxious thermal stimuli; (2) that there are proportionally fewer IB4-binding neurons and proportionally more NF200-expressing neurons in $Lgals1^{-/-}$ DRGs; (3) that the increase in large-diameter DRG neurons is correlated with an increase in the number of myelinated dorsal root axons; (4) that the terminal fields of nociceptive DRG neurons is deeper in the dorsal horn of $Lgals1^{-/-}$ mice than $Lgals1^{+/+}$ mice; and (5) that transmission of thermal nociceptive information to second order neurons in the dorsal horn is reduced in $Lgals1^{-/-}$ mice. These results suggest that Gal1 is required for the appropriate developmental specification of neuronal phenotypes in the DRG, axonal targeting in the spinal cord, and nociception in the adult.

Nociception serves to prevent actual or potential tissue damage through the detection of noxious thermal, chemical, and mechanical stimuli. These diverse sensory inputs are transmitted along two major nociceptive pathways that terminate primarily in laminae I and II (Snider and McMahon, 1998). The segregation of these small-diameter afferent fibre projections into a laminar-specific topology occurs during development and suggests different functional roles. Different classes of sensory neurons enter the spinal cord in sequence: large-diameter sensory fibres enter the spinal cord at E14.5, followed by small-diameter fibres at E15.5 (Ozaki and Snider, 1997). Interestingly, Gal1 expression was first demonstrated in the rat DRG at E14, just as small-diameter fibres were approaching the spinal cord (Regan et al., 1986). In the spinal cord, Gal1 is expressed in superficial laminae from P0, with most intense staining occurring between P0 and P7. This corresponds to the time at which appropriate connections are
established in the dorsal horn of the spinal cord (Regan et al., 1986). These observations led to speculation that Gal1 was involved in either axonal outgrowth or synaptic stability of nociceptive fibres during development (Dodd and Jessell, 1986). Once connections are made, these fibres continue to express Gal1 at lower levels.

2.4.2 Gal1 distribution

Since this is the first report of Gal1 expression in mouse DRGs, these results can only be compared to previous findings in rat DRGs. Approximately 68% of all neurons within the cervical DRGs are Gal1-IR in \( Lgals1^{+/+} \) mice. These results are in agreement with those for adult rats, in which 63% of DRG neurons were Gal1-positive, and 46% of neurons were intensely stained (Regan et al., 1986). Others have reported that most DRG neurons from the 4th and 5th lumbar levels in the rat have some Gal1-IR, with 20-26% intensely Gal1-IR (Imbe et al., 2003; Sango et al., 2004).

There is a decrease in the proportion of IB4-binding DRG neurons, and an increase in the proportion of neurons that are NF200-IR in adult \( Lgals1^{-/-} \) mice. Since Gal1 is highly expressed in neurons that bind IB4 in the \( Lgals1^{+/+} \) mouse, this indicates that Gal1 is required to specify the survival of IB4-binding neurons and/or the switch to the IB4-binding phenotype during development. Since there no change in the total number of axons in the dorsal root, we infer that some DRG neurons, rather than taking on the IB4-binding phenotype, instead become NF200-expressing. This is supported by increased number of myelinated axons in dorsal roots of \( Lgals1^{-/-} \) mice. Regardless, Gal1 is required for proper determination of DRG neuron phenotype during development.

Gal1 also affects axonal targeting: projections of olfactory and nociceptive axon subpopulations are altered in \( Lgals1^{-/-} \) mice. During olfactory system development, Gal1
is expressed by *Dolichos biflorus agglutinin*-binding axons as they are growing towards their targets and this expression continues at lower levels once connections have been made (Puche et al., 1996). Furthermore, Gal1 increases olfactory neurite outgrowth *in vitro* (Puche et al., 1996). During development in *Lgals1*⁻/⁻ mice, these axons do not reach their appropriate targets (Puche et al., 1996). In parallel with the altered olfactory system, small-diameter primary afferents (which normally express Gal1) have a significantly different terminal distribution in the dorsal horn of *Lgals1*⁻/⁻ mice compared to *Lgals1*⁺/⁺ mice. The altered depth profile of nociceptive axons in the dorsal horn and the decrease in Fos activation after noxious thermal stimulation in mice lacking Gal1 suggest that the significant increase in noxious pain threshold that is observed is in part due to the inappropriate connections of the small-diameter fibres in lamina II. The fact that IB4-positive axons overshoot their normal targets in the *Lgals1*⁻/⁻ superficial dorsal horn is interesting, given that Gal1 is also implicated in axonal elongation: simplistically, one might expect the Gal1-negative axons to fall short of their targets.

Gal1 could promote proper sensory neuron function and axon targeting by affecting axonal growth, phenotype specification, and/or survival. Adult rat peripheral axon growth is enhanced by Gal1 application: Gal1 stimulates neurite outgrowth from DRG explants and increases the rate of sensory and motor regeneration *in vivo*. This occurs by increasing Schwann cell migration and eliciting the release of an unidentified growth-stimulating factor from macrophages (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003; Horie et al., 2004). In addition, we found that neuronal Gal1 expression is correlated with regenerative potential in spinal motoneurons and rubrospinal neurons (McGraw et al., 2005). Schwann cells and dorsal root ganglion neurons secrete Gal1 via a non-classical pathway (Sango et al., 2004); secreted Gal1 may then act in an autocrine/paracrine fashion on sensory neurons and/or glial cells.
within the DRG during development and following axotomy (although a neuronal Gal1 receptor has not been identified). Therefore, the lack of Gal1 in the null-mutant mouse may lead to reduced trophic support within the DRG, resulting in a decreased number of IB4-binding neurons.

Gal1 also promotes axonal growth by altering adhesive properties of the extracellular matrix. For example, Gal1 mediates self-aggregation of primary olfactory neurons through the cross-linking of carbohydrate ligands, and facilitates fasciculation of DRG neurites in vitro (Outenreath and Jones, 1992; Mahanthappa et al., 1994). The absence of Gal1 in the null mutant mouse may thus lead to inappropriate targeting by either hindering axonal fasciculation or altering cellular adhesion during innervation of the spinal cord by nociceptive axons.

The developmental expression of Gal1 — high until target connection — and the misdirection of axons in the Lgals1<sup>−/−</sup> mouse olfactory system support a role for Gal1 both in target selection early in development, and in peripheral nerve injury-induced neurite outgrowth in the adult.

### 2.4.3 Role of IB4-binding neurons in thermal nociception

Both peptidergic (CGRP-IR) and non-peptidergic (IB4-binding) small-diameter sensory neurons are implicated in thermal nociception, although their precise roles remain elusive (Snider and McMahon, 1998). These two neuronal populations are functionally distinct in their response to heat (Stucky and Lewin, 1999), and heterogeneity in thermal responses exists within the non-peptidergic neurons: only half of the IB4-binding nociceptors are sensitive to noxious heat (Stucky and Lewin, 1999). This is most likely due to differences in their expression of thermally sensitive receptors. Although 78% of
rat IB4-binding neurons express the capsaicin- and heat-sensitive vanilloid receptor TRPV1 (VR1), which has been implicated in thermal hyperalgesia (Garcia-Martinez et al., 2002; Jarvis et al., 2002), the function of this protein in the mouse is less clear. TRPV1 is expressed in only 2-3% of IB4-binding neurons in mice (Zwick et al., 2002). In addition, heat-sensitive nociceptors from mice lacking both TRPV1 and TRPV2 have normal thresholds (Woodbury et al., 2004). Thus, although IB4-binding neurons are implicated in the sensation of noxious heat, the full complement of proteins involved in heat transduction in these afferents has not been elucidated. Regardless, our data indicate that IB4-binding neurons do play a role in thermal nociception. The reduction in the proportion of IB4-binding cells observed in this report as well as the alteration in their axons’ laminar termination suggest that this contributes to the observed attenuated thermal nocifensive responses in Lgals1−/− mice.

There have been two ion channels that have been implicated in sensing noxious cold, TRPA1 (ANKTM1, Story et al., 2003) and TRPM8 (CMR1, McKemy et al., 2002). TRPA1 is activated by noxious cold (<17°C), and is expressed by a subset of TRPV1-positive, TRPM8-negative, CGRP-IR somatic neurons (Story et al., 2003). TRPM8 is activated by a range of temperatures that include noxious and innocuous stimuli (8-29°C), and is present in about 15% of all DRG neurons (McKemy et al., 2002). This non-selective cation channel is thought to be present in a subpopulation of nociceptors (McKemy et al., 2002), although the distribution of TRPM8 in IB4-binding and CGRP-IR neurons in mice is currently not known. We suggest that the decrease in the number of IB4-binding (and potentially TRPM8-containing) neurons is one possible mechanism by which the threshold for noxious cold may be increased in Lgals1−/− mice.
2.4.4 Conclusions

In summary, the results of the present work show that the absence of Gal1 expression leads to subtle but significant shifts in the phenotypic distribution of primary afferent neurons, their terminal fields in the spinal cord, and substantial changes in the responses to noxious thermal stimuli.
2.5 References


Mahanthappa NK, Cooper DN, Barondes SH, Schwarting GA (1994) Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. Development 120: 1373-84.


ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112: 819-29.


3 Regulation of neuronal and glial galectin-1 expression by peripheral and central axotomy of rat primary afferent neurons

3.1 Introduction

Galectins are highly-conserved proteins that are distinguished by homologous carbohydrate recognition domains and specificity for galactoside-containing oligosaccharides (Cooper and Barondes, 1999; Hernandez and Baum, 2002; Gray et al., 2004). These proteins, which may be found in the extracellular matrix and on cell membranes, have the ability to promote cell-cell or cell-substrate interactions. Galectin-1 (Gal1) was the first galectin to be identified, and is widely expressed throughout the body: it is found in most organs, and in a variety of cells including nerve, placental, and muscle cells (Regan et al., 1986; Wasano et al., 1990; Poirier et al., 1992; Perillo et al., 1998). In the developing nervous system, Gal1 expression is initiated in DRG neurons, spinal motoneurons, cranial motoneurons, and olfactory neurons after the last cell division and remains elevated until their axonal targets are reached (Regan et al., 1986; Hynes et al., 1990; St John and Key, 1999). Gal1 is required for the typical termination pattern of axons in olfactory and somatic primary afferent systems (Puche et al., 1996; McGraw et al., 2005), indicating that Gal1 is necessary for proper targeting of subpopulations of axons during development.

Given that Gal1 plays a role in developmental pathfinding of axonal subpopulations, its involvement in nerve repair in the adult would not be surprising. Horie et al. (1999) were the first to suggest that Gal1 has a role in the initiation of axonal regeneration following

---

peripheral nerve injury, and subsequent studies have elucidated some of the underlying mechanisms (Horie and Kadoya, 2000; Fukaya et al., 2003; Horie et al., 2004). For the most part, these studies focused on the effects of the application of exogenous Gal1 and function-blocking antibodies; however, the regulation of endogenous Gal1 by axotomy has received little attention. Recently, it has been found that Gal1 mRNA is increased in facial motoneurons following peripheral axotomy (Akazawa et al., 2004; McGraw et al., 2004a). We also found that the upregulation of Gal1 mRNA in neuronal somata of the rubrospinal tract was positively correlated with its regenerative potential (McGraw et al., 2004b).

Manipulation of the dorsal root ganglion (DRG) neuron, which has one peripheral and one central projection, provides a unique opportunity to study gene and protein regulation following injury. Whereas axons projecting peripherally from sensory neurons have the ability to regenerate and reconnect with peripheral targets following injury, those that project centrally cannot regenerate past the peripheral nervous system-central nervous system (PNS-CNS) interface (dorsal root entry zone, DREZ) to reconnect with central targets in the spinal dorsal horn or brainstem. The selective induction of a regenerative program in DRG neurons by peripheral (but not central) axotomy is characterized by changes in gene expression, including upregulation of growth-associated proteins, neuropeptides, and cytoskeletal proteins (Hokfelt et al., 1994; Donnerer, 2003; Fenrich and Gordon, 2004). Other proteins that are upregulated following axotomy have important roles in neuropathic pain, including the neurotrophin nerve growth factor (Ramer et al., 1998; Ro et al., 1999) and specific sodium channel subtypes (Hains et al., 2003; Chung and Chung, 2004).
DRG neurons can be divided into modality-specific subgroups based on soma (and axon) size and on expression of specific protein markers. Approximately 70% of sensory neurons are small diameter (Snider and McMahon, 1998). Small-diameter neurons can be sub-classified as peptidergic, which express the neuropeptide calcitonin gene-related peptide (CGRP); or non-peptidergic, which bind the lectin Bandeiraea simplicifolia (IB4) and express the glial cell-line derived neurotrophic factor signaling receptor c-Ret (Chen et al., 1995; Molliver et al., 1997; Bradbury et al., 1998). Larger DRG neurons are identifiable by their expression of the heavy neurofilament NF200 (Lawson et al., 1984). Although most DRG neurons are Gal1-immunoreactive (-IR), small-diameter sensory neurons that express c-Ret mRNA have been shown to have the highest Gal1-IR in the adult (Regan et al., 1986; Sato and Perl, 1991; Sango et al., 2004).

Here we examine the distribution of Gal1 expression in peptidergic small-diameter (CGRP-expressing), non-peptidergic small-diameter (IB4-binding), and large-diameter (NF200-expressing) neurons. We subsequently characterize the regulation of Gal1 protein and mRNA expression in these neurons and their associated glial environments after peripheral axotomy and after dorsal rhizotomy.
3.2 Materials and methods

3.2.1 Animals and surgery

A total of 30 adult male Wistar rats (University of British Columbia Animal Care Facility, weight 200-250 g) were used for this study. All surgery was performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Rats were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (72 mg/kg; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (9 mg/kg; Bayer Inc, Etobicoke, ON), and all surgery was carried out under sterile conditions. To reduce post-operative pain and lessen blood flow to muscle during surgery, 0.4 mL of 2% lidocaine with epinephrine (Vétoquinol, Quebec, QC) was injected into the exposed superficial musculature around the spinal column. Dorsal rhizotomy or a peripheral axotomy of primary afferents was performed unilaterally at the 6th cervical level (C6) to the 1st thoracic level (T1). Dorsal rhizotomy was performed as described in Ramer et al. (2001b). Briefly, small pieces of vertebrae from C6-T1 were removed, exposing the dorsal roots. The roots were transected midway between the DRG and DREZ. For unilateral peripheral axotomy, nerves exiting the spinal column at C6-T1 were transected and a 5 mm section of nerve was removed, ensuring that all the neurons within a particular DRG were axotomized.

3.2.2 Tissue processing

At either 7 or 14 days after injury (at least 4 animals per group and 2 spinal segments per animal), rats were injected with a lethal dose of chloral hydrate (1.75 g per kg body weight). Upon loss of nociceptive reflexes, animals were perfused intracardially with PBS followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brain and spinal cord were removed and the tissue post-fixed for 24 hours in 4%
paraformaldehyde at 4°C. Tissue was cryoprotected in 22% sucrose solution in 0.1M PB. After cryoprotection, tissue was rapidly frozen in dry ice-cooled 2-methylbutane and 16 µm transverse cryosections of DRGs or spinal cords were cut, cold-mounted onto glass slides (Superfrost Plus), and stored at -80°C.

3.2.3 In situ hybridization (ISH)

The rat Gal1 probe, corresponding to bases 393-443, was a 51-mer oligonucleotide complementary to the 3'-untranslated sequence of Gal1 5’-CAC TCA AAG GCC ACA CAC TTA ATC TTG AAG TCA CCA TCC GCC GCC ATG TAG-3’ (GenBank accession number NM019904). The probes were end-labeled with [33P]-dATP (Perkin-Elmer, Woodbridge, ON) using deoxynucleotide terminal transferase according to a standard protocol (Kobayashi et al., 1997). Perfusion-fixed sections were hybridized to 1.2 x 10^6 cpm of probe for 16-18 h at 44°C. The slides were dipped in Kodak NTB-2 emulsion and exposed for 2 days (DRGs) or 4 days (dorsal horn and DREZ). Slides were then dehydrated in a series of alcohols and stored at room temperature. Spinal cord sections were later re-hydrated in dH2O for 1 hour and counterstained with a fluorescent Nissl stain (Neurotrace, 1:200, Molecular Probes Inc. Eugene, OR) and then coverslipped with Entallen (Fisher Scientific, Nepean, ON).

3.2.4 Immunohistochemistry

For Gal1 immunohistochemistry of DRGs, slides were washed in 0.1M phosphate-buffered saline (PBS) for 20 minutes, after which rabbit anti-Gal1 (1:4000) (Horie et al., 1999b) and one of mouse anti-CGRP (1:2000, Sigma, Oakville, ON), mouse anti-NF200 (clone N52, 1:500, Sigma), or IB4 (1:50, Sigma) was applied to the slides overnight. The slides were then washed for 30 minutes in PBS and a blocking solution of 10% goat
serum and 0.1% Triton-X100 in PBS was added for 20 minutes at room temperature. The slides were then exposed to a donkey anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch Inc, West Grove, PA) for one hour. For Gal1 immunohistochemistry, a tyramide signal amplification step was then employed as per the manufacturer's instructions (PerkinElmer Lifescience, Boston, MA). Cy3-conjugated Steptavidin (1:500, Sigma) was used to visualize the Gal1 antibody and Alexa 488-conjugated donkey anti mouse (1:300, Jackson Immunological Research) or Alexa 488-conjugated donkey anti goat (1:300, Jackson Immunological Research) was used to visualize the other primary antibodies. Slides were coverslipped with a 3:1 solution of glycerol: PBS.

3.2.5 Image Analysis

Quantification of immunohistochemistry in the DRG: Profile-based DRG quantification was carried out as described by Ramer et al. (2001a). Briefly, triple-labeled images of Gal1, CGRP and IB4 were imported into SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL). The DRG cell bodies were outlined creating an image overlay. The average intensity and Feret diameter of each object identified by the overlay was automatically measured. The threshold for immunopositivity for Gal1, IB4 and CGRP was determined by averaging three cell bodies in each section that were judged to be minimally positive. We used recursive translation, a stereological counting method which reconstructs cell populations based on size-distribution of profiles (Rose and Rohrlich, 1988), to calculate soma sizes and the proportion of cells expressing an antigen.

Quantification of Gal1-IR in the dorsal horn: Dorsal horn quantification was carried out as described previously (Ramer et al., 2001a; Gaudet et al., 2004). Briefly, for each rat cervical spinal cord level, three Gal1-IR images were imported into SigmaScan Pro 5.0
where an omni-directional edge-detection filter was applied. This eliminated variations in background intensity. Gal1-IR axon density (irrespective of staining intensity) was measured from a subsequent threshold overlay along three non-overlapping 50 µm-wide strips starting from uppermost border of grey matter and extending 450 µm ventrally. Measurements over each 10 µm depth were averaged across sections in rats and mean ± SEM axonal density was plotted as a function of depth.

Quantification of Gal1-ISH in degenerating sensory tracts: At least three sections per spinal cord level were analyzed. Nissl and darkfield (silver grain) images were taken of spinal cord ipsilateral and contralateral to injury using a digital camera attached to a fluorescent microscope (Carl Zeiss, Axioskop, Toronto ON, Canada) in combination with Northern Eclipse software (Empix Inc., Mississauga ON, Canada). All images were analyzed with SigmaScan Pro 5 software (SPSS Inc.). The proportional area of the cuneate fasciculus and dorsal horn occupied by silver grains was determined. Background autoradiographic signal was determined from the uninjured corticospinal tract. For each section, the silver grain density was determined for both the axotomized and contralateral (uninjured) side (Fig. 3.8A). These data were expressed as percent of the background signal.

Quantification of Gal1-ISH at the DREZ: Analysis of Gal1 ISH at the DREZ was similar to the ISH analysis of degenerating sensory tracts described above. At least three sections per DREZ were analyzed. Nissl images aided in determining the DREZ boundary so that similar sizes of both PNS and CNS tissue could be delineated (Fig. 3.9I). An
intensity threshold was applied to the silver grain image to determine grain area fraction of both PNS and CNS tissue at the DREZ.

3.2.6 Statistics

Quantification was performed blind with respect to injury. All data is represented as mean ± standard error of the mean (SEM), and all tests were carried out using SigmaStat 3.0 (SPSS Inc.). Unless otherwise indicated, a 1-way ANOVA with Holm-Sidak post-hoc test was used to determine significance between groups. Significance was assigned at $p < 0.05$. 
3.3 Results

3.3.1 Altered Gal1 mRNA and protein expression in the DRG following peripheral, but not central axotomy

We first examined Gal1 expression in uninjured DRGs. Using Gal1 ISH, we found that 57 ± 6% (mean ± SEM) of neuronal somata within uninjured DRGs had a grain density 5 times background (n=9; Fig. 3.1B; 3.2A,F). Using immunohistochemistry to visualize Gal1 protein, we found that 48 ± 1% of uninjured somata were Gal1-IR (n=9, Fig. 3.6A,F). Gal-IR was observed in both the cytoplasm and nucleus of sensory neurons (Fig. 3.3A; 3.4A; 3.5A). Of the Gal1-IR somata, 28 ± 2% were CGRP-IR (Fig. 3.3A), 33 ± 2% were IB4-binding (Fig. 3.4A), and 5.9 ± 0.2% were NF200-IR (Fig. 3.5A).

In DRG neurons injured by dorsal rhizotomy seven days earlier, there were no significant changes in Gal1 mRNA expression (64 ± 6%, n=9; Fig. 3.1D; 3.2B,F), Gal1-IR (Fig. 3.6B,F) or Gal1 co-localization with CGRP, IB4 or NF200 (Fig. 3.3C,D; 3.4C,D; 3.5C,D). Similarly, there were no significant changes in either mRNA expression (60 ± 4%, n=9; Fig. 3.1F; 3.2C,F), Gal1-IR (49 ± 2%; Fig. 3.6C,F), or Gal1 co-localization with CGRP, IB4 or NF200 (Fig. 3.3E,F; 3.4E,F; 3.5E,F) in DRGs 14 days after dorsal rhizotomy.

After spinal nerve lesion, in which all the peripherally-projecting sensory fibres for a particular DRG were axotomized, there were significant increases in expression of Gal1 mRNA and protein. We observed a significant increase in Gal1 mRNA expression in injured somata 7 days post-axotomy; the proportion of somata with high silver grain density was 82 ± 2% of all somata (n=9). This increase was most apparent in large-diameter cells (Fig. 3.1H; 2D). The proportion of Gal1-IR cells at 7 days post-axotomy was increased to 65 ± 3% (n=9, Fig. 3.6F). The increase in Gal1-IR somata was
greatest in large-diameter NF200-IR cells (Fig. 3.6D): 12 ± 2% of Gal1-IR somata were also NF200-IR 7 days post-axotomy (Fig. 3.5G,H), compared to 5.9 ± 0.2% of cells in the uninjured DRG (Fig. 3.5A,B). There was also a significant decrease in CGRP and IB4 co-labeling with Gal1 (CGRP: 4 ± 2%, Fig. 3.3G,H; IB4: 23 ± 3%, Fig. 3.4G,H) as a result of the injury-induced decrease in CGRP-IR and IB4-binding.

The expression of Gal1 mRNA and protein and colocalization of Gal1 with neuronal markers in DRGs 14 days after axotomy were similar to that observed at 7 days post-axotomy. In somata that were injured by spinal nerve transection 14 days earlier, 76 ± 6% of cells had high ISH levels (Fig. 3.1J; 3.2E,F), and 71 ± 4% of injured neurons contained Gal1-IR (Fig. 3.6E,F). Of the Gal1-IR cells, 4 ± 2% were CGRP-positive (Fig. 3.3I,J), 19 ± 2 % bound IB4 (Fig. 3.4I,J) and 12 ± 1% were NF200-IR (Fig. 3.5I,J). Thus, dorsal rhizotomy did not affect Gal1 mRNA or protein expression in injured DRG neurons significantly; however, peripheral axotomy induced increases in Gal1 mRNA and protein expression in injured DRG neurons, mainly in large-diameter, NF200-IR cells.

3.3.2 Novel Gal1 expression patterns in the dorsal horn following dorsal rhizotomy and peripheral axotomy

Gal1-IR was observed in the superficial laminae of the uninjured dorsal horn (n=9, Fig. 3.7A,C,E,G). At both 7 and 14 days following dorsal rhizotomy, there was a complete absence of Gal1-IR in the dorsal horn (Fig. 3.7B,D), indicating that Gal1 protein is anterogradely transported to primary afferent terminal fields in the dorsal grey matter of the uninjured spinal cord. Following peripheral axotomy, we observed a significant increase in Gal1-IR within deeper laminae of the dorsal horn (Fig. 3.7F,H). This was
most likely due to the augmented Gal1 expression in large-diameter somata induced by peripheral axotomy (Fig. 3.5).

Dorsal rhizotomy induced a significant increase in Gal1 mRNA in the degenerating sensory fibre tracts within the spinal cord (cuneate fasciculus and the medial dorsal horn where large diameter fibres invade the grey matter) at 7 and 14 days post-injury (Fig. 3.8). Seven days post-dorsal rhizotomy, these tracts contained a density of silver grains $3.3 \pm 0.2$ times higher than background, and at 14 days the density was $4.7 \pm 0.3$ times higher than background. These values were significantly greater than the uninjured, contralateral sides, which had densities of $1.3 \pm 0.2$ and $1.2 \pm 0.2$ times background at 7 and 14 days, respectively. These results suggest that cells endogenous to the spinal cord, which transcribe relatively small amounts of Gal1 mRNA in the uninjured situation, upregulate Gal1 mRNA in response to deafferentation.

### 3.3.3 Dorsal rhizotomy-induced Gal1 mRNA increase is restricted to the peripheral portion of the DREZ

We also studied Gal1 mRNA expression in the region of the DREZ. This consists of Schwann cell-containing peripheral and astrocyte- and oligodendrocyte-containing central compartments of the cord-adjacent dorsal roots. Seven and 14 days following dorsal rhizotomy, Gal1 silver grain density in the peripheral portion of the injured root rose to $4.3 \pm 0.5$ and $5.0 \pm 1.0$ times that of the contralateral side, respectively ($n=9$, Fig. 3.9). On the rhizotomized side, signal in the CNS compartment was $1.2 \pm 0.2$ and $1.4 \pm 0.1$ times the contralateral side after 7 and 14 days, respectively ($n=9$). Therefore, dorsal rhizotomy caused an increase in Gal1 mRNA in the peripheral, but not the central compartment of the DREZ.
Figure 3.1. DRG neuron Gal1 mRNA expression is regulated by axotomy of their peripheral, but not central projections. In the uninjured DRG, silver grains were observed over neuronal cell bodies (A,B). At 7 and 14 days after dorsal rhizotomy, there was no change in silver grain density (C-F). In contrast, there was a significant increase in silver grain density at both 7 and 14 days after peripheral axotomy (G-J). All sections were counterstained with fluorescent Nissl. Scale bar, 100 µm.
Figure 3.2. Gal1 autoradiographic signal (silver grain density) quantification of uninjured, axotomized, and rhizotomized DRGs. In uninjured cervical DRGs (C6-C8), 57 ± 6% (mean ± SEM) of the neurons had 5 times greater signal than the background (F). High silver grain densities were observed in somata of all sizes, but were predominately located in small diameter somata (A). Following dorsal rhizotomy (rhiz), there was no significant change in the proportion of cells expressing Gal1 mRNA: 64 ± 6% of cells expressed Gal1 mRNA at 7 days post-rhizotomy and 60 ± 4% of cells at 14 days (F). There was a significant increase in the proportion of cells with silver grains both at 7 (82 ± 2% of total cells) and 14 days post-axotomy (76 ± 6%) (F). Increased expression of Gal1 mRNA in large-diameter cells was mainly responsible for the increase in overall proportion of Gal1-expressing cells (D,E). Asterisks indicate significant differences compared to uninjured group.
(No text content available)
Figure 3.3. Gal1- and calcitonin gene-related peptide (CGRP)-IR in cervical DRGs after peripheral axotomy and dorsal rhizotomy. In the uninjured (control) DRG, 28 ± 2% of Gal1-IR somata also expressed CGRP (A,B). At 7 and 14 days after dorsal rhizotomy, 27 ± 2% and 26 ± 2% of Gal1-IR somata expressed CGRP, respectively (C,D,E,F). After peripheral axotomy, only 6 ± 1% of Gal1-IR cells expressed CGRP at 7 days and 4 ± 2% of somata expressed both Gal1 and CGRP at 14 days (G,H,I,J). These were both significantly less than control values. Graph shows proportion of Gal1-positive cells expressing CGRP. Arrows indicate cells that are both Gal1- and CGRP-IR; arrowheads show cells that are Gal1-, but not CGRP-IR. Asterisks indicate significant differences compared to DRGs contralateral to injury. Scale bar, 50 µm.
Figure 3.4. Gal1-IR and isolectin *Bandeiraea simplicifolia* (IB4)-binding in cervical DRG neurons following peripheral axotomy and dorsal rhizotomy. In the uninjured, contralateral DRG, 33 ± 2% of the Gal1-IR somata also bound IB4 (A,B). At 7 and 14 days post-dorsal rhizotomy, 36 ± 1% and 34 ± 1% of Gal1-IR somata also bound IB4, respectively (C,D,E,F). After peripheral axotomy, 23 ± 3% of Gal1-IR cells bound IB4 at 7 days, and 19 ± 2 % of Gal-IR somata also bound IB4 at 14 days (G,H,I,J). These were both significantly less than in DRGs contralateral to injury. Arrows indicate cells that are both Gal1-IR and IB4-binding; arrowheads show cells that are Gal1-IR, but not IB4-binding. Graph shows proportion of Gal1-positive cells binding IB4. Asterisks indicate significant differences compared to uninjured DRGs. Scale bar, 50 µm.
Figure 3.5. Gal1- and neurofilament (NF) 200-IR in cervical DRGs following peripheral and central axotomy. In the uninjured, contralateral DRG, 5.9 ± 0.2% of Gal1-positive somata also expressed NF200 (A,B). At 7 and 14 days after dorsal rhizotomy, 6.4 ± 0.1% and 7.1 ± 0.2% of the Gal1-IR somata expressed NF200, respectively (C,D,E,F). After peripheral axotomy, there was an increase in the proportion of Gal1-IR cells expressing NF200 to 12 ± 2% after 7 days, and to 12 ± 1% after 14 days (G,H,I,J). These proportions were both significantly greater than in cells of contralateral DRGs. Arrows indicate cells that are both Gal1- and NF200-IR; arrowheads show cells that are Gal1-, but not NF200-IR. Graph shows proportion of Gal1-IR cells expressing NF200. Asterisks indicate significant differences compared to DRGs contralateral to injury. Scale bar, 50 μm.
140

Gal1

control

7d rhiz

14 rhiz

7d ax

14d ax

merge

proportion Gal1 profiles

uninjured 7d rhiz 14 rhiz 7d axo 14d axo

*
Figure 3.6. Summary of Gal1-IR cell size distribution compared to the size distribution of the entire neuronal population in uninjured and injured DRGs. In the uninjured DRG, 48 ± 1% of somata were Gal1-IR (F). While Gal1-IR was found in cells of all sizes (A), a greater proportion of small-diameter CGRP- and IB4-positive cells expressed Gal1 than large-diameter NF200-IR cells (see Fig. 3.3, 3.4, 3.5). Seven days after dorsal rhizotomy, 52 ± 2% of cells were Gal1-IR and, at 14 days post-rhizotomy, 49 ± 1% of somata were Gal1-IR (B,C). Peripheral nerve injury induced an increase in the proportion of large diameter somata that contained Gal1-IR (D,E). At 7 days after peripheral axotomy, there was a significant increase in Gal1-IR cells (65 ± 3%) and after 14 days 70.8 ± 4.3% of somata were Gal1-IR. Asterisks indicate significant differences compared to DRGs contralateral to injury.
A

Gal1 +ve cells
all cells

B

7d rhizotomy
uninjured

C

14d rhizotomy
uninjured

D

7d axotomy
uninjured

E

14d axotomy
uninjured

F

proportion of Gal1 +ve cells

uninjured
7d rhiz.
14d rhiz.
7d axo
14d axo

proportional frequency

cell diameter (microns)
Figure 3.7. Differential regulation of Gal1-IR distribution in the C7 dorsal horn by central and peripheral axotomy. Dorsal rhizotomy induced a near-complete loss of dorsal horn Gal1-IR at both 7 and 14 days compared to the uninjured, contralateral side (A-D). At 7 and 14 days after spinal nerve lesion (peripheral axotomy), Gal1-IR axon density was higher in deeper dorsal horn laminae (E-H). At all points between the arrows, there was a significant difference in the density of Gal1-positive axons between ipsilateral and contralateral dorsal horns. Scale bar, 200 µm.
**Figure 3.8.** Gal1 mRNA increased significantly in degenerating sensory tracts in the spinal cord following dorsal rhizotomy. The major landmarks of the dorsal spinal cord can be identified using a cervical spinal cord section stained for NF200 (A). This animal underwent dorsal rhizotomy 7 days prior to fixation. On the rhizotomized (right) side, the intact gracile fasciculus (GF) and corticospinal tract (CST), and degenerating cuneate fasciculus (CF) and dorsal horn (DH) are identified. Arrows indicate the trajectories of large-diameter axons entering the deeper laminae. Silver grain density was quantified within dotted area on the contralateral and rhizotomized sides and expressed as a multiple of background (uninjured CST). After dorsal rhizotomy, the Gal1 autoradiographic signal increased significantly in the ipsilateral gracile fasciculus and dorsal horn to 3.3 ± 0.2 times background, compared to 1.3 ± 0.2 times on the uninjured side after 7 days. Signal was increased by 4.7 ± 0.3 times background on the rhizotomized side, and 1.2 ± 0.2 times on the contralateral side at 14 days post-rhizotomy. Asterisks indicate significant differences compared to DRGs contralateral to injury. Scale bar, 200 µm.
**Figure 3.9.** Gal1 mRNA increased significantly in the peripheral, but not central, compartment of the dorsal root entry zone (DREZ) at 7 (A-D) and 14 (E-H) days following dorsal rhizotomy. Sections were co-labeled with Gal1 autoradiographic signal (A,B,E,F) and fluorescent Nissl stain (C,D,G,H), which was used to demarcate glial cell bodies within the DREZ. Arrows indicate the PNS-CNS interface, which is also illustrated schematically (I). In the peripheral compartment of the DREZ, Gal1 silver grain density increased to 4.3 ± 0.5 times the contralateral side at 7 days post-rhizotomy, and to 5 ± 1 times contralateral after 14 days (n=9; A,B,J). In the central compartment of the DREZ, the autoradiographic signal was not significantly different from the contralateral side (7 days: 1.2 ± 0.2 times contralateral; 14 days: 1.4 ± 0.1) (n=9; E,F,J). Asterisks indicate significant differences compared to DRGs contralateral to injury. Scale bar, 100 µm.
3.4 Discussion

3.4.1 Overview

In the present study, we characterized the localization of Gal1 mRNA and protein in the DRG and dorsal spinal cord following peripheral and central axotomy. In the uninjured animal, Gal1 mRNA and protein was expressed mainly in small-diameter DRG neurons, and Gal1 protein was localized to superficial dorsal horn layers. We found that peripheral axotomy resulted in the upregulation of Gal1 mRNA and protein, most notably in large-diameter DRG neurons, whereas dorsal rhizotomy did not induce a significant change in Gal1 expression in the DRG. Spinal nerve lesion also resulted in augmented Gal1-IR in deeper laminae of the dorsal horn. Interestingly, deafferentation of the spinal cord resulted in significant depletion of Gal1-IR in the dorsal horn, but also caused an increase in Gal1 mRNA signal in degenerating sensory tracts. Finally, we found that dorsal rhizotomy induced a significant increase in Gal1 mRNA in the peripheral compartment of the DREZ, but did not affect Gal1 mRNA expression in its central portion.

3.4.2 Gal1 expression in the naïve DRG

In the adult rat cervical DRG, approximately 57% of neuronal somata had an ISH signal for Gal1 mRNA (silver grain density) that was at least 5 times greater than background levels. The majority of these profiles were small-diameter neurons. To our knowledge, this is the first report to quantify the size distribution of Gal1 mRNA expression in the DRG, but our results are consistent with previous reports that demonstrated expression of Gal1 mRNA in small-diameter sensory neurons (Hynes et al., 1990; Sango et al., 2004). We also found that about 48% of DRG neurons were Gal1-IR, which agrees well with previous findings: Regan et al. (1986) established that 63% of rat cervical DRGs
had some Gal1-IR, including 46% with intense Gal1-IR. Like cervical DRG neurons, most lumbar DRG neurons were Gal1-IR; however, only 20-26% of these somata were intensely Gal1-IR (Imbe et al., 2003; Sango et al., 2004). Such phenotypic differences between cervical and lumbar DRGs are not unprecedented: significant variations in the rostro-caudal distribution of proteins have been described in sensory ganglia at cranial and caudal levels. (Ramer et al., 2001a).

We also found that the majority of Gal1-IR somata were either IB4-binding (33%) or CGRP-IR (28%) small-diameter neurons. In lumbar DRGs, Gal1 co-localized mainly with c-Ret mRNA and also with a limited number of TrkA mRNA-expressing neurons (Imbe et al., 2003). In lumbar DRGs, only 63% of c-Ret mRNA-expressing neurons bind IB4 (Molliver et al., 1997) and approximately 50% of the IB4-binding neurons also have CGRP-IR (Wang et al., 1994; Bergman et al., 1999). Thus, the binding of IB4 to both CGRP-IR and c-Ret mRNA-expressing neurons is consistent with both the report of Gal1-IR occurring mainly in c-Ret mRNA-expressing lumbar DRG neurons and Gal1-IR occurring in both IB4-binding and CGRP-IR cervical DRG neurons.

### 3.4.3 Gal1 expression after injury

We used two models of nerve injury to study the differential responses of primary afferents to peripheral and central axotomy. After peripheral nerve injury, which normally elicits a robust cell body response (Fernandes and Tetzlaff, 2000), we observed a significant increase in the proportion of DRG neurons positive for Gal1 mRNA and protein. This increase can be attributed mainly to an injury-induced upregulation of Gal1 in large-diameter, NF200-IR somata. A concomitant increase in Gal1-IR was observed in deeper laminae of the dorsal horn, an area that corresponds to the central projections of large-diameter fibres. Our observations of augmented Gal1-IR in the cervical spinal
cord after peripheral nerve injury is consistent with published results, which describe increased Gal1-IR in deeper layers of the dorsal horn of the lumbar spinal cord following either sciatic nerve transection or spared nerve injury (Imbe et al., 2003).

We performed dorsal rhizotomy to study the effect of central axotomy on Gal1 expression. Dorsal rhizotomy did not induce any significant shifts in the proportion of somata expressing Gal1 mRNA or protein but it did result in diminished Gal1-IR in the dorsal horn. This latter finding indicates that uninjured primary afferents transport Gal1 protein to their central terminals in the dorsal horn. Dorsal rhizotomy also resulted in an upregulation of Gal1 mRNA in areas undergoing Wallerian degeneration. The only known CNS glial cell to express Gal1 is the reactive astrocyte (Camby et al., 2001). However, if rhizotomy induced Gal1 mRNA in reactive astrocytes, we would have expected ISH signal to be distributed more widely throughout the dorsal horn and central compartment of the DREZ, since astrocytes become reactive in response to dorsal rhizotomy wherever axonal degeneration occurs. Since high silver grain density was observed mainly along the paths of large-diameter, myelinated afferents in the medial dorsal horn, it is possible that activated microglia or, more likely, denervated oligodendrocytes upregulated Gal1 mRNA in response to deafferentation. It is possible that rhizotomy-induced upregulation of Gal1 by denervated glia (astrocytes, oligodendrocytes, or microglia) activates local microglia in a similar manner to the in vitro Gal1-mediated activation of macrophages described by Horie and colleagues (2004).

In addition, we determined that Gal1 mRNA was differentially regulated within the PNS and CNS portions of the cord-adjacent dorsal root (containing the DREZ) after dorsal rhizotomy. We observed a large rhizotomy-induced increase in Gal1 mRNA expression in the PNS portion of the DREZ, whereas its expression in the CNS changed little.
Schwann cells may be responsible for the increase in Gal1 mRNA that was observed in response to injury, since Schwann cells and sensory neurons express Gal1 in the PNS (Sango et al., 2004). Another potential source of Gal1 at these timepoints are macrophages, which are recruited to the injury site in large numbers after injury. These cells show a much more robust response in the PNS than the CNS after dorsal rhizotomy (Ramer et al., 2001b), and have been shown to express high levels of Gal1 upon activation (Rabinovich et al., 1999). It is interesting to note that injured axons have the capability to regenerate through the peripheral portion of the dorsal root, which contains increased Gal1 mRNA levels, but these axons fail to re-grow through the PNS-CNS interface, which is nearly devoid of Gal1 mRNA.

3.4.4 Conclusions

Although Gal1 seems to have important roles in axonal guidance during development (Puche et al., 1996; McGraw et al., 2005), and in motor axon regeneration following injury (McGraw et al., 2004a; McGraw et al., 2004b), its function in adult primary afferent neurons remains largely undefined. We confirmed that over half of uninjured DRG neurons express Gal1 (Regan et al., 1986; Imbe et al., 2003; Sango et al., 2004), and showed that Gal1 is transported to central terminals. Gal1 is secreted into the extracellular milieu, and can bind astrocytes facilitating the release of brain-derived neurotrophic factor (BDNF) (Sango et al., 2004; Sasaki et al., 2004). The actions of neuron- or glia-derived Gal1 are likely to contribute to BDNF-dependent processes, including synaptic plasticity and nociceptive processing (Thoenen, 2000; Pezet et al., 2002).

Following axotomy, successful regeneration depends on processes both intrinsic and extrinsic to the neuron. Peripheral axotomy induces multiple neuronally-expressed
regeneration-associated genes, including Gal1. Peripheral nerve injury also results in
large increases in Gal1 expression in reactive Schwann cells. Dorsal rhizotomy has little
effect on neuronal Gal1 expression, and glial Gal1 expression along the central
projection of primary afferent axons is much higher in the peripheral compartment
(where regeneration succeeds) than in the central compartment (where regeneration
fails). The manipulation of endogenous Gal1 levels may therefore serve to improve the
outcome of peripheral or central nervous system trauma.
3.5 References


4 The effect of galectin-1 on neurite outgrowth from cultured adult dorsal root ganglion neurons

4.1 Introduction

Peripheral axons have the extraordinary ability to regenerate and reinnervate targets following injury; regrowth succeeds due to the effective responses of both the injured neuron and the cells surrounding the neuron. Injured peripheral neurons upregulate regeneration-associated genes (RAGs) and enter a growth mode similar to that observed during development (Fu and Gordon, 1997). Cells within the axon’s environment also facilitate its regeneration: Schwann cells and macrophages phagocytose debris and secrete trophic factors, and Schwann cells form permissive tubes that guide axons (Fu and Gordon, 1997; Chen et al., 2007; Vargas and Barres, 2007). Unfortunately, both the neuronal growth response and the support provided by Schwann cells diminishes with time (Fenrich and Gordon, 2004), meaning that functional recovery following peripheral nerve injury is often limited – especially when the axons must grow great distances.

Galectin-1 (Gal1), a 14.5 kDa protein that has roles in fundamental cellular processes (Camby et al., 2006), promotes peripheral axon regeneration. Gal1 affects developmental growth of sensory (McGraw et al., 2005b) and olfactory (Puche et al., 1996) axon subpopulations, and regeneration of peripheral (Horie et al., 1999; Fukaya et al., 2003) and retinal ganglion cell (Okada et al., 2005) axons (reviewed by Gaudet et al., 2005). The mechanisms underlying Gal1’s role in axon regeneration remain largely unknown. Using peripheral nerve and dorsal root injuries, we found that Gal1 expression both within the neuron and its environment is correlated with an axon’s

propensity for regrowth (McGraw et al., 2005a). Therefore, Gal1 could promote axonal regeneration through mechanisms extrinsic and/or intrinsic to the neuron.

Extrinsically, Gal1 may affect neurons (Inagaki et al., 2000), and is known to bind to macrophages (Horie et al., 2004). Gal1 elicits increased neurite outgrowth from DRG explants after six days in culture (Inagaki et al., 2000). Given that there are multiple cell types present in the explants, it is unclear whether Gal1/Ox was acting directly on neurons or on other cell types present in the explant. Medium from macrophages treated with oxidized Gal1 (Gal1/Ox) enhances neurite outgrowth from rat DRG explants (Horie et al., 2004), and Gal1/Ox facilitates Schwann cell migration directly (Echigo et al., 2010); thus, other cell types could mediate Gal1/Ox’s effects on regeneration. Therefore, the action of extracellular Gal1/Ox on isolated DRG neurons is undefined.

When used as a substrate, Gal1 influences neuritic growth from isolated neurons. A Gal1 substrate promotes adhesion and aggregation of DRG (early postnatal; Outenreath and Jones, 1992) and olfactory neurons (Mahanthappa et al., 1994), fasciculation of DRG neuron axons (Outenreath and Jones, 1992), and neurite extension from olfactory neurons (Puche et al., 1996). It is not known how exogenous Gal1 affects neurite outgrowth from adult DRG neurons.

It is also not clear whether Gal1 acts as a neuronal RAG. Interestingly, Gal1 interaction with the monomeric GTP-binding protein H-Ras is required for neurite outgrowth from PC12 cells (Rotblat et al., 2004), suggesting that Gal1 may act within peripheral neurons to promote axon extension.
Here, we culture DRG neurons from wild-type and Gal1 null mutant mice to establish whether Gal1 acts as a RAG intrinsic to neurons. In addition, we determine whether extracellular Gal1 is sufficient to elicit neurite outgrowth from dissociated rat DRG neurons. Surprisingly, we find that neither intrinsic nor extrinsic Gal1 promotes neurite outgrowth from these cells, implying that Gal1 does not effect axon regeneration directly.
4.2 Materials and methods

4.2.1 Animals

For mouse experiments, 15 adult age-matched 129P3/J (Lgals1\(^{+/+}\) Jackson Labs, Bar Harbor, Maine) and 15 129P3/J Lgals1 null mutant (Lgals1\(^{-/-}\) (Poirier and Robertson, 1993)) were used. We also used 10 Wistar rats (UBC Centre for Disease Modeling, Vancouver, BC; Charles River, Willmington, MA). All experiments conformed to Canadian Council for Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee.

4.2.2 Dissociated dorsal root ganglion neuron culture

Neurons were cultured as described previously (Gardiner et al., 2005). Adult mouse and rat neurons were treated identically. Animals were killed by overdose with chloral hydrate (1.75 g per kg body weight). After harvesting DRGs and placing them in a Petri dish containing Leibovitz’s L-15 (Invitrogen, Carlsbad, CA), connective tissue and nerve branches were cleared. Collagenase (Sigma-Aldrich, Oakville, ON) was added to 0.125%, and the DRGs were incubated at 37°C and 5% CO\(_2\) for 40-45 minutes, at which point the solution was aspirated and a fresh 0.125% collagenase mixture (in F-12; Invitrogen) was added for another 40 minutes. After removing the collagenase, 0.25% trypsin (Sigma-Aldrich) was added to the DRGs for 20-25 minutes. Trypsin was inactivated by washing the cells with 30% fetal bovine serum (Invitrogen), and the DRGs were washed three times with F-12 before being dissociated by trituration in 1 mL F-12. The triturated cells were passed through a 70 \(\mu\)m filter (VWR, Mississauga, ON) and centrifuged at 0.1 rcf (900 rpm) for five minutes. The cells were resuspended in the last 500 \(\mu\)L of medium before being carefully pipetted onto a cushion of 15% bovine serum albumin (Sigma-Aldrich), which was then centrifuged at 900 rpm for 10 minutes. After
aspirating the supernatant, the pellet (which contained the neurons) was suspended in culture medium (enough volume for 100 µL per well), which comprised F-12, N2 additives (1:100; Sigma-Aldrich), insulin (10 pM; Sigma-Aldrich), and cytosine arabinoside (AraC, 0.01mM; Sigma-Aldrich). The cell suspension was then added to eight-chambered slides (VWR), which had been coated overnight with 2 µg/mL laminin (Sigma-Aldrich) in poly-D-lysine, washed with phosphate-buffered saline (PBS), and pre-filled with 280 mL culture medium. Neurons from a single mouse were divided amongst the wells of one slide, whereas enough neurons were harvested from one rat to coat four slides at the appropriate cell density: for this reason, we cultured rat neurons whenever possible. The cells were incubated for 40 minutes to allow settling before 20 µL of growth factors or other treatments were added. These treatments included media alone, nerve growth factor (NGF) (2.5S) (10 ng/mL; Sigma-Aldrich), glial cell line-derived growth factor (GDNF) (10 ng/mL; gift from Regeneron Pharmaceuticals, Tarrytown, NY), and/or various concentrations of Gal1/Ox (100, 1000 ng/mL) (production described by Inagaki et al., 2000). The cells were incubated for 20-24 hours before fixation in 4% paraformaldehyde (20-30 minutes). The slides were placed in PBS and were stored at 4°C overnight (or longer). We completed four experiments with mouse DRGs (all with NGF and GDNF included as positive controls), five experiments with rat DRGs and NGF added, and three experiments with rat DRGs and GDNF added.

4.2.3 Immunocytochemistry

Non-specific binding sites were blocked by incubating slides with 10% normal donkey serum for one hour. Next, the slides were incubated with mouse anti-βIII tubulin (Sigma-Aldrich; 1:500) and Alexa 546-conjugated phalloidin (1:200; Molecular Probes, Carlsbad, CA) for two hours. After three PBS washes, we added Alexa 488-conjugated donkey
anti-mouse (1:250; Molecular Probes) for one hour. All antibodies were diluted with PBS containing 0.2% Triton X-100 and 0.1% sodium azide. The slides were then exposed to the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; 1:50) for five minutes and were rinsed in PBS before coverslipping.

4.2.4 Image analysis

Images were captured using an Axioplan 2 microscope (Zeiss, Jena, Germany), QImaging digital camera (Burnaby, BC), and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). All images for a given marker were captured under the same exposure settings. To facilitate an unbiased analysis of various parameters of neurite outgrowth from DRG neurons (especially proportion of cells bearing neurites), 15-20 images were taken of random fields of view scattered throughout each well. An image was only captured if two or more neurons were present in one of these random fields of view.

Images were analyzed using SigmaScan Pro 5 software (SPSS, Chicago, IL). Three parameters were measured for neurite outgrowth analysis: proportion of cells bearing neurites, length of longest neurite, and neuritic branching. For the proportion of cells bearing neurites, the number of cells bearing one or more neurites that were at least 1.5 times the diameter of its cell body was divided by the total number of cells for that treatment. For the length of the longest neurite, the length of a line drawn along the longest neurite for each neuron was recorded (only if the longest neurite exceed 1.5 times the diameter of the cell body). For neuritic branching, concentric circles were superimposed on a thresholded image of the cells centred around the neuron of interest, and the number of neuritic crossings of each circle was counted. We also tabulated the total number of crossings for each treatment. Only neurons with one or more neurites
extending at least 66 µm were included in the branching analysis. There were at least two wells for each treatment in each experiment; data collected from these treatment wells were combined for each experiment for analysis. Averages presented herein represent the means of all experiments performed for each parameter.

SigmaPlot 2001 and SigmaStat 3.0 (both SPSS) were used to graph and statistically analyze the data, respectively. Two-way ANOVAs and Holm-Sidak post-hoc tests were used to establish whether treatment groups were significantly different from one another. Significance was set at $p < 0.05$. All graphs show mean ± standard error of the mean.
4.3 Results

4.3.1 Neurite outgrowth in the absence of Gal1

First, we sought to establish whether neuronal Gal1 acts intracellularly to stimulate neurite elongation from cultured DRG neurons; that is, whether it is a RAG in these cells. To test this, we compared neurite outgrowth from dissociated $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mouse DRG neurons. The mean results of four experiments are presented here (Fig. 4.1, 4.2). As expected, NGF and GDNF both had positive effects on parameters of neuritic outgrowth from dissociated DRG neurons when compared to the control (media only) group. Treatment of $Lgals1^{+/+}$ and $Lgals1^{-/-}$ neurons with either NGF or GDNF increased the proportion of neurons bearing neurites, increased the average length of the longest neurite extended, and enhanced neuritic branching (Fig. 4.2).

Neurons that lacked Gal1 did not display any abnormalities with respect to neurite outgrowth (Fig. 4.1). There were no significant differences in any of the three parameters studied between the two genotypes (Fig. 4.2). These results suggest that Gal1 does not act as an intrinsic factor within neurons to promote axonal regeneration.

4.3.2 The effects of Gal1/Ox treatment on neurite outgrowth

Although Gal1 does not appear to act as a RAG within DRG neurons, it is possible that extracellular Gal1/Ox could effect peripheral axonal growth directly. To test this, we cultured rat DRG neurons on laminin in the presence or absence of neurotrophic factors and/or Gal1/Ox (Fig. 4.3, 4.4). Treatment with growth factors augmented neurite outgrowth from these rat cells. GDNF and NGF treatment elicited more neurons to extend neurites irrespective of the presence or absence of Gal1/Ox (except for NGF + 1 µg/mL Gal1/Ox (not significantly different)). Neurotrophic factors did not significantly
Figure 4.1. Inverted fluorescent photomicrographs of dissociated \textit{Lgals1}^{+/+} and \textit{Lgals1}^{-/-} dorsal root ganglion (DRG) neurons cultured in the absence or presence of trophic factors. Neurons were cultured for 20 to 24 hours in the presence of media alone, GDNF, or NGF before fixation and βIII-tubulin immunocytochemistry. These representative images show that there was no notable difference in the elaboration of neurites from cultured \textit{Lgals1}^{+/+} and \textit{Lgals1}^{-/-} DRG neurons (quantified in Fig. 4.2). As expected, growth factor addition elicited considerably more neurite outgrowth than did media alone from neurons of both genotypes. Scale bar, 200 μm.
Figure 4.2. Neuritic outgrowth from dissociated \textit{Lgals1}^{+/+} DRG neurons is not significantly different from outgrowth from \textit{Lgals1}^{-/-} neurons. We analyzed the proportion of cells bearing neurites (a), the length of the longest neurite elaborated (\(\mu m\); >1.5x cell body diameter) (b), and neuritic branching (c, c'-c'''). There were no significant differences between \textit{Lgals1}^{+/+} and \textit{Lgals1}^{-/-} neurons in any of the parameters measured, suggesting that Gal1 is not required for initiation, growth, or branching of neurites. However, growth factor addition affected neurite outgrowth. A higher proportion of \textit{Lgals1}^{+/+} neurons elaborated neurites when presented with either GDNF or NGF (a; \(p < 0.001\) vs media alone). Addition of GDNF or NGF to \textit{Lgals1}^{+/+} or \textit{Lgals1}^{-/-} neurons increased the length of the longest neurite (b; \(p < 0.001\)). To assess neuritic branching, we counted the number of times neurites crossed concentric circles overlaid at set distances from the cell body, and analyzed the total number of crosspoints (c) and the number of crosspoints at set distances from the cell body with different treatments (c': +media; c'': +GDNF; c'''': +NGF). Addition of either GDNF or NGF induced significantly more neurite branching from neurons of both genotypes compared to addition of media alone (c; \(p < 0.001\)).
**Figure a**
Proportion of cells bearing neurites:
- Lgals1+/+
- Lgals1/-

Growth factor treatment:
- none
- +GDNF
- +NGF

**Figure b**
Mean length of longest neurites (μm):
- none
- +GDNF
- +NGF

**Figure c**
Branching:
- none
- +GDNF
- +NGF

**Figure c’**
Crosspoints vs. distance from cell body (μm):
- Lgals1+/+
- Lgals1/-

**Figure c”**
Crosspoints vs. distance from cell body (μm):
- +GDNF

**Figure c”’**
Crosspoints vs. distance from cell body (μm):
- +NGF
affect the length of the longest neurite. NGF, but not GDNF, promoted neurite branching from the cultured DRG neurons (all conditions except NGF + 1 µg/mL Gal1/Ox (not significantly different from 1 µg/mL Gal1/Ox alone)) (Fig. 4.4).

We added Gal1/Ox at 100 ng/mL and 1 µg/mL to dissociated rat DRG neurons both alone and in combination with the growth factors. When added in the absence of neurotrophic factors, Gal1/Ox did not significantly affect the proportion of cells bearing neurites (Fig. 4.4a), the length of longest neurite (Fig. 4.4b), or neurite branching (Fig. 4.4c, c'). Concomitant application of Gal1/Ox and GDNF/NGF did not alter the proportion of cells with neurites, neurite length, or neurite branching compared to adding neurotrophic factors alone (Fig. 4.4b, c, c'-c'').

We found that Gal1/Ox had no significant effects on the three parameters of neuritic outgrowth, either when added to the normal media alone, or when added to the media containing NGF or GDNF. Therefore, extracellular Gal1/Ox, which promotes peripheral axon regeneration in vivo, does not appear to elicit axon growth directly from cultured neurons.
**Figure 4.3.** Inverted fluorescent photomicrographs of dissociated rat dorsal root ganglion (DRG) neurons cultured in the absence or presence of Gal1/Ox and/or trophic factors. Neurons were cultured for 20 to 24 hours with or without 1 µg/mL Gal1/Ox in the presence of media alone, GDNF, or NGF. These representative images show that there was no significant effect of adding Gal1/Ox to isolated DRG neurons. Neurotrophic factor addition enhanced neurite initiation from cultured neurons, and NGF treatment increased neurite branching. Scale bar, 200 µm.
Figure 4.4. Gal1/Ox treatment has no significant effect on neurite outgrowth from dissociated rat DRG neurons. We analyzed the proportion of cells bearing neurites (a), the length of the longest neurite elaborated (µm; >1.5x cell body diameter) (b), and neuritic branching (c, c’-c”’). Gal1/Ox had no significant effect on the proportion of neurons extending neurites (a), on the length of the longest neurite elaborated (b), or on neurite branching (c), whether in the absence or presence of neurotrophic factors. Neurotrophic factor addition had robust impact on neurite outgrowth: both GDNF and NGF caused more neurons to elaborate neurites (a; p < 0.05 vs appropriate media control, except for NGF + 1 µg/mL Gal1/Ox (not significantly different)). Neurotrophic factors had no significant effect on the length of the longest neurite (b). NGF generally elicited significantly more branching from cultured neurons than did GDNF or media alone (c; p < 0.05 vs appropriate media control and GDNF-treated group, except for NGF + 1 µg/mL Gal1/Ox (not significantly different from 1 µg/mL Gal1/Ox alone)). We also quantified the number of crosspoints at set distances from the cell body with different treatments (c’: +media; c”: +GDNF; c”’: +NGF).
**a** proportion

<table>
<thead>
<tr>
<th>Growth factor treatment</th>
<th>Proportion of cells bearing neurites</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.1</td>
</tr>
<tr>
<td>+GDNF</td>
<td>0.3</td>
</tr>
<tr>
<td>+NGF</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**b** length

<table>
<thead>
<tr>
<th>Growth factor treatment</th>
<th>Mean length of longest neurites (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>200</td>
</tr>
<tr>
<td>+GDNF</td>
<td>300</td>
</tr>
<tr>
<td>+NGF</td>
<td>400</td>
</tr>
</tbody>
</table>

**c** branching

<table>
<thead>
<tr>
<th>Growth factor treatment</th>
<th>Total crosspoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>20</td>
</tr>
<tr>
<td>+GDNF</td>
<td>40</td>
</tr>
<tr>
<td>+NGF</td>
<td>60</td>
</tr>
</tbody>
</table>

**c’** +media

<table>
<thead>
<tr>
<th>Added substance</th>
<th>Crosspoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>+media</td>
<td>30</td>
</tr>
<tr>
<td>+Gal1/Ox (100 ng/ml)</td>
<td>20</td>
</tr>
<tr>
<td>+Gal1/Ox (1 μg/ml)</td>
<td>10</td>
</tr>
</tbody>
</table>

**c’’** +GDNF

<table>
<thead>
<tr>
<th>Distance from cell body (μm)</th>
<th>Crosspoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>15</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>400</td>
<td>25</td>
</tr>
</tbody>
</table>

**c’’’** +NGF

<table>
<thead>
<tr>
<th>Distance from cell body (μm)</th>
<th>Crosspoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>15</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>400</td>
<td>25</td>
</tr>
</tbody>
</table>
4.4 Discussion

4.4.1 Overview

We studied Gal1’s effects on neurite outgrowth from isolated DRG neurons. First, we used \textit{Lgals}\textsubscript{1}\textsuperscript{-/-} neurons and demonstrated that intracellular Gal1 is not required for neurite outgrowth: \textit{Lgals}\textsubscript{1}\textsuperscript{-/-} neurons did not exhibit any deficiencies in neurite extension or branching, whether cultured in the presence or absence of trophic factors. Second, we cultured dissociated rat DRG neurons in the presence of Gal1/Ox, and found that this protein did not have significant effects on neurite outgrowth. These results suggest that Gal1 does not promote axon growth directly from adult DRG neurons, and that non-neuronal cells likely play a more central role in the enhancement of axon regeneration by Gal1.

4.4.2 Potential links between intracellular Gal1 and the neuronal switch to growth mode

Evidence from previous studies implied that Gal1 might have a direct role in axon elongation: Gal1 promotes peripheral axon growth \textit{in vivo} (Horie et al., 1999; Fukaya et al., 2003) and \textit{in vitro} (Horie et al., 1999; Inagaki et al., 2000; Horie et al., 2004); it is required for the normal timecourse of recovery of motor function after facial nerve injury (McGraw et al., 2004a); and its expression within neurons is correlated with their capacity for regrowth (McGraw et al., 2004b; McGraw et al., 2005a).

Functional evidence for the potential role of Gal1 as a RAG was compiled using PC12 cells (Rotblat et al., 2004), which are adrenal medulla-derived pheochromocytoma cells that have the potential to differentiate into neurons. Gal1 interacts with and enhances the activity of the activated form of the G-protein Ras (H-Ras-GTP and K-Ras-GTP), and
activated Ras is implicated in neurite elongation. A single point mutation in Gal1 inhibited its ability to sustain H-Ras-GTP activation and localization to the plasma membrane, and thereby attenuated neurite outgrowth. Conversely, overexpression of wild-type Gal1 (normally expressed at low levels in PC12 cells) led to H-Ras activation, as well as the elaboration of longer neurites from PC12 cells (but did not alter the number of neurites per cell). Gal1 levels were also positively correlated with ERK phosphorylation. In addition to signaling via the Raf-1-MEK-ERK pathway, activated Ras can signal downstream through the PI3-kinase (PI3K)-Akt pathway. Elad-Sfadia and colleagues (2002) showed that Gal1 acts on Ras to preferentially activate Raf-1 over PI3K, and therefore may modulate the morphology of neuritic arbours.

Interestingly, the Raf-1 and PI3K pathways affect distinct properties of cultured neurons, and also seem to have differential effects on developmental and regenerative axon growth. Markus et al. (2002) used apoptosis-resistant Bax<sup>-/-</sup> embryonic DRG neurons to circumvent their requirement for trophic factors, and manipulated the activation of the Ras-Raf-1-MEK-ERK and PI3K-Akt pathways in these cells. They found that the Ras-Raf-1-ERK cascade was necessary and sufficient for axon extension, but had no effect on axon branching. In contrast, they showed that PI3K and Akt were necessary but not sufficient to increase axon length, that overexpression of either protein increased axon calibre, and that Akt overexpression induced neuritic branching. It is possible, however, that these signaling pathways may have different effects in adult neurons. Using cultured adult mouse neurons, Liu and Snider (2001) found that the MEK and PI3K pathways were required for NGF-induced axon extension – as in embryonic neurons – but also found that the JAK-STAT pathway, and not MEK or PI3K, were required for the remarkable extending phenotype induced by conditioning lesion (also see Qiu et al., 2005).
Given that previous work implicated Gal1 in Ras and Raf-1 activation at the expense of the PI3K pathway, one might expect that cultured \textit{Lgals1}^{-/-} neurons would extend shorter neurites and/or have more branched arbours. We found that these neurons had no significant abnormalities in neurite length or branching. In addition, there was no difference in the proportion of neurons extending neurites between genotypes. There are several possible explanations for these results. First, Gal1 may not be acting within these cells in the same manner as it does in PC12 cells: it is not known how well signaling pathways in PC12 cells represent those underlying axon growth in primary neuron cultures. Second, H- and K-Ras may not be expressed in adult DRG neurons (H-Ras expression has been demonstrated in embryonic, but not adult DRG neurons (Borasio et al., 1993; Vogel et al., 2000)); in that case, Gal1 might not modulate Raf-1-MEK-ERK activation. Third, compensatory mechanisms associated with development in the absence of Gal1 may allow \textit{Lgals1}^{-/-} neurons to extend neurites normally.

In summary, our data suggest that intracellular Gal1 is not required for \textit{in vitro} neurite outgrowth in the adult.

\textbf{4.4.3 Potential effects of extracellular Gal1 on axon growth}

In peripheral nerves, Gal1 is expressed and secreted by neurons and Schwann cells (Sango et al., 2004), and likely macrophages (Rabinovich et al., 1998; Correa et al., 2003; Gaudet et al., 2009), and Gal1 mRNA and protein levels increase in the injured nerve (McGraw et al., 2005a; Gaudet et al., 2009). Extracellular Gal1/Ox causes increased neurite elaboration from DRG explants (Inagaki et al., 2000; Horie et al., 2004), and is known to induce macrophage activation (Horie et al., 2004) and
accumulation (Gaudet et al., 2009), and Schwann cell migration (Echigo et al., 2010); however, the effects of Gal1/Ox on isolated peripheral axons were not known.

Previous studies showed that Gal1 could act as a substrate to enhance the adhesive properties of cultured embryonic DRG neurons and early post-natal olfactory neurons (Outenreath and Jones, 1992; Mahanthappa et al., 1994). Cultured olfactory neurons also elaborated longer neurites on a Gal1 substrate than on bovine serum albumin-coated slides (Puche et al., 1996). Some – but not all – of these effects of Gal1 were attributed to the protein’s lectin activity (only present in its reduced form, Gal1/Red).

Gal1 is known to interact with a plethora of extracellular proteins (Camby et al., 2006), including the extracellular matrix molecule laminin (Ozeki et al., 1995) and its integrin family receptors (Gu et al., 1994). Gal1 has dual effects on laminin-integrin signaling: Gal1 prevents the association of specific integrins with laminin (Gu et al., 1994; Moiseeva et al., 1999), but can lead to increased β1-integrin activation (Moiseeva et al., 2003). It is possible that Gal1/Red promotes the interaction between laminin and axonal integrin receptors, thereby enhancing adhesive properties and potentially increasing axon growth (Mahanthappa et al., 1994). These interactions could be responsible for defective developmental targeting of subpopulations of sensory (McGraw et al., 2005b) and olfactory (Puche et al., 1996) axons in Lgals1<sup>-/-</sup> mice.

Although Gal1/Red interacts with various extracellular and membrane-bound proteins in a carbohydrate-dependent fashion, it is only Gal1/Ox that enhances peripheral axon regeneration from cultured DRG explants (Inagaki et al., 2000). We added Gal1/Ox to the media to establish whether the protein could act as a soluble growth factor that promotes axon extension from isolated cultured DRG neurons, much like the robust growth induced from DRG neurons by neurotrophic factors. We reasoned that Gal1/Ox
might bind a receptor that activates intracellular signaling pathways that control axon
extension (analogous to the unidentified Gal1/Ox receptor in macrophages; Horie et al.,
2004). Extracellular Gal1 promotes ariginase activation and polyamine production in
cultured peritoneal macrophages (Correa et al., 2003); in neurons, activation of the
arginase-polyamine pathway is associated with axon growth (Cai et al., 2002; Deng et
al., 2009). Therefore, if exogenous Gal1/Ox modulated this pathway in neurons as well,
it might enhance neurite outgrowth. However, we found that Gal1/Ox treatment had little
effect on cultured DRG neurons.

Two studies have shown that Gal1/Ox might act as an extracellular factor that enhances
neurite outgrowth directly in vitro (Inagaki et al., 2000; Horie et al., 2004). Gal1/Ox
elicited growth of up to 60 percent more neurites from adult rat DRG explants compared
to control cultures, which is in stark contrast with the results presented here. There are
at least two possible explanations for these apparent differences. First, these
researchers used DRG explants, which contain a variety of cell types (e.g. neurons,
satellite cells, Schwann cells, macrophages, fibroblasts) and leave neurons in close
apposition, where cells can provide each other with growth factors at high local
concentrations. The use of dissociated DRG neurons is more relevant for defining
whether a factor directly affects neurite growth, since there are fewer confounding
factors (and cells) that could hinder an appropriate interpretation. Second, they
quantified neurite outgrowth at three, six, and/or 10 days after culture, which could
exaggerate any differences in growth compared to what would be observed at 24 hours
after culture of dissociated neurons.

The limited effectiveness of exogenous Gal1/Ox for promoting axon growth directly is
underscored by conspicuous differences between the robust growth induced by
neurotrophic factors and the absence of effects of Gal1/Ox when added in the absence of growth factors. We showed here that exogenous Gal1/Ox has no significant effect on neurite outgrowth from dissociated DRG neurons, whether added in the absence or presence of neurotrophic factors.

4.4.4 Conclusions

Discovering and understanding factors that enhance peripheral axon regeneration could lead to the development of more effective therapies for peripheral nerve and spinal cord injuries. The protein Gal1 represents one potential treatment, as it has been implicated in peripheral nerve repair. Using cultured DRG neurons, we established that Gal1 does not significantly effect axon growth by acting directly on neurons: Gal1 is not necessary to enhance axon growth from these cells, and exogenous Gal1/Ox does not appear to have growth-promoting effects. Future experiments could establish whether neuronal Gal1 is involved in neurite outgrowth from embryonic DRG neurons and whether Gal1 is involved in the rapid axon extension elicited by prior peripheral conditioning lesions.

Our results suggest that Gal1 does not effect significant axon growth directly by regulating the growth state of neurons. By effectively eliminating this possibility, our results imply that Gal1 promotes axon regeneration predominantly by acting on cells within the environment of regrowing axons, which include macrophages and Schwann cells.
4.5 References


Mahanthappa NK, Cooper DN, Barondes SH, Schwarting GA (1994) Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. Development 120: 1373-1384.


5 A role for galectin-1 in the immune response to peripheral nerve injury

5.1 Introduction

Lectins are proteins that have the ability to bind specific carbohydrate motifs. Galectin-1 (Gal1) is a 14 kDa lectin whose structure and function depend on its oxidation state: reduced Gal1 is a homodimer that binds β-galactosides, whereas oxidized Gal1 (Gal1/Ox) is monomeric and lacks lectin activity (Inagaki et al., 2000). Gal1 is expressed in various tissues throughout development and into adulthood in mammals, and has been implicated in numerous fundamental cellular processes, including apoptosis, proliferation, and cell adhesion. Gal1 is found both in the peripheral and central nervous systems (PNS and CNS) during rodent development, but its expression is restricted mainly to the PNS in the adult (Dodd and Jessell, 1986; Hynes et al., 1990; Regan et al., 1986). In the nervous system, Gal1 has roles in axonal pathfinding during development (Puche et al., 1996; McGraw et al., 2005b), in neuropathic pain (Imbe et al., 2003), and in the regeneration of axons following injury (Horie et al., 1999) (see Gaudet et al. (2005) for review). Gal1 is expressed by axons and nonneuronal cells in peripheral nerves (Sango et al., 2004), and its expression is increased following injury. Extracellular Gal1 in the nerve is thought to act exclusively on macrophages (Horie et al., 2004).

Damage to any type of tissue initiates a characteristic response from immune cells that assist with and may regulate the repair of the wound (Martin and Leibovich, 2005). Although cells resident to the injured tissue contribute to the repair process, the vast majority of effective immune cells emigrate from nearby blood vessels. Neutrophils are

---

usually the first immune cells to invade the injury site, followed by macrophages and mast cells. Macrophages play an important role in phagocytosis upon arrival, and provide multiple cytokines and growth factors to the surrounding tissue. In the injured PNS, macrophages cooperate with Schwann cells to break down detached myelin and axonal debris (Wallerian degeneration) and initiate nerve repair (Hirata and Kawabuchi, 2002). Several soluble factors thought to be secreted by Schwann cells following injury are involved in the recruitment of hematogenous macrophages to the injured nerve, including monocyte chemoattractant protein-1 (CCL2) (Toews et al., 1998; Perrin et al., 2005), leukemia inhibitory factor (Tofaris et al., 2002), and pancreatitis-associated protein-III (Namikawa et al., 2006) (for review, see Martini et al. (2008)).

Gal1 is also involved in the macrophage response to peripheral nerve injury. Recent studies have shown that extracellular Gal1/Ox may expedite repair of injured nerves by binding to macrophages and activating intracellular signaling pathways leading to the release of an unidentified factor that promotes Schwann cell migration and axonal regeneration (Horie et al., 2004; Okada et al., 2005). Since Gal1 is upregulated in the injured peripheral nerve following axotomy, and since Gal1/Ox binds a receptor on macrophages, we hypothesized that Gal1 may have an effect on the injury-induced accumulation of macrophages. Here, we show that exogenous Gal1/Ox is sufficient to facilitate accumulation of macrophages in uninjured sciatic nerve, and that Gal1 is necessary for the normal accumulation of macrophages following peripheral axotomy.
5.2 Materials and methods

5.2.1 Preparation of oxidized galectin-1

Production of oxidized recombinant human galectin-1 (Gal1/Ox) has been described previously (Inagaki et al., 2000). Briefly, Gal1 was expressed by *Escherichia coli*, and was isolated from the supernatant by diethylaminoethyl high-performance liquid chromatography (HPLC). Purified Gal1 underwent air oxidation in the presence of CuSO₄: Gal1 was diluted 20x with 20mM Tris-HCl (pH 8.0), CuSO₄ was added (final concentration: 0.0001%, w/v), and the solution remained at 4°C overnight to promote disulphide bond formation. Gal1/Ox was then purified using reverse-phase HPLC (YMC-Pack Protein RP column; YMC, Kyoto, Japan) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. This Gal1/Ox did not degenerate after a 10-day incubation at 37°C in PBS (5 µg/ml), as determined by SDS-PAGE and HPLC.

5.2.2 Animals and surgery

A total of 181 adult (2-4 month old) 129P3/J (*Lgals1*⁺/⁺, Jackson Labs, Maine) and 171 adult 129P3/J *Lgals1*null mutant (*Lgals1*⁻/⁻ (Poirier and Robertson, 1993); *Lgals1* is the gene that encodes Gal1) mice were used for these experiments. All experiments were performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Mice were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (80 mg/kg; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (40 mg/kg; Bayer Inc, Etobicoke, ON), and surgeries were carried out under sterile conditions. Lacri-Lube ophthalmic ointment was used to lubricate the animals’ eyes while under anesthesia. Mice were treated with buprenorphine (0.05-0.10 mg/kg; Animal Resources Centre, McGill University, Montreal, QC) intramuscularly to relieve pain and administered 1.5 mL Ringer’s solution
subcutaneously to rehydrate following surgery. Daily monitoring was carried out thereafter.

All surgeries involved exposure of the sciatic nerve by blunt dissection of the overlying thigh muscle. To test the effect of injection of oxidized Gal1 (Gal1/Ox), sciatic nerves of $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mice were exposed just distal to the greater sciatic notch at the hip. In most mice, the sciatic was divided into two branches at this point; one branch was injected with 0.2 µL of either human immunoglobulin G (IgG), zymosan, or Gal1/Ox (all 5 µg/mL) using a fine-tipped Hamilton (Reno, NV) syringe, while the other branch was tied tightly with a 7-0 silk suture to mark the site of injection. Animals in this group ($n = 6$ per treatment, per strain) were killed three days later. Other surgeries involved tight ligation of the entire sciatic nerve just distal to the greater sciatic notch using 7-0 silk suture. To study the effect of Gal1-specific function-blocking antibody (Gal1-Ab; host goat) on injury-induced macrophage accumulation, we injected 1.5 µL of human IgG or Gal1-Ab into the nerve just distal to ligation, immediately following injury ($n = 5$ $Lgals1^{+/+}$ mice per treatment). These mice survived for three days after axotomy. To determine whether macrophage accumulation is altered in mice lacking Gal1, we harvested $Lgals1^{+/+}$ and $Lgals1^{-/-}$ tissue from animals that were uninjured, and those that had received tight sciatic nerve ligation 1, 3, 7, 14, 21, and 28 days previously ($n = 5$ per group, per strain). In sciatic nerve transplant experiments, we excised 1 cm-long pieces of sciatic nerve from $Lgals1^{+/+}$ or $Lgals1^{-/-}$ mice. Excised nerves were washed and maintained briefly in phosphate-buffered saline (PBS) to prevent them from drying out during the procedure. We grafted nerve explants into gaps created by resecting the sciatic nerve in host mice, so that both ends of the donor nerve abutted and stuck to the transected proximal and distal ends of the recipient nerve. Animals survived for 3 or 7
Dorsal rhizotomy was performed unilaterally from the 4th cervical level (C4) to the 2nd thoracic level (T2). Dorsal rhizotomy was performed as described in Ramer et al. (2001). Briefly, the dorsal roots of C3-T2 segments were exposed by removing small pieces of vertebrae, and the roots were transected midway between the DRG and dorsal root entry zone. Cervical spinal cords from rhizotomized $Lgal5^{+/+}$ and $^{-/-}$ mice were collected at 7, 14, and 21 days post-injury (dpi).

5.2.3 Tissue processing / immunohistochemistry
At the appropriate time, animals were injected with a lethal dose of chloral hydrate (1.75 g per kg body weight). Upon the loss of nociceptive reflexes, animals were perfused transcardially with 0.1 M PBS (pH 7.4) followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. Collected tissue was placed in 4% paraformaldehyde at 4°C overnight. The tissue was then cryoprotected for at least one day in 20% sucrose in PBS. Tissue was cut into longitudinal (sciatic nerve and DRG, 10 µm) or transverse (spinal cord, 16 µm) sections, thaw-mounted onto glass slides (Superfrost Plus; VWR, Mississauga, ON), and stored at -80°C. After blocking in 10% normal donkey serum, slides were incubated with rat anti-F4/80 (MCAP497, 1:200; Serotec, Raleigh, NC), and/or rabbit anti-galectin-1 (1:400; Kirin Breweries, Gunma, Japan) primary antibodies overnight. The next day, slides were washed three times in PBS and were incubated with Alexa 488-conjugated donkey anti-rat (A21208, 1:250; Molecular Probes, Carlsbad, CA) and/or Cy3-conjugated donkey anti-rabbit (711-165-152, 1:400; Jackson
ImmunoResearch, West Grove, PA) secondary antibodies for two hours. All antibodies were diluted with PBS, 0.2% Triton X-100, and 0.1% sodium azide.

### 5.2.4 Image analysis

Images were captured using an Axioplan 2 microscope (Zeiss, Jena, Germany), QImaging digital camera (Burnaby, BC), and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). All images for a given marker were captured under the same exposure settings. Images were analyzed using SigmaScan Pro 5 software (SPSS, Chicago, IL). We estimated the density of macrophages in a particular section of tissue by using an intensity threshold to analyze the area of the tissue occupied by F4/80 immunoreactivity. The area of interest was identified and an intensity threshold was applied on the entire image (the threshold was consistent for all images). For injured or injected sciatic nerves, we analyzed areas that were 3-6 mm distal to treatment; and for DRGs, we analyzed the entire area bounded by the DRG capsule. The ‘overlay math’ function allowed us to determine the density of F4/80-immunoreactive (-IR) cells and their processes within these areas of interest. Using SigmaPlot 2001 (SPSS) we calculated macrophage density by dividing the total area of F4/80-IR in a given image by the total area of interest. Thresholds defining F4/80-IR areas were used for analyzing the density of macrophages, since F4/80-IR cells were often grouped together and were therefore impossible to count reliably. For all analyses, four images per animal were analyzed and averaged. Data are expressed as mean proportion ± standard error of the mean. Data were analyzed using GraphPad InStat (San Diego, CA): Tukey-Kramer multiple comparisons tests were used to calculate significance ($p < 0.05$).
5.3 Results

5.3.1 The density of Gal1-immunoreactive cells is increased in distal nerve following sciatic nerve injury

Although we have shown previously that Gal1 mRNA is upregulated in the peripheral portion of the dorsal root following dorsal rhizotomy (McGraw et al., 2005a), the up-regulation of Gal1 protein following peripheral nerve injury has never been confirmed immunohistochemically. We characterized Gal1-IR at various timepoints after tight sciatic nerve ligation in the distal nerve of \textit{Lgals1}^{+/+} mice (Fig. 5.1). The density of Gal1-IR cells was higher in distal nerve at three dpi compared to uninjured control nerve (\( p < 0.05 \)). Gal1-IR partially co-localized with F4/80-positive macrophages in both uninjured and axotomized nerve (arrowheads, Fig. 5.1), and the intensity of Gal1-IR in these cells was enhanced following injury. This suggests that macrophages, in addition to Schwann cells and neurons, may contribute to the expansion of the Gal1 pool in the injured peripheral nerve.

5.3.2 Endogenous Gal1 is necessary for the typical injury-induced macrophage response during Wallerian degeneration

To establish whether extracellular Gal1 expressed within the sciatic nerve has a role in macrophage accumulation following peripheral axotomy, we compared macrophage density in sciatic nerves distal to ligation three days post-operation. \textit{Lgals1}^{+/+} mice were divided into three groups: injury only, injury plus IgG injection, and injury plus injection of Gal1-Ab (Fig. 5.2). Macrophage density was very low in the unmanipulated contralateral sciatic nerve (Fig. 5.2; cont.). Sciatic nerve ligation led to a massive accumulation of macrophages by three dpi (Fig. 5.2; ispi.). Injection of IgG solution significantly increased injury-induced macrophage accumulation (\( p < 0.001 \); Fig. 5.2, +IgG) (cf. Li et al. (2007)).
Figure 5.1. Galectin-1-immunoreactive (Gal1-IR) cell density is increased in the distal nerve following peripheral axotomy. The density of Gal1-IR cells was examined in the uninjured \( Lgals1^{+/+} \) sciatic nerve, and at various timepoints following injury in the distal nerve. The peak Gal1-IR density was observed at 3 days post-injury (dpi). Gal1-IR (red) partially co-localized with F4/80-IR (green) at all timepoints (arrowheads), and intensity and density of both proteins were increased in the distal nerve by axotomy. Scale bar, 200 µm.
**Figure 5.2.** The axotomy-induced increase in macrophage density in the *Lgals1*+/+ sciatic nerve distal to ligation was attenuated by injection of Gal-1-specific function-blocking antibody (Gal1-Ab) at 3 days post-injury. Photomicrographs show F4/80-IR in sciatic nerve contralateral to injury (cont.); ligated, untreated sciatic nerve (uninj.); ligated, IgG-treated sciatic nerve (+IgG); and ligated, Gal1-Ab-treated sciatic nerve (+Gal1-Ab). Axotomy-induced macrophage accumulation in the Gal1-Ab-treated nerve was significantly less than both positive controls. Dagger indicates significant difference between IgG-injected and ipsilateral untreated groups. Asterisk indicates significant difference between Gal1-Ab treated group and all other groups. Scale bar, 200 µm.
Sciatic nerve, 3 days post-axotomy

Density of F4/80-IR
In contrast, Gal1-Ab injection into the nerve just distal to ligation at the time of injury attenuated the axotomy-induced increase in F4/80-IR density by more than 50% ($p < 0.001$; Fig. 5.2, +Gal1-Ab), which is especially remarkable given the increase in macrophage density induced by injection of non-specific IgG. Therefore, extracellular Gal1 is required for the normal macrophage response to nerve injury in wild-type mice.

5.3.3 Mice lacking Gal1 exhibit impaired macrophage responses following peripheral axotomy

Since Gal1-Ab injection had a significant effect on macrophage accumulation following sciatic nerve ligation, we were interested in whether the macrophage response is altered in $Lgals1^{+/+}$ mice following sciatic nerve ligation. We completed a timecourse of injury-induced macrophage accumulation in the sciatic nerve, L5 DRG, and L5 ventral horn of $Lgals1^{+/+}$ and $Lgals1^{-/-}$ mice. In the sciatic nerve distal to ligation (Fig. 5.3), macrophage density had increased significantly in both strains by one dpi, and density remained elevated for at least 21 dpi. Macrophage accumulation in the nerve distal to axotomy peaked at three dpi in $Lgals1^{+/+}$ mice ($p < 0.05$). This was significantly different from the highest density in $Lgals1^{-/-}$ mice ($p < 0.001$), which occurred around 14 dpi. F4/80-IR density in the nerve distal to injury differed in $Lgals1^{-/-}$ compared to $Lgals1^{+/+}$ mice at various timepoints: at one dpi ($p < 0.05$); at three dpi ($p < 0.001$); at 14 dpi ($p < 0.01$); and at 21 dpi ($p < 0.01$). Thus, injury-induced macrophage accumulation in the distal sciatic nerve is both delayed and diminished in $Lgals1^{-/-}$ mice.

A similar, but less pronounced phagocytic cell accumulation occurs in the L5 DRG following sciatic nerve ligation (Hu and McLachlan, 2002; Hu and McLachlan, 2003; Lu and Richardson, 1993). The L5 DRG contains a large sensory neuron population that
Figure 5.3. Axotomy-induced accumulation of macrophages was delayed and diminished in sciatic nerves of $Lgals1^{-/-}$ mice, as visualized by F4/80-IR. We examined the nerve distal to sciatic nerve ligation. In the $Lgals1^{+/+}$ sciatic nerve ipsilateral to injury, peak macrophage density was observed at 3 days following injury. In $Lgals1^{-/-}$ mice, maximal macrophage accumulation was not achieved until 14 days after lesion, and the peak macrophage density was attenuated in these mice relative to $Lgals1^{+/+}$ mice. ‘cont’, contralateral. Unless indicated otherwise, photomicrographs represent tissue ipsilateral to injury. Asterisks indicate significant differences between $Lgals1^{-/-}$ nerves and $Lgals1^{+/+}$ nerves at same timepoint; dagger indicates significant difference between peak densities in $Lgals1^{-/-}$ (14 days) and $Lgals1^{+/+}$ (3 days) groups. Scale bar, 200µm.
projects to peripheral targets via the sciatic nerve. In the L5 DRG (Fig. 5.4), F4/80-IR density increased significantly by seven dpi in \textit{Lgals1}^{+/+} mice ($p < 0.001$). This was also the timepoint at which macrophage density peaked in these mice ($p < 0.005$), and this density differed significantly from that in \textit{Lgals1}^{--} mice ($p < 0.001$). Macrophage accumulation peaked at 14 dpi in \textit{Lgals1}^{--} DRGs ipsilateral to injury ($p < 0.05$); this peak was not significantly different from that induced by injury in \textit{Lgals1}^{+/+} mice. Therefore, the induction of macrophage accumulation by sciatic nerve injury was delayed (but not diminished) in the L5 DRG of mice lacking Gal1.

Immune cells also accumulate in regions associated with degeneration following dorsal root injury. We found that F4/80-IR density in the dorsal horn and dorsal column ipsilateral to septuple cervical dorsal rhizotomy was significantly lower in \textit{Lgals1}^{+/+} compared to \textit{Lgals1}^{--} mice at 21 dpi ($p < 0.05$; Fig. 5.7 [Supplementary Fig. 1 in published paper] and data not shown). There was no difference between genotypes in the uninjured dorsal horns and columns, nor was there a difference between genotypes on the ipsilateral side at 7 or 14 dpi. F4/80-IR density in the \textit{Lgals1}^{+/+} dorsal horn was increased after both sciatic nerve ligation and dorsal rhizotomy, but the increase induced by sciatic nerve injury was more pronounced (Fig. 5.8 [Supplementary Fig. 2 in published paper]).

### 5.3.4 Exogenous Gal1/Ox facilitates macrophage accumulation in uninjured \textit{Lgals1}^{+/+}, but not \textit{Lgals1}^{--} sciatic nerve

In order to determine whether Gal1/Ox effects macrophage accumulation, we injected 1 ng of either human IgG (negative control), the yeast particulate zymosan (positive control) or Gal1/Ox into one uninjured branch of \textit{Lgals1}^{+/+} and \textit{Lgals1}^{--} sciatic nerves
Figure 5.4. Injury-induced macrophage accumulation was delayed in L5 dorsal root ganglia (DRGs) of Lgals1\(^{+/+}\) mice, as visualized by F4/80-IR. In the Lgals1\(^{+/+}\) DRG ipsilateral to injury, maximal macrophage accumulation was observed at 7 days following sciatic nerve ligation. In contrast, Lgals1\(^{+/+}\) DRGs associated with injury exhibited maximal macrophage density at 14 days after injury. The peak macrophage densities were not significantly different between strains. ‘cont.’, contralateral; ‘ipsi.’, ipsilateral. Unless indicated otherwise, photomicrographs represent tissue ipsilateral to injury. Asterisk indicates significant difference between Lgals1\(^{+/-}\) and Lgals1\(^{+/+}\) DRGs at same timepoint. Scale bar, 200\(\mu\)m.
**Lgals1** +/+

uninj.

1d

3d

7d

14d

21d

**Lgals1** −/

F4/80

---

**Graph:**

<table>
<thead>
<tr>
<th>Days post-axotomy</th>
<th>Density of F4/80-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninj.</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
</tr>
<tr>
<td>14</td>
<td>0.03</td>
</tr>
<tr>
<td>21</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Legend:**

- **Lgals1** +/+
- **Lgals1** −/

*Significant difference.*
Injection of human IgG had little effect on F4/80-IR density compared to zymosan three days after injection. Gal1/Ox's effect on macrophage accumulation differed between strains: Gal1/Ox injection caused significantly more accumulation in $Lgals1^{+/+}$ nerves than did IgG ($p < 0.001$); however, exogenous Gal1/Ox did not increase macrophage density higher than control levels in $Lgals1^{-/-}$ nerves. Whereas macrophage density in zymosan-treated $Lgals1^{+/+}$ and $Lgals1^{-/-}$ nerves was not significantly different, macrophage density was significantly higher in Gal1/Ox-treated $Lgals1^{+/+}$ nerves than in $Lgals1^{-/-}$ nerves ($p < 0.01$). In addition, macrophage density in Gal1/Ox-treated $Lgals1^{+/+}$ nerves did not differ significantly from zymosan-treated $Lgals1^{+/+}$ nerves, although F4/80-IR was more widespread in zymosan-treated sciatic nerves. These results suggest that: (1) Gal1/Ox is sufficient to direct the accumulation of $Lgals1^{+/+}$ macrophages in the absence of injury, and (2) macrophages from $Lgals1^{-/-}$ mice have a lower Gal1/Ox sensitivity than wild-type macrophages. It is possible that Gal1 is required for normal macrophage development: when differentiating monocytic cells do not express or are not exposed to Gal1 (in the $Lgals1^{-/-}$ mouse), they may not develop sensitivity to this protein or express its cognate receptor.

5.3.5 Nerve transplantation in $Lgals1^{-/-}$ mice: compensation and (lack of) responsiveness to Gal1

We performed a transplant experiment in order to establish whether macrophage accumulation in injured $Lgals1^{-/-}$ mice could be rescued by nerve grafts from wild-type animals. We transplanted $Lgals1^{+/+}$ and $Lgals1^{-/-}$ donor nerves into $Lgals1^{+/+}$ and $Lgals1^{-/-}$ host mice, and studied the density of F4/80-IR at three and seven dpi in the transplanted nerve (Fig. 5.6). At three dpi, $Lgals1^{+/+}$ transplant nerves in $Lgals1^{+/+}$ host mice had a higher macrophage density than any other group ($p < 0.05$; Fig. 5.6a-d, i).
This suggests that Gal1 is required for the typical macrophage response to injury at this
timepoint, and that Gal1 in the Gal1-positive nerve is not sufficient to rescue the \textit{Lgals1}^{-/-} nerve phenotype. By seven dpi, the F4/80-IR density was significantly higher in both
groups that received a \textit{Lgals1}^{+/+} nerve than at three dpi, and macrophage density in these
nerves was not significantly different from the \textit{Lgals1}^{+/+} nerve in the \textit{Lgals1}^{+/+} host.
Interestingly, macrophage density in \textit{Lgals1}^{+/+} nerves in \textit{Lgals1}^{-/-} hosts was significantly
different from all other groups at seven dpi ($p < 0.001$; Fig. 5.6e-i), and the density in the
\textit{Lgals1}^{+/+} nerve into \textit{Lgals1}^{-/-} host group at seven dpi was not different from that at three
dpi. Taken together, these results suggest that Gal1 is required for the typical
macrophage response to injury, that Gal1 in wild-type nerve is not sufficient to rescue
macrophage accumulation in \textit{Lgals1}^{-/-} nerve, and that there are compensatory
mechanisms in place within the \textit{Lgals1}^{-/-} nerve that allow for delayed macrophage
accumulation up to \textit{Lgals1}^{+/+} levels.
Figure 5.5. Oxidized galectin-1 (Gal1/Ox) injection into uninjured sciatic nerve was sufficient to facilitate macrophage accumulation in Lgals1<sup>+/+</sup> (but not Lgals1<sup>-/-</sup>) mice. F4/80-IR was used to assess macrophage density in uninjured Lgals1<sup>+/+</sup> and Lgals1<sup>-/-</sup> mice injected with human IgG, zymosan, and Gal1/Ox three days after injection. As expected, nerves injected with zymosan had a significantly higher macrophage density in both strains relative to the group that received IgG injection. In Lgals1<sup>+/+</sup> sciatic nerves, injection of Gal1/Ox induced greater accumulation of macrophages than IgG injection. In contrast, macrophage density in Lgals1<sup>-/-</sup> nerves injected with Gal1/Ox was not significantly different from those that were injected with IgG. Asterisks indicate significant differences between zymosan- or Gal1/Ox-treated groups and the IgG group; dagger indicates significant differences between Lgals1<sup>-/-</sup> Gal1/Ox-treated nerves and both Lgals1<sup>+/+</sup> Gal1/Ox-treated and Lgals1<sup>-/-</sup> zymosan-treated groups. Scale bar, 100 µm.
Figure 5.6. Macrophage accumulation in segments of sciatic nerve transplanted to Lgals1\(^{+/+}\) mice (left panels) or Lgals1\(^{-/-}\) mice (right panels) three and seven days post-transplantation. At three days post-transplant, macrophage accumulation was greatest in Lgals1\(^{+/+}\) mice that received Lgals1\(^{+/+}\) transplants (a, i). Interestingly, transplantation of a Lgals1\(^{+/+}\) nerve segment into Lgals1\(^{-/-}\) mice did not normalize macrophage accumulation at three days post-transplantation (d, i). At seven days post-transplantation, F4/80-IR density in the Lgals1\(^{+/+}\) nerve segment transplanted into Lgals1\(^{-/-}\) mice was significantly lower than all other transplant groups (h, i). ‘trans’, nerve transplanted during surgery. Asterisks indicate significant differences between Lgals1\(^{+/+}\) \(\rightarrow\) Lgals1\(^{+/+}\) transplant and all other groups at 3 days; dagger indicates significant difference between Lgals1\(^{+/+}\) \(\rightarrow\) Lgals1\(^{-/-}\) group and all other groups at 7 days. Scale bar, 200\(\mu\)m.
**Figure 5.7.** Dorsal rhizotomy-induced microglial/macrophage accumulation in the dorsal horn was reduced at 21 dpi in *Lgals1*<sup>−/−</sup> mice. The dorsal horn ipsilateral to rhizotomy consistently had a higher density of F4/80-IR than did the contralateral side in both mouse strains. The only significant difference in accumulation between the ipsilateral dorsal horns of *Lgals1<sup>+/+</sup>* and *Lgals1<sup>−/−</sup>* mice occurred at 21 dpi. ‘cont.’, contralateral; ‘ipsi.’, ipsilateral. Asterisk indicates *p* < 0.05 compared to contralateral side. † indicates *p* < 0.05 compared to contralateral side in *Lgals1*<sup>+/+</sup> mice at 21 dpi. ‡ indicates *p* < 0.05 compared to ipsilateral side in *Lgals1*<sup>+/+</sup> mice at 21 dpi. Scale bar, 100 µm.
Figure 5.8. Comparison of macrophage/microglial accumulation in the dorsal horn of Lgals1^{+/+} mice after sciatic nerve ligation or dorsal rhizotomy. The dorsal horn ipsilateral to injury consistently had a higher F4/80-IR density than did the contralateral side. There was a greater macrophage/microglial accumulation in the dorsal horn after sciatic nerve ligation compared to dorsal rhizotomy. Microglial/macrophage accumulation in the contralateral dorsal horn at 21 dpi was significantly higher than in the contralateral dorsal horn at 7 and 14 dpi for both injuries. ‘axo’, axotomy of the sciatic nerve; ‘rhiz’, dorsal rhizotomy; ‘cont.’, contralateral; ‘ipsi.’, ipsilateral. Asterisk indicates $p < 0.05$ compared to contralateral side. † indicates $p < 0.05$ compared to ipsilateral side after sciatic nerve ligation. ‡ indicates statistically significant increase in contralateral density.
5.4 Discussion

5.4.1 Overview

In the present study, we have established that Gal1 activity in peripheral nerve positively regulates accumulation of immune cells in the nervous system following peripheral axotomy. Using a peripheral nerve injury model in transgenic mice, we showed that: (1) Gal1 is required for normal injury-induced macrophage accumulation in the nerve distal to injury, as well as to the ipsilateral L5 DRG; (2) exogenous Gal1/Ox facilitates macrophage accumulation in uninjured \( Lgals1^{+/+} \) sciatic nerve; (3) Gal1/Ox is not sufficient to direct macrophage accumulation in \( Lgals1^{-/-} \) sciatic nerve; and (4) compensatory mechanisms in \( Lgals1^{-/-} \) mice allow for injury-induced macrophage accumulation to occur at later timepoints. These results show that Gal1, which is upregulated transiently in the distal nerve around three days post-axotomy, accelerates the early macrophage response to nerve injury.

5.4.2 Gal1 and the immune response

Gal1 is expressed by many leukocytes (dendritic cells and activated macrophages, B cells and T cells) (Blaser et al., 1998; Rabinovich et al., 1998; Zuniga et al., 2001), and has critical immunoregulatory functions (Rabinovich et al., 2007). Although Gal1 is generally thought to act as an immunosuppressive agent, previous studies have described conflicting roles for Gal1 in the modulation of the immune response. For instance, Gal1 regulates the survival of activated T cells, but not naïve T cells or monocytes (Perillo et al., 1995; Rabinovich et al., 1998; Barrionuevo et al., 2007). Moreover, Gal1 treatment increases phagocytosis by untreated monocytes, but decreases phagocytosis by monocytes activated by interferon-\( \gamma \) (Barrionuevo et al., 2007). Thus, the response of a particular immune cell to Gal1 \textit{in vivo} is the net result of
5.4.3 Role of Gal1 following peripheral nerve injury

Previous studies have shown that Gal1 promotes axonal regeneration following injury in a variety of models: mixed (sensory and motor) peripheral nerve transection and tubulization (Horie et al., 1999), facial (motor) nerve injury (McGraw et al., 2004a; McGraw et al., 2004b), and optic nerve injury (Okada et al., 2005). There are four mechanisms that may underlie the Gal1’s actions on nerve repair. First, Gal1 may be acting directly on the regenerating axon itself (Horie et al., 1999; Inagaki et al., 2000; Horie et al., 2004; Miura et al., 2004). Second, Gal1 promotes Schwann cell migration following injury (Horie et al., 1999; Fukaya et al., 2003), presumably allowing these cells to expedite Wallerian degeneration and to form bands of Büngner for regrowing axons. Third, Gal1/Ox activates macrophages by binding to a cell surface receptor, and may recruit them to the site of injury through this ligand-receptor interaction (Horie et al., 2004). Finally, Gal1 may indirectly enhance regeneration by causing degeneration of detached axons following axotomy (Plachta et al., 2007). We will consider the latter two processes in more detail, since these involve or cause the accumulation of macrophages.

Gal1 may act directly on macrophages to elicit their accumulation following peripheral axotomy. This injury-induced accumulation is the result of both hematogenous monocyte invasion and resident macrophage proliferation. Since blood-derived macrophages constitute the majority of the macrophage population in the injured nerve soon after injury (Mueller et al., 2003), it is possible that Gal1 is affecting the homing-in or extravasation of these cells. Gal1/Ox activates macrophages by binding an
unidentified receptor that initiates an intracellular tyrosine phosphatase phosphorylation cascade (Horie et al., 2004), and a similar action could underlie axotomy-induced recruitment of these cells. However, Gal1 inhibits trans-migration of bone marrow progenitor cells, granulocytes and monocytes across an endothelial cell layer in vitro and prevents their mobilization from bone marrow in vivo (Kiss et al., 2007), and Gal1 treatment inhibits neutrophil extravasation, chemotaxis, and transendothelial migration (Rabinovich et al., 2000; La et al., 2003). These results suggest that Gal1 actually inhibits the mobilization, chemotaxis and migration of these immune cells, and would attenuate their accumulation. Thus, Gal1 may not recruit hematogenous macrophages directly, and another mechanism might underlie ability to promote phagocyte accumulation following nerve injury.

Gal1-induced degeneration of axons could also have a key role in immune cell accumulation following injury. Gal1 has recently been implicated in the axon degeneration: this process involves signaling intrinsic to the neuron, as Gal1 treatment of isolated embryonic stem cell-derived neurons leads to the degeneration of their neurites (Plachta et al., 2007). These authors also showed that the elimination of peripheral nerve endings is delayed in Lgals1−/− mice following sciatic nerve injury. Interestingly, we found that Gal1/Ox injection induced axon degeneration in the sciatic nerve in our model, as axons (identified by PGP-9.5-IR) were discontinuous and beaded up more in areas surrounding Gal1/Ox injection than in areas adjacent to IgG injection (data not shown). Thus, the oxidized form of Gal1 may be able to cause local axonal degeneration, which would lead to the release of cytokines and other factors that promote immune cell activation and recruitment. The pro-inflammatory cytokine cascades initiated by Gal1-induced degeneration may overcome Gal1’s signal to prevent immune cell extravasation, leading to the net influx of neutrophils and macrophages.
Degeneration of detached axon and myelin debris is crucial for efficient axonal regeneration following peripheral axotomy. The inability to remove this debris is the major factor that impedes axon regeneration in Wallerian degeneration slow (Wld^S) mice: fewer macrophages accumulate in the nerve distal to injury, and this results in delayed clearance of myelin and axonal debris (Brown et al., 1994). Interestingly, assisting degeneration by crushing the distal nerve at the time of injury increases the rate of regeneration in these mice, suggesting that the residual intact debris acts as a more robust barrier for regrowing axons. Thus, delayed and diminished macrophage accumulation in the distal nerves of injured Lgals1^-^- mice may underlie delayed peripheral axon regeneration in nerves of these mice.

The timecourse of peripheral axotomy-induced macrophage accumulation has been characterized extensively. For instance, Bendszus and Stoll (2003) examined axotomy-induced macrophage infiltration in rat using ED1-IR and found that macrophage density increased in the injured nerve up to 14 days after sciatic nerve crush. Likewise, Avellino et al. (2004) found the highest density of macrophages present between 14 and 21 days after sciatic nerve transection in rat. In contrast, Perry et al. (1987) and de la Hoz et al. (2003) showed that macrophage density peaked around five days after mouse sciatic nerve crush and transection, respectively. We found that macrophage accumulation peaked around three dpi in our wild-type mice, although we did not have a five day timepoint. As mentioned above, some previous studies showed a more protracted wave of macrophage accumulation. We attribute the disparities between reported timelines of macrophage infiltration to differences in techniques used: the animal model, injury model, antibody, and analysis method used all have important roles in defining how we observe macrophages' response to nerve injury (time and intensity of response).
The results of our nerve transplant experiment (Fig. 6) proved to be intriguing. Not surprisingly, macrophage density was highest in $Lgals1^{+/+}$ hosts with $Lgals1^{+/+}$ donor nerves at 3 dpi. $Lgals^{+/+}$ transplant nerves into $Lgals1^{-/-}$ recipients did not rescue the knockout phenotype at this timepoint. This finding is congruent with the results of our gain-of-function experiment, which showed that $Lgals1^{-/-}$ macrophages are not responsive to Gal1/Ox. The fact that macrophage density in $Lgals1^{+/+}$ and $Lgals1^{-/-}$ animals that received $Lgals1^{-/-}$ transplants was at the same level as $Lgals1^{+/+}$ hosts with $Lgals1^{+/+}$ transplant nerves at 7 dpi suggests that a compensatory mechanism is initiated in the absence of Gal1, which promotes macrophage accumulation at this later timepoint. Interestingly, this compensatory mechanism does not seem to be present in Gal1-positive nerves: macrophage density in $Lgals1^{+/+}$ nerves transplanted into $Lgals1^{-/-}$ hosts remains lower than in other groups at 7 dpi. Macrophage density in this group is lower than in others at both timepoints, which suggests that $Lgals1^{-/-}$ macrophages might not be responsive to Gal1. Taken together, these results suggest that Gal1 may be involved in a cytokine cascade evoked by nerve injury.

5.4.4 Conclusions

Although peripheral axons have the ability to regenerate following injury, repair and functional recovery in humans is usually incomplete and inadequate. Gal1 is one factor that may improve nerve repair following injury: Gal1 may act both within the injured neuron, and within its axon’s environment, to promote regeneration. In the current study, we have defined a role for Gal1 in the axonal environment. We have shown that Gal1 is necessary and sufficient to direct typical macrophage accumulation following peripheral nerve injury, and our data suggest that Gal1 may enhance the efficiency of Wallerian degeneration in the PNS by promoting phagocyte accumulation following nerve injury.
5.5 References


6 Discussion: exploring galectin-1’s impact on axon growth

In summary, every sectioned nerve regenerates its axons by means of sprouts from the central stump which, as Tello proved, cross the scar and assail the peripheral stump to reach the external sensory and muscular terminations. Arriving at their destination, attracted no doubt by some substance (or physical influence as yet unknown) arising from the nuclei of the terminal apparatus, the destroyed motor arborization moulds itself anew.

- Ramon y Cajal, 1991, p. 92

6.1 Overview

In this dissertation, I have studied the effects of Gal1 on developmental and regenerative axon growth and associated cellular events. I found that Gal1 has critical roles in the proper development of sensory neurons and their central connections (chapter two). I also established that Gal1 expression – both within DRG neurons and their environment – is correlated with the regenerative capacity of sensory axons (chapter three). I then defined whether Gal1 intrinsic or extrinsic to the injured neuron likely played a role in enhancing axon growth: whereas Gal1 had no significant direct effect on isolated DRG neurons (chapter four), the protein was necessary for typical PNI-induced macrophage accumulation and Gal1/Ox was sufficient to direct macrophage accumulation even in the uninjured nerve (chapter five).

In chapter two, I established that Gal1 has an important role in the development of DRG neuron subpopulations, and is required for proper targeting of central terminals of primary afferents in the spinal dorsal horn. The central terminal fields of nociceptive subpopulations were shifted ventrally in dorsal horns of Lgals1-/- mice. I demonstrated that fewer dorsal horn neurons in Lgals1-/- mice were activated following noxious stimulation of their forepaw, despite having a similar complement of total dorsal horn
neurons as in \textit{Lgals1}^{+/+} mice. Interestingly, these anatomical alterations were accompanied by significant sensory deficits: \textit{Lgals1}^{-/-} mice were less sensitive to noxious heat and cold compared to \textit{Lgals1}^{+/+} mice. These data suggest that Gal1 has a critical role in specifying development of DRG neuron subtypes, and that Gal1 is essential for the proper wiring of connections between nociceptive primary afferents and target second order neurons in the spinal dorsal horn.

In chapter three, I examined the regulation of Gal1 by axotomy of peripheral and central processes of DRG neurons. I found that Gal1 mRNA and protein expression were correlated with axons’ regenerative capacities in all cases examined. Gal1 mRNA and protein were expressed in a higher proportion of DRG neurons following PNI, but not DRI. In addition, Gal1 mRNA was increased in the growth-permissive PNS portion of the dorsal root, but not in the inhibitory CNS compartment of the root. Therefore, the data imply that Gal1 may act within the neuron as a RAG, and/or in the environment of the regrowing axon to enhance axon regeneration.

In chapter four, I sought to establish whether Gal1 affects axon growth directly. Using dissociated DRG neurons from \textit{Lgals1}^{-/-} mice, I demonstrated that Gal1 is not necessary for axonal extension from these peripheral neurons. In addition, I examined neurite growth from rat DRG neurons in the presence of exogenous Gal1/Ox, and found that the protein had no significant effects on any of the parameters studied. My results imply that Gal1 likely has little direct effect on DRG neurons, and that Gal1’s effects on axon regeneration \textit{in vivo} probably involve cells within the neuron’s surroundings.

In chapter five, I explored the influence of Gal1 on macrophage accumulation in peripheral nerves. Using Gal1-Ab, I showed that Gal1 is required for the typical PNI-
induced accumulation of macrophages at an early stage after injury. I then studied macrophage density in injured $Lgals1^{+/+}$ and $Lgals1^{-/-}$ sciatic nerves, and established that Gal1 is required for the typical timecourse of macrophage accumulation following PNI. Interestingly, Gal1/Ox was sufficient to facilitate macrophage accumulation in the uninjured nerve. My results show that Gal1 has a key role in mediating the accumulation of these immune cells following injury. The data imply that Gal1/Ox-mediated macrophage accumulation may be one mechanism that underlies the enhancement of axon regeneration by Gal1 $\textit{in vivo}$ (Fig. 6.1).

Overall, these results point to an important role for Gal1 in developmental and regenerative axon growth. The pattern of expression of Gal1 – both during development and after nerve injury in the adult – suggest that Gal1 might positively regulate axon growth, and there is accumulating functional evidence that support a role for Gal1 in the process. In this dissertation, I established an important function for Gal1 in the development of specific axon subpopulations, and demonstrated that Gal1 likely acts by mechanisms extrinsic to the injured neuron to promote axon regeneration in the adult rodent.
Figure 6.1. Schematic representing mechanisms that underlie Gal1/Ox-mediated peripheral axon regeneration. The major cell types resident to the peripheral nerve – Schwann cells, neurons, and macrophages – all secrete Gal1 (Rabinovich et al., 1998; Sango et al., 2004). Following peripheral nerve injury (lightning bolt), release of Gal1 into the extracellular milieu is increased: extracellular Gal1 is then free to bind its (unidentified) receptor on macrophages (Horie et al., 2004). Binding of Gal1/Ox to this receptor alters intracellular signaling pathways within macrophages. In this dissertation, I established that Gal1/Ox also effects macrophage accumulation following injury, possibly through binding the aforementioned receptor. The Gal1/Ox-activated macrophages – which are present in large numbers due, in part, to Gal1/Ox – then release an unknown factor that enhances Schwann cell migration and axon regeneration (Horie et al., 2004). Gal1/Ox also promotes Schwann cell migration directly (not shown) (Echigo et al., 2010). I also found that Gal1 did not elicit axon growth directly from cultured sensory neurons. Thus, Gal1/Ox enhances peripheral axon regeneration indirectly by recruiting and activating monocytes/macrophages. Hamburger represents axon/myelin debris in the distal nerve.
6.2 Fateful moments: galectin-1 and DRG neuron development

Previous studies characterized the spatiotemporal regulation of developmental Gal1 expression in the DRG and spinal cord (Dodd and Jessell, 1986; Regan et al., 1986): their results suggested that Gal1 might influence the development of DRG neurons and/or their connections. In addition, Gal1 has been implicated in axon targeting of subpopulations of olfactory axons to appropriate targets in the olfactory bulb (Puche et al., 1996); therefore, Gal1 might have a similar role in the guidance of DRG neuron projections. In chapter two, I used \textit{Lgals1}^{+/-} mice to demonstrate that Gal1 has a critical role in the proper development of DRG neuron subpopulations and their central projections. I also showed that these mice had corresponding deficits in sensitivity to noxious heat and cold stimuli.

6.2.1 Galectin-1 is required for the development of specific DRG neuron subpopulations

\textit{Lgals1}^{+/-} DRGs contain approximately 20 percent fewer IB4-binding neurons and about 10 percent more NF200-IR neurons relative to \textit{Lgals1}^{+/+} DRGs (there was no significant difference in the proportion of CGRP-IR DRG neurons between genotypes). The differences in the proportion of smaller IB4-binding and larger NF200-IR DRG neurons between genotypes was also associated with anatomical differences in the dorsal root: there were significantly more large-diameter fibres in the dorsal root. These results suggest that Gal1 regulates the phenotype of DRG neurons: Gal1 be involved in a switch that controls the development or differentiation of specific neuronal subtypes.

Recent studies have uncovered details of the transcriptional program that controls sensory neuron neurogenesis and subtype specification. Neurogenesis in DRGs is
initiated by the transcription factors Neurogenin-1 and Neurogenin-2 (Ma et al., 1999; Fode et al., 1998), which then lead to the expression of other transcription factors called Neurod1 and Neurod4 (Fode et al., 1998). After the neurogenic phase (around E9.5-10.5; Anderson, 1999), prospective neurons become post-mitotic and begin to express genes that define their ultimate function. The pan-sensory transcription factors Islet-1 and Brn3a control genes that are essential for the development of sensory neuron subpopulations: development in the absence of Islet-1 is associated with a massive loss of TrkA-positive DRG neurons (Sun et al., 2008); conditional knockout of Brn3a in trigeminal sensory neurons results in a profound decrease in the proportion of TrkA- and TrkC-expressing neurons, and a concomitant increase in the proportion of TrkB-expressing neurons (Dykes et al., 2010). Rather than controlling Trk expression directly, Islet-1 and Brn3a likely regulate the expression of Runx transcription factors (Dykes et al., 2010).

Runx transcription factors have key roles in the development of DRG neuron subclasses. Soon after their formation during embryogenesis, all nociceptors express TrkA (Farinas et al., 1998) and the transcription factor Runx1 (Chen et al., 2006a). Within the first three postnatal weeks, a major Runx1-dependent shift in nociceptive neuron phenotype occurs. Some neurons downregulate TrkA and upregulate Ret; these generally represent the IB4-binding population in the adult. Meanwhile, those neurons that maintain TrkA expression downregulate Runx1 (Chen et al., 2006a); these are mostly CGRP-IR neurons in the adult. Neuronal \(\text{trkC}\) expression is activated by the transcription factor Runx3 (Kramer et al., 2006). In contrast, the proportion of neurons expressing TrkB is restricted by over-expression of either Runx1 or Runx3 and is increased by loss of either Runx (Levanon et al., 2002; Kramer et al., 2006; Inoue et al., 2007), suggesting that the TrkB phenotype is a default pathway for DRG neuron
Both TrkC- and TrkB-expressing neurons are generally large-diameter and NF200-IR.

Differences in the distribution of DRG neuron phenotypes in \( Lgals1^{-/-} \) mice might be explained by altered expression of Runx transcription factors in their neurons. \( Lgals1^{-/-} \) mouse DRGs contain a smaller proportion of IB4-binding neurons, which are those that express Runx1 throughout later development. Their DRGs also have a higher proportion of NF200-IR neurons, which are those that either express Runx3 (TrkC) or neither Runx1 nor Runx3 (TrkB). It is therefore possible that a smaller proportion of \( Lgals1^{-/-} \) neurons upregulate Runx1 during development compared to \( Lgals1^{+/+} \) neurons. These Runx1-negative \( Lgals1^{-/-} \) neurons could instead develop into different subtypes: they might not upregulate Runx3 either and therefore develop into TrkB-expressing, NF200-IR neurons; and/or they might upregulate Runx3 instead of Runx1, and develop into TrkC-expressing, NF200-IR neurons. It is also possible that Gal1 regulates expression of the transcription factors that control Runx expression.

6.2.2 Wiring before firing: galectin-1’s role in developmental axon targeting

Previous studies using \( Lgals1^{-/-} \) mice had described a role for Gal1 in the typical targeting of olfactory axon subpopulations – those that project from the olfactory epithelium to the caudal olfactory bulb (Puche et al., 1996). In chapter two, I established a similar role for Gal1 in the targeting of the central projections of DRG neuron subpopulations to correct targets in the spinal dorsal horn. In \( Lgals1^{-/-} \) mice, nociceptive primary afferent axons – which comprise the IB4-binding and CGRP-IR subpopulations – had terminal fields that extended further ventrally than they did in \( Lgals1^{+/+} \) mice. I also
demonstrated that these anatomical alterations in axon targeting had important functional and behavioural consequences. Therefore, Gal1 is required for the proper development and targeting of DRG neuron subpopulations. Furthermore, Gal1 is required for typical sensitivity to noxious heat and cold stimuli.

The mechanisms underlying Gal1’s effects on axon targeting remain undefined. As with axon regeneration, developmental axon targeting is regulated by factors intrinsic and extrinsic to the axon; Gal1 could be acting in either or both areas to refine axon pathfinding. Rotblat and colleagues’ (2004) finding that intrinsic Gal1 expression promotes neurite growth from PC12 cells supports a potential role for the protein within neurons. Conversely, studies from the 1990s suggest that environmental Gal1 may modulate axon growth from DRG neurons: Gal1 increases adhesion and fasciculation of embryonic DRG neurons (Outenreath and Jones, 1992), and enhances adhesion of and neurite growth from olfactory neurons (Mahanthappa et al., 1994; Puche et al., 1996). Gal1 also enhances the interaction between laminin and integrins (Mahanthappa et al., 1994). Therefore, Gal1 could act on central branches of embryonic nociceptive primary afferents to promote their fasciculation and/or outgrowth, ultimately defining their terminal zone in the spinal dorsal horn.

In addition to regulating terminal differentiation of DRG neurons during embryonic development, Runx transcription factors define the terminal zones of developing central DRG neuron branches. Runx1 confines nociceptive afferents to their appropriate dorsal horn laminae during outgrowth: development in its absence results in targeting defects of CGRP-IR (deeper projections; Yoshikawa et al., 2007) and/or IB4-binding (more superficial projections; Chen et al., 2006b). These two studies reported conflicting results on alterations in nociceptor projections that might be explained by the use of
different strains of Runx1 conditional knockout mice. Interestingly, Chen and colleagues (2006b) also found that the Runx1 mutants were less sensitive to noxious heat and cold stimuli, but had normal thresholds for mechanical stimuli. Although Runx1’s specific effects on axon targeting are not completely clear, the transcription factor controls central targeting of nociceptive afferents and sensitivity to noxious thermal stimuli; therefore, neuronal Gal1 might be involved in the expression or activity of Runx1. The Runx protein expressed specifically in TrkC-positive proprioceptors, Runx3, controls the targeting of these neurons’ central projections. In mice lacking Runx3, the central processes of proprioceptive axons fail to project to the ventral horn, and these mice have severe limb ataxia and abnormal posture (Inoue et al., 2002; Levanon et al., 2002).

Another unresolved issue concerns the oxidation state of the Gal1 that affects developmental axon growth. Although Gal1/Ox, but not Gal1/Red, seems to improve regenerative axon growth (Inagaki et al., 2000), the requirement for Gal1’s lectin activity in in vitro studies on developmental axon growth was not consistent. Outenreath and Jones (1992), who used embryonic DRG neurons, found that Gal1 promoted cell adhesion and axon fasciculation, and that this activity was independent of its lectin activity. These results suggest that Gal1/Ox (and perhaps Gal1/Red) could elicit these effects. In contrast, studies on early postnatal olfactory neurons, which showed that Gal1 caused cell adhesion and neurite outgrowth, demonstrated that Gal1’s lectin activity was required to regulate these processes (Mahanthappa et al., 1994; Puche et al., 1996). Future studies could establish which version(s) of Gal1 act on developing axons. Given that Gal1/Ox seems to have little direct effect on adult axons, and that Gal1/Red has such a wide array of potential binding partners, one might surmise that Gal1/Red could mediate DRG axon targeting during development.
In summary, Gal1 has intriguing effects on the development of DRG neurons and their connections. Defects in DRG axon targeting observed in mice lacking Gal1 could result from effects on the neuron and/or its environment, as in regenerative axon growth.
6.3 Good as new? Enhancing axon growth with galectin-1

Previous studies have shown that Gal1 is necessary and sufficient for typical axon regeneration using various *in vitro* and *in vivo* models of nerve injury (Fukaya et al., 2003; Horie et al., 1999; Horie et al., 2004; Inagaki et al., 2000; McGraw et al., 2004a; Okada et al., 2005). In chapters three, four and five, I sought to uncover the mechanisms underlying Gal1-mediated regeneration.

6.3.1 Gal1 expression is correlated with the regenerative capacity of injured DRG neurons

It was not previously known whether Gal1 acted within the neuron and/or within the axon’s environment to promote axon regrowth. Gal1-Ab treatment tempers axon regeneration (Horie et al., 1999), implying that extrinsic Gal1 may play an important role in regrowth. Other *in vitro* functional (Rotblat et al., 2004) and *in vivo* correlative (McGraw et al., 2004a) data support a role for intraneuronal Gal1 in regeneration.

In chapter three, I showed that Gal1 expression is correlated with an axons regenerative ability after PNI and DRI. A higher proportion of DRG neurons was positive for Gal1 mRNA and -IR after PNI, but not DRI, relative to uninjured controls. In addition, Gal1 mRNA was upregulated in the peripheral, but not central compartment of the dorsal root. Therefore, the results implied that Gal1 might act both within the neuron (as a RAG), and within its axon’s environment, to promote regeneration after PNI.

These findings fit well with previous studies on Gal1 expression and regenerative capacity. First, Gal1 seems to be expressed at higher levels in the CNS during development – when it is more growth-permissive – but at low levels in most CNS
regions in the adult (Akazawa et al., 2004; Hynes et al., 1990). In addition, Gal1 expression in neurons is correlated with their regenerative capacity in at least three other injury models. Following spinal nerve injury, cervical motor neurons upregulate Gal1 mRNA; this upregulation is maintained for at least 14 dpi (McGraw et al., 2004b). Interestingly, rubrospinal neurons, which are CNS neurons and cannot normally regenerate their axons, downregulate Gal1 mRNA following rubrospinal tract lesion; BDNF infusion (for one week) facilitates both axon regeneration (Kobayashi et al., 1997; Plunet et al., 2002) and Gal1 upregulation above control levels (McGraw et al., 2004b). Finally, peripherally-projecting facial motor neurons upregulate Gal1 mRNA after facial nerve injury; this upregulation is attenuated upon reconnection with peripheral targets by 14 dpi, whereas neurons with resected axons maintain high Gal1 levels at 14 dpi (McGraw et al., 2004a). Thus, Gal1 expression is correlated with the growth state of the injured neuron. Collectively, these results suggest that Gal1 might act as a factor intrinsic or extrinsic to the neuron to promote axon regeneration.

6.3.2 Assessing intrinsic value: galectin-1 as a potential neuronal regeneration-associated gene

As outlined above, accumulating expression data imply that Gal1 may act within neurons to promote axon regeneration; however, few studies have addressed the possibility by manipulating Gal1 levels.

In chapter four, I studied the effects of Gal1 on neurite outgrowth from cultured adult DRG neurons. If Gal1 were a RAG, one would expect that \( Lgals1^{-/-} \) neurons would not elaborate neurites as well as those from \( Lgals1^{+/+} \) mice. Interestingly, I found that \( Lgals1^{-/-} \) neurons were not significantly different from \( Lgals1^{+/+} \) neurons in any of the
neurite outgrowth parameters studied. Similarly, rat DRG neurons treated with exogenous Gal1/Ox displayed no significant improvement in neurite extension or branching. Therefore, Gal1 does not seem to act as a RAG within adult DRG neurons, and extracellular Gal1/Ox has insignificant effects on neurite outgrowth.

One study has demonstrated a potential role for Gal1 as a RAG. Rotblat et al. (2004) manipulated Gal1 expression in PC12 cells, and found that Gal1 was necessary and sufficient to elicit neurite outgrowth from this cell line. As discussed in the chapter, there are several possibilities that could explain the apparent discrepancy between the Rotblat study and that presented in chapter five: first, the signaling pathways in the PC12 cell line might not represent what occurs in differentiated neurons (Zhou and Snider, 2006); second, the types of the G-protein Ras that Gal1 interacts with might not be expressed in adult DRG neurons; and third, compensatory mechanisms in Lgals1−/− neurons may conceal the potential growth-promoting effects of Gal1 (future experiments could test the effects of conditional Gal1 knockdown).

Future studies might establish whether intracellular Gal1 is sufficient for increasing neurite outgrowth, or whether Gal1 is involved in embryonic or conditioning lesion-induced axon growth; however, my data from chapter four showed that intracellular and extracellular Gal1 likely have little direct effect on neurons. By elimination, these results imply that Gal1 probably acts on non-neuronal cells to promote axon regeneration.

6.3.3 Galectin-1 is good for the environment

When considering potential non-neuronal cellular targets of Gal1 in the nerve, the macrophage emerged as a particularly attractive candidate. Gal1/Ox binds to
macrophages \textit{in vitro}, and these cells respond to Gal1/Ox by releasing an unidentified factor that promotes axon regeneration and Schwann cell migration (Horie et al., 2004).

In chapter five, I identified a role for Gal1 in the accumulation of macrophages in the nerve. Gal1-IR was significantly increased in the nerve distal to injury at three dpi, which coincides with the time of peak macrophage accumulation in my model. I found that exogenous Gal1/Ox was sufficient to drive macrophage accumulation into the uninjured nerve, and Gal1 was necessary for injury-induced macrophage accumulation.

PNI-induced macrophage accumulation results from a combination of initial proliferation of resident nerve macrophages and later recruitment of many hematogenous monocytes that differentiate into macrophages upon entering the nerve. Although our data did not clarify whether Gal1 modulates macrophage proliferation or recruitment, previous studies established that invading hematogenous macrophages constitute the majority of the total macrophage population during the immune response (Mueller et al., 2003); it therefore seems most likely that Gal1 affects monocyte and/or macrophage recruitment. In a more recent study, Malik et al. (2009) confirmed that Gal1 does indeed influence monocyte recruitment. Interestingly, they used \textit{in vitro} transwell assays to show that Gal1 induced chemotaxis of monocytes, but not macrophages, and they found that this activity of Gal1 was lectin activity-dependent, required a G-protein-coupled receptor, and likely signaled through the MEK MAP kinase pathway. Taken together, these results suggest that Gal1, which is upregulated in peripheral nerves after injury, acts as a chemoattractant for monocytes. The monocytes could then enter the nerve, where they would differentiate into macrophages before removing debris and assisting with the repair process. It is not known whether Gal1 affects macrophage proliferation in the nerve as well.
Gal1’s modulation of the inflammatory response is complex: in addition to its roles in macrophage activation and monocyte chemoattraction, Gal1 has been implicated in various other pro- and anti-inflammatory processes (Rabinovich et al., 2002; Toscano et al., 2007). For instance, Gal1 differentially affects phagocytosis by macrophages depending on their activation state: whereas Gal1 increases phagocytosis by untreated cultured monocytes, the protein dampens the phagocytic response of interferon-γ-activated monocytes (Barrionuevo et al., 2007). Gal1 decreases activated macrophage production and activity of inducible nitric oxide synthase (Correa et al., 2003; Echigo et al., 2010), which catalyzes the formation of nitric oxide that can damage neurons when released by activated macrophages (Dawson and Dawson, 1996; Zochodne et al., 1997). Gal1 treatment also elicits a Th2 response (Motran et al., 2008; Toscano et al., 2006), which is implicated in the production of anti-inflammatory cytokines (D’Elios and Del Prete, 1998), and can preferentially induce apoptosis of pro-inflammatory Th1 T cells (Toscano et al., 2006). Therefore, Gal1 has seemingly contradictory roles in the regulation of the typical inflammatory response. Following PNI, Gal1 may recruit monocytes and activate certain macrophage functions, while still limiting potentially damaging aspects of the immune response by producing anti-inflammatory factors.

Gal1/Ox has several effects on non-neuronal cells of the nerve that probably account for the majority of Gal1-mediated enhancement of axon regeneration. As mentioned above, Gal1/Ox binds an unidentified receptor on macrophages, which respond by releasing an unknown large protein that promotes both Schwann cell migration and axon regeneration (Horie et al., 2004). A more recent study has elucidated more detail on the mechanisms underlying Gal1/Ox’s actions. Echigo and coworkers (2010) confirmed that conditioned medium from Gal1/Ox-treated macrophages elicited Schwann cell migration
In addition, they showed that Gal1/Ox alone facilitated Schwann cell migration (though not to the degree of the conditioned medium), despite the fact that Horie et al. (2004) showed that fluorescent Gal1/Ox did not directly bind Schwann cell membranes. The authors also established that Gal1/Ox acted through a G-protein-coupled receptor and the MAP kinase pathway in cultured macrophages.

Taken together, these results suggest that Gal1/Ox promotes axon regeneration by acting on two non-neuronal cell types: first, Gal1/Ox binds monocytes/macrophages, promoting their accumulation and stimulating the release of a factor that enhances axon regeneration and Schwann cell migration; and second, Gal1/Ox acts directly on Schwann cells to promote their migration, which could lead to more effective formation of permissive bands of Büngner. Future studies could clarify the receptors and pathways through which Gal1/Ox acts, and attempt to reconcile the ultimate effects of Gal1’s pro- and anti-inflammatory actions.

6.3.4 Branching out: galectin-1-mediated regeneration placed into context

Gal1 promotes axon regeneration: that is relatively well-established. The statement is an over-simplification, however. Obviously, different potential treatments, and even different concentrations of the same factor, will enhance axon growth to varying degrees. In order to determine whether Gal1/Ox is worthy of being termed a “potential therapy” for patients with PNI, I will compare the robustness of the effects of Gal1/Ox with that of other potential therapies, and I will discuss limitations associated with studies on Gal1 that have been completed thus far.

Much of the evidence supporting a role for Gal1 in axon regeneration is derived from a single in vitro model of axon growth: the adult rat DRG explant cultured in a collagen gel.
Multiple studies over the past decade have used the explants to define roles of Gal1 on neurite outgrowth. As noted in chapter four, one of the limitations associated with this model is that the neurons are not isolated, so one cannot identify which cell types are responsible for any effects observed. Regardless, these studies consistently show that Gal1 increases the number of neurites elaborated from DRG explants by 40 to 100 percent relative to control explants (Horie et al., 1999; Horie et al., 2004; Inagaki et al., 2000; Miura et al., 2004).

Interestingly, these numbers compare favourably with the effects of neurotrophic factors and of conditioning lesion. Tonge’s group has studied neurite growth from collagen gel-embedded DRG explants in the presence of growth factors. Leclere et al. (2005) found that NGF and GDNF elicited robust neurite outgrowth from sensitive subpopulations of adult mouse DRG neurons (NGF increased the number of CGRP-IR axons by more than 200 percent; GDNF increased the number of IB4-binding axons by more than 100 percent). NGF treatment also doubles the mean length of neurites elaborated (Ozturk and Tonge, 2001). When adult mouse DRG neurons are primed for growth by a conditioning lesion three days prior to explant culture, they extend ~70 percent more neurites compared to control explants (Ekstrom et al., 2003). Therefore, based on these in vitro data, Gal1/Ox appears to be a decent therapeutic candidate for PNI.

There are several limitations with the in vitro experiments on Gal1’s effects. First, all of the in vitro experiments prior to that described in chapter four were completed using the exact same model: DRG explants cultured in collagen gel with serum-free medium, in the absence of growth factors. Establishing whether Gal1 affects axon growth in other well-characterized culture models could reinforce the data that support its role in regeneration. Another related (potential) limitation is that all of the previous in vitro
studies were completed in associated laboratories. Given that Gal1’s effects on peripheral axon regeneration have been known for more than ten years, one might expect that other groups would attempt to define whether Gal1 affects axon growth. A third limitation of the previous studies is that none of them included positive controls; it is therefore difficult to objectively compare the effects of Gal1 on neurite outgrowth to the effects of other outgrowth-promoting treatments. Addition of a neurotrophic factor, or culturing DRGs that experienced prior conditioning lesions, would provide context that would allow for a more meaningful comparison between treatments. Finally, assessing the effects of Gal1 on neurite outgrowth on inhibitory substrates, such as myelin or CNS glial cell types, could be instructive for repair after SCI or DRI. The experiments described in chapter four address many of these concerns: they were performed using a different model and by an independent laboratory group, and positive controls (NGF and GDNF) were used. Earlier experiments suggested that Gal1 may elicit neurite outgrowth directly from DRG neurons; however, my data showed that Gal1/Ox does not directly promote neurite outgrowth.

Although the balance of the *in vitro* data are promising, robust effects shown by *in vivo* studies would provide more compelling evidence that would support a potential therapeutic role for Gal1 after PNI. Only five published studies have characterized the regulation of peripheral axon regeneration by Gal1 anatomically or behaviourally. Horie and colleagues’ (1999) pioneering study showed that Gal1 promoted axon regeneration in a peroneal nerve injury and tubulization model: there were four times as many axons at 0.5 mm distal to the lesion site at seven dpi when the tube contained Gal1. Similarly, Fukaya et al. (2003) demonstrated that Gal1/Ox, continuously delivered by osmotic minipump, promoted axon regeneration through an acellular autograft (freeze-killed sciatic nerve), with approximately twice as many axons 7 mm distal to injury compared to
animals treated with PBS. Using recovery of whisker function as a behavioural measure of axon regeneration, McGraw and coworkers (2004a) established that $Lgals1^{-/-}$ mice recover from facial nerve axotomy approximately one day after $Lgals1^{+/+}$ mice (12 versus 11 dpi, respectively). This result was in line with alterations in recovery of whisker function in p75$^{NTR}$ hypomorphic mice (one to two days quicker than wild-type; 12 versus 13-14 dpi, respectively; Ferri et al., 1998) and severe combined immunodeficient mice (two days slower than wild-type; 12 versus 10 dpi, respectively; Serpe et al., 2002).

After DRI, McGraw (2004) found that Gal1/Ox infusion promotes significant, but limited regrowth of small-diameter primary afferents into the CNS portion of the dorsal root. Gal1 treatment did not affect the regeneration of large-diameter fibres, which stalled at the DREZ. Kadoya et al. (2005) showed that Gal1/Ox-treated rats recovered slightly more function beginning at 21 days after sciatic nerve injury, as measured by toe spread of the hindpaw. Finally, Okada et al. (2005) established that intravitreal injection of Gal1/Ox could promote axon regeneration after optic nerve injury: Gal1/Ox treatment increased the number of axons regenerating 0.5 and 1 mm beyond the injury site at 14 dpi. Interestingly, Gal1/Ox’s effects on regeneration of retinal ganglion cell axons were comparable (or even more robust than) those of zymosan and forskolin, which are factors known to elicit optic nerve regeneration.

I will now discuss several other potential treatments that promote axon regeneration in vivo, although it is difficult to compare their effects with those of Gal1/Ox as a variety of injury models and methods have been used for measuring axon outgrowth. Moreover, the effects of many of the most promising candidates for regenerative therapies have been studied more intensively in CNS than in PNS repair.
Conditioning lesion of the sciatic nerve at 14 days prior to experimental sciatic nerve lesion increases the rate of peripheral axon regeneration by 22 to 25 percent (Oblinger and Lasek, 1984; Jacob and McQuarrie, 1993), an effect that has been attributed to the increased rate of slow component b transport of cytoskeletal proteins (Fu and Gordon, 1997; Hoffman and Cleveland, 1988; McQuarrie and Lasek, 1989; Tetzlaff et al., 1988; Wujek and Lasek, 1983). Gershenbaum and Roisen (1980) found that cAMP has extraordinary effects on axon regeneration after sciatic nerve crush: cAMP-treated animals fully recovered by 16 dpi, which was 12 days before saline-treated animals. They attributed the result both to effects on the neurons and on Wallerian degeneration. In contrast, other studies (McQuarrie et al., 1977; Knoops et al., 1990) have found that cAMP does not regulate peripheral axon regeneration. In fact, Knoops et al. (1990) found that when dibutyryl-cAMP (a cAMP analogue) was included in the tubulization model after sciatic nerve injury (similar to the model used in the Gal1 studies), axon regeneration was attenuated by 40 percent mid-way through the bridge. Thus, the effects of cAMP on peripheral regeneration are somewhat unclear, and depend on the model used. More recent studies have focused on the role of cAMP in CNS regeneration, where it seems to have important effects on axon extension and functional recovery (Hannila and Filbin, 2008; Neumann et al., 2002; Nikulina et al., 2004; Pearse et al., 2004; Qiu et al., 2002; Spencer and Filbin, 2004).

IL-6 is another factor that promotes functional recovery following PNI. Expression of IL-6 and its receptor IL-6R are increased in peripheral nerves following injury (Hirota et al., 1996). IL-6 loss of function – either by addition of an IL-6R function-blocking antibody (Hirota et al., 1996) or by knocking out IL-6 in mice (Zhong et al., 1999; Inserra et al., 2000; Galiano et al., 2001) – tempers peripheral axon regeneration. The protein has a critical role in functional recovery after sciatic nerve crush, as IL-6 knockout mice have a
significantly lower sciatic functional index at 30 dpi compared to wild-type mice (wild-type mice recover fully by 24 dpi) (Zhong et al., 1999). Anatomically, IL-6 knockout mice have about 13 percent less regeneration at four days after facial nerve crush, as assessed by CGRP- and galanin-IR (Galiano et al., 2001). IL-6 is also implicated in the conditioning lesion-induced regeneration of DRG axons in the dorsal columns (Cafferty et al., 2004; Cao et al., 2006); this effect likely also involves cAMP (Cao et al., 2006). Even these relatively well-characterized factors that promote axon regeneration affect regrowth differentially depending on the model used; therefore, it is important to establish whether Gal1 promotes regeneration in other models of PNS and CNS injury.

Although McGraw’s (2004) findings that Gal1/Ox promotes axon regeneration after DRI appear promising, its effects are not as robust as are those elicited by neurotrophic factor treatment. Gal1/Ox treatment facilitates the growth of small-diameter fibres (identified by CGRP-IR and wheat-germ agglutinin tracing) across the DREZ into the CNS: the density of axons present in the CNS compartment of the DREZ increased two-fold with Gal1/Ox treatment, to about 1.5 percent for CGRP-IR fibres. In contrast, Ramer and colleagues (2000) showed that neurotrophic factors had more significant effects on axon regrowth. Intrathecal infusion with NGF led to a four-fold increase in CGRP-IR axon density to about 10 percent of the total area of the CNS portion of the DREZ. GDNF treatment had remarkable effects on P2X3-IR axons: axon density in the CNS was increased about 20-fold to approximately 20 percent of the total area. In addition, GDNF elicited outgrowth of CGRP- and NF200-IR subpopulations. Both NGF and GDNF (as well as NT-3) stimulated long-distance regeneration and functional recovery (as measured by electrophysiology and behavioural testing) (Ramer et al., 2000; Ramer et al., 2002). Therefore, although Gal1/Ox promotes regeneration of small-diameter
primary afferents, it does not promote axon growth into the CNS comparable to the
distance or density facilitated by neurotrophic factor treatment.

Again, there are several limitations to the *in vivo* studies of Gal1-mediated regeneration
that make it difficult to compare its effectiveness with that of other treatments. As with
the *in vitro* studies, few groups have studied Gal1’s activity in animal models. The lack
of positive controls is another issue: it precludes comparison with other potential
treatments. Only one of the five experiments testing *in vivo* regeneration included
groups that acted as positive controls (Okada et al., 2005). Another limitation is that only
one study has characterized the effects of Gal1 in a model of CNS injury (optic nerve
injury; Okada et al., 2005). Many of the most promising factors for eliciting peripheral
axon regeneration also promote axon regeneration or repair following SCI, and can
facilitate neurite growth from cultured DRG neurons on inhibitory CNS myelin substrates
(although Gal1 does not seem to elicit axon growth directly). Therefore, future studies
could address whether Gal1 affects CNS repair in animal models of SCI.
6.4 Summary

Axon growth is a complex process that involves both the neuron itself and myriad factors and cells that surround the neuron and axon. In this dissertation, I explored some of the effects of Gal1 on developmental and regenerative axon growth.

Previous studies had shown that the spatiotemporal expression pattern of Gal1 was consistent with it having a role in axon guidance during development of DRG neurons’ central terminals (Dodd and Jessell, 1985; Dodd and Jessell, 1986; Hynes et al., 1990; Regan et al., 1986). In addition, Gal1 has a key role in the development of olfactory axon subpopulations (Puche et al., 1996). In chapter two, I established that Gal1 is required for the development of the typical distribution of DRG neuron subpopulations. In addition, I demonstrated that Gal1 is necessary for central projections of nociceptive primary afferents to connect appropriately with targets in the superficial dorsal horn: development in the absence of Gal1 is associated with remarkable deficits in sensitivity to noxious thermal stimuli. Gal1 therefore has critical roles in the development of DRG neurons and their connections.

I subsequently studied the regulation of Gal1 by axotomy of peripheral and central branches of DRG neurons, and found that the regulation of Gal1 expression is correlated with the regenerative capacity of these neurons, both within these neurons and their surrounding environment. These results suggested that Gal1 might improve regeneration by acting directly within neurons and/or by acting on non-neuronal peripheral nerve cells.
In chapters three and four, I examined the mechanisms that could underlie Gal1-mediated axon regeneration. Gal1 did not have a significant direct effect on neurons, suggesting that it might act on other cells within the nerve. In chapter five, I established that Gal1/Ox enhances injury-induced macrophage accumulation in peripheral nerves.

From these latter two chapters, it seems that Gal1 promotes axon regeneration mainly through non-neuronal cells within the nerve. More specifically, Gal1/Ox facilitates macrophage accumulation after injury; these cells are activated by Gal1/Ox to secrete a factor that promotes Schwann cell migration and axon regeneration (Echigo et al., 2010; Horie et al., 2004).

Interestingly, it is quite possible that different mechanisms underlie Gal1’s effects on developmental and regenerative axon growth. How Gal1 affects the proper development of DRG neurons and their axons is largely undefined, but the mechanism almost certainly does not involve macrophages. In contrast, regenerative growth supported by Gal1 seems to be largely dependent on its effects on macrophages.

It is still unclear whether Gal1 (or Gal1/Ox) is truly a viable candidate as a therapy for improving functional recovery after nerve injury in humans. Future studies should explore Gal1’s potential for promoting regrowth in various models of axon regeneration in the PNS and CNS by comparing its effects with other potential treatments. In addition, examining neurite outgrowth from dissociated neurons, whether isolated or co-cultured with other cell types, will help define whether this small protein can have considerable effects in the pathological nervous system.
6.5 References


Mahanthappa NK, Cooper DN, Barondes SH, Schwarting GA (1994) Rat olfactory neurons can utilize the endogenous lectin, L-14, in a novel adhesion mechanism. Development 120: 1373-1384.


ANIMAL CARE CERTIFICATE

Application Number: A09-0617

Investigator or Course Director: Matt S. Ramer

Department: ICORD

Animals:

Start Date: January 1, 2009

Approval Date: December 21, 2009

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Exploiting the innate plasticity of spread axons to improve the

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Axonal plasticity and the p75 neurotrophin receptor

Funding Agency: Michael Smith Foundation for Health Research
Funding Title: Sensory function and dysfunction in neurotrauma: Models, molecules and mechanisms

Unfunded title: N/A
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A09-0452

Investigator or Course Director: Matt S. Ramer

Department: ICORD

Animals:

- Mice GFP-expressing and wild-type controls 50
- Mice galectin-1 knockout 200
- Rats Long-Evans 20
- Rats Wistar 300
- Rats Sprague-Dawley 80
- Mice CD1 100
- Mice 129/svjae 50
- Mice p75 knockout and wild-type controls 100

Start Date: July 1, 2009
Approval Date: October 19, 2009

Funding Sources:

- Funding Agency: Canadian Institutes of Health Research (CIHR)
- Funding Title: Exploiting the innate plasticity of spared axons to improve the outcome of spinal deafferentation
- Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A04-1459

Investigator or Course Director: Matt S. Ramer

Department: Zoology

Animals:

- Mice C57/Bl6, CD-1, 129SVJae, 129P35 500
- Rats Sprague Dawley, Long Evans, Wistar 400

Start Date: October 1, 2001

Approval Date: February 24, 2009

Funding Sources:

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Exploiting the innate plasticity of spared axons to improve the outcome of spinal deafferentation

- Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
  Funding Title: Axonal plasticity and the p75 neurotrophin receptor

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Exploiting the innate plasticity of spared axons to improve the outcome of spinal deafferentation
<table>
<thead>
<tr>
<th>Funding Agency</th>
<th>Funding Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Sciences and Engineering Research Council of Canada (NSERC)</td>
<td>Axonal plasticity and the p75 neurotrophin receptor</td>
</tr>
<tr>
<td>Natural Sciences and Engineering Research Council of Canada (NSERC)</td>
<td>Axonal regeneration across glial interfaces</td>
</tr>
<tr>
<td>Christopher Reeve Paralysis Foundation</td>
<td>Improving neurotrophin-mediated sensory regeneration in the spinal cord through antagonism od the p75 receptor</td>
</tr>
<tr>
<td>Michael Smith Foundation for Health Research</td>
<td>Primary deafferentation of the spinal cord: consequences and repair strategies</td>
</tr>
</tbody>
</table>

| Unfunded title: | N/A |

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093