# GALECTIN-1 IN INJURY AND REGENERATION

by

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## ABSTRACT

In higher vertebrates, injury to both sensory and motor axons within the central nervous system (CNS) results in permanent loss of sensation and paralysis. In contrast, peripheral nervous system (PNS) injuries are often followed by functional recovery. This disparity in the regenerative ability of the PNS and CNS is attributed to both the intrinsic gene response of injured neurons and the environment across which an injured axon must extend in order for successful regeneration to occur.

Recent reports demonstrate that the carbohydrate-binding protein galectin (Gal)-1, which is expressed during the development of sensory and motor neurons and in the mature adult, is involved in regrowth of axotomized neurons following a peripheral nerve injury. Specifically, *exogenous* application of recombinant Gal1 to injured peripheral nerves increased the rate of axonal growth into acellular grafts. However, the axotomy-induced changes of *endogenous* neuronal Gal1 expression have not been examined. In this thesis, I demonstrate that axotomized neurons that were able to regenerate or initiate a growth response also had increased Gal1 expression. Gal1 returned to uninjured levels upon target re-innervation suggesting that the target may partially regulate Gal1 expression. Furthermore, homozygous Gal1 null mutant (-/-) mice showed an attenuated rate of functional recovery after a nerve crush.

I also examined sensory responses of Gal1 -/- mice, since Gal1 is expressed during sensory neuronal development as well as within the adult dorsal root ganglion (DRG). The absence of Gal1 in the Gal1 -/- mouse led to an increased threshold for thermal nociceptive stimuli. This correlated with differences in nociceptive neuron proportions and their pattern of termination within the spinal cord.

Taken together these data underscore an important contribution of endogenous Gal1 to the regenerative process and to sensory neuronal development and/or maintenance.

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# Abbreviations

| | |
|---|---|
| -/- | homozygous null mutation |
| -/+ | heterozygous mutation |
| ANOVA | analysis of variance |
| BBB | blood brain barrier |
| BDNF | brain derived growth factor |
| °C | degree Celsius |
| C | cervical level |
| $Ca^{++}$ | calcium ion |
| CAP-23 | cortical-associated protein – 23 kDa |
| CGRP | calcitonin gene-related peptide |
| CNS | central nervous system |
| CNTF | ciliary neurotrophic factor |
| CRD | carbohydrate recognition domain |
| CSPG | chondroitin sulfate proteoglycan |
| CST | corticospinal tract |
| CTB | cholera toxin sub unit B |
| DREZ | dorsal root entry zone |
| DRG | dorsal root ganglia |
| E | embryonic day |
| ECM | extracellular matrix |
| ERK | extracellular signal-regulated kinases |
| EtBr | ethidium bromide |
| F-actin | filamentous actin |
| FAK | focal adhesion kinase |
| FGF | fibroblast growth factor |
| Gal1 | galectin-1 |
| Gal1-Ox | oxidized galectin-1 |
| Gal1-Red | reduced galectin-1 |
| GAP - 43 | growth associated protein - 43 kDa |
| GDNF | glial cell line-derived neurotrophic factor |
| GFAP | glial fibrillary acidic protein |
| GFR | GDNF receptor |
| GTPase | guanosine triphosphatases |
| IB4 | isolectin *Bandeiraea simplicifolia* |
| IHC | immunohistochemistry |
| IL | Interleukin |
| IR | immunoreactivity |
| ISH | *in situ* hybridization |
| kDa | kilodalton |
| L | lumbar |
| µm | micrometer |
| MAPK | mitogen-activated protein kinase |
| MEK | MAPK/ERK kinase |
| mRNA | messenger ribonucleic acid |
| NF | neurofilament |
| NGF | nerve growth factor |
| NK-1 | substance P receptor |
| NOS | nitric oxide synthase |
| NT | neurotrophin |
| PB | phosphate buffer |
| PBS | phosphate buffer saline |
| PFA | paraformaldehyde |
| rh | recombinant |
| P | post natal day |
| PBS | phosphate buffered saline |
| PN | peripheral nerve |
| PNS | peripheral nervous system |
| RAG | regeneration associated gene |
| redox | reduction oxidation |
| RN | red nucleus |
| RST | rubrospinal tract |
| SCI | spinal cord injury |
| SEM | standard error of the mean |
| SMN | spinal motoneuron |
| T | thoracic level |
| thermo | temperature-activated transient |
| TRPs | receptor potential ion channels |
| Trk | tropomyosin receptor kinase or tyrosine kinase |
| TSA | tyramide signal amplification |
| WGA | wheat germ agglutin |
| *wt* | wild type |

## STATEMENT OF ORIGINAL CONTRIBUTIONS

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The thesis author, John McGraw, was the primary researcher for all the results presented in the above articles and this thesis. L.W. Oschipok provided expertise with *in situ* hybridization. J. Liu performed the surgeries and the tissue processing for the rubrospinal tract experiments. L.T. McPhail performed the all of the facial nerve surgeries and processed the CD-1 mice tissue. F. Poirier generated the galectin-1 null mutant mice. T. Kadoya of Pharmaceutical Research Laboratory, Kirin Brewery Co. kindly provided recombinant oxidized galectin-1 protein and galectin-1 antibodies for use in these experiments. A.D. Gaudet, C.F.W. Mak, F. Zhang and S.J. Williams kindly provided technical assistance with computer tracings analysis. All work was performed under the supervision and experimental design provided by the thesis author.

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# CHAPTER 1: GENERAL INTRODUCTION

## *Traumatic Nerve Injuries*

Traumatic nerve injury, particularly in the central nervous system (CNS), often results in a permanent devastating disability. Two examples of traumatic nerve injuries are spinal cord injury (SCI) and brachial plexus injury. One of the most persistently pursued goals in the field of neuroscience is the full restoration of neuronal function after a traumatic SCI. This is due in part to the frequency with which such injury occurs, and its profound impact on those injured and on society. Approximately 3 in every 100,000 people living in developed countries sustain an SCI each year (Dincer et al., 1992; Levi et al., 1995; Nobunaga et al., 1999; Dryden et al., 2003). In Canada, 78% of these injuries occur in people between 15 and 34 years of age whose average life expectancy is almost equivalent to that of an able-bodied person (CPA, 2000). SCI and associated permanent loss of neurological function, in some cases followed by a lifetime of chronic pain, can be economically, socially and physically debilitating for those who sustain an SCI. There is also great financial cost; indeed, the total direct cost of SCI in Canada can amount to as much as $25 million over the lifetime of the injured person (Dijkers, 1997; Kennedy et al., 1997; McColl et al., 1997; CPA, 2000; ICORD, 2003).

Brachial plexus injury is another type of commonly occurring nerve injury involving damage to the complex of nerves innervating the arms. The brachial plexus has both a peripheral and centrally projecting branch. When injury occurs to the central branch, permanent monoplegia, anesthesia and often the onset of severe intractable pain of the upper extremities can result (Berman et al., 1998). In the United States, this type of injury occurs as a complication of childbirth in 3 out of every 1000 births (Dunham, 2003) and in 4 out of every 100,000 young healthy individuals as a result of accidents related to motorcycle and snowmobile use, or sustained during manual labour (Midha, 1997). It is significant that when injury occurs in the PNS component of the brachial plexus, the results can be less devastating. This is due in large part to one of the disparities between the central nervous system and the peripheral nervous system: regenerative ability.

*Limitations to CNS Regeneration*

Since medical observations were first recorded, the most consistent and striking difference noted between the peripheral and central nervous systems has been that the permanent deficit that is a result of a traumatic CNS injury appears never to be restored, whereas either partial or full recovery is common after a peripheral nerve injury. Although the presence of this distinction remained "received wisdom" among medical professionals and scientists up until the early 20$^{th}$ century, scientific discoveries within the last century have begun to challenge this notion. In their landmark series of experiments published in 1928, F. Tello and S. Ramon y Cajal demonstrated growth into peripheral nerve grafts of a limited number of axotomized CNS axons. Although these experiments clearly disproved historical notions of the impossibility of CNS regeneration, their significance was not recognized until more than fifty years later, when they were revisited and expanded upon by Richardson and colleagues using more modern techniques. The efforts of Richardson and colleagues galvanized the field of neuronal regeneration research (Richardson et al., 1980; David and Aguayo, 1981; Richardson et al., 1982; Richardson et al., 1984). These elegant experiments clearly demonstrated that there is intrinsic growth capacity within the injured CNS when a growth-supportive environment is present, and laid the initial foundation for our present understanding of the processes required for axonal regeneration. Subsequent research in this field has focused on either the intrinsic growth capacity of injured neurons or the environment across which an injured axon must extend in order for successful regeneration to occur in the CNS (Tetzlaff and Steeves, 2000).

This thesis sets out to examine whether the intrinsic neuronal expression of galectin (Gal)-1 correlates with the growth potential of neurons and whether Gal1 promotes neuronal regeneration. In order to arrive at this hypothesis I will first briefly summarize both the extrinsic (environmental) and intrinsic factors that inhibit axonal regeneration. Then I will examine the dorsal root ganglion (DRG) system in order to examine strategies to promote axonal regeneration and to describe the discovery of Gal1 as a growth promoting protein. The introduction is concluded by a brief overview of my hypothesis and how it will be addressed in chapters 2-5. Finally Chapter 6 concludes with a general discussion of results and limitations of these experiments, potential mechanisms that may explain the reported results, and future studies.

*Environmental inhibition of regeneration*

The work of Richardson and Tello demonstrated that glial (non-neuronal) cells and the associated extracellular matrix molecules of the adult CNS (commonly called the extrinsic environment) do not support regenerative growth. In contrast, during initial development of the CNS, axonal outgrowth must readily occur. This perceived shift from a growth-supportive environment during development to a growth-restrictive environment after development coincides with axonal myelination by glial cells called oligodendrocytes (Hasan et al., 1993), suggesting that myelin is a growth inhibitor.

Myelin was one of the earliest characterized axonal growth inhibitors within the CNS (Caroni and Schwab, 1988a). In order to promote axonal growth by injured CNS axons, four main approaches have been employed to overcome myelin's growth-inhibiting effects. The earliest approach involved the use of functional blocking antibodies to promote regrowth and sprouting of the axotomized corticospinal tract (CST) (Schnell and Schwab, 1990; Bregman et al., 1995). These antibodies blocked specific myelin epitopes found at 35 and 250 kDa within a myelin fraction, or what is now thought of as Nogo (Caroni and Schwab, 1988b; Spillmann et al., 1998). Upon identification of the Nogo receptor, a second approach was to create specific receptor antagonists that also promoted CST regrowth (GrandPre et al., 2002). A third approach involves immunologically killing oligodendrocytes to reduce all myelin epitopes around the injury site. Through the use of complement proteins and oligodendrocyte-specific antibodies, some functional axonal growth and recovery was reported to be observed after partial or complete injury (Keirstead et al., 1995; Dyer et al., 1998). In the fourth and most novel approach, S. David and colleagues used a myelin vaccination approach, which elicits an immunological response against myelin in order facilitate growth or promote sparing of axotomized CST axons (Huang et al., 1999). These diverse *in vivo* approaches have all supported the notion that myelin contributes significantly to regenerative failure in the CNS.

However, the idea that myelin is the principal barrier to regeneration in the CNS was recently challenged. Previous *in vitro* data demonstrated that myelin strongly inhibited DRG growth (Schwab et al., 1993). Davies and colleagues carefully transplanted dissociated

mouse dorsal root ganglion neurons into the heavily myelinated rat corpus collusm (Davies et al., 1997) and surprisingly, these neurons grew long distances. The mouse axons appeared to only stop growing in proteoglycan rich areas (Davies et al., 1997; Davies et al., 1999). Previous *in vitro* studies have demonstrated clearly that these proteoglycans inhibit DRG outgrowth and are a known component not only of the barrier between the PNS and CNS, but also of the astroglial scar (McKeon et al., 1991; Pindzola et al., 1993; Asher et al., 2001; Zhang et al., 2001).

The astroglial scar forms after a penetrating injury to the CNS, and is comprised of various glial and other non-neuronal cells. In vertebrates, any major disruption of tissue such as dermis or CNS tissue often results in a fibrotic scar with associated extracellular matrix (ECM) molecules (Fitch and Silver, 1997a; Martin, 1997). This scar serves to quickly re-seal the wound by walling off the area from further damage and to restore homeostasis. Although the scar that develops following CNS trauma is beneficial in that it serves to contain the damaged area and restore the barrier between the periphery and CNS, it unfortunately also creates a barrier to regenerating axons (Davies et al., 1999). To overcome this regenerative obstacle, researchers have sought to reduce or remove the instigators of astrogliotic scar formation or the associated inhibitory ECM molecules (reviewed in McGraw et al., 2001). Perhaps the most promising results were observed when the proteoglycans at the scar site were enzymatically degraded. In axotomized ascending sensory fibers within the spinal cord, this treatment enhanced the growth into a predegenerated peripheral nerve graft in one model as well as increased both anatomical plasticity and functional recovery after a dorsal column crush (Bradbury et al., 2002). Although the reductions of inhibitory myelin epitopes or glial scar ECM molecules seem promising, only very limited axonal growth has ever been achieved to date. Accordingly other factors are likely to be involved that are actively inhibiting regeneration or that are required for regeneration but are absent. Examining a regenerating system, such as the PNS, gives further insight into the potential reasons for the lack of repair within the CNS.

*PNS development and regeneration*

The differing abilities of the CNS and PNS to regenerate are in part explained by the differing glial response to injury. In the injured CNS, oligodendrocyte myelin and astroglial

scar formation inhibit axonal regrowth. In stark contrast to the CNS, in a peripheral nerve injury, Schwann cells (the myelinating cells of the PNS) and macrophages actively reduce inhibitory barriers to neuronal repair. Schwann cells proliferate, retract their processes, and form linear arrays (bands of Büngner) in the distal segment in preparation for nerve regrowth (Ramon y Cajal, 1928; Bunge, 1987). In addition, by releasing proteases to degrade the ECM, Schwann cells actively degrade inhibitory proteoglycans (Fu and Gordon, 1997). Within the first few days after injury occurs, blood-borne macrophages invade the injury site and along with the resident macrophages, serve to remove growth-inhibitory myelin debris (Perry et al., 1987). These macrophages also attenuate protease release from Schwann cells. Excessive protease expression in Schwann cells could damage surviving neurons and thus must be regulated to prevent over degradation (La Fleur et al., 1996). The need for this macrophage response is clearly evident when macrophages are unable to clear debris. For example, in the C57BL/Wld$^s$ mutant mouse, delayed Wallerian degeneration occurs due to mutation in an ubiquitination factor (Conforti et al., 2000), in turn causing the failure of macrophages to clear axonal debris and inhibitory proteins (Perry et al., 1990; Chen and Bisby, 1993; Glass et al., 1993). A consequence of this mutation is delayed peripheral regeneration. As opposed to the glial cells in the CNS that form barriers to growth, non-neuronal cells within the PNS actively participate in neuronal regeneration by providing a more permissive growth environment.

Schwann cells are not only involved in preparing the environment for peripheral growth, but also in providing as a source of neurotrophic factors that aid in neuronal survival and regeneration of the injured neuron. The discoveries of nerve growth factor (NGF) and its tropomyosin receptor kinase (Trk) A led to the identification of a family of neurotrophins and their respective receptors. Some time later, other members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5 and glial cell-line derived neurotrophic factor (GDNF), and their associated receptors (see table 1) where discovered (Levi-Montalcini, 1966; Barde et al., 1978; Kaplan et al., 1991; Lin et al., 1993). The DRG system has proven to be an excellent model for understanding the neurotrophins and their effects on neurodevelopment and repair, since an increasing amount of evidence

suggests that distinct populations of sensory neurons require different target-derived neurotrophins for survival during development and phenotypic maintenance in the adult.

Rodent expression patterns of neurotrophic receptor mRNA and protein support the idea that neurons of different functional classes are regulated by different members of the neurotrophin family. For example, the NGF receptor TrkA localizes to 70-80% of all DRG neurons during development and in about 40% of medium and small-sized unmyelinated DRGs (Molliver et al., 1995; Molliver and Snider, 1997). Null mutations (-/-) to the NGF or TrkA gene led to a 70-80% decrease in sensory neuronal survival in the adult and a loss of pain sensation demonstrating that NGF signaling via the TrkA receptor is required for survival of these nociceptive neurons (Crowley et al., 1994; Smeyne et al., 1994; Silos-Santiago et al., 1995). However, as development proceeds, some neurons switch their neurotrophic factor requirement. Starting at embryonic (E) 15, continuing until postnatal (P) 7 and lasting until adulthood, half of NGF-dependent neurons lose their TrkA expression and begin to express RET, the signaling receptor for GDNF (Molliver and Snider, 1997; Molliver et al., 1997). This leads to an adult DRG population comprised of 40% TrkA and 30% Ret expressing neurons. Even though both of these populations are mainly small diameter C-fiber neurons responding to nociceptive stimuli, they overlap minimally. Although the specific functional nociceptive roles of these fiber populations remains elusive, that these fibers terminate in separate spinal laminae, and that they express different catalogues of molecules offer some indication of discrete functions. The third population of sensory neurons possessing medium to large somata are responsible for mechano- and proprioception, and express the NT-3 receptor, TrkC (McMahon et al., 1994). 10% of all DRG neurons express mRNA for the BDNF receptor TrkB in a population of cells that convey mechanoreceptive information (Mu et al., 1993; Wright and Snider, 1995; Carroll et al., 1998).

| Sensory modality | Neurotrophic dependence | Expressed neurotrophic receptor | Phenotypic marker | Cell body size | % |
|---|---|---|---|---|---|
| nociceptors | NGF | TrkA | CGRP<br>galanin | small | 40 |
| nociceptors | GDNF | RET, GFRα1<br>GFRα2, GFRα3 | IB4<br>P2X3 | small | 30 |
| proprioceptors | NT3 | TrkC | NF200<br>NPY | large | 25 |
| mechanorecptors | BDNF | TrkB | NF200 | large | 10-30 |
| hair follicle receptor | NT-4/5 | TrkB | | med-large | |

**Table 1. A summary of the diversity of sensory neurons within the DRG.**

After an axotomy, neurotrophins also play an important role in axonal regeneration. Following axonal injury, Schwann cells within the distal stump upregulate the expression of NGF, BDNF and NT-4 (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993; Anand et al., 1997). However this change in expression is not sufficient to compensate for the lack of target-derived factors and as a result, the injured DRG undergoes a number of changes (Heumann et al., 1987; Knyihar-Csillik et al., 1991). These involve the down-regulation of the peptides calcitonin gene-related peptide (CGRP) (Noguchi et al., 1990) and substance P (Nielsch et al., 1987), and the up-regulation of the peptides vasoactive intestinal polypeptide (Shehab and Atkinson, 1986), galanin (Hokfelt et al., 1987) and neuropeptide Y (Wakisaka et al., 1991). Furthermore, an up-regulation of the enzyme nitric oxide synthase (NOS) (Verge et al., 1992) and growth-associated protein (GAP)-43 (Woolf et al., 1990) are observed after axotomy. Together, these changes are thought to represent adaptive responses serving to reduce the deleterious effects of peripheral nerve damage and to promote survival and regeneration of the lesioned sensory afferents (Hokfelt et al., 1994). While peripheral nerve injury changes the expression of a variety of genes in the DRG, this response is attenuated or nonexistent after a rhizotomy (Chong et al., 1996; Andersen and Schreyer, 1999).

*Intrinsic gene response correlates with regeneration in the DRG*

The outcome of sensory nerve lesions may vary considerably, depending upon the extent and location of injured neurons. For instance, a lesion between the dorsal root ganglion and the periphery (axotomy) typically results in the most successful recovery (Fu and Gordon, 1997). In contrast, recovery never occurs after a rhizotomy, a lesion occurring between the CNS and the DRG (Carlstedt et al., 1988; Bradbury et al., 2000). Dorsal root axons regenerate at half the rate of injured peripheral processes (Wujek and Lasek, 1983; Oblinger and Lasek, 1984). This disparity in regenerative ability of the DRG is due, in part, to the differing gene responses elicited by these two injuries. For example, within one day of a peripheral axotomy the transcription factor c-jun is upregulated and remains elevated until target innervation (Leah et al., 1991; Herdegen et al., 1997). In contrast, a rhizotomy only elicits a slight increase in c-jun expression (Jenkins et al., 1993; Broude et al., 1997). Because of this

difference in regenerative ability, the DRG is an excellent system in which to examine both the changes in gene expression and regenerative ability of axotomized neurons *in vitro* and *in vivo*.

In culture, DRG neurons extend long processes only after they have been axotomized *in vivo* 2-7 days prior to culture, whereas only short-branched processes occur without prior axotomy (Smith and Skene, 1997). The reversal of this axotomy-induced growth mode by transcriptional inhibitors suggests the requirement of mRNA synthesis for successful *in vitro* growth (Smith and Skene, 1997). These findings are confirmed *in vivo*. Axotomized ascending sensory fibers in the dorsal column do not sprout nor do they grow into a peripheral nerve graft. However, these fibers will both sprout or grow into a CNS tissue or a pre-degenerated peripheral graft if their peripheral process is axotomized one week before their central process is cut (Richardson and Issa, 1984; Richardson and Verge, 1987; Neumann and Woolf, 1999). Peripheral axotomy initiates many gene changes within the DRG, but a few well-characterized genes have garnered much of the attention. For example, GAP-43 (also known as F1, B-50 and neuromodulin) is used as a marker of an enhanced intrinsic growth state (Plunet et al., 2002). Usually only detected within subpopulations of neurons within the mature nervous system, GAP-43 increases when neurons enter a regenerative state (Skene, 1989; Schreyer and Skene, 1991; Tetzlaff et al., 1991; Chong et al., 1992; Fernandes et al., 1999). For example, rhizotomy in the DRG does not lead to changes in GAP-43, whereas, peripheral axotomy increases GAP-43 expression in neuronal somata from whence it is transported into the uninjured central branch of the nerve (Schreyer and Skene, 1991; Chong et al., 1994b). Thus this well-characterized protein is also used as a marker for regenerative growth ability within the CNS. Cervically axotomized rubrospinal neurons capable of limited growth into a predegenerated peripheral nerve graft have only a mild and transitory gene (GAP-43, Tα1 tubulin) response (Tetzlaff et al., 1991; Kobayashi et al., 1997). With this meager response, a small population of injured rubrospinal neurons (43 of a total of 1200 rubrospinal neurons) readily grows into a degenerated peripheral nerve (Kobayashi et al., 1997). Application of brain-derived neurotrophic factor (BDNF) to the red nucleus increases regenerative-associative gene expression and also increases the growth propensity of these injured axons as indicated by the increased number of neurons (131 out

of 1200 rubrospinal neurons) able to grow into the graft (Kobayashi et al., 1996; Kobayashi et al., 1997).

The link between GAP-43 and growth was mainly correlational. GAP-43-deficient mice develop normally, but their neurons fail to grow in the proper pathways in the optic chiasm (Strittmatter et al., 1995). Furthermore, GAP-43 over-expressing mice fail to promote the growth of injured ascending dorsal column fibers into permissive PN transplants (Bomze et al., 2001). Only with the combined over-expression of cortical-associated protein (CAP)-23, another growth-associated protein, and GAP-43 were the ascending fibers able to grow into PN grafts. However, the growth that occurred was less than that of a priming lesion (Bomze et al., 2001). These experiments indicate that a multitude of intrinsic factors are required to achieve even a limited amount of growth within the CNS environment.

*DRG regeneration strategies*

Several strategies exist to enhance the injury response to rhizotomy. Exogenous growth factor application has had the most success in rescuing changes in neurochemistry, conduction velocity and gene expression after both axotomy and rhizotomy (Verge et al., 1995; Munson and McMahon, 1997; Munson et al., 1997; Bennett et al., 1998). Not surprisingly, specific neurotrophins affect those neurons expressing the appropriate receptors. For example, rhizotomized large-diameter sensory neurons expressing TrkC can regrow through the dorsal root entry zone (DREZ), the PNS-CNS interface for sensory neurons, to make functional connections through the exogenous application of NT-3 (Ramer et al., 2000). Likewise, GDNF promotes growth of the neurons expressing GFR$\alpha$1; however, BDNF has little effect even though 10-25% of the neurons express TrkB (Ramer et al., 2000). Importantly, regenerating neurons make appropriate and functional connections. For example, after rhizotomy, NGF treatment causes small diameter nociceptive fibers to regenerate back to the dorsal horn and thus allow rats to sense painful stimuli and respond appropriately (Ramer et al., 2000). NT3 treatment results in the restoration of proprioception (Ramer et al., 2002). Delaying NT3 treatment by 1 or 2 weeks after rhizotomy results in a considerable decrease in growth into the DREZ with no functional connections (Ramer et al., 2001b). These data indicate that there are many putative barriers to entry into the CNS. Research has begun to focus on the interplay of intrinsic and extrinsic factors limiting

regeneration but now time appears to alter the amount each one of these factors contributes to regenerative failure.

In order to further elucidate other putative factors involved in neuronal repair, Horie *et al* (1999) used a DRG cell culture assay combined with cells derived from monkey kidney. Kidney cells secrete a wide variety of factors that it was hoped would play a role in axonal repair. Using COS1 conditioned media in DRG explants, Horie *et al* observed that a 14.5 kDa fraction had some growth-promoting effect. After purification and sequencing, they identified the lectin, Galectin (Gal1)-1 as the growth-promoting protein. Using *E. coli* as expression vectors of human Gal1, recombinant (rh) Gal1 was then obtained to create functional blocking antibodies. It was observed that Gal1 protein expression was elevated at the injury site in both Schwann cells and neuron terminals. When rhGal1-Ox was applied to axotomized rat sciatic nerves, an increase in neuronal growth into an acellular environment was noted, whereas Gal1 functional blocking antibodies reduced the amount of growth when compared to control antibody application (Horie et al., 1999). A new class of proteins had been found to have growth-promoting effects within the DRG.

***Lectins***

Lectins are carbohydrate-binding proteins that recognize specific oligosaccharide structures and, most notably, cause agglutination and/or lysis of erythrocytes and leukocytes (termed "lectin activity") (Barondes, 1988). The earliest identification of a lectin was in 1872 when crystalline structures, termed Charcot-Leyden crystals, were grown from the sputum of asthmatics (reviewed in Kilpatrick, 2002). These crystals have been recently identified as galectin-10 (reviewed in Kilpatrick, 2002). Not until 1902 was the first lectin activity described (Flexner and Noguchi, 1902). Today the lectin family is classified on the basis of its members' dependence on $Ca^{++}$ for binding ligands. The C-type lectins, of which there are seven subfamilies, require $Ca^{++}$ for carbohydrate binding, whereas the S-type, known as the galectins, bind independently of $Ca^{++}$ (Barondes et al., 1994b).

*Galectins*

Galectins are members of a highly conserved family of lectins widely distributed throughout the animal kingdom. These proteins occur in organisms ranging from viruses, plants, nematodes, and invertebrates to humans (Cooper, 2002). To date, fourteen mammalian

forms, galectin-1 through to 14 respectively, have been identified in a wide variety of tissues (Barondes et al., 1994b; Rabinovich et al., 2002). Of these galectins, only galectin-1, 3 and 5 were found biochemically and then cloned. The other galectins have been identified using sequence homology, since galectins share structural similarities in their carbohydrate recognition domain (CRD). Using similar sequence-based searching techniques within human genomic data, seven additional mammalian candidates have been identified that may be included in the galectin family (Cooper and Barondes, 1999; Cooper, 2002). Although similarities exist between galectins' CRDs, the overall structure linking the CRDs together further classifies the galectins into specific groups (Figure 1.1) (Hirabayashi and Kasai, 1993). Galectin-1, 2, 5, 7, 10, 11 and 13 are termed prototypical galectins because they exist as monomers or non-covalent homodimers consisting of two identical CRDs. Gal3 exists only as a monomer with two functional domains and Gal4, 6, 8, 9 and 12 exist as high order oligomers (Barondes et al., 1994b; Barondes et al., 1994a; Leffler et al., 2004). Gal1 was first identified in electric eels in 1975 (Teichberg et al., 1975). It is one of the most extensively studied galectins since its expression occurs in most organs (Perillo et al., 1998) as well as a wide range of cells including placenta (Poirier et al., 1992) and muscle cells (Wasano et al., 1990) and neurons (Regan et al., 1986).

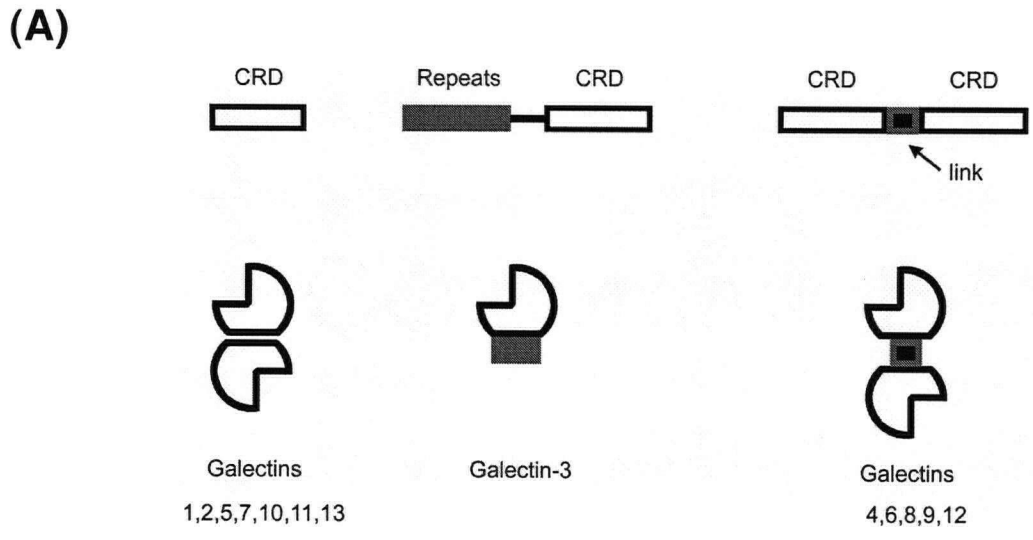



**Figure 1.1 Galectin structures**

(A) Homologous carbohydrate recognition domain (CRD) present in all galectins. The primary protein structure is represented along the top row of the diagram. The quaternary structure is represented in the second row. Galectin-1 can exist either as a monomer or a homodimer indicated by the space between the two circular structures. (B) Structure of a human Gal1 dimer. 40% of the galectin-1 protein is a β-pleated sheet (arrows) and 10% α-helix with random coils (wire).

*Galectin-1: structure*

Gal1 exists as a monomeric or homodimeric protein comprised of subunits of 135-amino-acid long-chains, each such chain having a molecular weight of 14.5 kDa (Barondes et al., 1994b). The structure of a human Gal1 monomer consists of β-pleated sheets (40%), α-helixes (10%) and random coils. As a dimeric molecule, Gal1 possesses two galactoside binding sites allowing either intramolecular or intermolecular crosslinking by binding more than one sugar residue. Gal1 has been reported to be released from myogenic cells (Cooper and Barondes, 1990), Chinese hamster ovary cells (Cho and Cummings, 1995), human leukemia cell lines (Lutomski et al., 1997b), Schwann cells and dorsal root ganglions (Sango et al., 2004).

GALECTIN-1: 2 REDOX FORMS

Gal1 exists in two known conformations, each of which has a different biological effect. These conformations are dictated by Gal1's reduction/oxidation (redox) state. Under reducing conditions, reduced Gal1- (Red) exists as a homodimer (Lobsanov et al., 1993). In the reduced conformation there are two CRD that facilitate both *intra* and *inter*molecular binding (Liao et al., 1994; Perillo et al., 1998). This binding then promotes cellular adhesion, fasciculation and/or agglutination via β-galactoside binding (Perillo et al., 1998). β-galactoside binding is termed lectin activity. *In vitro,* lectin activity can be inhibited either by the addition of specific sugar residues that would competitively inhibit β-galactoside binding or by Gal1 oxidization (Tracey et al., 1992; Brewer, 2004). The change from a reduced to oxidized state was then termed "oxidative inactivation" (Tracey et al., 1992), yet this oxidative inactivation switched Gal1 from allowing β-galactoside binding (lectin activity) to promoting neuronal outgrowth (Inagaki et al., 2000). When oxidized, Gal1 changes conformation and exists only as a monomer. Examining Gal1's molecular structure gives further insight into how the switch from a homodimer to a monomer occurs. Within each Gal1 subunit, six cysteines can form three intramolecular disulfide bonds ($Cys^{2}$-$Cys^{130}$, $Cys^{16}$-$Cys^{88}$ and $Cys^{42}$-$Cys^{60}$) (Gupta et al., 1996; Horie and Kadoya, 2000). When these bonds form, Gal1 changes from a reduced state to an oxidized state (Gal1-Ox) (Horie and Kadoya, 2000). Gal1 is believed to exist mainly in a reduced state within cells, since free radicals that can be generated in oxidizing environments can be deleterious to normal cellular

functions. When secreted, a proportion of the released Gal1 becomes oxidized due to the oxidizing conditions in the extracellular environment.

*Galectin-1: location and secretion*

Although Gal1 is present on the cell surface or ECM like all other galectins, it lacks a recognizable secretion sequence and accordingly does not pass though the standard Golgi/ER pathway. Instead, Gal1 has an acetylated N-terminus, free sulfhydryls and lacks the glycosylation that is most often associated with cytoplasmic proteins. Cellular localization is predominately cytoplasmic with some nuclear staining, but is never observed inside the classic secretory pathways. Similar to that of fibroblast growth factor (FGF)-1 and –2, the secretion of Gal1 has been demonstrated though a novel non-classical secretory mechanism possibly through an ABC transporter (Cleves et al., 1996; Cleves and Kelly, 1996) and not via the classical vesicle-mediated exocytosis (Cooper and Barondes, 1990; Schafer et al., 2004).

*Galectin-1: non-neuronal tissue*

MUSCLE TISSUE

Gal1 expression occurs in both striated and smooth muscle cells (Watt et al., 2004). *In vitro* studies demonstrate that the fusion of myoblasts is reduced if the cells originate from Gal1 -/- mice (Poirier and Robertson, 1993). Striated muscle from Gal1 -/- mice has more connective tissue than that of Gal1 wild type (*wt)* animals. After muscle injury, Gal1 *wt* muscle cells demonstrate an increase of Gal1 protein expression. The fact that striated muscle in Gal1 -/- mice does not regenerate as quickly as Gal1 *wt* muscle tissue after injury suggests that the injury induced increase of Gal1 expression in Gal1 *wt* mice is involved in muscle regeneration (Watt et al., 2004). Therefore Gal1 is important for both muscle development and repair after injury.

KIDNEY DEVELOPMENT

Kidney cells are known to express Gal1, 3 and 9 (Hughes, 2002). Gal1 particularly is important for ECM and connective tissue organization (Wasano et al., 1990). There are no reports of differences in kidney development or organization between Gal1 -/- and *wt* mice.

## T CELL REGULATION

Research has identified immunomodulatory properties for Gal1. In three experimental autoimmune diseases, the T-cell-mediated autoimmune disease experimental encephalomyelitis (EAE), experimental autoimmune myasthenia gravis (EAMG) in rabbits and collagen induced rheumatoid arthritis, Gal1 either prevents the initiation of the disease as in the case with EAE or attenuates the disease's effects in the case of EAMG or rheumatoid arthritis (Levi et al., 1983; Offner et al., 1990; Rabinovich et al., 1999). Although the precise mechanisms (including Gal1's redox state) remain unclear, Gal1 is implicated in regulating immune activation through T cell apoptosis (Perillo et al., 1995; Nguyen et al., 2001; Zuniga et al., 2001). It is suggested that the reduction of active Gal1 is correlated with immune activation imbalance (Lutomski et al., 1997a).

Gal1 may also regulate immune responses under normal physiological conditions (Rabinovich et al., 2002). Gal1 expression occurs in immune-privileged sites such as the placenta and the eyes (Hirabayashi and Kasai, 1984; Iglesias et al., 1998; Ogden et al., 1998). At such sites, inflammation could have devastating consequences, so multiple factors ensure rapid elimination of inflammatory cells. Recently, FasL expression in sites of immune privilege has been shown to selectively kill T cells by apoptosis to maintain the immune barrier (Ferguson and Griffith, 1997a, b; Griffith and Ferguson, 1997; Stuart et al., 1997). Accordingly, Gal1 expression might aid in maintaining immune barriers.

## CANCER

In multicellular organisms, homeostasis is maintained through a balance of proliferation and death. Gal1 can limit T cell infiltration by inducing apoptosis, but Gal1 expression also occurs within constantly rejuvenating or growing tissues, such as smooth muscle, the adult olfactory system or other cells undergoing rapid growth such as tumour cells (Puche et al., 1996; Moiseeva et al., 2000; Camby et al., 2001). For example, the malignancy of astrocytomas, prostate carcinomas and metastatic pancreatic cancer correlates with high Gal1 expression (Berberat et al., 2001; Camby et al., 2001; van den Brule et al., 2001). In one of the few experiments demonstrating a direct effect of Gal1 on metastatic growth, the application of Gal1 anti-sense mRNA to cultured astrogliomas significantly reduced the cells' malignancy (Yamaoka et al., 2000). It is proposed that Gal1 changes the adhesive

properties of cell-cell and cell-matrix interactions of the tumours while attenuating the immune response to increase their ability to proliferate and spread.

*Galectin-1: neuronal expression*

The first publication relating to Gal1 expression within the rat CNS was presented in 1986 (Regan et al., 1986). Using antibodies against a Gal1 epitope, the authors reported Gal1 (then termed RL-14.5) protein expression in 63% of primary sensory somata as well as expression in spinal motor neurons (Regan et al., 1986). The initial expression began at E13-14 as these sensory neurons finished their final mitotic division and began their growth towards their targets within the dorsal horn of the spinal cord. When Gal1-expressing neurons reached their targets, Gal1 expression remained elevated, albeit at lower levels (Regan et al., 1986; Hynes et al., 1990; St John and Key, 1999; Sango et al., 2004). *In situ* hybridization studies later revealed that the Gal1 protein within the neurons reflects the synthesis by the neurons themselves and not the accumulation of the protein from other central or peripheral cells (Hynes et al., 1990). Gal1 expression was also observed in the olfactory bulb, cranial motoneurons and sympathetic ganglia (Hynes et al., 1990). However, although studies using Gal1 -/- mutant mice have offered further insight into this lectin's potential role in axonal growth and development, the precise role of Gal1 during development remains elusive.

*Galectin-1: neuronal function*

Gal1 expression occurs in a specific class of olfactory neurons. In the olfactory system, nerve fibers originating in the olfactory epithelium converge on spatially-defined glomeruli in the olfactory bulb (St John and Key, 1999). The ensheathing glial cells along this pathway synthesize and express Gal1 or laminin (Mahanthappa et al., 1994; Crandall et al., 2000). Although these two molecules associate in the same tract, the function of their interaction remains uncertain (Crandall et al., 2000). *In vitro*, Gal1 promotes the fasciculation of primary olfactory neurons by facilitating the binding axon-axon and axon-matrix (such as laminin) interactions in an integrin-independent mechanism (Mahanthappa et al., 1994). Using Gal1 -/- mice, generated by inserting a neo cassette containing a stop codon into the Gal1 gene by homologous recombination in embryonic stem cells (Poirier and Robertson, 1993), the effects of Gal1 on olfactory bulb development were examined. In the adult Gal1 -/- mice, the

*Dolichos biflorus agglutinin*-binding neurons came close to their appropriate targets but failed to reach them and make proper connections (Puche et al., 1996). In these animals, immunohistochemistry revealed a normal distribution of Schwann cells, NCAM, and laminin at the olfactory bulb (Puche et al., 1996). These data suggested that Gal1 may be involved in neuronal outgrowth and/or synaptic connectivity of specific neuronal populations and/or has a putative role in neuronal maintenance.

*Galectin-1: neuropathological conditions*

PAIN

Partial nerve injury is frequently associated with hyperalgesia (increased pain sensitivity), allodynia (pain from non-noxious stimuli) and general ongoing pain. As previously indicated, high levels of Gal1 are associated with increased growth and oxidized Gal1 promotes outgrowth from peripheral as well as central branches of DRG neurons *in vitro* (Horie et al., 1999; Berberat et al., 2001; van den Brule et al., 2001). Using a model for neuropathic pain, in the spinal nerve ligation model, Gal1 immunoreactivity increases in the dorsal horn (Camby et al., 2001; Imbe et al., 2003). Using a cDNA array to examine changes in the gene expression 2d-28d after a sciatic nerve axotomy reveals that Gal1 mRNA increases as much as two to five times after injury at all time points examined (Xiao et al., 2002). The increase in Gal1 expression elicits some neuropathic effects, since intrathecal administration of function-blocking Gal1 antibodies attenuates mechanical hyperalgesia (Cameron et al., 1997; Imbe et al., 2003). In summary, Gal1 correlates with increased excitation and sensitization of neurons in a neuropathic pain state, whereas reduction of Gal1 activity reduces both the anatomical and functional changes associated with this increased activity.

NEURONAL REGENERATION: GAL1-OX

The growth-promoting effects of Gal1 were discovered using kidney cells combined with an *in vitro* DRG assay (Horie et al., 1999). Horie and colleagues demonstrated that recombinant Gal1-Ox monomers, and not the reduced dimers, specifically promoted DRG outgrowth *in vitro* and accelerated injured peripheral nerve growth into silastic tubes *in vivo* (Horie et al., 1999). This work was later repeated using acellular autografts with the retrograde tracer FluoroGold applied at the ends of the grafts to label growing axons (Fukaya et al., 2003). For both DRG and spinal motor neurons, there were an increased number of FluoroGold-labeled somata with rhGal1-Ox infusion as compared to vehicle alone treatment (Fukaya et al.,

2003). Conversely, Gal1 functional-blocking antibodies significantly reduced the number of labeled DRGs and SMN when compared to control rabbit IgG application alone (Fukaya et al., 2003). In both of these experiments, the increased growth of axotomized neurons was closely associated with increased Gal1 immunoreactivity in Schwann cells as well as increased migration into the tubes or autografts (Horie et al., 1999; Fukaya et al., 2003). In these experiments, only complete nerve resections attached to either a silastic tube or an acellular graft were used to examine nerve regeneration. This injury model prevents the assessment of *functional* regeneration through the use of specific sensory and motor assessment because the regenerating axons are unable to reach their targets. Accordingly, these experiments demonstrate that Gal1-Ox increases the rate of neuronal regrowth *in vitro* and *vivo*, but not whether appropriate and functional connections are made.

***Hypothesis, relevance and models***

Although all of the results discussed above supported the hypothesis that exogenously-applied recombinant Gal1-Ox promotes axonal regeneration in Gal1-expressing neurons, I believed that the more basic questions of 1) whether neuronal Gal1 expression changed after axotomy or 2) whether the intrinsic Gal1 expression had a role in axonal injury and/or regeneration had not been answered. After surveying the literature, ***I hypothesized that endogenous neuronal Gal1 expression influences the growth potential of axotomized neurons.*** Testing this hypothesis required a clinically relevant model that would further our understanding of regenerative neuronal processes in traumatic injury. Since most of the Gal1 expression data indicated that both the SMN and DRG neuronal populations would yield some results, I chose the brachial plexus as a model system to attempt to examine Gal1 expression in a medically relevant injury.

Brachial plexus injuries are medically complex, due to associated anesthesia, paralysis and complex pain of differing degrees (Berman et al., 1998). Scientifically, these injuries are intriguing. Depending on the severity and location of injury, recovery of brachial plexus motor and sensory deficits varies considerably. Here, lesion classification can aid in determining functional outcome. Clinically, an upper limb rhizotomy with associated motor nerve avulsion or axotomy injury often results in one of three recognized syndromes: Duchenne-Erb or Erb's syndrome (with the levels C5-C6 being injured), Klumpke or

Duchenne-Aran syndrome (C8-T1 injured) or the most devastating when the roots from C5-T1 are damaged. Increased understanding of regenerative failure may lead to strategies to promote regenerative success in rodents and will hopefully aid in better treatments and recovery for humans.

*Experimental outline addressing my hypothesis*

To ascertain whether Gal1 expression correlates with growth propensity, I examined two well-established neuronal regeneration models. I compared changes in Gal1 mRNA expression in axotomized SMN (which are known to successfully regenerate back to their targets), to the non-regenerating axotomized rubrospinal neurons. These results are presented in **Chapter 2.**

To further support this hypothesis I then examined DRG neurons, since they have different regenerative responses depending on lesion location. Here I examined whether Gal1 expression changes between a peripheral axotomy (increased regenerative response) and a rhizotomy (limited or no regenerative response). Using rhGal1, I artificially increased the available Gal1-Ox to determine whether this would facilitate growth into the CNS after rhizotomy. These results are presented in **Chapter 3.**

Although the application of Gal1 or functional blocking antibodies addresses questions about Gal1 involvement in regeneration, it does not address the role of the endogenous production of Gal1. Thus I have examined Gal1 -/- mice and their possible functional deficits in both the naïve and injured animals. Since Gal1 has a putative role in neuropathic pain, **Chapter 4** examines whether Gal1 -/- mutant mice display differences in nociceptive behavior and sensory anatomy.

Finally, in **Chapter 5**, using Gal1 -/- mice, I tested whether the complete absence of Gal1 changes the regenerative ability of neurons. Although the medial nerve of the brachial plexus is interesting to study because it contains sensory, motor and sympathetic components, it is also difficult to assess functional regeneration. Separating sensory and motor behavior responses can be potentially challenging. Accordingly, I chose to examine the facial nucleus, which primarily contains motoneurons. Since this nerve innervates the

mouse whisker pad, I examined the rate of functional recovery as determined by analyzing whisker movement between Gal1 -/- and wild type mice.

Overall, in the following four chapters I attempted to assess injury-induced changes in neuronal Gal1 expression and whether these changes are important for functional regeneration.

# CHAPTER 2: GALECTIN-1 EXPRESSION CORRELATES WITH THE REGENERATIVE POTENTIAL OF RUBROSPINAL AND SPINAL MOTONEURONS

*Introduction*

A striking disparity exists between the regenerative ability of axons within the CNS, and neurons that project to the periphery. Extrinsic factors such as the glial response and the intrinsic gene expression of the injured neurons are implicated in the lack of regeneration within the CNS (Steeves and Tetzlaff, 1998; McGraw et al., 2001). In particular, axonal regeneration of peripherally axotomized motor neurons is associated with a cell body response that includes the increased expression of immediate-early genes, trophic factors, trophic factor receptors, neuropeptides and cytoskeletal proteins (Herdegen et al., 1992; Tetzlaff et al., 1994; Fernandes et al., 1999; Fernandes and Tetzlaff, 2000). Recently, exogenous application of rhGal1-Ox has been shown to promote both the rate and success of axonal elongation of peripherally-projecting neurons; however, both the expression and role of the endogenous neuronal Gal1 expression in the CNS injury is unknown (Horie and Kadoya, 2000; Fukaya et al., 2003).

Lectins are carbohydrate-binding proteins that recognize specific oligosaccharide moieties involved in promoting immune cell agglutination and cell signaling processes (Barondes, 1988). Gal1 is a member of the galectin subfamily of lectin proteins whose sequence is conserved from *Caenorhabditis elegans* through to humans (Barondes et al., 1994b). The growth state of various tissues from this diverse range of organisms is tightly correlated with Gal1 expression. For example, in adult rodents and mammals, Gal1 expression occurs within tissues that constantly rejuvenate or grow, such as smooth muscle, the adult olfactory system, and tumour cells (Puche et al., 1996; Moiseeva et al., 2000; Camby et al., 2001).

Within the developing nervous system, high levels of Gal1 expression are also observed (reviewed in Perillo et al., 1998; Moiseeva et al., 2000). In embryonic rats, Gal1 mRNA expression increases within the somata of spinal motoneurons (SMN) until they reach their target muscles, following which, expression is maintained at a lower level throughout adult life (Hynes et al., 1990). After a nerve injury, axons re-enter a growth mode to re-establish their connections. Gal1 mRNA increases within 6 to 24 hours in motoneurons following

facial nerve axotomy (Akazawa et al., 2004). Here I examined the expression of Gal1 in regenerating and non-regenerating neurons. Specifically, I compared the Gal1 mRNA level within the somata of injured SMN, a population that does regenerate to its peripheral targets (Nguyen et al., 2002), to that of injured rubrospinal neurons (RSN), a CNS neuronal population that does not typically exhibit axonal regeneration following spinal axotomy (Barron et al., 1975; Tetzlaff et al., 1994). I also assessed the effects of brain derived growth factor (BDNF) on Gal1 expression within the red nucleus, since BDNF application to the red nucleus increases both the expression of genes associated with regeneration, and the ability of axotomized rubrospinal neurons to grow into a permissive peripheral nerve graft (Kobayashi et al., 1997; Kwon et al., 2002b).

***Materials and Methods***

*Surgery*

Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, weight 200-250 g) were used. 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 under sterile conditions.

*Rubrospinal tract lesion*

Under anesthesia, 15 Sprague-Dawley rats underwent a transection of the dorsolateral funiculus at the level of the fourth cervical vertebra (C4), severing the rubrospinal tract unilaterally. The lesion was made with a pair of fine iris scissors. Six animals underwent intracranial implantation of a 28-gauge cannula into the vicinity of the rubrospinal neurons (6.3 mm posterior to bregma, 1.7 mm to the right of midline, and 6.5 mm deep to the dural membrane) as described previously (Kobayashi et al., 1997). An osmotic minipump (Alzet no. 2001, 1 μg/h, Palo Alto, CA) was connected to the cannula by silastic tubing. Three animals received 12 μl/d of BDNF (gift from Regeneron Pharmaceuticals, Tarrytown, NY) in a vehicle solution of 20 mM sterile PBS, 100 units of Penicillin/Streptomycin (Gibco BRL, Burlington, ON), and 0.5% rat serum albumin (Sigma-Aldrich, Oakville, ON) while three animals received vehicle alone.

*Spinal nerve lesions*

12 (n=4 for each time point) rats were anesthesitized, and the spinal nerves were exposed on the left side from C6 to C8. Nerves were cut at their exit points from the spinal column and a 5mm section of peripheral nerve was removed. The wound was closed in layers with silk sutures.

*Perfusion / cryosectioning*

At either 7 or 14 days, animals were injected with a lethal dose of chloral hydrate and monitored. Upon the loss of nociceptive reflexes, animals were perfused intracardially with PBS followed by cold 4% paraformaldehyde. 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 a 22% sucrose solution in PB. After cyroprotection, tissue was rapidly frozen in

supercooled 2-methylbutane and, later, 16 μm cryosections of brain or spinal cords were cut, cold-mounted onto glass slides (Superfrost plus) and stored at -80°C.

*Immunohistochemistry*

For Gal1 immunohistochemistry of spinal motoneurons, slides were washed in 0.1m PBS for 20 minutes, after which rabbit anti-Gal1 (1:6000 in 0.1M PBS, 0.2% Triton X-100, and 0.1% sodium azide) was applied to the slides overnight. Two separate Gal1 antibodies were used for the analysis showing identical results. One Gal1 antibody was generous gift from Dr. D. N. Cooper at the University of California San Francisco (Regan et al., 1986) and the other was previously generated (Horie et al., 1999). Both antibodies are shown to be specific for Gal1 alone (Regan et al., 1986; Horie et al., 1999). 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. A tyramide signal amplification step was then employed as per the manufactures instructions (PerkinElmer Lifescience, Boston, MA). Cy3-conjugated Steptavidin (1:500, Sigma) was used to visualize the Gal1 antibody. A fluorescent Nissl stain (Neurotrace, 1:200, Molecular Probes Inc. Eugene, OR) was then applied to visualize nissl substance. Slides were coverslipped with a 3:1 solution of glycerol: PBS.

*In situ hybridization*

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 [$^{33}$P]-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 3 days (SMN) or 5 days (red nucleus). Slides were then dehydrated in a series of alcohols and stored at room temperature. Spinal cord sections were later re-hydrated in $dH_20$ for 1 hour and counterstained with Neurotrace (Molecular Probes. 1:200). For the red nucleus, sections were stained in 0.01% ethidium bromide (EtBr) for 1 hr and rinsed under running

$H_2O$ for 1 hr. Slides were then dehydrated in a series of alcohols and coverslipped with Entallen (Fisher Scientific, Nepean, ON).

*Image analysis*

Determination of immunoreactivity within somata has been previously described (Ramer et al., 2003). Briefly, for spinal motoneuron immunohistochemistry, a fluorescent microscope (Axioskop, Carl Zeiss, Toronto, ON) was used to visualize chromophore labeled tissue, and images were captured using a digital camera (Carl Zeiss) in combination with Northern Eclipse software (Empix Inc, Mississauga ON). Neurotrace and Gal1 images were taken of both the injured and the contralateral sides at the same image capture settings. Motoneuron cell bodies were outlined on the neurotrace image. The resulting drawn layer was then used to determine the staining intensity of Gal1 labeled profiles in the same double-labeled sections. An area in close proximity to the traced images but without neuronal cell bodies was also traced to determine background (non-specific) labeling. The intensity of this layer was subtracted from the motoneuron intensity values. To determine proportions of positively labeled motoneurons, the threshold for positive immunoreactivity was determined subjectively and recorded in each section so that variations in intensity could be accounted for on a section-by-section basis. Injured somata were compared to the uninjured contralateral somata.

For in situ hybridization (ISH), Neurotrace (spinal motoneurons) or EtBr (rubrospinal neurons) and darkfield images of silver grains were taken of both the injured and the contralateral sides using a digital camera attached to a fluorescent microscope in combination with Northern Eclipse software. All images were analyzed with SigmaScan Pro 5 software (SPSS Inc, Chicago, IL). The percentage area occupied by silver grains of each neuronal cell body determined the somal grain area fraction. This was measured by outlining the individual neuronal cell bodies using the neurotrace or EtBr image and applying the resulting cell profile layer to the darkfield (silver grain) image. For each animal, the percentage area occupied by ISH signal per soma was determined for both the axotomized and contra lateral (uninjured) side. The difference between background autoradiographic signal and the mean ISH signal per soma was calculated. At least three sections, >100 $\mu$m apart, were quantified

per ventral horn or red nucleus, and the relative ISH signal was represented as a multiple of background.

*Statistics*

Quantification was performed blind with respect to the treatment groups. All data are represented as mean ± standard error of the mean (SEM), and all tested were carried out using SigmaStat 3.0 (SPSS Inc, Chicago, IL). Unless otherwise indicated, a Student's t-test was applied to the average area covered by ISH signal per somata to detect significant changes between axotomized and contralateral neurons and an ANOVA test was used to determine significant differences between groups. If significance was found then a post hoc Holm-Sidak test was used. Significance was assigned at $P<0.05$.

***Results***

*Spinal nerve axotomy increases motoneuron Gal1 mRNA and Protein Levels*

Axotomy of cervical spinal nerves from the 6$^{th}$ cervical (C6) to 8$^{th}$ cervical (C8) level was performed in order to examine changes in Gal1 mRNA in spinal motoneurons, 7 and 14 days (n=4 animals per group) after injury. Spinal nerves were lesioned at their exit point from the spinal column to ensure that all motoneurons in a particular segment were completely severed. Seven days after axotomy, the average percentage cell soma area occupied by silver grains was 16.6 ± 1.7 (mean ± SEM) times greater than background on the injured side and 9.3 ± 0.9 on the contralateral uninjured side, representing a significant increase ($p<0.05$, Figure 2.1). 14 days after injury, this expression remained significantly elevated to 15.0 ± 2.1 times background signal on the axotomized side and 6.6 ± 0.8 on the contralateral uninjured side ($p<0.05$, Figure 2.1). Gal1 immunohistochemistry on the same tissue revealed an increase in the proportion of motoneurons possessing high levels of Gal1 staining intensity compared to the uninjured controls (Figure 2.2), and a cumulative sum chart (Figure 2.2 right inset) reveals a significant increase (as observed by a right shift of the curve) of the proportion of Gal1 positive SMN soma at both 7 and 14 days after injury ($p < 0.05$ Kolmogorov-Smirnov test).

*BDNF reverses RSN axotomy induced decrease of Gal1 mRNA expression*

RSNs within the central projecting rubrospinal tract were transected at the C4 level, and the red nucleus was examined 3, 7 and 14 days after injury (n=3 animals per group). The expression of Gal1 mRNA in the uninjured red nucleus was lower than that of uninjured SMN as indicated by the longer (3 days for SMN, 5 days for RSN) exposure time required to obtain comparable silver grain densities. In contrast to SMNs, axotomized RSN exhibited a decreased Gal1 mRNA expression. At 3 days post-lesion, the axotomized RSN mean silver grain proportional area was 10.2 ± 0.9 times greater than background compared to 11.2 ± 1.2 times background on the uninjured (contralateral) side (not significant, $p>0.05$, Figure 2.3). At 7 days post-lesion, the axotomized RSN somata had a significantly lower average silver grain proportional area of 7.7 ± 0.7 times background compared to 12.0 ± 0.8 on the uninjured (contralateral) side, ($p<0.02$, Figure 3). At 14 days post-lesion, there was an even greater decrease in Gal1 average grain area fraction over individual somata from 9.7 ± 0.7

times background on the uninjured side to 5.3 ± 0.6 times background on the injured side ($p<0.05$, Figure 2.3).

To test the hypothesis that Gal1 is associated with regenerating systems, the regenerative response of RSNs was stimulated via infusion of BDNF into the vicinity of the injured RSNs (Kobayashi et al., 1997; Plunet et al., 2002). In the present study, 12 μg/day infusion of BDNF for seven days after injury resulted in a significant increase in the Gal1 silver grain proportional area, which was 9.0 ± 1.3 times background on the uninjured contralateral RSN compared to 13.9 ± 1.0 times background on the axotomized RSN ($p<0.05$, Figure 2.4). No significant differences in Gal1 expression were observed between the axotomized and uninjured contralateral RSN in vehicle-treated control animals (Figure 2.4). However, between treatment group comparisons showed that there was a significant difference between the BDNF treated group and both the vehicle treated group ($p=0.02$, Figure 4) and untreated lesioned somata at 7d ($p<0.02$, Figure 2.3 and 2.4). The vehicle treatment effect has been previously described in the same paradigm when examining changes of GAP-43 mRNA within the red nucleus (Kobayashi et al., 1997). Sense oligonucleotides did not reveal any specific autoradiographic signal within SMN or the red nucleus.

**Figure 2.1 Gal1 ISH signal in cervical spinal motor neurons**

The proportional area of motoneuron somata covered by silver grains was quantified 7 and 14 days (n=4 per group) after a C6-C8 spinal nerve lesion and compared to background signal. Seven days after axotomy the average proportional area was 16.6 ± 1.7 (mean ± SEM) times greater than background on the injured side, which was significantly higher than the uninjured contralateral side of 9.3 ± 0.9 times greater than background ($p<0.02$). 14 days after injury, this expression remained elevated at 15.0 ± 2.1 times greater than background on the axotomized side and 6.6 ± 0.8 on the contralateral uninjured side representing a significant increase ($p<0.02$). * indicates $p<0.05$, Scale bar = 50 microns.

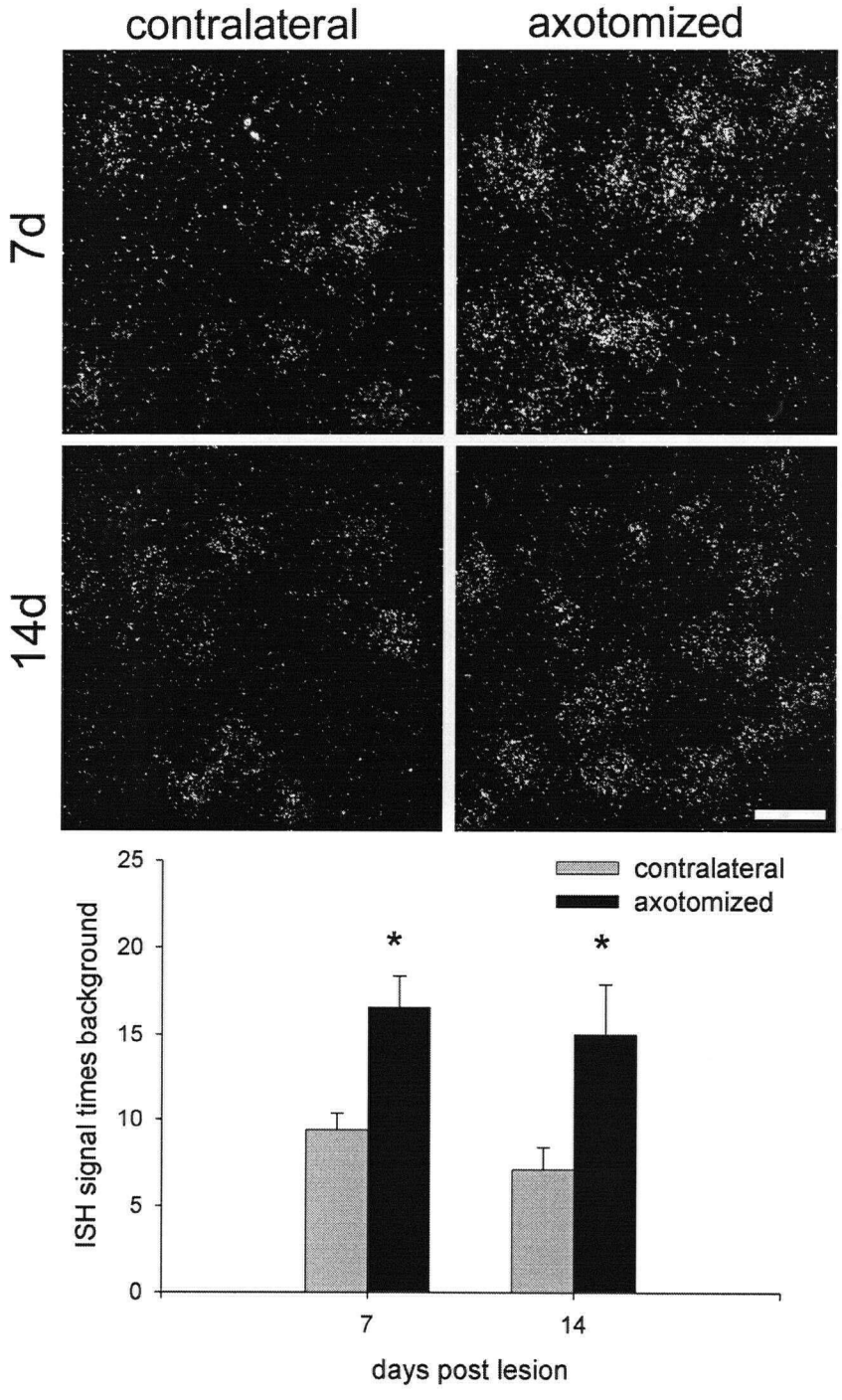

contralateral
axotomized
7d
14d
25
20
15
10
5
0
ISH signal times background
contralateral
axotomized
*
*
7
14
days post lesion

**Figure 2.2 Gal1-IR of spinal motor neurons**

Seven and fourteen days after injury an increase in Gal1 immunoreactivity was observed compared to the uninjured contralateral side. Histograms of immunoreactivity (0-255 grayscale) revealed a significant increase in Gal1 immunoreactivity (graph on far right). Cumulative sum (Qsum) plots were used to determine statistical significance (chart inset far right, $p < 0.05$ Kolmogorov-Smirnov test). Scale bar = 50 microns

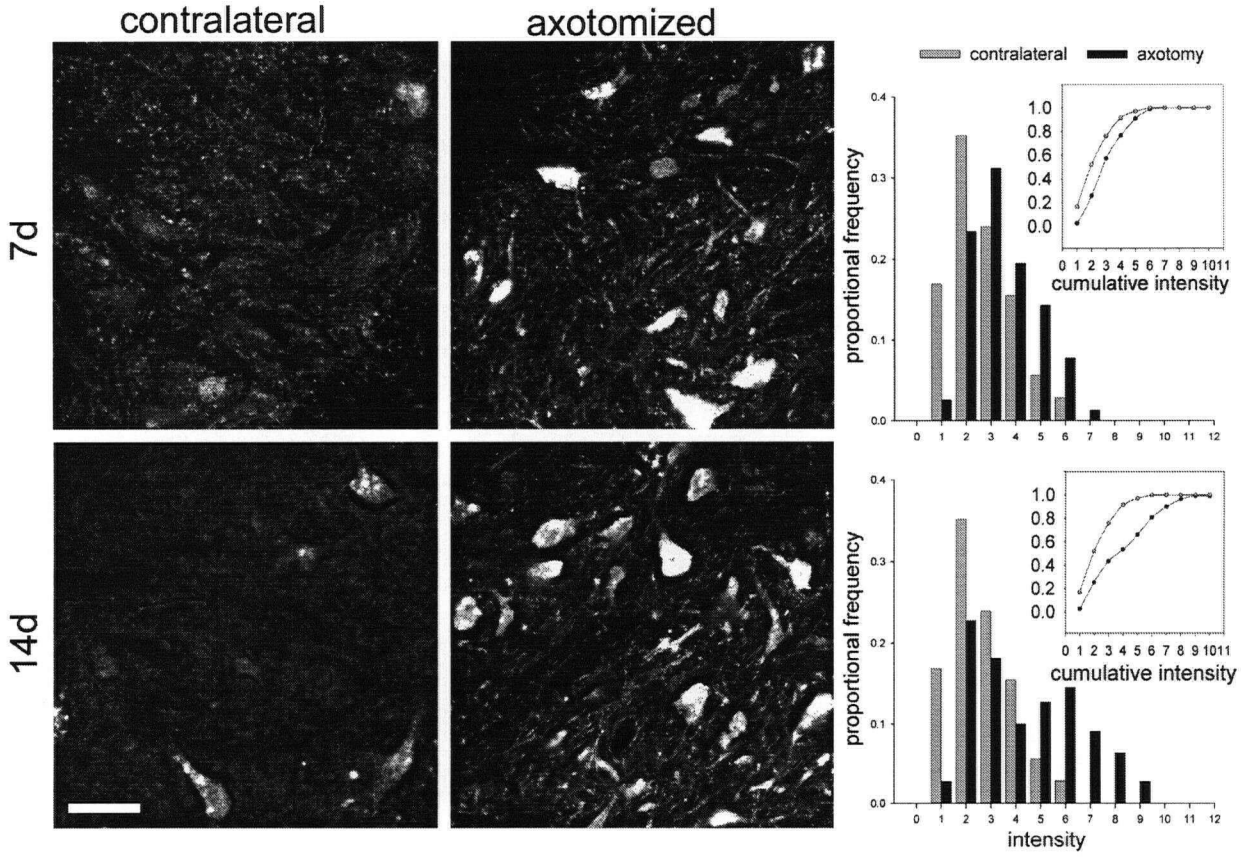

contralateral
axotomized
7d
14d
contralateral
axotomy
proportional frequency
cumulative intensity
intensity

**Figure 2.3 Gal1 ISH signal in the red nucleus**

Gal1 *in situ* hybridization signal in the red nucleus 3, 7 and 14 days (n=3 per group) after a transection of the dorsolateral funiculus (which includes the rubrospinal tract) at the fourth cervical level. At 3 days post lesion, the axotomized RSN had an average proportional area of somata covered by silver grains was 10.2 ± 0.9 (mean ± SEM) times greater than background compared to 11.2 ± 1.2 times background on the contralateral side ($p>0.05$, top panel). At 7 days post lesion, the axotomized RSN somata had a significantly lower average proportional area covered of 7.7 ± 0.7 times background compared to 12.0 ± 0.8 on the uninjured contralateral side ($p<0.02$, middle panel). At 14 days post lesion, there was a significant decrease in Gal1 average grain area fraction over individual somas from 9.7 ± 0.7 times background on the uninjured side to 5.3 ± 0.6 times background on the injured side ($p=0.01$). * indicates $p<0.05$, scale bar = 50 microns.

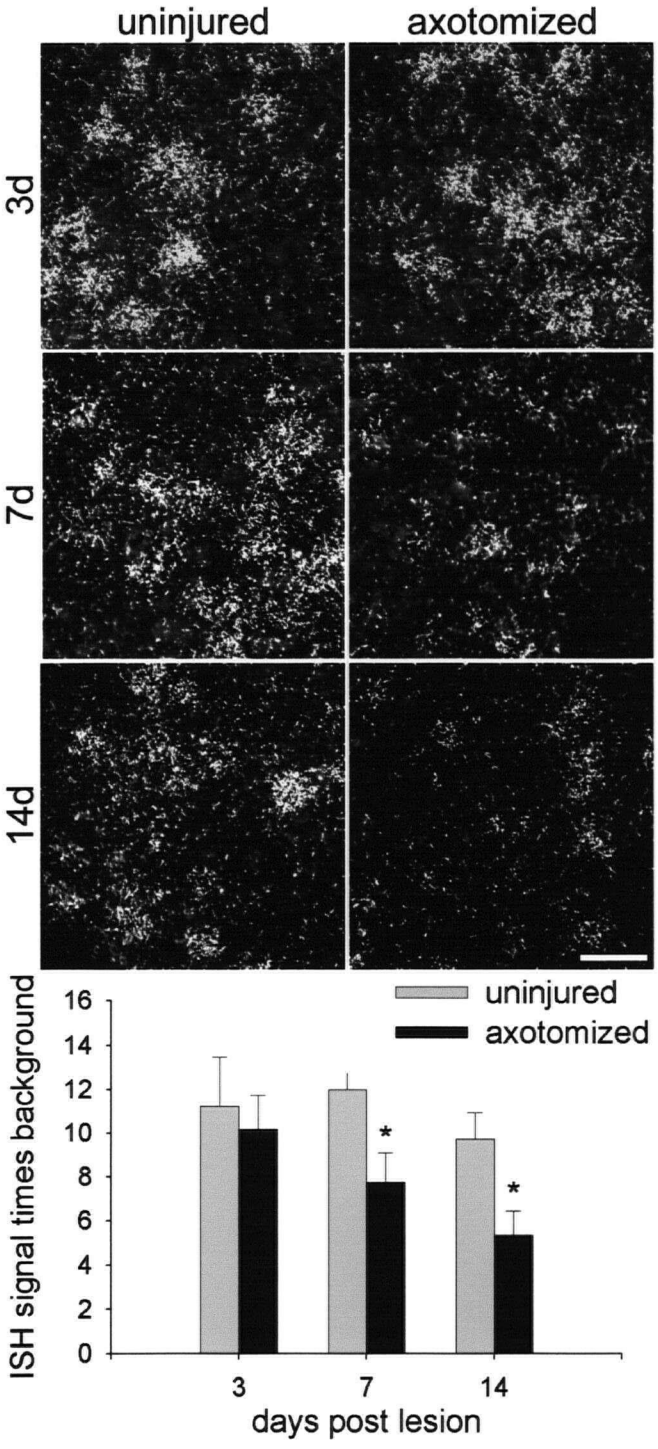
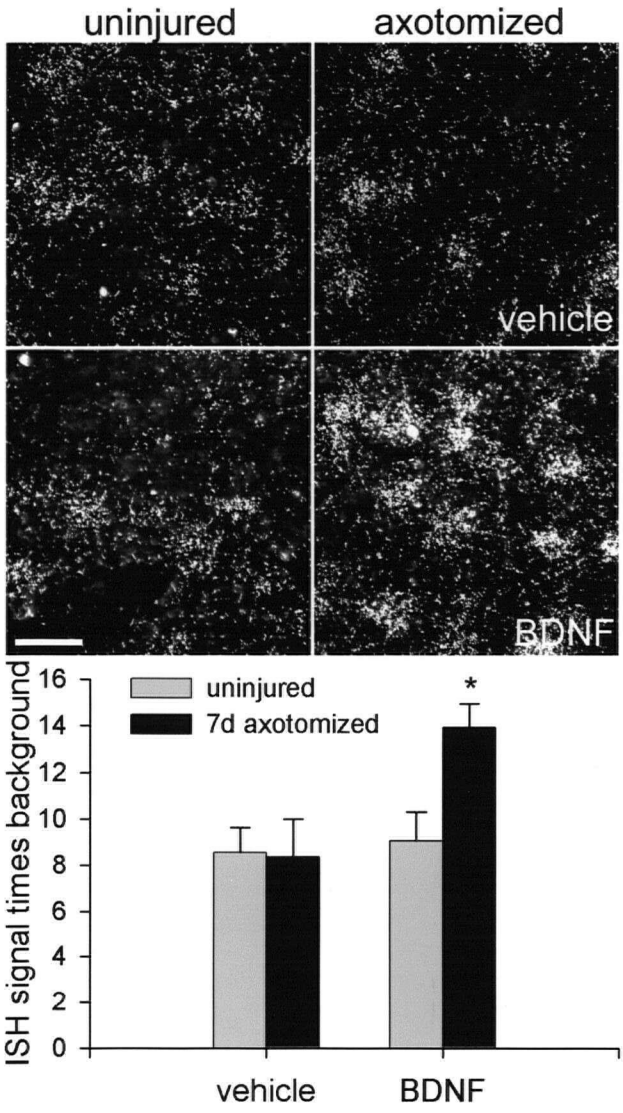

uninjured
axotomized
3d
7d
14d
ISH signal times background
16
14
12
10
8
6
4
2
0
uninjured
axotomized
*
*
3
7
14
days post lesion

**Figure 2.4 Gal1 ISH signal in the red nucleus following BDNF treatment**

Gal1 *in situ* hybridization signal in the red nucleus seven days after transection of the dorsolateral funiculus (including the rubrospinal tract) at the forth cervical level, plus a one-week infusion of either vehicle or BDNF (n=3 per group) into the vicinity of the red nucleus. Here I show that after a 12 μg/day infusion of BDNF there is a significant increase in the average proportional area of somata covered by silver grains from 9.0 ± 1.3 (mean ± SEM) times background on the uninjured contralateral RSN compared to 13.9 ± 1.0 times background on the axotomized RSN ($p<0.05$, middle panel and histogram). No significant differences in Gal1 expression were observed between the injured (8.4 ± 1.6 times background) and uninjured contralateral (8.6 ± 1.1 times background) RSN in vehicle-treated control animals ($p>0.05$, top panel and histogram). There was a significant difference between the BDNF treated group and the untreated lesioned somata at 7d compared ($p<0.02$ Figure 2.3) or to the vehicle treatment group ($p=0.02$). * indicates $p<0.05$). Scale bar = 50 microns.


uninjured
axotomized
vehicle
BDNF
ISH signal times background
16
14
12
10
8
6
4
2
0
uninjured
7d axotomized
*
vehicle
BDNF

*Discussion*

The repertoire of molecules whose expression increases in successfully regenerating neurons includes trophic factors, cytokines, cytoskeletal and guidance molecules (Fernandes and Tetzlaff, 2000; Kwon et al., 2002a). Changes in these molecules are thought to represent adaptive responses to reduce the deleterious effects of peripheral nerve damage and promote survival and regeneration of lesioned axons. Previous work has shown that high Gal1 expression correlates with both growing SMN and DRG neurons during neuronal development (Regan et al., 1986). Furthermore, in the adult, application of rhGal1-Ox increases both the regenerative rate and success for both sensory and SMN regeneration (Regan et al., 1986; Fukaya et al., 2003). However, changes in the endogenous neuronal expression of Gal1 are currently unknown. Here, I examined whether endogenous Gal1 expression changes following axonal injury, and how its expression correlates with the regenerative propensity of the injured neurons.

A large increase in Gal1 mRNA and protein occurs within the neuronal somata of SMN 7 and 14 days following peripheral axotomy. I then asked whether Gal1 expression was correlated with the muted regenerative potential of CNS neurons in the well-characterized rubrospinal system. When injured at the cervical level, these neurons are unable to regenerate but can initiate a weak regenerative response (Tetzlaff et al., 1991). In contrast to SMN, Gal1 mRNA expression decreased after axotomy. These results are similar to the expression profiles of regeneration-associated genes such as interleukin-6, which shows a decrease of mRNA in the red nucleus after axotomy but an increase in the facial nucleus after a peripheral injury (Streit et al., 2000). Unlike SMNs, the upregulation of genes associated with regeneration in RSNs is meager (Fernandes et al., 1999; Fernandes and Tetzlaff, 2000). This modest change in gene expression correlates with the limited ability of a small number of neurons to grow into a pre-degenerated peripheral nerve graft (Kobayashi et al., 1997). BDNF application to the red nucleus not only enhances the regeneration associated gene response of these axotomized neurons, but also leads to a three-fold increase in the number of RSNs able to grow into a peripheral nerve graft (Kobayashi et al., 1997). Similar to other regeneration-associated genes, Gal1 expression in injured RSNs increased in response to BDNF application. This is the first demonstration of Gal1 expression in the red nucleus,

injury-induced changes in Gal1 expression within motoneurons, differential Gal1 responses to injury, and a neurotrophic factor-induced change in Gal1 mRNA expression. These data, as summarized in Table 2, indicate that Gal1 mRNA expression strongly correlates with the regenerative potential of injured neuronal populations. Although the expression pattern of Gal1 is suggestive of a significant effect in axonal regeneration, its precise role remains elusive.

While previous reports show that the manipulation of exogenous Gal1-Ox around a peripheral nerve injury site promotes both rate and success of peripheral nerve regeneration only recently has this mechanism begun to be elucidated (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). Gal1 has been hypothesized to act as a cytokine and/or as an adhesion molecule. This potential dual role for Gal1 may be attributed to the differing redox states in which Gal1 can exist. Oxidized and reduced Gal1 may have different functional roles within the nervous system. Previous studies have reported that exogenously applied, rhGal1-Ox can promote DRG outgrowth and regeneration *in vitro* and *in vivo,* as well as spinal motoneuron regrowth *in vivo* (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). Conversely, application of anti-Gal1 antibody reduces the number of successfully regenerating SMNs and decreases DRG neurite outgrowth (Horie et al., 1999; Fukaya et al., 2003). Although the Gal1-Ox receptor has not been identified, recent observations indicate that Gal1 binds to macrophages *in vitro,* leading to the release of an unidentified factor that facilitates both Schwann cell migration and DRG neurite outgrowth (Horie et al., 2004). This corresponds to the findings that recombinant oxidized galectin-1 (rhGal1-Ox) promotes Schwann cell migration into an acellular graft or empty tube and that Schwann cells and peripherally projecting (vigorously regenerating) neurons contain Gal1 protein (Horie et al., 1999; Fukaya et al., 2003). These data indicate that Gal1-Ox may be acting as a cytokine via macrophages to promote axonal regrowth.

| neuronal population | Gal1 change following axotomy |
|---|---|
| spinal motoneurons | ↑ |
| red nucleus | ↓ |
| red nucleus + vehicle | ↔ |
| red nucleus + BDNF | ↑ |

**Table 2. A summary of Gal1 changes in SMN and RSN following axotomy.**

Neuronally expressed Gal has been shown to be released into the extracellular environment, where it is readily oxidized (Cooper and Barondes, 1990; Avellana-Adalid et al., 1994; Imbe et al., 2003; Schafer et al., 2003; Sango et al., 2004). Therefore the increased endogenous Gal1 expression in axotomized SMN observed in this report could be released into the extracellular space, where it becomes oxidized, and potentially acts in a similar manner as applied rhGal1-Ox. The released Gal1 may facilitate peripheral glial cells to promote axonal regeneration, whereas the lack of specific signals within the CNS may contribute to regenerative failure of the injured RSN. Some Gal1 is not released into the extracellular environment but remains within the cell, most likely in its reduced form. Here, Gal1 may also influence the injury response through effects on second messenger cascades of neurotrophin signaling. Reduced Gal1 has recently been shown to stabilize the binding of the GTPase H-Ras to microdomains, resulting in the alteration of intracellular signaling cascades that may modify the neuronal trophic response (Paz et al., 2001; Elad-Sfadia et al., 2002). Accordingly, both redox states of Gal1 may lead to an increase of neuronal growth propensity and motility.

The results of the present study demonstrate a differential response of Gal1 mRNA expression to axonal injury of PNS versus CNS projecting neuronal populations. Furthermore, increased expression of Gal1 correlates with the regenerative propensity of injured neurons.

# CHAPTER 3: REGULATION OF GALECTIN-1 EXPRESSION BY AXOTOMY IN RAT PRIMARY AFFERENT NEURONS

*Introduction*

Injury to the peripheral or central branches of primary sensory neurons illustrates the difference in regenerative capacity of the peripheral and central nervous systems. Axotomy of a sensory neuron's peripheral branch can result in successful anatomical and functional regeneration. This is in contrast to the complete absence of recovery observed if the axotomy occurs to the centrally projecting branch that terminates in the spinal cord. Both the extrinsic CNS environment and the intrinsic neuronal injury response contribute to this disparity (Steeves and Tetzlaff, 1998; Fernandes and Tetzlaff, 2000; McGraw et al., 2001). Axonal regeneration of peripherally axotomized sensory neurons is associated with a cell body response that serves to reduce the deleterious effects of the peripheral nerve damage and to promote survival and growth of the lesioned sensory afferents (Hokfelt et al., 1994). Recently, application of rhGal1-Ox has been shown to promote both the rate and of axonal elongation of peripherally projecting neurons and the success of reinnervation; however, little is known regarding the changes in endogenous neuronal Gal1 expression after primary afferent axonal injury (Horie and Kadoya, 2000; Fukaya et al., 2003; Imbe et al., 2003).

Gal1 is one of 14 known members of the galectin family of β-galactoside-binding proteins (Barondes et al., 1994b). Gal1 is one of the most extensively studied galectins since its expression occurs in most organs as well as in a wide range of cells including neurons, placenta and muscle cells (Wasano et al., 1990; Poirier et al., 1992) (Regan et al., 1986; Perillo et al., 1998). High Gal1 expression in these tissues correlates with cellular growth and rejuvenation. For example, in astrogliomas, elevated Gal1 expression occurs in highly malignant tumors (Yamaoka et al., 2000; Camby et al., 2001). This metastatic growth was attenuated by application of anti-sense Gal1 mRNA (Yamaoka et al., 2000). Within the developing nervous system, Gal1 expression in dorsal root ganglia (DRG), spinal motor neurons, cranial and olfactory neurons begins after the last cell division occurs and remains elevated, albeit at lower levels, until their targets are reached (Regan et al., 1986; Hynes et al., 1990; St John and Key, 1999). These observations suggest that Gal1 is associated with cellular growth.

Within the lumbar DRG almost all neurons display some Gal1-IR but the small diameter sensory neurons that express c-RET mRNA have the highest Gal1-IR in the adult (Regan et al., 1986; Sato and Perl, 1991; Sango et al., 2004). Approximately 70% of all sensory afferents are small diameter neurons (Snider and McMahon, 1998). These small diameter nociceptive neurons can be further classified based on their neurochemistry. About half of the small diameter neurons are peptidergic since they express CGRP, whereas, the other half of these neurons are non-peptidergic neurons, bind IB4 and express the GDNF signaling receptor c-Ret (Chen et al., 1995; Molliver et al., 1997; Bradbury et al., 1998). Large-caliber axons carrying proprio- and mechanoceptive information are identifiable within the DRG by their expression of the large molecular weight neurofilament NF200 (Lawson et al., 1984). The distribution of Gal1 expression in peptidergic (CGRP expressing), non-peptidergic (IB4 binding) and large diameter (NF200 expressing) neurons is investigated. Furthermore I examined the changes in Gal1 protein and mRNA expression in these neurons after an axotomy and after a rhizotomy. The promotion of sensory fiber regrowth into the CNS after a dorsal rhizotomy was attempted through the application of rhGal-Ox.

***Materials and Methods***

*Surgery*

A total of 40 adult male wistar rats (University of British Columbia's animal care facility, weight 200-250 g) were used for this study. See Chapter 2 Materials and Methods for anesthetic details. To reduce post-operative pain and lessen blood flow to the muscle, 0.4 mL of 2% lidocaine with epinephrine (Vétoquinol, Quebec, QC) was then injected into the exposed superficial musculature around the spinal column. Rhizotomy or an axotomy of primary afferents were performed on the left side at the C5 to the 2$^{nd}$ thoracic level (T1). For the rhizotomy, details of the procedure are found in (Ramer et al., 2001b). Briefly, small pieces of vertebrae from C4-T2 were removed and the dorsal roots were exposed. The roots were crushed midway between the DRG and DREZ. For the axotomy, nerves exiting the spinal column at C5-T1 were cut and a 5mm section of nerve was removed. This ensured that all the peripheral nerves for a particular DRG were axotomized.

*Gal1 infusion*

Directly after a C5-T1 rhizotomy (as described above), some animals received either recombinant galectin-1 (described previously Horie et al., 1999), or vehicle delivered by 2 week osmotic minipumps (n=5 per group, 3 DREZ per animal analyzed, Alzet model 2002) (previously described in Ramer et al., 2000; Ramer et al., 2001b). To trace growing axons, the left median nerve of adult male Wistar rats was injected with a tracer cocktail containing 1% cholera toxin sub unit B (CTB, for the large diameter fibers, List Biological Inc, Campbell, CA), 1% wheat germ agglutatin (WGA, both peptidergic and non-peptidergic nociceptive fibers, Vector Labs, Burlingame CA) 11 days after the rhizotomy. One or two weeks following the injury, the C5-T1 spinal cord was harvested.

*Perfusion / cryosectioning*

See Chapter 2, materials and methods for details

*In situ hybridization*

See Chapter 2 Materials and methods for ISH details. Slides were dipped in Kodak NTB-2 emulsion and exposed for 2 days (DRGs) or 4 days (dorsal horn and DREZ). Sections were counter stained with Nissl as described in Chapter 2.

*Immunohistochemistry*

For Gal1 immunohistochemistry of DRGs, slides were washed in 0.1M PBS for 20 minutes, after which rabbit anti-Gal1 (1:4000) (Horie et al., 1999) and one of mouse anti-CGRP (1/2000, Sigma), mouse anti NF200 (clone N52, 1/500, Sigma), rabbit anti-GFAP (1/1000, Sigma) or IB4 (1/50, Sigma), goat anti-WGA (1/200, Vector Laboratories) 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 manufactures 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.

*DRG quantification-ISH*

DRG quantification was carried out as described by Ramer et al. (2001b), Bennett (2000) and in Chapter 2 materials and methods. Briefly, Nissl-stained DRGs cell bodies were outlined creating an image overlay. The percentage area occupied by silver grains determined the somal grain area fraction. The somal grain area fraction was measured by outlining the individual neuronal cell bodies using the neurotrace and applying the resulting cell profile layer to the darkfield image. For each animal, the percentage area occupied by ISH signal per soma was determined for both the axotomized and contra lateral (uninjured) side. The difference between background autoradiographic signal and the mean ISH signal per soma was calculated. Using recursive translation, a stereological counting method which reconstructs cell populations based on size-distribution of profiles (Rose and Rohrlich, 1988), the proportion of cells having a signal 5 times background was calculated. This threshold was arbitrarily chosen to observe changes in mRNA levels in different sub-populations within the DRG. At least three sections, >100 $\mu$m apart, were quantified per DRG.

*DRG quantification-immunoreactivity*

DRG quantification was carried out as described by Ramer et al. (2001b). Briefly, labeled images of Gal1 and either CGRP, IB4 or NF200 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, CGRP and NF200 was determined by averaging three cell bodies in each section that were judged to be minimally positive. Using recursive translation, a stereological counting method which reconstructs cell populations based on size-distribution of profiles (Rose and Rohrlich, 1988), the proportion of cells expressing an antigen and the soma sizes were calculated. At least three sections, >100 $\mu$m apart, were quantified per DRG.

*Dorsal horn quantification- Gal1- immunoreactivity*

Dorsal horn quantification was carried out as described by Ramer et al. (2001a). Briefly, for each rat cervical spinal cord level, three Gal1-IR images were imported into SigmaScan Pro 5.0 (SPSS Inc.) where a threshold was applied. Staining was measured along three non-overlapping 50-micron wide strips starting from uppermost border of grey matter and extending 450 microns ventrally. Measurements at each depth were averaged across sections in mice and mean ± SEM axonal density was plotted as a function of depth. For every10 microns of depth, the average axonal density was determined. Using a student's t-test, differences between Gal wt and -/- mice were determined at 10-micron intervals.

*Dorsal horn quantification Gal1-ISH*

At least two sections per spinal cord level were analyzed. Nissl and darkfield (silver grain) images were taken of both the injured and the contralateral non-injured side using a digital camera attached to a microscope (Carl Zeiss, Axioskop) in combination with Northern Eclipse software (Empix Inc, Mississauga ON). All images were analyzed with SigmaScan Pro 5 software (SPSS Inc.). The percentage area in the dorsal horns (see Figure 3.8E) occupied by silver grains determined the grain area fraction. Background autoradiographic signal was determined from the uninjured corticospinal tract. For each section, the percentage area in the dorsal horn occupied by ISH signal was determined for both the

axotomized and contralateral (uninjured) side (see Figure 3.8E). These data were expressed as percentage of the ISH background signal.

*DREZ ISH quantification*

Analysis of Gal1 ISH in the DREZ was similar to the dorsal horn ISH analysis as previously described. Briefly at least two 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 as indicated in Figure 3.9I. A threshold was applied to the silver grain image to determine grain area fraction of both PNS and CNS tissue at the DREZ.

*DREZ tracer quantification*

Axonal regeneration into the CNS was quantified densitometrically as previously described in Ramer et al (2000; 2001b): a threshold was applied to each of three nonadjacent images from each DREZ at C7 or C8, and the axonal density for either NF200, CTB, CGRP or WGA was determined in the DREZ as delineated by GFAP-IR.

*Statistics*

Quantification was performed blind with respect to the treatment groups. 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 stated, a Student's t-test was used to determine significance. All results are stated as mean ± standard error of the mean (SEM)Significance was assigned at $p<0.05$.

***Results***

*Changes in the Gal1 expression in the DRG following injury*

In the uninjured DRG 56.8 ± 5.6% (mean ± SEM) of neuronal somata had a grain area density 5 times greater than background (n=3, Figure 3.1A, 3.2 A and 3.2 F). Immunohistochemistry revealed that only 47.5 ± 1.1% of somata were Gal1-immunoreative (IR) (n=3, Figure 3.6 A and 3.6 F). Gal1-IR was observed in both the cytoplasm and nucleus of sensory neurons (Figures 3.3 C, 3.4C, 3.5C). Of the Gal1-IR somata 28.2 ± 2.4% were CGRP –IR (Figure 3.3A, histogram), 33.3 ± 1.77% were IB4 binding (Figure 3.4A, histogram) and 5.9 ± 0.2% were NF200 –IR (Figure 3.5A, histogram). 7 days following a rhizotomy, there were no significant changes in either Gal1 mRNA expression (n=3, 64.5 ± 5.5% somata, Figure 3.1B, 2B and F), Gal1-IR (Figure 3.6B and F) or co-localization with CGRP, IB4 or NF200 (Figure 3.3F, 4F, 5F) when compared to uninjured DRGs. Again at 14 days after a rhizotomy I did not observe any significant changes in either mRNA expression (n=3, 60.1 ± 4.2%, Figure 3.1C, 3.2C and 3.2D), Gal1-IR (49 ± 1.8% somata, Figure 3.6C and 3.6D) or co-localization with CGRP, IB4 or NF200 (Figure 3.3I, 3.4I, 3.5I) when compared to uninjured DRGs.

After a spinal nerve lesion in which all the sensory fibers for the particular DRG were axotomized, there were significant increases in both Gal1 mRNA and protein expression. I observed a significant increase in the proportion of somata with high silver grain density at 7 days to 82.4 ± 2.3% of all somata (n=3). This increase was mainly restricted to the large diameter cells (Figure 3.1D, 3.2D and F). Gal1-IR also was increased at this time point to 65 ± 3.2% (n=3, Figure 3.6F) of all somata with some nuclei displaying greater Gal1-IR than control levels (Figure 3.4M and O). Again this increase was mainly in the large diameter (NF200-IR) cells (Figure 3.6E) since 11.6 ± 2% of Gal1-IR somata were also NF200 positive (Figure 3.4L) compared to 5.9 ± 0.2% of Gal1-IR and NF200-IR cells (Figure 3.4C). There was also a significant decrease in CGRP and IB4 co-labeling with Gal1 (4.4 ± 1.7%, Figure 3.3L and 23.1 ± 3.0%, Figure 3.4L respectively) as a result of the decrease in the overall CGRP-IR and IB4 binding of somata. 14 days after axotomy, these results did not significantly change from 7 days after axotomy.

**Figure 3.1 Photomicrographs of Gal1 *in situ* hybridization signal**

All sections were counter stained with fluorescent Nissl stain (Figure 3.1, left column). In the uninjured DRG, silver grains could clearly be observed over cell soma (A and B). At 7 and 14 days after rhizotomy, there was no apparent change in the number of silver grains over cell bodies (D and F). However, at both 7 and 14 days after a spinal nerve lesion (peripheral axotomy), an increase in silver grains over somata was observed (H and J). Scale bar = 100 microns.

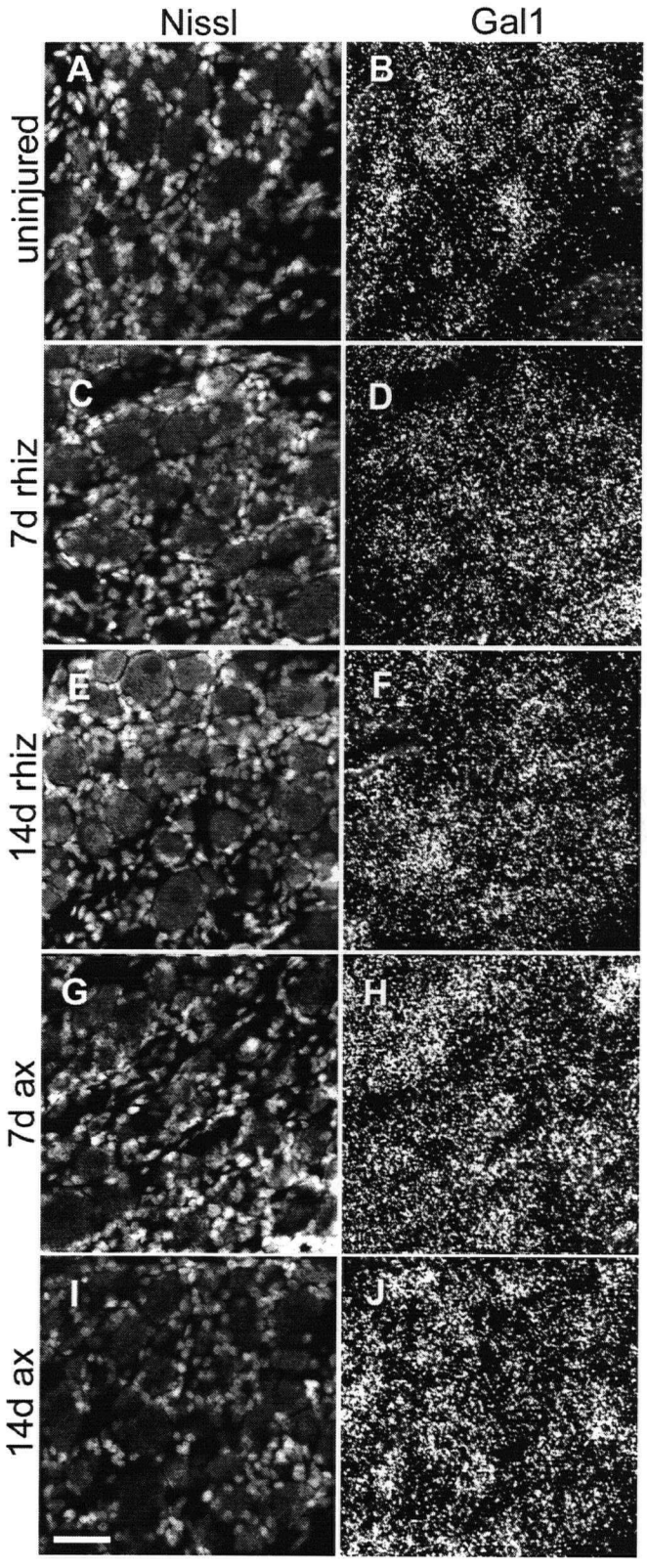

Nissl
Gal1
uninjured
7d rhiz
14d rhiz
7d ax
14d ax
A
B
C
D
E
F
G
H
I
J

**Figure 3.2 Quantification of Gal1 autoradiographic signal in the DRG**

A baseline of 5 times background (intense signal) silver grain signal was arbitrarily set as the threshold for somata positive for Gal1 mRNA. In uninjured cervical DRG (C6-C8), 56.8 ± 5.6% (mean ± SEM) of the cells had 5 times greater signal than the background (F). High levels Gal1 mRNA (A, black bars), as indicated by silver grains, were observed in somata of all sizes when compared to the total cellular population (A, white bars) but were predominately located in small diameter somata (A). Following a rhizotomy (rhiz), there was a small increase to 64.5 ± 5.5% at 7 days and 60.1 ± 4.2% at 14 days of the total number of cells with high proportion of silver grains (F). There appeared to be a slight increase in the larger diameter cells (grey bars) when compared to the uninjured control animals (black bars, B and C). Only after a spinal nerve lesion (axo) was there a significant increase in the number of cells highly expressing Gal1 mRNA (F). There was a significant increase in silver grains both at 7 days after an axotomy (82.4 ± 2.3% of total cells), and at 14 days after axotomy (75.7 ± 6.2%) when compared to uninjured DRGs (F). In particular an increased number of larger diameter cells expressed Gal1 mRNA (D and E). * indicates $p<0.05$ compared to uninjured animals.

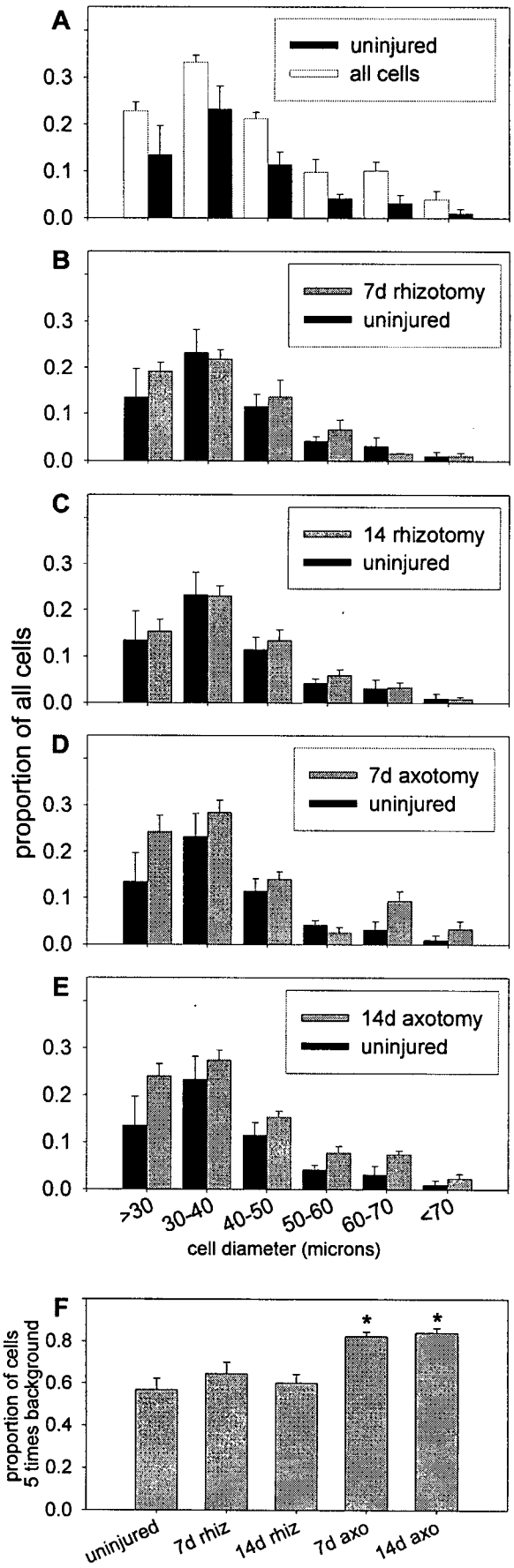

A
uninjured
all cells
B
7d rhizotomy
uninjured
C
14 rhizotomy
uninjured
D
7d axotomy
uninjured
E
14d axotomy
uninjured
proportion of all cells
0.0
0.1
0.2
0.3
>30
30-40
40-50
50-60
60-70
<70
cell diameter (microns)
F
proportion of cells 5 times background
0.0
0.2
0.4
0.6
0.8
*
*
uninjured
7d rhiz
14d rhiz
7d axo
14d axo

**Figure 3.3 Gal1-IR and CGRP-IR in the DRG**

Photomicrographs of immunohistochemistry of Gal1 and CGRP immunoreactivity of cervical DRGs. In the uninjured (control) DRG, 28.2 ± 2.4% of the Gal1-immunoreactive (IR) somata also expressed CGRP (Figure 3.3 C). At 7 and 14 days after rhizotomy 27.0 ± 2.4% and 26.1 ± 2.3% of the Gal1-IR somata expressed CGRP respectively (Figure 3.3F and I). After axotomy, only 6.5 ± 1.3% of Gal1-IR cells expressed CGRP at 7 days and 4.4 ± 1.7% of Gal-IR somata also expressed CGRP at 14 days (Figure 3.3L and O). These are both significantly less than control values. Scale bar = 50 microns, * indicates $p<0.001$ when compared to uninjured DRGs.

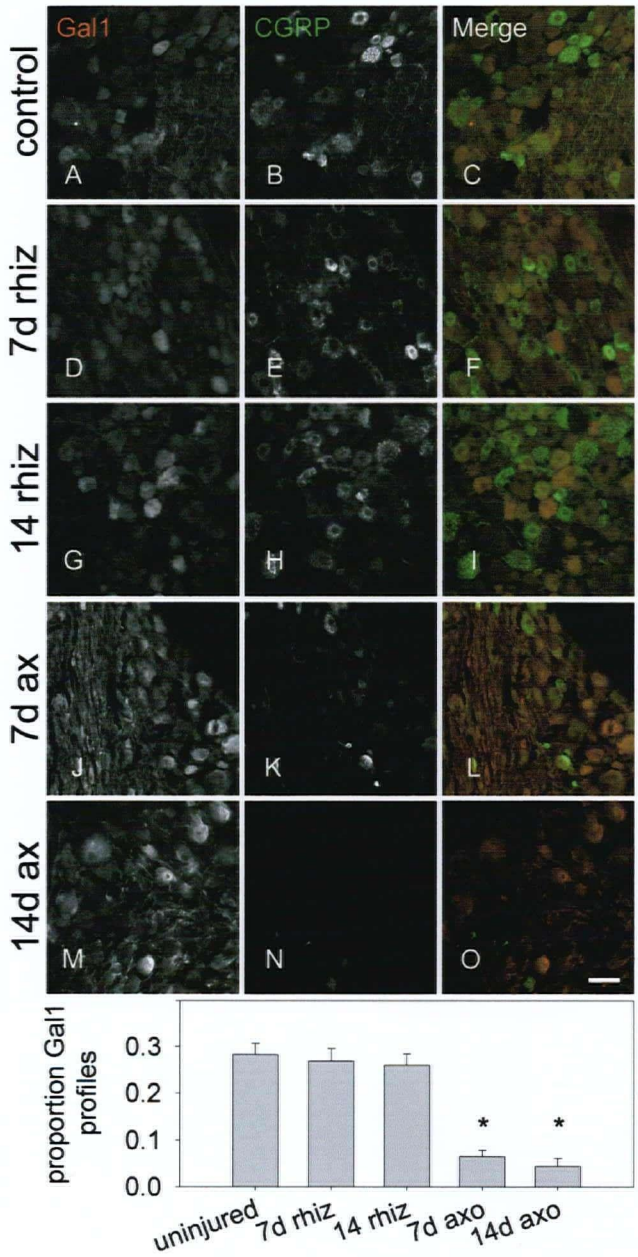

Gal1
CGRP
Merge
control
7d rhiz
14 rhiz
7d ax
14d ax
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
proportion Gal1 profiles
0.0
0.1
0.2
0.3
*
*
uninjured
7d rhiz
14 rhiz
7d axo
14d axo

**Figure 3.4 Gal1-IR and IB4-IR in the DRG**

Photomicrographs of immunohistochemistry of Gal1 immunoreactivity and IB4-binding of cervical DRGs. In the uninjured (control) DRG, 33.3 ± 1.77% of the Gal1-IR somata also bound IB4 (Figure 3.4 A,B,C). At 7 and 14 days after rhizotomy 36.0 ± 1.1% and 34.5 ± 0.9% of Gal1-IR somata bound IB4 respectively. After axotomy, 23.1 ± 3.0% of Gal1-IR cells bound IB4 after 7 days and 18.9 ± 2.0 % of Gal-IR somata also bound IB4 at 14 days (Figure 3.4L and O). These are both significantly less than control values. Scale bar = 50 microns, * indicates $p<0.001$ when compared to uninjured DRGs.

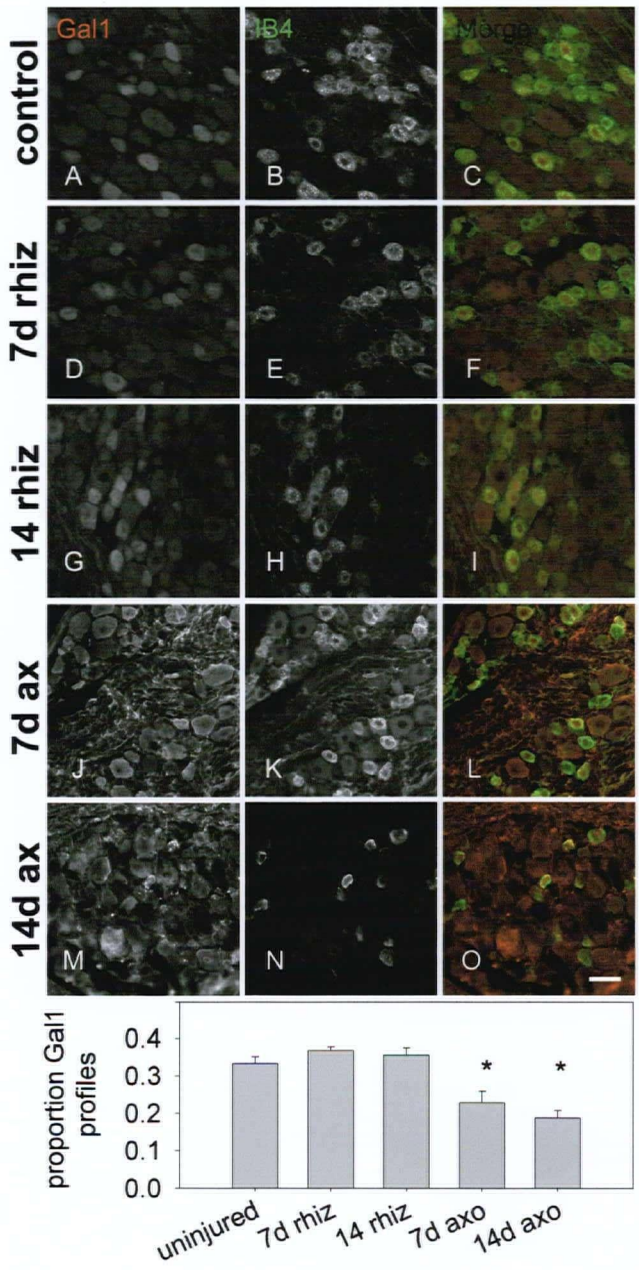

Gal1
IB4
Merge
control
7d rhiz
14 rhiz
7d ax
14d ax
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
proportion Gal1 profiles
0.4
0.3
0.2
0.1
0.0
*
*
uninjured
7d rhiz
14 rhiz
7d axo
14d axo

**Figure 3.5 Gal1-IR and NF200-IR in the DRG**

Photomicrographs of immunohistochemistry of Gal1 and NF200 immunoreactivity in cervical DRGs. In the uninjured (control) DRG, 5.9 ± 0.2% of the Gal1-IR somata also expressed NF200 (Figure 3.5 C). At 7 and 14 days after rhizotomy 6.4 ± 0.1% and 7.1 ± 0.2% of the Gal1-IR somata expressed NF200 respectively (Figure 3.3F and I). After axotomy, there was an increase to 11.6 ± 2% of Gal1-IR cells that expressed NF200 after 7 days and 12.0 ± 1.2% of Gal-IR somata also expressed NF200 at 14 days (Figure 3.3L and O). These were both significantly greater than control values. Scale bar = 50 microns, * indicates $p<0.001$ when compared to uninjured DRGs.

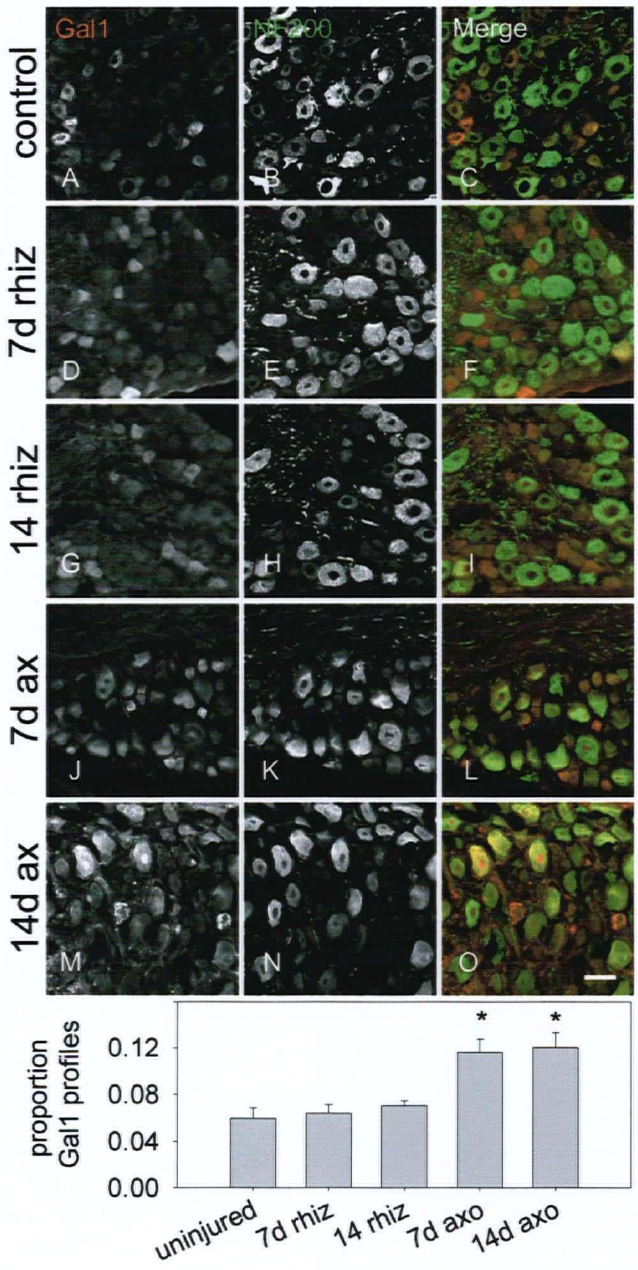
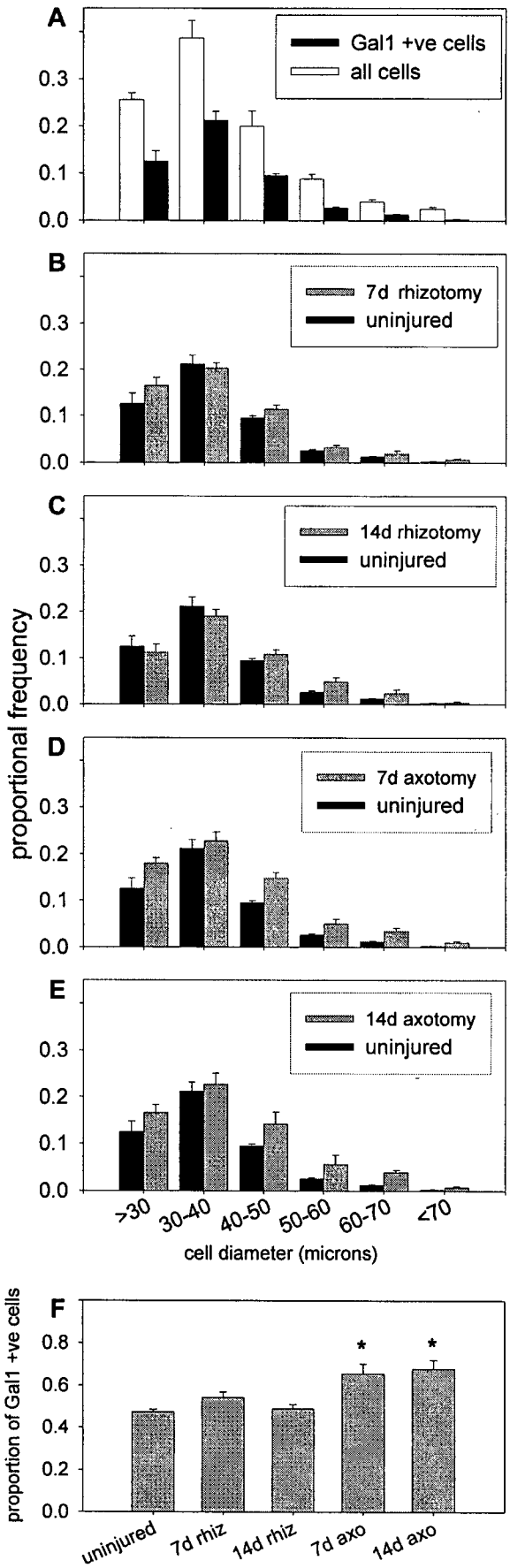

Gal1
NF200
Merge
control
7d rhiz
14 rhiz
7d ax
14d ax
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
proportion Gal1 profiles
0.12
0.08
0.04
0.00
*
*
uninjured
7d rhiz
14 rhiz
7d axo
14d axo

**Figure 3.6. Proportion of Gal1-IR cells compared to the total proportion of somata within the DRG.** In the uninjured DRG, 47.5 ± 1.1% of somata were Gal1-IR (6F). Gal1-IR was mainly observed in both small and medium diameter cells (black bars, A) when compared to the entire population (white bars, A). While Gal1-IR was found in cells of all sizes, a greater proportion of small diameter CGRP and IB4-positive cells expressed Gal1 than large diameter NF200 positive cells. 7 days after rhizotomy, 52.0 ± 2.1% of cells were Gal1-IR and after 14 days 49 ± 1.8% of somata were Gal1-IR. While these were not significantly different from control values there appeared to be an increase in the proportion of large diameter somata that had Gal1-IR (C and D). 7 days after axotomy there was a significant increase in Gal1-IR cells (65 ± 3.2%) and after 14 days 70.8 ± 4.3% of somata were Gal1-IR. * indicates $p<0.05$ compared to uninjured DRGs.


A
Gal1 +ve cells
all cells
B
7d rhizotomy
uninjured
C
14d rhizotomy
uninjured
D
7d axotomy
uninjured
E
14d axotomy
uninjured
0.0
0.1
0.2
0.3
proportional frequency
>30
30-40
40-50
50-60
60-70
<70
cell diameter (microns)
F
proportion of Gal1 +ve cells
0.0
0.2
0.4
0.6
0.8
*
*
uninjured
7d rhiz
14d rhiz
7d axo
14d axo

At 14 days post-axotomy, 75.7 ± 6.2% of axotomized somata had high ISH levels (Figure 3.1E, 3.2E, 3.2F). 70.8 ± 4.3% of the somata were also Gal1-IR (Figure 3.6E, F). Of these Gal1-IR somata, 4.4 ± 1.7% were CGRP positive (Figure 3.3O), 18.9 ± 2.0 % bound IB4 (Figure 3.4O) and 12.0 ± 1.2% were NF200 positive (Figure 3.5O). Overall few changes were observed in Gal1 mRNA or protein expression after a rhizotomy but after an axotomy larger diameter NF200-IR increase their Gal1-IR.

*Gal1 IHC expression in the dorsal horn*

Gal1-IR was present in the uninjured dorsal horn (n=3, Figure 3.7A,C,E, D). Both 7 and 14 days following a rhizotomy there was a complete absence of Gal1-IR in the dorsal horn (Figure 3.7B, D). This data indicates that Gal1 protein is normally anterogradely transported to primary afferent terminals fields in the dorsal grey matter. Following a peripheral axotomy, I observed a significant increase in Gal1-IR deeper within the laminae (Figure 3.8F and H). This was most likely due to the increased Gal1 expression in large-diameter somata (Figures 3.5O, 6E).

In the intact dorsal horn Gal1 mRNA was not detectable (Figure 3.8). However, 7 and 14 days after rhizotomy silver grains were observed in the degenerating fiber tracts within the spinal cord (cuneate fasciculus and the medial dorsal horn where large diameter fibres invade the grey matter) indicating Gal1 mRNA expression (Figure 3.8). 7 days following a rhizotomy, these degenerating fiber tracts had 3.3 ± 0.2 times background and at 14 days it was 4.7 ± 0.3 times background which were significantly greater than the uninjured sides of 1.3 ± 0.2 and 1.2 ± 0.2 times background at 7 and 14 days respectively ($p<0.05$).

After both 7 and 14 days following a rhizotomy, an increase in autoradiographic signal occurred. At 7 days following a rhizotomy there was an increase to 3.3 ± 0.2 times background compared to 1.3 ± 0.2% on the uninjured side. 14 days following a rhizotomy there was an increase to 4.7 ± 0.3% of background compared to the uninjured side of 3.3 ± 0.2% of background (Figure 3.8 A and C).

**Figure 3.7 Distribution of Gal1- IR in the C7 dorsal horn.**

Compared to the contralateral (uninjured) dorsal horn (A and C), there was a complete absence of Gal1-IR at both 7 and 14 days after rhizotomy (B and D). At 7 and 14 days after a spinal nerve lesion (axotomy), Gal1-IR was observed at increased depths in the dorsal horn (Figure 3.7F and H) when compared to the uninjured side (E and G). When Gal1-IR is plotted against depth, the axotomy-induced shift in depth of Gal1-IR is apparent at both 7 and 14 days (gray lines) in the dorsal horn ipsilateral to the uninjured contralateral side (black lines). At all points between the arrows, there is a significant difference in Gal1-IR between axotomized and uninjured sides. Scale bar = 200 microns.

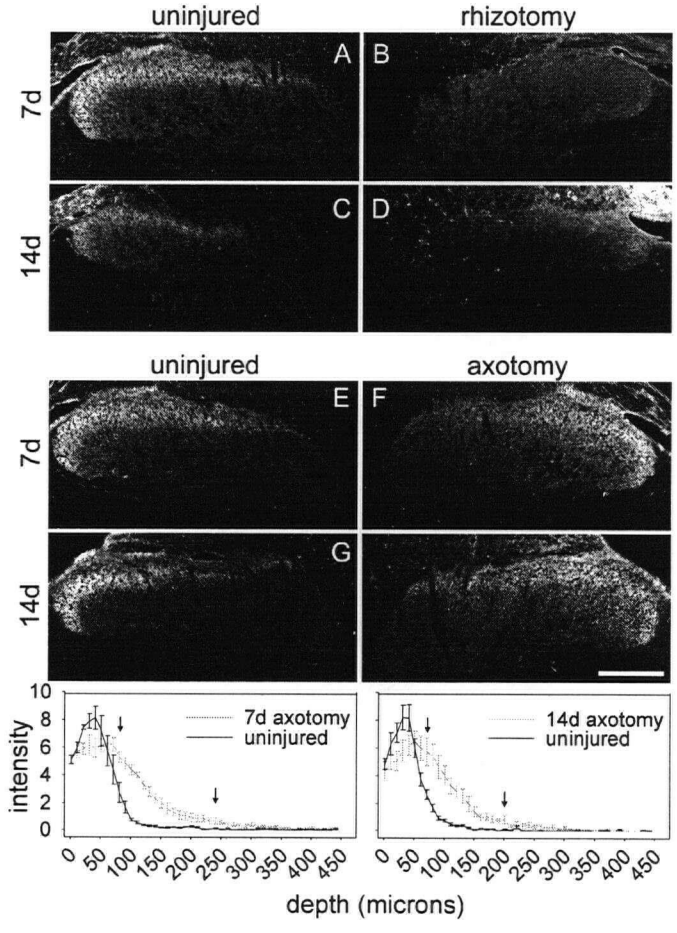
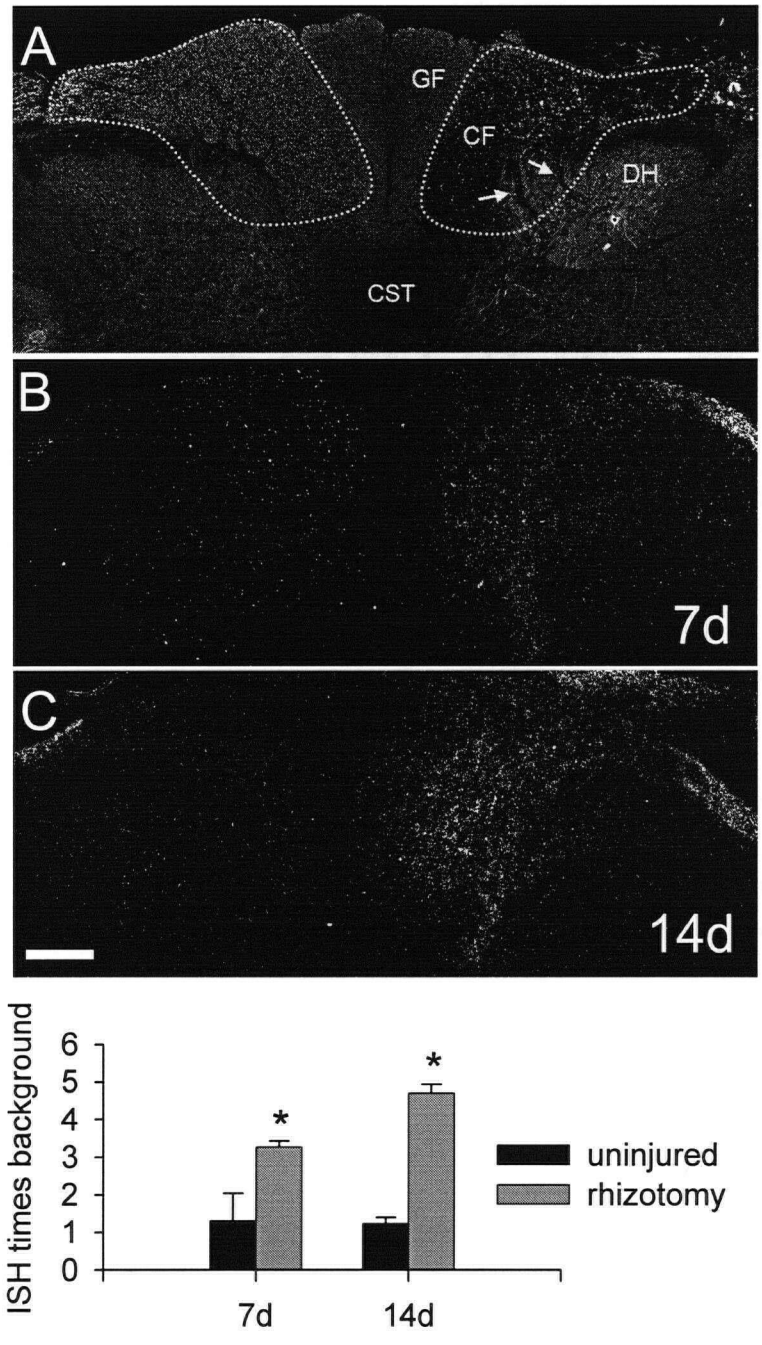

uninjured
rhizotomy
A
B
7d
C
D
14d
uninjured
axotomy
E
F
7d
G
14d
intensity
7d axotomy
uninjured
14d axotomy
uninjured
depth (microns)

**Figure 3.8 Gal1 ISH in the dorsal horn following rhizotomy**

Gal1 mRNA significantly increased in the dorsal horn following a rhizotomy. Using a cervically spinal cord section one week after rhizotomy that has been stained for NF200, the major landmarks of the dorsal spinal cord can be identified (A). On the rhizotomized side (right side) the intact gracile fasciculus (GF), corticospinal tract (CST) and degenerating cuneate fasciculus (CF) and dorsal horn (DH) can clearly be seen. The arrows indicate the large-diameter axons entering the deeper lamina. The dotted area on the rhizotomized (right) and uninjured contralateral (left) side on the autoradiographic sections (B and C) were quantified for ISH signal and expressed as a percentage of background (uninjured CST tract). After a rhizotomy, the Gal1 autoradiographic signal increased significantly to 3.3 ± 0.2 times background compared to 1.3 ± 0.2% on the uninjured side after 7 days and 4.7 ± 0.3% on the rhizotomized side 1.2 ± 0.2% on the contralateral side after 14 days. * Indicates $p<0.05$ when compared to the contra lateral side. Scale bar = 200 microns


A
GF
CF
DH
CST
B
7d
C
14d
ISH times background
6
5
4
3
2
1
0
*
*
7d
14d
uninjured
rhizotomy

*Gal1 mRNA expression at the DREZ*

After a rhizotomy, an increase was observed in Gal1 autoradiographic signal to 4.3 ± 0.5 times greater than the contralateral side after 7 days and 5.3 ± 1.0 after 14 days on the PNS side (n=3, Figure 3.9A,B and J). On the CNS side of the DREZ, an increase in autoradiographic signal 1.2 ± 0.2 times the contralateral side after 7 days and 1.4 ± 0.1 after 14 days was observed (n=3, Figure 3.9E,F and J).

*Intrathecal Gal1-Ox has little effect on central regeneration of rhizotomized afferents*

Gal1-Ox has previously been demonstrated to increase the regenerative rate after a peripheral axotomy (Horie et al., 1999; Fukaya et al., 2003). To test whether Gal1 promotes sensory neuron growth into the CNS after a rhizotomy rhGal1-Ox (10 μg/ml) was infused for 2 weeks directly after a cervical rhizotomy. There were no significant differences in NF200 staining central to DREZ between vehicle and Gal1 infusions (n=5 per group, Figure 3.10). Staining for the large diameter neuronal tracer CTB showed a non-significant increased trend of staining between the two treatment groups. CGRP-IR in the DREZ increased after Gal1 treatment (n=5 per group, 0.016 ± 0.001% of DREZ) compared to vehicle alone (0.005 ± 0.0007%, Figure 3.11). Immunohistochemistry for the small diameter neuronal tracer WGA also revealed a slight significant increase in immunoreactivity for the Gal1 treated animals (0.006 ± 0.001% of area) compared to vehicle alone (0.002 ± 0.0002%). While these increases were significant, they were small.

**Figure 3.9 Gal1 ISH at the DREZ after rhizotomy**

Gal1 mRNA significantly increases in the PNS after a rhizotomy. Figure 3.9A, B, E and F are photomicrographs of autoradiographic signal (silver grains) following a Gal1 ISH of the DREZ. Figures 9C, D, G and H are the same sections counter stained with fluorescent Nissl stain. Arrows indicate the DREZ boundary. Figure 3.9I is a schematic illustrating the PNS and CNS interface. After rhizotomy, the Gal1 autoradiographic signal increased to 4.3 ± 0.5 times greater than the contralateral side after 7 days and 5.3 ± 1.0 after 14 days on the PNS side (n=3, Figure 3.9A,B and J). On the CNS side of the DREZ, the autoradiographic signal increased to 1.2 ± 0.2 times the contralateral side after 7 days and after 14 days, an increase of 1.4 ± 0.1 (n=3, E, F and J). Scale bar = 100 microns, * indicates $p<0.001$ compared to the uninjured contralateral side.

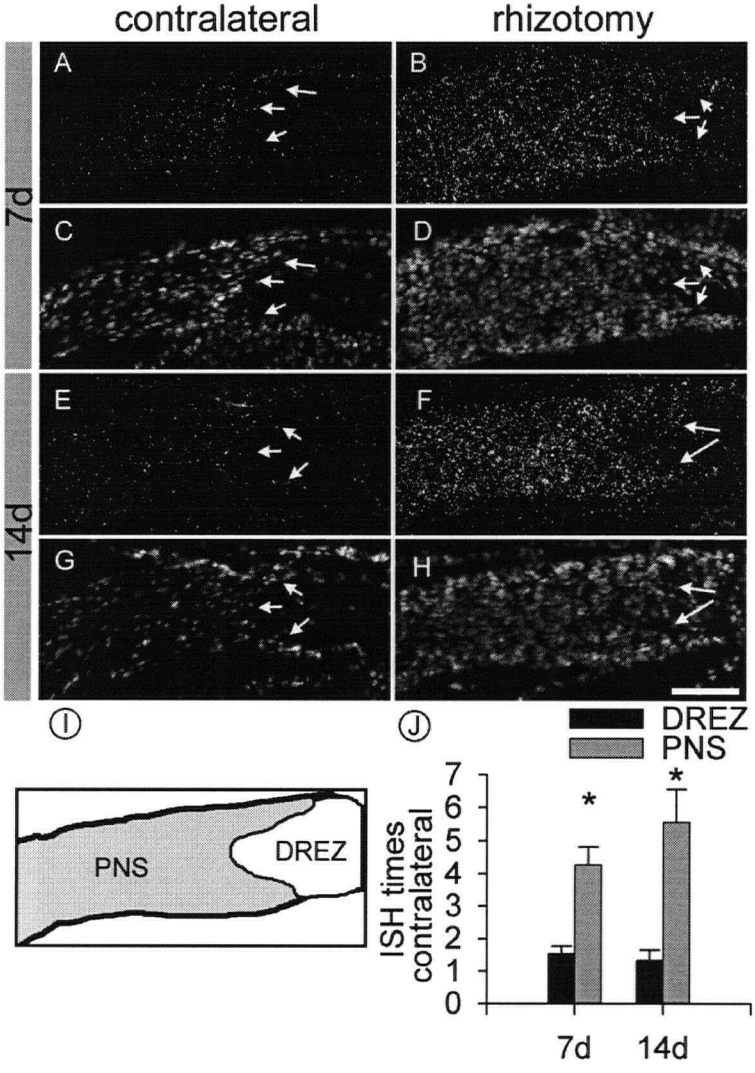

contralateral
rhizotomy
A
B
C
D
E
F
G
H
7d
14d
I
J
PNS
DREZ
DREZ
PNS
ISH times contralateral
0
1
2
3
4
5
6
7
*
*
7d
14d

**Figure 3.10 rhGal1-Ox does not promote growth of NF200 and CTB labeled fibers**
In triple-labeled images of GFAP (to delineate the DREZ), NF200 (large diameter fibers) and CTB (neuronal tracer), I observed that 1.0 ± 0.2% of the DREZ area (delineated by GFAP, see methods) was covered by NF200-IR after rhGal1-Ox infusion: this was not significantly different from the CTB-covered area of the DREZ in vehicle-treated animals (0.9 ± 0.3% area covered). Using CTB to trace large diameter neurons, rhGal1-Ox-treated animals did not have a significantly greater amount of CTB staining in the DREZ (0.002 ± 0.001% of DREZ) than vehicle treated animals (0.001 ± 0.0002%). Scale bar = 100 microns.

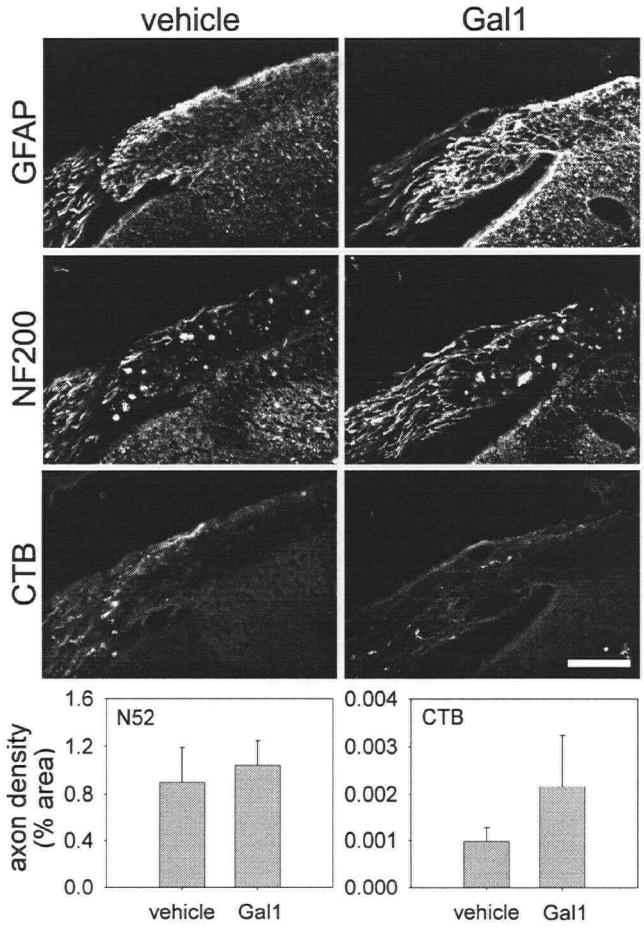
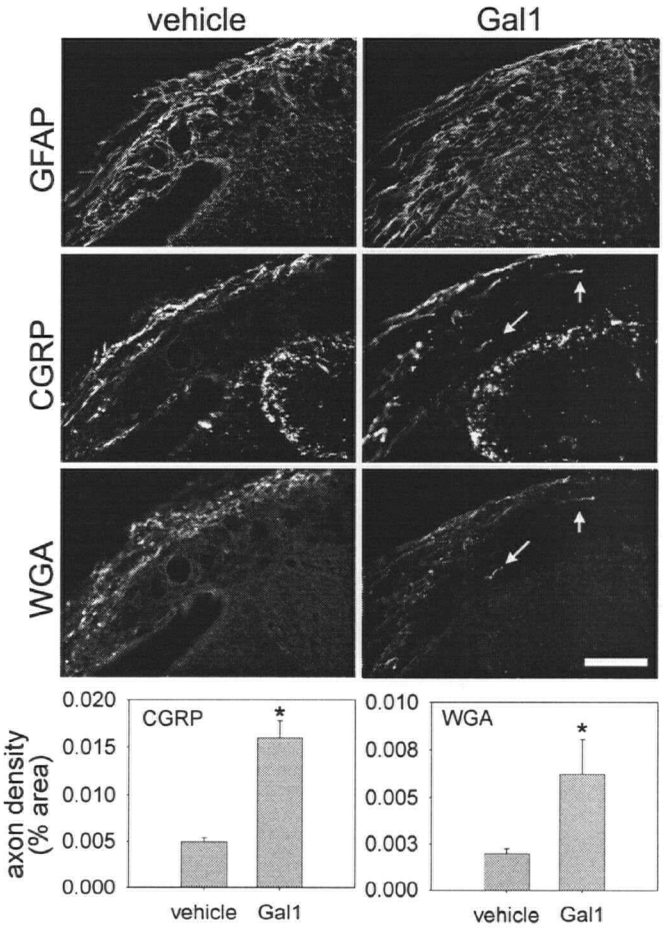

vehicle
Gal1
GFAP
NF200
CTB
axon density (% area)
N52
CTB
1.6
1.2
0.8
0.4
0.0
0.004
0.003
0.002
0.001
0.000
vehicle
Gal1
vehicle
Gal1

**Figure 3.11 rhGal1-Ox promotes growth of CGRP and WGA labeled fibers**

rhGal1-Ox promotes limited growth of small diameter fibers across the DREZ 14 days after rhizotomy. In triple-labeled images of GFAP (to delineate the DREZ), CGRP (peptidergic fibers) and WGA (neuronal tracer), 0.016 ± 0.001% of the DREZ area (delineated by GFAP, see methods) was covered by CGRP-IR after rhGal1-Ox infusion: this was significantly greater than the CGRP-covered area of the DREZ in vehicle-treated animals (0.005 ± 0.0007%). Using WGA to trace small diameter neurons, rhGal1-Ox treated animals had a significantly greater amount of WGA staining (0.006 ± 0.001% of DREZ) than vehicle-treated animals (0.002 ± 0.0002%). Arrows indicate fibers double-stained for both CGRP and WGA. Scale bar = 100 microns, * indicates $p<0.05$.


vehicle
Gal1
GFAP
CGRP
WGA
axon density (% area)
CGRP
WGA
0.020
0.015
0.010
0.005
0.000
0.010
0.008
0.005
0.003
0.000
*
*
vehicle
Gal1
vehicle
Gal1

***Discussion***

*Gal1 expression in the naïve DRG*

In the adult cervical DRG, 56.8 ± 5.6% of the sensory nuclei had an ISH signal for Gal1 mRNA 5 times greater than background levels. The majority of these profiles were small diameter neurons. This is the first report to quantify the size distribution of Gal1 mRNA expression in the DRG, and these results are consistent with previous reports that show small diameter sensory neurons with high Gal1 mRNA expression (Hynes et al., 1990; Sango et al., 2004). I show 47.5 ± 1.1% of the nuclei had some Gal1-IR which is similar to a previous finding of 63% of all DRGs having some Gal1-IR with 46% having strong Gal1-IR (Regan et al., 1986). In lumbar DRGs most cells also have Gal1-IR but only 20-26% of these somata were intensely Gal1-IR (Imbe et al., 2003; Sango et al., 2004). These differences between cervical and thoracic Gal1-IR cellular proportion are not surprising. Significant variations in the rostro-caudal distribution of sensory phenotypes exist in DRGs. For example, a greater overall proportion and size distribution of $P2X_3$-IR somata occurs in cervical DRGs when compared to lumbar DRGs (Ramer et al., 2001a).

The majority of Gal1-IR neurons were either IB4-binding (33%) or CGRP-IR (28%) small diameter neurons within cervical DRGs. In the lumbar DRG, Gal1-IR cells co-localized mainly to c-Ret mRNA (94%) expressing somata and also to a limited number of TrkA mRNA (6.8%) expressing neurons (Imbe et al., 2003). In these lumbar DRGs, only 63% of the 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). Accordingly, 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 neurons and Gal1-IR occurring in both IB4-binding and CGRP-IR cervical neurons (this report).

*Gal1 expression after injury*

Using two different injury paradigms, peripheral axotomy and rhizotomy, two different injury responses were elecited within the DRG and spinal cord. These results are summarized in Table 3. After a peripheral nerve lesion, which normally elicits a strong cell body response, I observed a significant increase of both Gal1 mRNA and –IR, mainly within the large diameter NF200–IR sensory neurons. Within the spinal cord, increased Gal1-IR

occurred within the deeper layers of the dorsal horn. This is consistent with previous findings in both an L4/L5 spared nerve injury model (SNL) of neuropathic pain and sciatic nerve transection increased Gal1-IR within deeper layers of the dorsal horn (Imbe et al., 2003).

Following rhizotomy, Gal1 mRNA or –IR within the DRG was not significantly different than control animals but there was a complete absence of Gal1-IR within the dorsal horn, demonstrating that Gal1 protein is normally transported to the nerve terminals in uninjured sensory neurons. Gal1 mRNA expression and not Gal1-IR was observed within the degenerating fiber tracts within the spinal cord; however, Gal1 mRNA was more readily detected in the DRG than Gal1 protein. A semi-quantitative polymerase chain reaction for Gal1 mRNA on rhizotomized spinal cord would further demonstrate these increases. Reactive astrocytes could be expressing Gal1 mRNA in the deafferented spinal cord since astrocytomas are the only known Gal1 expressing glial cells within the CNS (Camby et al., 2001). However, since astrocytes become reactive throughout the dorsal horn following rhizotomy, and since silver grain density was present mainly along the paths of large diameter (myelinated) afferents in the medial dorsal horn, it may also be that activated microglia or oligodendrocytes upregulated Gal1 mRNA. Using antibodies to either GFAP (astrocytes) or Rip (oligodendrocyte cell bodies) combined with a Gal1 ISH may aid in resolving which cells express Gal1 mRNA after rhizotomy in the dorsal horn. On the PNS side of the DREZ there was a large increase in Gal1 mRNA expression. Schwann cells were most likely expressing Gal1 mRNA here since both sensory neurons and Schwann but not macrophages express Gal1 in the PNS (Sango et al., 2004).

| | changes in Gal1 expression | |
|---|---|---|
| **location** | **rhizotomy** | **axotomy** |
| DRG | ↔ | ↑ |
| DREZ | ↑ (PNS side) | |
| dorsal horn | ↓ (IR) | ↑ |
| cuneate fasciculus | ↑ (mRNA) | |

**Table 3. A summary of changes in Gal1 expression in sensory neurons following injury.**

*Redox state affects Gal1 functions*

When secreted from cells, some of the Gal1 changes from a reduced (Gal1-Red) to an oxidized state (Gal1-Ox), transforming Gal1 from a lectin to a trophic molecule (Tracey et al., 1992; Horie and Kadoya, 2000). Within neurons, Gal1-Red has been identified as a component of the Survival of Motor Neuron complex which is involved in neuronal growth and survival (Park et al., 2001; Rossoll et al., 2003). Once released into the extracellular space, Gal1-Red facilitates axonal growth by increasing adhesion (Mahanthappa et al., 1994). Exogenous application of Gal1-Ox but not Gal1-Red increases axonal outgrowth by acting as a growth factor and/or a cytokine to stimulate DRG outgrowth *in vitro*, and increases the rate of sensory and motor growth into acellular nerve grafts and tubes *in vivo* (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). This occurs by increasing Schwann cell migration and eliciting the release of a yet unidentified growth-promoting factor from macrophages (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003; Horie et al., 2004). These data suggest that both endogenous Gal1 redox states increase neuronal outgrowth, but only the exogenous application of Gal1-Ox increases the rate of peripheral nerve regeneration. Accordingly, I attempted to promote sensory neuron axonal regeneration after a rhizotomy by exogenous Gal1-Ox application. Significant numbers of large diameter fibers were not observed regenerating into the spinal cord. This suggests that Gal1 does not promote large diameter neuronal growth into the CNS or a reduction in Gal1's efficacy. Future experiments should also repeat Horie et al., (1999) tube ligation study to confirm Gal1-Ox activity. However Gal1-Ox did promote some regeneration of small diameter nociceptive CGRP and/or WGA labeled fibers suggesting that some Gal1-Ox was active. Unlike the long distance and robust growth of rhizotomized sensory neuronal growth observed after neurotrophin delivery (Ramer et al., 2000; Ramer et al., 2002), Gal1-Ox induced only a very limited amount of nociceptive fiber growth into the CNS. The Gal1-induced growth in the DREZ was more reminiscent of a localized sprouting response than that of robust regeneration.

The precise role of Gal1 expression in the uninjured nervous system remains uncertain. Over half of the uninjured DRGs express Gal1 (Figure 3.2) (Regan et al., 1986; Imbe et al., 2003; Sango et al., 2004). Once expressed, Gal1 is released from central nerve

terminals where it can bind to astrocytes which then release BDNF (Sango et al., 2004; Sasaki et al., 2004). BDNF has been implicated in such diverse roles as synaptic plasticity to nociception modulation (Thoenen, 2000; Pezet et al., 2002a). Interestingly, sensory neurons of Gal1 -/- mice do not make appropriate central connections during development. For example, olfactory neurons in Gal1 -/- mice that would normally express Gal1 grow towards their targets but do not make the final appropriate connections (Puche et al., 1996). Small-diameter primary sensory neurons of Gal1 -/- mice also grow to the dorsal horn but terminate within deeper laminae, an observation which correlates with reduced nocifensive responses to noxious thermal stimuli compared to Gal1 wt mice (Chapter 4). These data suggest that the intrinsic expression of Gal1 is not critical for neuronal outgrowth in the CNS during development but may rather be involved in making or maintaining appropriate connections.

# CHAPTER 4: INCREASED THERMAL-PAIN THRESHOLDS IN GALECTIN-1 NULL MUTANT MICE: CORRELATION WITH ALTERED NOCICEPTIVE PRIMARY AFFERENT NEURONAL NUMBER AND SPINAL TERMINAL FIELDS

Small diameter DRG afferents transmit nociceptive information from the periphery to the CNS. These thinly-myelinated and unmyelinated small diameter neurons comprise approximately 70% of all cells within the DRG (Snider and McMahon, 1998). The small-caliber group can be further subdivided based on neurochemistry and termination pattern within the spinal cord: neurons expressing the neuropeptide CGRP terminate in laminae I and II outer (IIo) (Averill et al., 1995); and, neurons that express the ATP receptor $P2X_3$ (non-peptidergic neurons) and bind IB4 terminate in lamina II inner (IIi) of the spinal cord (Chen et al., 1995; Molliver et al., 1997; Bradbury et al., 1998). Large-caliber axons carrying proprio- and mechanoceptive information terminate in deeper laminae (III-X) are identifiable within the DRG by their expression of the large molecular weight neurofilament NF200 (Lawson et al., 1984).

During rodent development, different neurotrophic factors regulate distinct functional classes of sensory neurons. For example, the 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 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, the signaling receptor for the GDNF family of neurotrophic factors (Molliver and Snider, 1997; Molliver et al., 1997) leading to an adult DRG population comprised of 40% TrkA and CGRP and 30% Ret-expressing and IB4-binding neurons. In the rat, during development and in the adult, these small diameter neurons also express the carbohydrate-binding protein Gal1 (Regan et al., 1986). Following loose ligation of the rat sciatic nerve, which causes peripheral neuropathy and thermal allodynia, Gal1 immunoreactivity increases in laminas I and II of the spinal cord (Cameron et al., 1997). Decreasing this activity using intrathecal application of Gal1 functional blocking antibodies leads to reduced mechanical allodynia (Imbe et al., 2003). Furthermore, exogenously applied 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 nociceptive sensory neuronal outgrowth and maintenance. However, to date no studies have attempted to examine the role of Gal1 in sensory neuronal development.

The Gal1 -/- null mutant mouse is viable without obvious phenotypic abnormalities (Poirier and Robertson, 1993). Interestingly, in these mice, a neuronal subpopulation within the olfactory bulb that normally expresses Gal1 does not reach appropriate targets in olfactory glomeruli (Puche et al., 1996). These data suggest that Gal1 may be required for axonal growth or pathfinding. Here I attempted to further elucidate the potential role of Gal1 by examining Gal1 expression in the DRG and spinal cord of Gal1 *wt* mice, changes in neuronal populations and primary afferent terminations in Gal1 null-mutants, and correlate these changes in nociceptive behaviour.

***Materials and Methods***

Animals

A total of 22 adult age-matched 129P3/J (wild-type, Jackson Labs, Maine), 22 adult 129P3/J galectin-1 homozygous null mutant mice (Gal1 -/-)(Poirier and Robertson, 1993) and 7 CD-1 mice (University of British Columbia's animal care facility) were used for these experiments. The generation of the Gal1 -/- mice has been described (Poirier and Robertson, 1993). All experiments were performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee.

*Behavioral testing*

A total of 7 CD-1 mice, 7 Gal1 *wt*, 7 Gal1 -/- mice were used for the behavioral testing. Two tests (punctate pressure and either radiant heat or cold plate) 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-test was 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 force was set at 20g increasing with ramp set to 7 seconds. Upon nocifensive withdrawal (involving some or all of: sustained elevation, biting, licking or shaking the paw), the instantaneous force applied to the plantar surface eliciting the withdrawal was recorded automatically. Random (non-nocifensive) 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, a UV laser connected to a timer was turned on underneath a mouse paw (model #7371 UGO Basile Biological Research, Comerio VA). Latency to withdrawal was recorded. Only nocifensive movements were recorded.

Cold plate test: A mouse was placed on a 1°C cold plate (model # 0134-0021 Columbus Instruments, OH) and a timer was immediately started. The latency to the initial nocifensive response was recorded.

*c-fos activation*

Fos protein expression is used as a marker for neuronal activation in the spinal cord (Hunt et al., 1987). A total of 8 Gal1 *wt* and 8 Gal1 -/- mice were used to determine if there are differences between Gal1 -/- or *wt* mice 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 seconds with a 30 second delay between each submersion. Animals were killed 2 hours after thermal stimulation when c-fos activation in the dorsal horn has peaked (Hunt et al., 1987; Dai et al., 2001).

*Perfusion / cryosectioning*

Perfusion was carried out as described in Chapter 2, Material and Methods Perfusion.

*Immunohistochemistry*

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 then blocked for 20 minutes in 10 % normal goat serum and then either goat anti mouse-Gal1 (1:500 R&D systems, MN), rabbit anti-NeuN (1:100, Chemicon, Temecula CA), rabbit anti-CGRP (1:2000, Sigma, Oakville, ON), followed by biotin-conjugated IB4 (1/50, Vector Labs), βIII tubulin (1:500, Sigma) or mouse anti c-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 either 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.

*DRG - IR quantification*
The same DRG quantification technique was used as described in Chapter 3, Materials and Methods: DRG quantification.

*Dorsal horn - IR quantification*
Dorsal horn quantification for CGRP and IB4 was carried out as described by Ramer et al. (2001a) and Chapter 3 Materials and Methods, dorsal horn quantification.

*c-fos-IR quantification*
Three c-fos 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 remove any background staining and then c-fos positive cells were automatically counted.

*NeuN-IR quantification*
For each animal, three images of NeuN-IR cells 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 lamina I –II was 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.

*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-Elmer, 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 $10^6$ 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 $dH_20$ 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).

*Image analysis 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, a Student's t-test was used to determine significance. All results are stated as mean ± standard error of the mean (SEM).

***Results***

*Functional differences between Gal1 null mutant and wild type mice*

The responses to both noxious and non-noxious stimuli of Gal1 -/- were compared to that of the inbred Gal1 *wt* mice. In addition, the responses of an outbred line of mice were examined (CD-1) to ensure that the particular line of 129P3/J *wt* mice responds similarly to thermal stimulation. When placed on a 1°C cold plate, null mutant mice displayed significantly longer latencies before displaying a nociceptive withdrawal response (involving some or all of: sustained elevation, biting, licking or shaking the paw) when compared to either Gal1 wt or CD-1 mice (75.4 ± 6.8 for Gal1 -/-, mean ± SEM, compared to 18.4 ± 1.3 seconds for Gal1 *wt*, or 21.1± 1.5 seconds for CD-1; ANOVA, $p<0.001$, Figure 4.1). The CD-1 mice were significantly different from Gal1 -/- mice (ANOVA, $p<0.001$) but not from Gal1 *wt* mice. Gal1 -/-mice had increased withdrawal latency from radiant heat (8.3 ± 0.3 seconds, front paw; 8.7 ± 2.6 seconds, hind paw) compared to Gal1 *wt* mice (5.3 ± 0.3 seconds, front paw; 5.4 ± 2.6 seconds, hind paw, ANOVA, $p<0.012$) and CD-1 mice (6.1 ± 0.6 seconds, front paw; 6.2 ± 0.7 seconds, hind paw $p<0.025$).

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 Gal1-/- (front: 6.7 ± 0.6 hind paw: 7.5 ± 2.6 grams of force) compared to Gal1 *wt* groups (front: 5.7 ± 1.3 hind paw: 6.7 ± 0.6 grams of force, Figure 4.1).

*Gal1 expression in Gal1 wt mice*

Small diameter nociceptive afferents are known to express the ATP receptor $P2X_3$, terminate in lamina II inner of the dorsal horn in the spinal cord, and require the neurotrophin GDNF for neuronal development (McMahon and Moore, 1988; Chen et al., 1995; Molliver et al., 1997). Gal1 -/- mice displayed reduced thermal nocifensive responses when compared to either Gal1 wt or CD-1 mice. Using immunohistochemistry, Gal1 protein expression occurs in 68 ± 8.3% of all somata within the C7 and C8 DRGs and that this expression is not limited to any particular size class (Figure 4.2, histogram). Peptidergic neurons, as indicated by CGRP immunoreactivity, showed 32 ± 3.8% staining overlap with Gal1 (Figure 4.2, histogram inset).

**Figure 4.1 Gal1 -/- and wt sensory tests**

Gal1 -/- mice have reduced nocifensive thermal response compared to Gal1 wt mice and CD-1 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: Gal1 -/- mice (grey bar) could remain on a 1°C cold plate for a significantly longer period of time when compared to Gal1 *wt* and CD-1 mice. Gal1 -/- mice remain on the cold plate for 75.4 ± 6.8 seconds (mean ± SEM) before exhibiting a nociceptive cold response as compared to Gal1 wt mice which lasted for 18.4 ± 1.3 ($p<0.001$) seconds and CD-1 mice (white bar) which lasted 21.1± 1.5 seconds ($p<0.001$). No significant difference was found between the Gal1 wt and CD-1 mice strains. The * indicates $p<0.05$ compared to either Gal1 wt or CD-1 mice.

*Radiant heat test,* middle graph: Gal1 -/- mice (grey bars) withstand radiant heat 8.3 ± 0.3 seconds for the front and 8.7 ± 2.6 seconds for the hind paw which is significantly longer than the time seen for either the Gal1 wt mice (black bars) of 5.3 ± 0.3 seconds ($p<0.012$) for the front and 5.4 ± 0.3 seconds for the hind paw ($p<0.025$) or the CD-1 mice which had a response of 6.1 ± 0.6 seconds ($p<0.012$) for the front and 6.2 ± 0.7 seconds for the hind paw ($p<0.025$). There was no significant difference observed between Gal1 wt or CD-1 mice. The * indicates $p<0.05$ compared to either Gal1 wt or CD-1 mice.

*Progressive punctate force*, bottom graph: There is no significant difference in the amount of non-noxious force required to illicit a response between Gal1 -/- mice (grey bars) and the Gal1 wt mice (black bars). Gal1 -/- 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 the Gal1 wt 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.

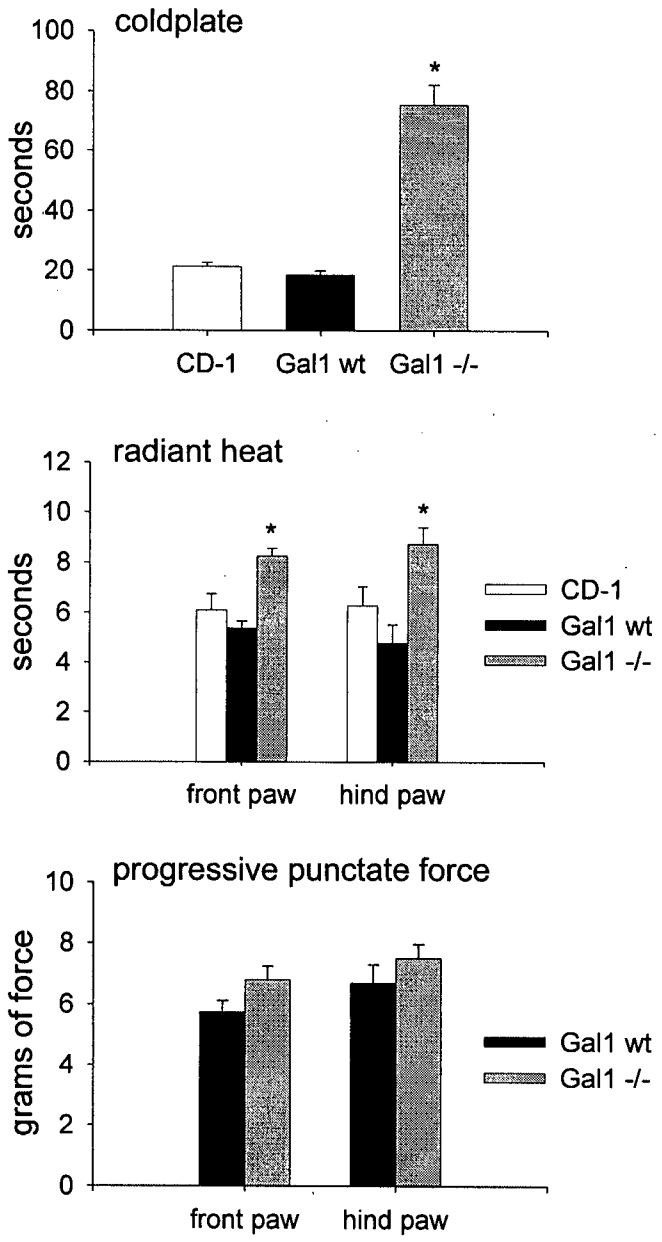
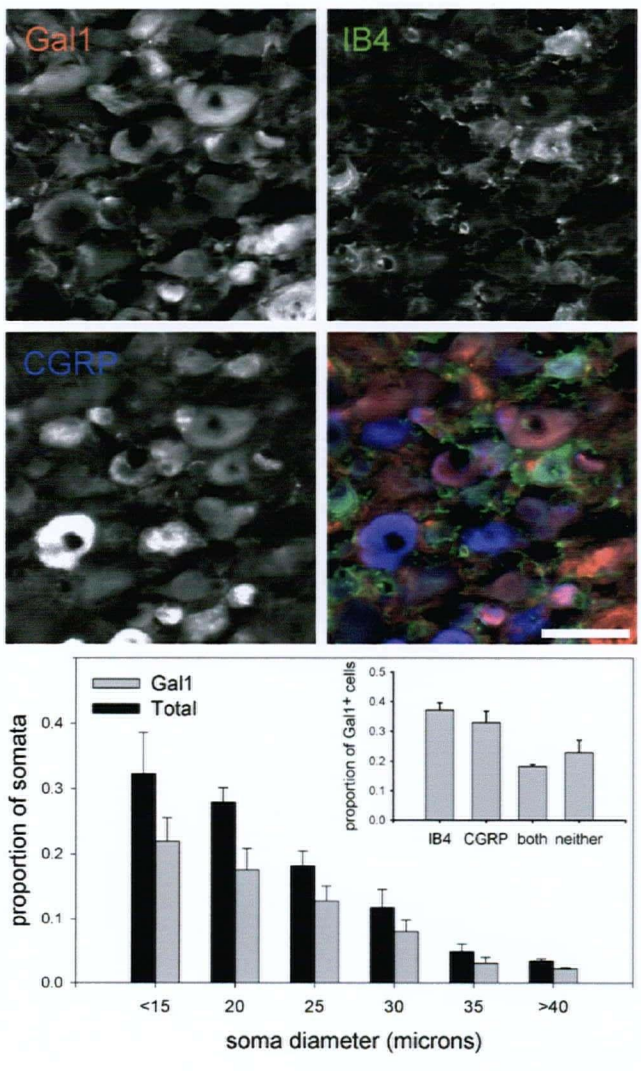

coldplate
100
80
60
40
20
0
seconds
*
CD-1
Gal1 wt
Gal1 -/-
radiant heat
12
10
8
6
4
2
0
seconds
*
*
front paw
hind paw
CD-1
Gal1 wt
Gal1 -/-
progressive punctate force
10
8
6
4
2
0
grams of force
front paw
hind paw
Gal1 wt
Gal1 -/-

**Figure 4.2 Gal1-IR in the C7 and C8 DRGs of Gal1 wt mice**

Representative photomicrographs of a C8 mouse DRG triple labeled for Gal1 (red), IB4 (green), CGRP (blue) and the merged picture of all three images (colour). Gal1 immunohistochemistry (red) shows that 68 ± 8.3% of all somata express Gal1 distributed evenly across somata size (grey bars, large histogram). Of this Gal1 expressing population, 37 ± 2.4 % also bind the lectin IB4, 32 ± 3.8% express CGRP, 18 ± 0.6% bind both IB4 and express CGRP and 23 ± 4.1% do not bind IB4 nor express CGRP (histogram bottom inset). Scale bar = 50 microns.


Gal1
IB4
CGRP
Gal1
Total
proportion of somata
0.0
0.1
0.2
0.3
0.4
<15
20
25
30
35
>40
soma diameter (microns)
proportion of Gal1+ cells
0.0
0.1
0.2
0.3
0.4
0.5
IB4
CGRP
both
neither

Non-peptidergic neurons that bind IB4 also co-express Gal1 with a 37 ± 2.4% frequency (Figure 4.2, histogram inset). 18 ± 0.6% of DRG neurons bind both IB4 and express CGRP and 23 ± 4.1% do not bind IB4 nor express CGRP (Figure 4.2, histogram inset).

*Anatomical differences*

Using radioactive *in situ* hybridization for Gal1 mRNA followed by autoradiography Gal1 mRNA was observed in the DRG somata (Figure 4.3) and the silver grains predominately co-localized to neurons within the DRG (Figure 4.3). Silver grain density was at background levels in Gal1 -/- tissue sections, even when exposed for 2 days longer than Gal1 *wt* sections, confirming that Gal1 mRNA expression was undetectable in the Gal1 -/- mice (Figure 4.3 middle panel).

Differences in peptidergic (CGRP expressing) and non-peptidergic (IB4 binding) nociceptive neurons within the DRG between -/- and Gal1 *wt* mice were examined. CGRP immunohistochemistry indicated a non-significant difference of 55 ± 5% CGRP-positive somata in Gal1 *wt* mice and 49 ± 4 % CGRP positive somata in -/- mice (Figure 4.4). Interestingly, there was a significant reduction in the proportion of cells binding IB4 from 59 ± 2% in Gal1 *wt* mice to 38 ± 4 % in Gal1 -/- ($p<0.05$, Figure 4.4). The co-localization of both IB4-binding and CGRP expressing neurons was also significantly decreased from 37 ± 3 % in Gal1 *wt* mice to 20 ± 2 % in -/- mice ($p<0.05$, Figure 4.4).

*Changes in sensory neuronal distribution and spinal termination in Gal1 null mutant mice*

Interestingly, when IB4 staining was examined in the dorsal horn, Gal1-/- mice showed a significant increase in the depth of IB4 binding within the dorsal horn (middle panel Figure 4.5). The difference between the distances of the red arrows illustrate that the binding of IB4 has not only shifted to deeper level, but expanded in depth in Gal1 -/- mice. Compared to Gal1 -/- mice, the peak IB4 binding density occurred between 70 - 80 microns deep to lamina I, whereas in Gal1 *wt* mice, the maximum binding intensity occurred 30-40 microns deep to lamina I (Figure 4.5 bottom right graph). CGRP immunohistochemistry also showed a significant difference of the depth of CGRP staining within the dorsal horn between -/- and *wt* mice (Figure 4.5, bottom left graph). Here, maximum CGRP immunoreactivity was also observed to be significantly deeper in Gal1 -/- mice when compared to Gal1 *wt* mice. When

the CGRP (green image) and IB4 (red image) are superimposed (Figure 4.5, colour picture), the white arrowheads illustrate the increased depth of IB4 binding within the dorsal horn.

Using c-fos expression as a marker for neuronal primary afferent-elicited activity, in second-order neurons in the dorsal horn, I tested whether the null mutant and wild type mice differed in their expression of c-fos after thermal stimulation (Hunt et al., 1987; Dai et al., 2001). Two hours after exposure of the front paw to noxious thermal stimuli, the dorsal horn of spinal segments C7 and C8 were examined. These two segments were examined since these two spinal levels receive the majority of the sensory input from the forepaw. After exposure to 1°C water (Figure 6, left panel) Gal1 -/- mice had 14.7 ± 0.9 c-fos positive cells at per section C7 and 13.1 ± 0.6 c-fos positive nuclei at C8 compared to Gal1 *wt* mice, which had 21.1 ± 1.9 c-fos positive nuclei per section at C7 and 24.1 ± 2.7 c-fos positive cells per section at C8 ($p<0.05$ compared at the same level between Gal1 -/- and Gal1 *wt* animals, Figure 4.6).

After exposure to 52°C water, Gal1 -/- mice had 18.7 ± 2.1 c-fos positive nuclei per section at C7 and 28.0 ± 1.9 c-fos positive cells per section at C8 compared to Gal1 *wt* mice, which had 27.5 ± 2.9 c-fos positive nuclei at per section C7 and 34.5 ± 1.5 c-fos positive nuclei per section at C8 (*$p<0.05$ compared at the same level between Gal1 -/- and *wt* animals, Figure 4.6). The neuronal specific antibody NeuN, as previously described by McPhail and colleagues (McPhail et al., 2004), was used to determine if the differences in c-fos expression can 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 Gal1 -/- or *wt* mice (figure 4.7).

**Figure 4.3 Gal1 ISH signal in the DRG**

Gal1 *in situ* hybridization signal (red) can clearly be seen in Gal1 wild-type (wt) mice (middle left panel) and that this localizes to neuronal somata (blue arrows, top right panel). Sections have been counter stained with the fluorescent nissl stain (green) within the mouse DRG in mice (top panels). Gal1 -/- (middle panel) do not show any ISH signal within the DRG or specific cell somata (vertical blue arrows) confirming that Gal1 -/- do not express Gal1 mRNA. Scale bar = 100 microns.

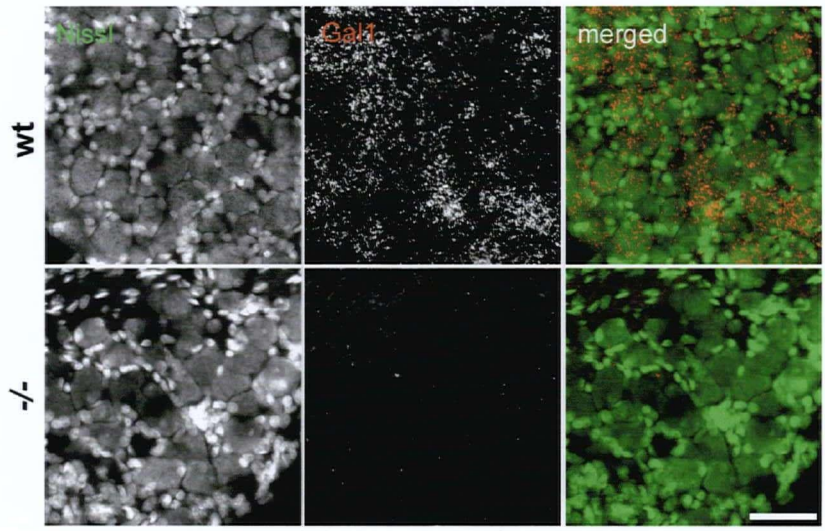

Nissl
Gal1
merged
wt
-/-

**Figure 4.4 CGRP and IB4 in Gal1 -/- and wt mice**

Distribution of CGRP (green) and IB4-binding (red) neurons in the DRG between Gal1 -/- and Gal wild type (wt) mice (n=4 for both groups). I observed approximately 55 ± 5% of cells expressing CGRP in cell bodies within the C7 and C8 dorsal DRG within Gal1 *wt* (top left panel) and 49 ± 4 % of cells in -/- mice (top right panel) and there was no significant difference between these two groups (bottom graph). There was 59 ± 2% of cells binding IB4 in cell bodies within the C7 and C8 dorsal DRG within Gal1 *wt* (middle left panel) which was significantly different to the 38 ± 4 % of cells binding IB4 in the -/-mice as indicated in the bottom graph. A significant decrease in the proportion of cells expressing both CGRP and binding IB4 decreased from 37 ± 3 % in Gal1 *wt* to 20 ± 2 % in Gal1 -/- neurons. * indicates $p < 0.05$, Scale bar = 100 microns

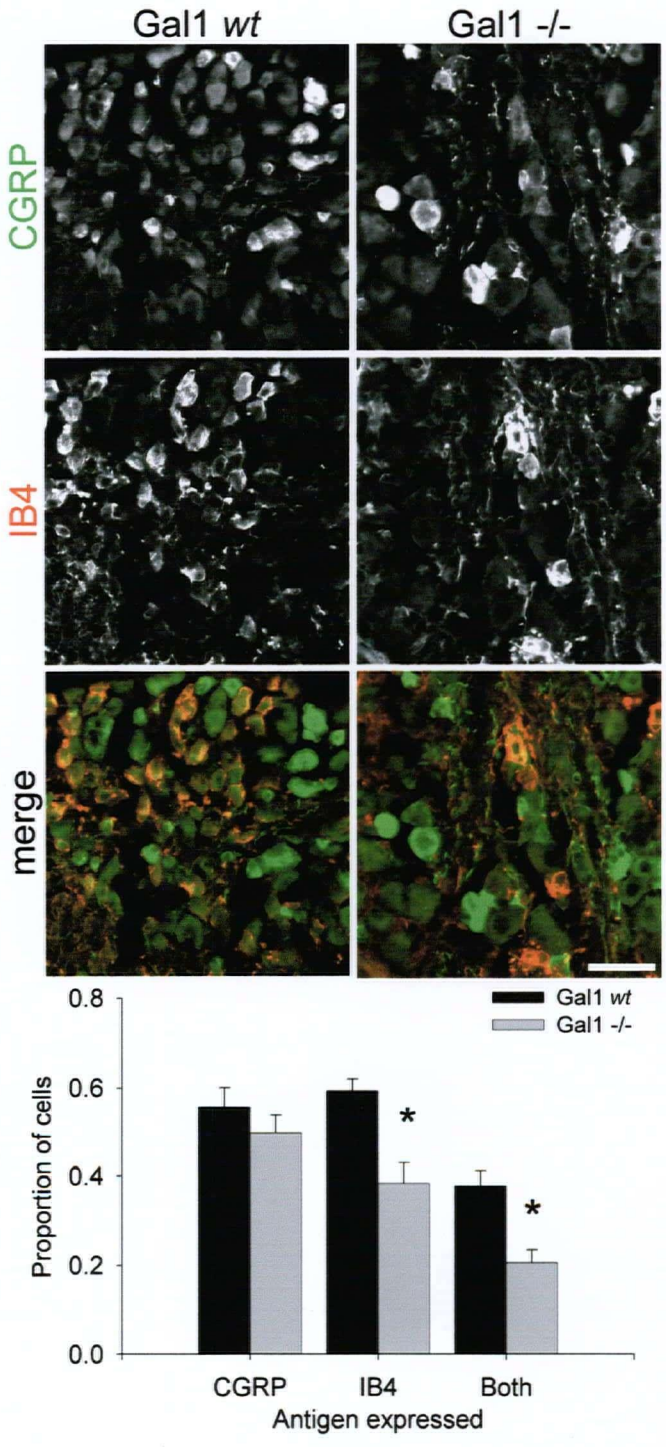

Gal1 wt
Gal1 -/-
CGRP
IB4
merge
Gal1 wt
Gal1 -/-
Proportion of cells
0.8
0.6
0.4
0.2
0.0
*
*
CGRP
IB4
Both
Antigen expressed

**Figure 4.5 CGRP and IB4 in the dorsal horn of Gal1 -/- and wt mice**

Differences in distribution of nociceptive fiber terminals within the dorsal horn of Gal1 -/- and Gal1 *wt* mice (n=4 for both groups). CGRP immunohistochemistry of -/- mice (top right panel) within the cervical dorsal horn reveals a significant increase of CGRP immunoreactivity within the deeper lamina of the dorsal horn when compared to *wt* mice (top right panel). This is clearly observed when the CGRP staining intensity of Gal1 *wt* (black line) and -/- (grey line) Gal1 mice are plotted as a function of depth (bottom left graph). IB4 binding neurons within the dorsal horn appear in deeper lamina in the -/- mice (middle right panel) when compared to the Gal1 wt mice (middle left panel). Red arrows (middle panel) illustrate the increase in depth of IB4 binding in Gal1 -/- mice when compared to wild-type mice. When IB4 binding was plotted against depth (bottom right graph), there was a significantly shift of IB4 binding intensity within deeper lamina of the spinal cord in -/- mice as compared to Gal1 wt mice. This was clearly demonstrated when IB4-binding intensity is plotted as a function of depth. Gal1 -/- (grey line) had increased staining in deeper lamina compared to wild-type (black line) mice. When the CGRP and IB4 images were overlapped (right panel), white arrowheads indicate the increased depth of IB4 binding (red) comparing Gal1 *wt* (bottom left) and -/- (bottom right) animals. * indicates $p<0.05$, scale bar = 100 microns, error bars of graphs represent SEM.

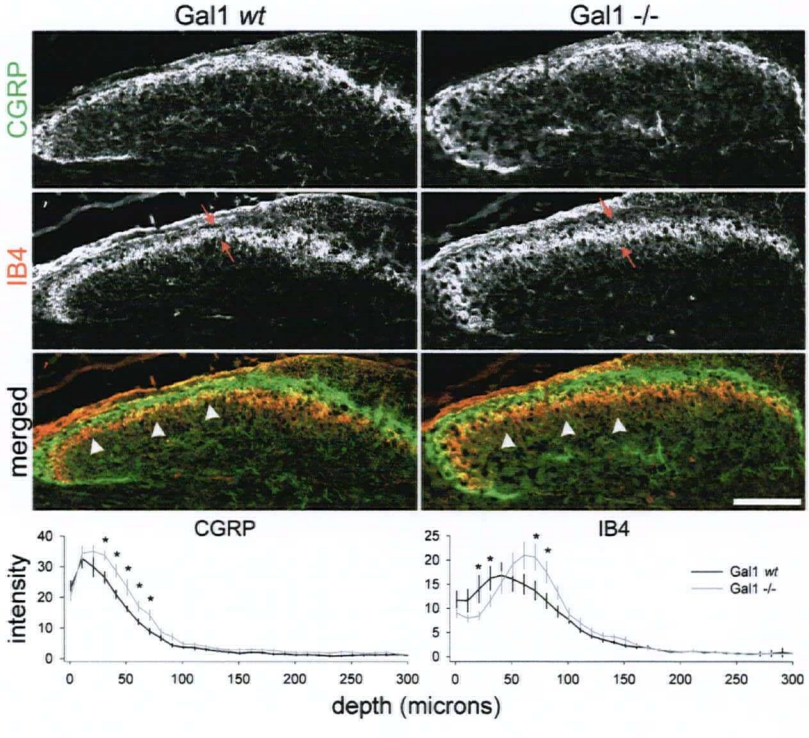

Gal1 wt
Gal1 -/-
CGRP
IB4
merged
CGRP
IB4
intensity
depth (microns)
Gal1 wt
Gal1 -/-

**Figure 4.6 c-fos-IR in the mouse dorsal horn after noxious temperature**

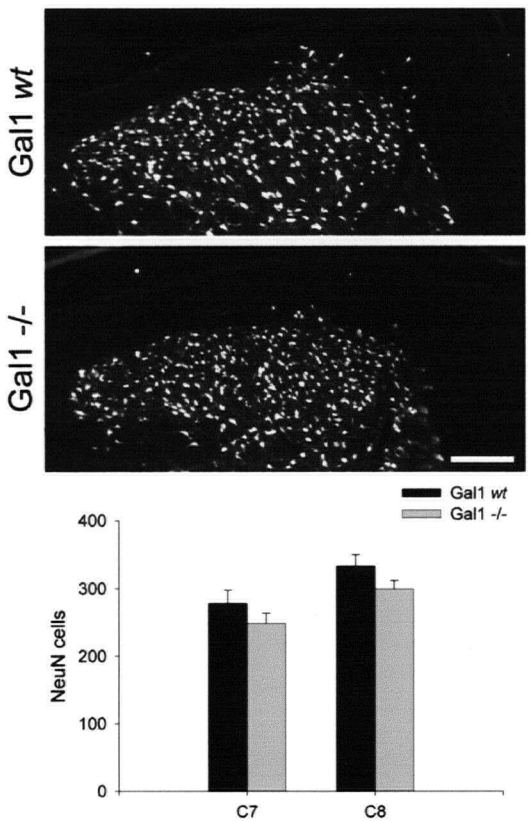
Two hours after thermal nociception, fewer c-fos positive cells in the dorsal horn of Gal1 -/- mice is observed when compared to Gal1 *wt* mice. After exposure to 1°C water (left panel) Gal1 -/- mice (grey bar, bottom left) had significantly less 14.7 ± 0.9 c-fos positive cells per section at C7 compared to 21.1 ± 1.9 c-fos positive cells per section in Gal1 *wt* ($p = 0.02$). At C8 there were 13.1 ± 0.6 c-fos positive cells per section in Gal1 -/- mice that was significantly less than Gal1 *wt* mice (black bar, bottom left), which had 24.1 ± 2.7 c-fos positive cells per section ($p = 0.007$). * indicates $p < 0.05$ compared at the same level between Gal1 -/- and Gal1 *wt* animals, n=4 for both -/- and Gal1 *wt* mice.

After exposure to 52°C water (right panel) Gal1 -/- mice (grey bar, bottom left) had significantly less 18.7 ± 2.1 c-fos positive cells per section at C7 compared to 27.5 ± 2.9 c-fos positive cells per section in Gal1 *wt* ($p < 0.05$). At C8 there were 28.0 ± 1.9 c-fos positive cells per section in Gal1 -/- mice which was significantly less than Gal1 *wt* mice (black bar, bottom left), which had 34.5 ± 1.5 c-fos positive cells per section ($p < 0.04$). * indicates $p < 0.05$ compared at the same level between Gal1 -/- and Gal1 *wt* animals, n=4 for both -/- and Gal1 *wt* mice.

Figure 4.6 c-fos in the mouse dorsal horn

**Figure 4.7 NeuN-IR in the mouse dorsal horn**

The reduced number of c-fos positive neurons in Gal1 -/- is not a result of a difference in the number of second order neurons. NeuN immunohistochemistry was performed on Gal1 -/- (top panel) and wild type (middle panel) spinal cords at C7 and C8 to quantify the number of neuronal cell bodies within the dorsal horn (n=4 for both groups). The graph (bottom panel) shows that there is no significant difference between the Gal1 -/- and Gal1 *wt* mice at either C7 or C8 levels. Gal1 -/- mice had 248 ± 15 NeuN positive cells at C7 and 299 ± 17 NeuN positive cells at C8 compared to wild-type mice (black bar, bottom right), which had 278 ± 20 NeuN positive cells at C7 and 333 ± 19 NeuN positive cells at C8. Scale bar = 100 microns.


Gal1 wt
Gal1 -/-
Gal1 wt
Gal1 -/-
NeuN cells
400
300
200
100
0
C7
C8

## *Discussion*

### *Neurodevelopment of nociceptive fibers*

Nociception is a basic requirement for the avoidance of actual or potential tissue damage. Not surprisingly, the majority of primary afferent fibers transmit a variety of nociceptive information ranging from thermal and chemical to mechanical sensitivity. These diverse nociceptive inputs are transmitted along two major nociceptive pathways terminating in laminas I and II (Snider and McMahon, 1998). The segregation of these small diameter afferent fiber projections into a laminar specific topology is a key event during neurodevelopment and suggests different functional roles. Different classes of sensory neurons enter the spinal cord in sequence. In the thoracic cord, large diameter sensory fibers enter the spinal cord at E14.5 followed by the small diameter fibers at E15.5 (Ozaki and Snider, 1997). Interestingly, in the rat Gal1 expression was first seen in the DRG at E14, just as small diameter fibers grew towards the spinal cord (Regan et al., 1986). At P0 in the superficial dorsal horn, Gal1 expression was seen in laminae I and II with the highest staining intensity seen from P0-P7. This correlates with the time at which appropriate connections are established in the dorsal horn of the spinal cord (Regan et al., 1986). These observations led to the speculation that Gal1 was involved in either axonal outgrowth or synaptic stability of nociceptive fibers during neurodevelopment (Dodd and Jessell, 1986). Once connections are made, these fibers continue to express Gal1 at lower levels. After a peripheral neuropathy in the rat, where increased nociception occurs, Gal1 mRNA increases in the DRG as well as protein levels increase in lamina I and II (Cameron et al., 1997; Xiao et al., 2002). Furthermore, Gal1 function blocking antibodies attenuate the mechanical allodynia associated with neuropathic pain (Imbe et al., 2003). Taken together, these data strongly suggest that Gal1 plays a significant role in both neurodevelopment and maintenance of small diameter sensory afferents. To compare with results in the rat, Gal1 expression was examined in *wt* mice and then determined if the lack of Gal1 in Gal1 -/- mice correlates with anatomical and functional differences in nociceptive behaviour.

Gal1 -/- mice displayed reduced nocifensive thermal responses compared to Gal1 wt mice. These attenuated responses correlates with differences in primary afferent distribution and termination within the dorsal horn of Gal1 -/- mice. Noxious thermal stimulation also

stimulated less second order neurons as indicated by c-fos activation. Taken together, these data suggest Gal1 is involved connecting and/or maintaining adult sensory neurons *in vivo*.

*Gal1 distribution*

Approximately 68% of all neurons within the cervical DRGs are Gal1-immunoreactivity (IR) in Gal1 *wt* mice. Since this is the first report of Gal1 expression in mice DRGs these results can only be compared to previous findings in rat DRGs. In adult rats, Regan et al (1986) report 63% of all DRGs examined (it is not stated from which spinal cord segment) are positive for Gal1-IR with 46% of these DRGs having strong Gal1-IR. Most DRG neurons from the 4$^{th}$ and 5$^{th}$ lumbar (L) levels in the rat have some Gal1-IR, but only 20-26% are intensely Gal1-IR positive (Imbe et al., 2003; Sango et al., 2004). The Gal1-IR within Gal1 wt mice is somewhat consistent with previous reports in rats given that there are observed differences in the portion of sensory neuron types between cervical and lumbar DRGs (Ramer et al., 2001a).

*Gal1 expressing neurons fail to make appropriate connections in Gal1 -/- mice*

Gal1 -/- mice have neither gross morphological differences from Gal1 wt mice, known compensatory changes in the expression of other galectins, nor any changes in immune cell numbers (Poirier and Robertson, 1993). During olfactory development, Gal1 is expressed by *Dolichos biflorus agglutinin* binding neurons as they are growing towards their target and this expression continues, albeit at lower levels, once connections have been made (Puche et al., 1996). Furthermore, when grown *in vitro*, Gal1 increases olfactory neurite outgrowth (Puche et al., 1996). In the Gal1 -/- mice, these *Dolichos biflorus agglutinin*-binding neurons do not reach their appropriate target during development (Puche et al., 1996). Somewhat reminiscent of the olfactory system, analogous abnormalities of small-diameter primary afferents (which express Gal1, Figure 4.2) have a significantly different terminal distribution in the dorsal horn of Gal1 -/- mice compared to Gal1 wt mice (Figure 4.5). The altered immunoreactivity of nociceptive markers in the dorsal horn (Figure 4.2) and the decrease in c-fos activation after noxious thermal stimulation in mice lacking Gal1 (Figure 4.6), suggest that the significant increase in noxious pain threshold that is observed (Figure 4.1) is in part due to the inappropriate connections of the small diameter fibers in lamina IIi (Figure 4.5), and not due to any potential differences in the number of second order neurons as indicated

by NeuN staining (Figure 4.7). There are many potential mechanism which are discussed below leading to the reduced proportion of IB4-binding cell bodies in the DRG, and the binding of IB4 and CGRP immunoreactivity within deeper layers of the dorsal horn.

*Neuronal Galectn-1 interactions*

There are many putative mechanisms by which Gal1 can maintain sensory neuronal function. Gal1 has been shown to act as a growth factor and/or a cytokine by stimulating DRG outgrowth *in vitro* and increasing the rate of sensory and motor regeneration *in vivo* by increasing Schwann cell migration and possibly eliciting the release of a yet unidentified factor from macrophages (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003; Horie et al., 2004). A neuronal Gal1 receptor has not been identified but under normal circumstances Gal1 is secreted, via the non classical pathway, (Cooper and Barondes, 1990) by Schwann cells and dorsal root ganglion neurons (Sango et al., 2004). This secreted protein may then act in an autocrine/paracrine fashion on sensory neurons and/or glial cells within the DRG during development. Therefore, the lack of Gal1 in the null mutant mouse may lead to a reduction of trophic support within the DRG, resulting in a decreased proportion of IB4-binding neurons.

*Gal1 alters ECM binding*

Gal1 also promotes axonal growth by altering adhesion properties of the extracellular matrix molecules (ECM). For example, Gal1 mediates self-aggregation of primary sensory olfactory neurons through the cross linking of carbohydrate ligands, and facilitates DRG fasciculation *in vitro* (Outenreath and Jones, 1992; Mahanthappa et al., 1994). This inappropriate neuronal targeting was previously reported in the olfactory system (Puche et al., 1996) and now in the primary sensory afferent neurons terminating on the dorsal horn of the spinal cord. The absence of Gal1 in the null mutant mice may then lead to inappropriate targeting by either hindering axonal fasciculation or altering cellular adhesion during small diameter sensory afferent growth into the spinal cord.

*IB4 neurons in thermal nociception*

Both peptidergic and non-peptidergic (IB4-binding) small diameter sensory neurons are implicated in thermal nociception although their precise role remains somewhat elusive (Snider and McMahon, 1998). These two neuronal populations are functionally distinct in

their response to heat, suggesting that they may respond to distinct aspects of noxious stimulation (Stucky and Lewin, 1999). 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 the differences in receptor expression on these neurons. Of all IB4-binding neurons approximately 78% express the capsaicin and thermal sensitive vanilloid receptor (VR1/TRPV1) and 67% also express the ATP-gated receptor $P2X_3$ (Bradbury et al., 1998; Guo et al., 1999). Application of a $P2X_3$ or VR1 antagonist to rats with inflammation leads to reductions in thermal hyperalgesia (Garcia-Martinez et al., 2002; Jarvis et al., 2002). IB4-binding neurons also express the neurturin co-receptor GFRα2 (Bennett et al., 1998). In GFRα2 -/- mice, a decrease in heat-evoked currents is observed when compared to GFRα2 +/+ but there were no observed changes in the number of IB4-postive cells (Stucky et al., 2002). Chemically killing non-peptidergic neurons via a single injection of IB4 conjugated to the toxin saporin leads to a temporary decrease in thermal nocifensive behavior (Vulchanova et al., 2001). These data indicate that IB4-binding neurons do play a role in thermal nociception. The reduction in the proportions of IB4-binding cells observed in this report as well as the alterations in laminar termination in these neurons correlate with the observed attenuated thermal nocifensive responses in Gal1 -/- mice.

Although Gal1 expression within the CNS was first reported in 1984 (Dodd et al.), we are only now beginning to understand its role in neurodevelopment, axonal injury and regeneration. In this chapter I show that Gal11 null mutant mice have anatomical and sensory deficits compared to Gal1 wt mice, and that these differences correlate with changes in behavioral responses to noxious stimuli. The precise role of Gal1 during neurodevelopment remains elusive, and more research is required to determine not only its role in potential inter- and intracellular signaling, but also the specific receptors involved in this signaling process.

# CHAPTER 5: ENDOGENOUS MOTONEURONAL GALECTIN-1 INCREASES AFTER AXOTOMY AND PROMOTES FUNCTIONAL RECOVERY AFTER FACIAL NERVE INJURY

***Introduction***

Neuronal Gal1 expression occurs within primary sensory neurons and motoneurons (Regan et al., 1986). During the period of motor axonal outgrowth in embryonic rats, Gal1 mRNA expression increases within somata of spinal motoneurons until target muscles are reached, following which, expression is maintained at a lower level throughout adult life (Hynes et al., 1990). Although the precise functions of this protein remain uncertain, Puche (1996) demonstrated that recombinant Gal1 modifies cellular adhesion of mouse olfactory neurons *in vitro*. Although Gal1 -/- mice are viable and do not display any overt phenotype, they also demonstrated that specific olfactory neurons that normally express Gal1 grew to inappropriate targets in the absence of the protein *in vivo* (Puche et al., 1996). RhGal1-Ox, but not the reduced Gal1 form, promotes axonal growth both *in vitro* and *in vivo* (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003; Horie et al., 2004). Specifically, after a sciatic nerve injury exogenously applied Gal1-Ox increased the rate and success of spinal motor and sensory axonal growth as indicated by either neurofilament staining or retrograde tracer application (Horie et al., 1999; Fukaya et al., 2003). This effect was reversed using Gal1 function-blocking antibodies that significantly reduced axonal regrowth. In both of these reports, the promotion of axonal growth by Gal1 was also associated with increased Schwann cell migration into an acellular environment. The neuronal expression of Gal1-Ox acts as a cytokine by stimulating macrophages to release an unidentified factor to promote both neuronal outgrowth and Schwann cell proliferation and migration *in vitro* (reviewed in Horie and Kadoya, 2000; Horie et al., 2004).

Although exogenous application of recombinant Gal1 facilitates motor axon regrowth and Gal1 mRNA expression occurs within naïve motoneurons, there is a paucity of information regarding changes in the endogenous expression of Gal1 mRNA following axonal injury. Previous studies used injured sciatic nerves as a model for assessing axonal regeneration. This nerve contains sensory, motor and sympathetic neurons and therefore does not allow for a clear assessment of functional regeneration within specific neuronal populations. Here I have used a facial motoneuron model of axonal injury. The facial nerve carries almost entirely motor fibers, and functional recovery can be assessed by movement of vibrissae (Paxinos, 1985; Isokawa-Akesson and Komisaruk, 1987). After a facial nerve

crush, whisker movement ceases until the injured axons begin to re-innervate their targets (Gilad et al., 1996; Ferri et al., 1998; Serpe et al., 2002; Kamijo et al., 2003). The amount and character of whisking movement, (amplitude and frequency) is directly proportional to the success of axonal regeneration (Tomov et al., 2002).

In the present study, I examined the endogenous Gal1 mRNA expression in the adult mouse facial nucleus after axonal injury. I used both a nerve resection and crush injury to examine Gal1 mRNA changes within the motoneuron cell bodies to ascertain whether Gal1 expression correlates with the regenerative state of the axons. To determine the significance of endogenous Gal1 for axonal regeneration, the recovery of whisking movement of Gal1 -/- to Gal1 *wt* mice was compared after facial nerve crush.

## *Materials and Methods*

### *Surgery*

For anesthetic procedures and information on Gal1 wt, -/- and CD-1 mice see Chapter 4 Materials and Methods.

### *Facial Nerve Lesion*

Facial nerve lesions were performed as described previously (McPhail et al., 2004). Briefly, under anesthesia, the facial nerve was exposed at its exit from the stylomastoid foramen. The buccal branch of the facial nerve was either transected and a 2-3 mm nerve segment was removed to prevent nerve regeneration, or the nerve was crushed twice for a period of 5 seconds with #5 forceps (Fine Science Tools, North Vancouver, BC) and the wound was closed with sutures.

### *Facial Nerve Injections*

Facial nerves were exposed as described above. Using a Hamilton syringe and a pulled glass micropipette, 1 µl of saline solution, 50 $\mu$M colchicine (Sigma, Oakville, ON) or 2 $\mu$g/$\mu$l of GDNF (gift from Regeneron Pharmaceuticals, Tarrytown, NY) was injected into the uninjured nerve at the same site where the nerve was crushed in previous experiments. The skin was sutured closed and the animals were allowed to survive for three days.

### *Perfusion / cryosectioning*

See Chapter 2 Materials and Methods for perfusion and cryosectioning. For mouse facial tissue all sections were cut at 14 microns.

### *In situ hybridization*

See Chapter 4 Materials and Methods for Gal1 ISH methods. Note for mouse facial tissue autoradiographic signal was exposed for 3 days.

### *Galectin-1 ISH analysis*

At least three sections per animal were analyzed, and to prevent a neuron being analyzed twice each tissue section was a least 100 $\mu$m apart. Nissl and darkfield (silver grain) images were taken of both the injured and the contralateral (uninjured) side using a digital camera attached to a fluorescent microscope (Carl Zeiss, Axioskop) in combination with Northern Eclipse software (Empix Inc, Mississauga ON). All images were analyzed with SigmaScan

Pro 5 software (SPSS Inc., Chicago, IL). The percent area occupied by silver grains was determined. This was accomplished by outlining the individual neuronal cell bodies using the nissl image and applying the resulting layer to the darkfield image. Background autoradiographic signal was then subtracted to obtain the corrected area occupied by silver grains. For each animal, the percentage area occupied by ISH signal per soma was determined for both the axotomized and contralateral (uninjured) side. The data were expressed as percentage of the mean ISH signal per soma on the contralateral uninjured side (as described previously by Fernandes et al., 1999).

*Mouse whisker movement analysis*

The Gal1 -/- (n=4) and wt (n=4) mice used in this study were born within 24 hours of each other and were 4 weeks old when surgery was performed. Prior to surgery and under light anesthesia, all but two whiskers in the caudal C-row were trimmed from the whisker pad as previously described by Tomov et al. (2002) and the wound was sutured closed. To analyze changes in whisker movement over time as an indicator of regeneration of the crushed facial nerve, before surgery and after the first 3 days following a facial nerve crush, 2-5 minutes of whisker movement was recorded using a digital video camera (Cannon, XR50MC) as described by (Tomov et al., 2002). Digital images were then transferred to a Macintosh computer (Apple Computer, Cupertino CA) and individual frames were obtained using iMovie 3.03 (Apple Computer, Cupertino CA). Individual frames of the maximal protraction were obtained (Figure 5.6A). Using Image J (1.30p, NIH, Bethesda, ML) a straight line was drawn between the tear ducts of the right and left orbits of the eyes (Figure 5.6B). This line represented the 0° angle. An angle was then measured and recorded between this line and the maximum forward sweep of the vibrissae (Figure 5.6C). Frequency was measured by counting the number of vibrissae sweeps per second. For each animal on each day until 14 days after surgery, 4 separate vibrissae movements were measured and averaged.

*Statistics*

Quantification was performed blind with respect to the treatment groups. All data are represented as mean ± standard error of the mean (SEM), and all tested were carried out using SigmaStat 3.0 (SPSS Inc, Chicago, IL). A 1-way ANOVA with Holm-Sidak post hoc test was used to determine significance between groups. Significance was assigned at $p<0.05$.

***Results***

*Gal1 mRNA expression in the facial nucleus*

In the facial nucleus of Gal1 *wt* (129P3/J) mice, *in situ* hybridization (ISH) for Gal1 mRNA followed by autoradiography was used to determine motoneuronal Gal1 mRNA expression (Figure 5.1). Silver grain density was at background levels in Gal1 -/- tissue sections, even when exposed for 2 days longer than Gal1 *wt* sections, confirming that Gal1 mRNA expression was undetectable in the Gal1 -/- mice (Figure 5.1). Gal1 *wt* mice are an inbred line and were used for comparison. Motoneurons in these mice show moderate amounts of silver grains that leveled around 7.23 ± 0.89 times background. In addition, Gal1 expression was examined in an outbred line of mice (CD-1) to ensure that this particular line of 129P3/J *wt* mice has similar levels of Gal1 mRNA expression and responds similarly to axonal injury. The baseline levels of Gal1 ISH signal in the uninjured facial motoneurons of these CD1 mice were comparable to the 129P3/J line (data not shown). Control experiments with a sense probe to Gal1 revealed only background levels of silver grains on the tissue sections in either the Gal1 *wt*, Gal1 -/- or CD-1 facial motor nuclei.

*Gal1 expression after crush or resection of the facial nerve*

After a facial nerve crush in Gal1 wt mice, ISH signals for Gal1 mRNA significantly increased in the axotomized motoneurons (Fig. 2). The silver grain density after 3 days was 307.3 ± 46.6% of contralateral and 360.9 ± 57.9% after 7 days. Statistically, these increased mRNA levels were significantly different from the levels found in uninjured motoneurons ($p<0.001$, $p<0.005$ respectively; Figure 5.2). By 14 days after a crush injury the nerves have successfully regenerated back to their targets as indicated by complete behavioral recovery (Figure 5.6). At this time, silver grain density decreased to 147.8 ± 8.1% of contralateral. This level of expression was no longer significantly different from the uninjured control animals ($p>0.05$, Figure 5.2 histogram).

**Figure 5.1 Gal1 is expressed in uninjured facial motoneurons**

Photomicrographs of Gal1 *in situ* hybridization signal (white middle panel, red bottom panel) that have been counter stained with the fluorescent nissl stain (green) within the mouse facial motor nucleus (FMN). Angled arrows on the Gal1 *wt* photomicrographs (left panels) indicate Gal1 mRNA is neuronally expressed. In Gal1 -/- mice (right panels) the photographic emulsion was left on 2 days longer than the Gal1 *wt* mice sections ensured the absences of mRNA signal was not due to a reduction in signal. Vertical arrows indicate no silver grains over Gal1 -/- neuronal cell bodies and that Gal1 mRNA does not show any greater ISH signal than background levels (right panels). Scale bar = 50 microns.

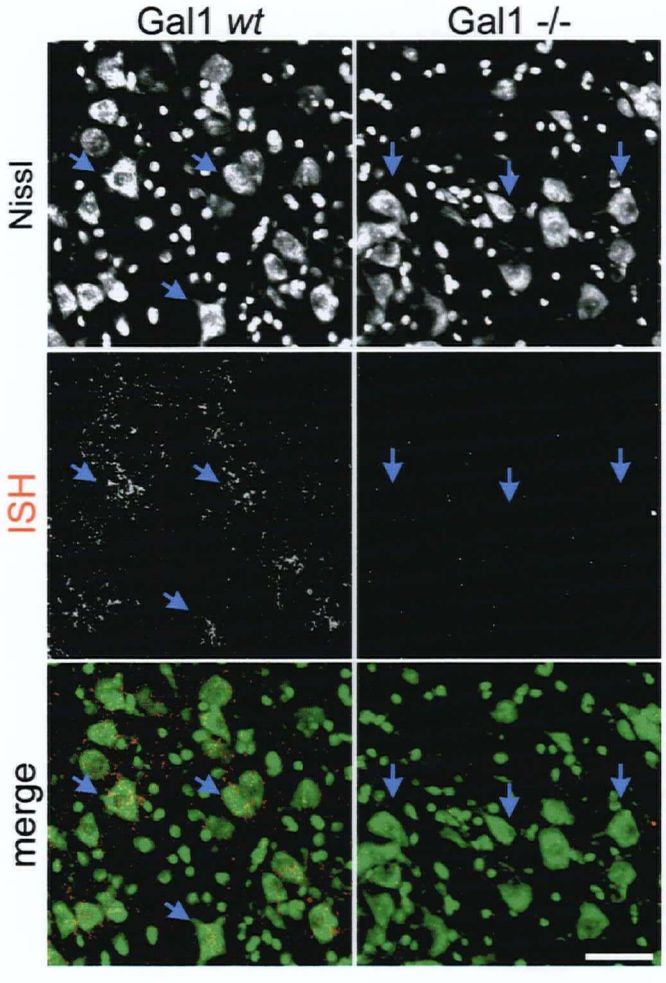
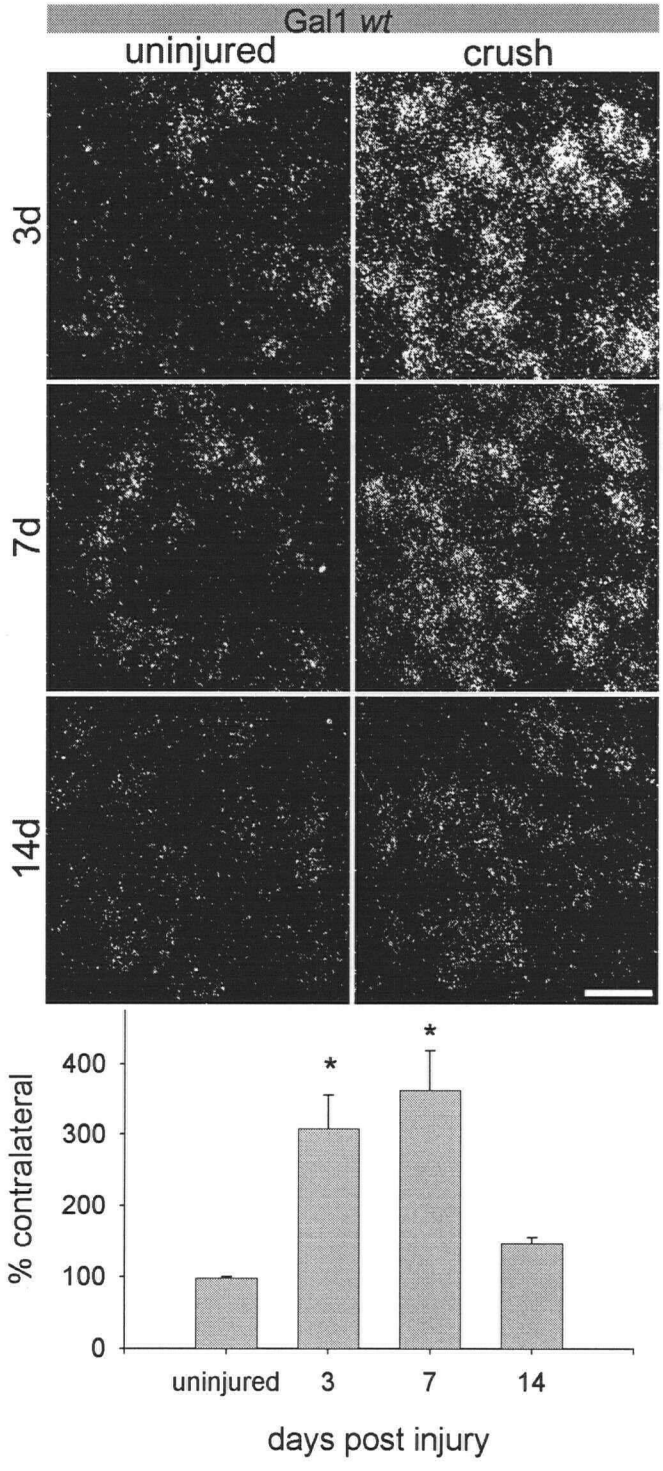

Gal1 *wt*
Gal1 -/-
Nissl
ISH
merge

**Figure 5.2 Gal1 ISH in Gal1 wt mice after a nerve crush**

At 3 days post lesion (top panel), the silver grain density over facial motoneurons after a crush was 307.3 ± 46.6% (mean ± SEM) of the contralateral and 360.9 ± 57.9% after 7days (middle panel). These increased mRNA levels were significantly different from the levels found in uninjured motoneurons ($p<0.001$, $p<0.005$ respectively). By 14 days after a crush (bottom panel), there was an increase of 147.8 ± 8.1% of contralateral which was not significantly different to uninjured animals ($p>0.05$, bottom histogram). * indicates $p<0.05$ compared to uninjured animals, scale bar = 50 microns.


Gal1 wt
uninjured
crush
3d
7d
14d
% contralateral
400
300
200
100
0
*
*
uninjured
3
7
14
days post injury

Numerous studies have demonstrated strain specific responses in mice to a variety of neurological insults (reviewed in Steward et al., 1999). In the present study, in addition to the inbred 129P3/J mice, Gal1 expression was examined in an outbred strain of mice (CD-1) to ensure that the axonal injury induced increase in Gal mRNA was not a strain specific response.

I observed a similar Gal1 mRNA expression after axonal injury in both mouse strains; compare the Gal-1 wt mouse in Figure 5.2 to the CD-1 mouse in Figure 5.3. After a facial nerve crush in CD-1 mice, an increase in grain density to 254.8 ± 27.9% of the contralateral side after 3 days and 346.6 ± 30.4% after 7 days was observed. When compared to the Gal-1 expression in uninjured motoneurons these increases were significant ($p<0.001$, $p<0.005$ respectively (Figure 5.3). At 14 days after crush a decrease in grain density to 191.1 ± 46.6% was observed. Similar to the Gal-1 wt strain this level of expression was no longer significantly different to the uninjured control animal ($p=0.05$, Figure 5.3 histogram).

After a facial nerve resection, an injury preventing axonal reconnection with the targets, a significant and rapid increase of Gal1 mRNA occurred within 3 days and persisted until at least 14 days after the injury (Figure 5.4), the longest time point examined. Quantification of these results at 3 and 7 days post injury revealed a increase in silver grain density to 257.3 ± 26.6% and 299.6 ± 29.1% of contralateral, (Figure 5.4). Compared to the levels in uninjured motoneurons these ISH signals were significantly different with p values smaller than $p<0.01$. At 14 days after a resection injury the Gal1 mRNA expression remained significantly elevated (309.8 ± 83.2% of contralateral) which contrasts with the reduced Gal1 expression at 14 days after a nerve crush (Figure 5.2 and 5.3).

**Figure 5.3 Gal1 ISH in CD-1 mice after a nerve crush**

Gal1 mRNA is transiently upregulated by facial nerve crush in outbred CD1 mice. At 3 days post lesion (top panel), the silver grain density over facial motoneurons after a crush was 254.8 ± 27.9% (mean ± SEM) of contralateral and 346.6 ± 30.4% after 7 days (middle panel). When compared to the Gal-1 expression in uninjured motoneurons these increases were significant ($p<0.001$, $p<0.005$ respectively). 14 days post axotomy (bottom panel), silver grain density was 191.1 ± 46.6% of contralateral (bottom histogram) and was not significantly different from the uninjured control animals ($p=0.05$, bottom histogram). * indicates $p<0.05$ when compared to uninjured animals, scale bar = 50 microns.

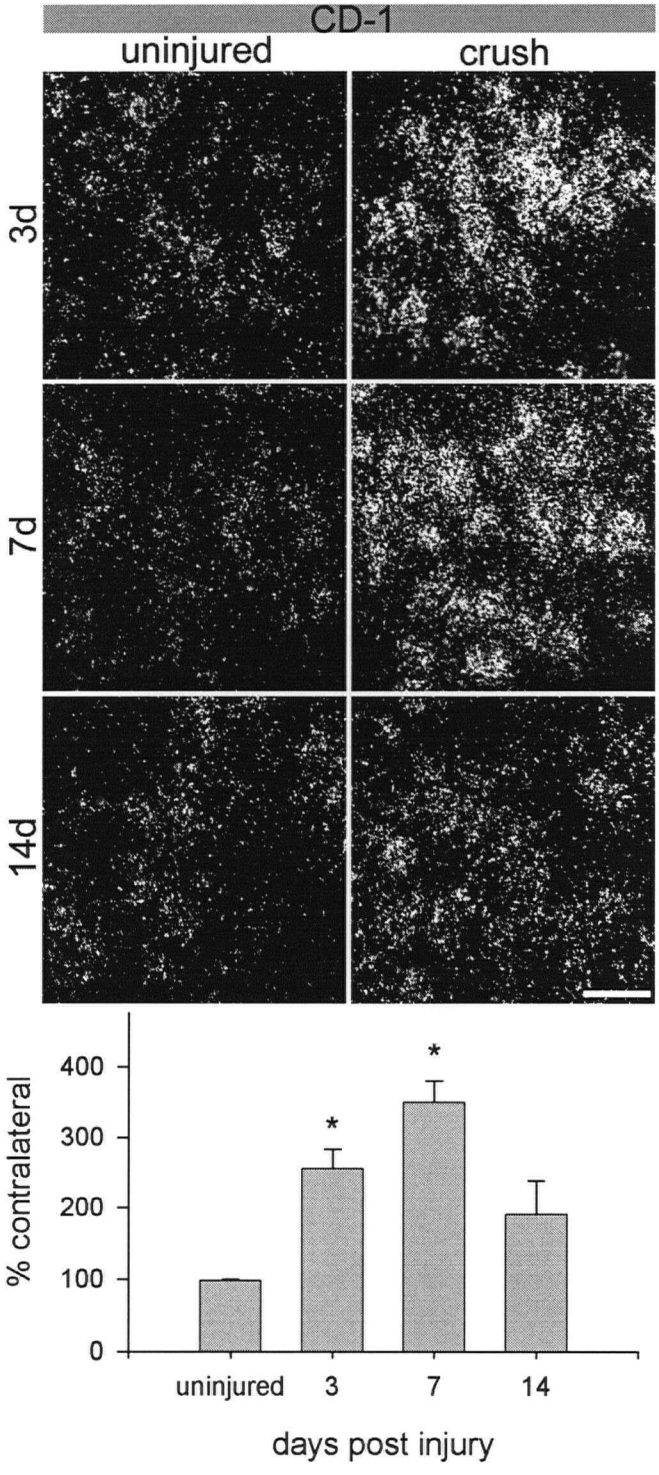
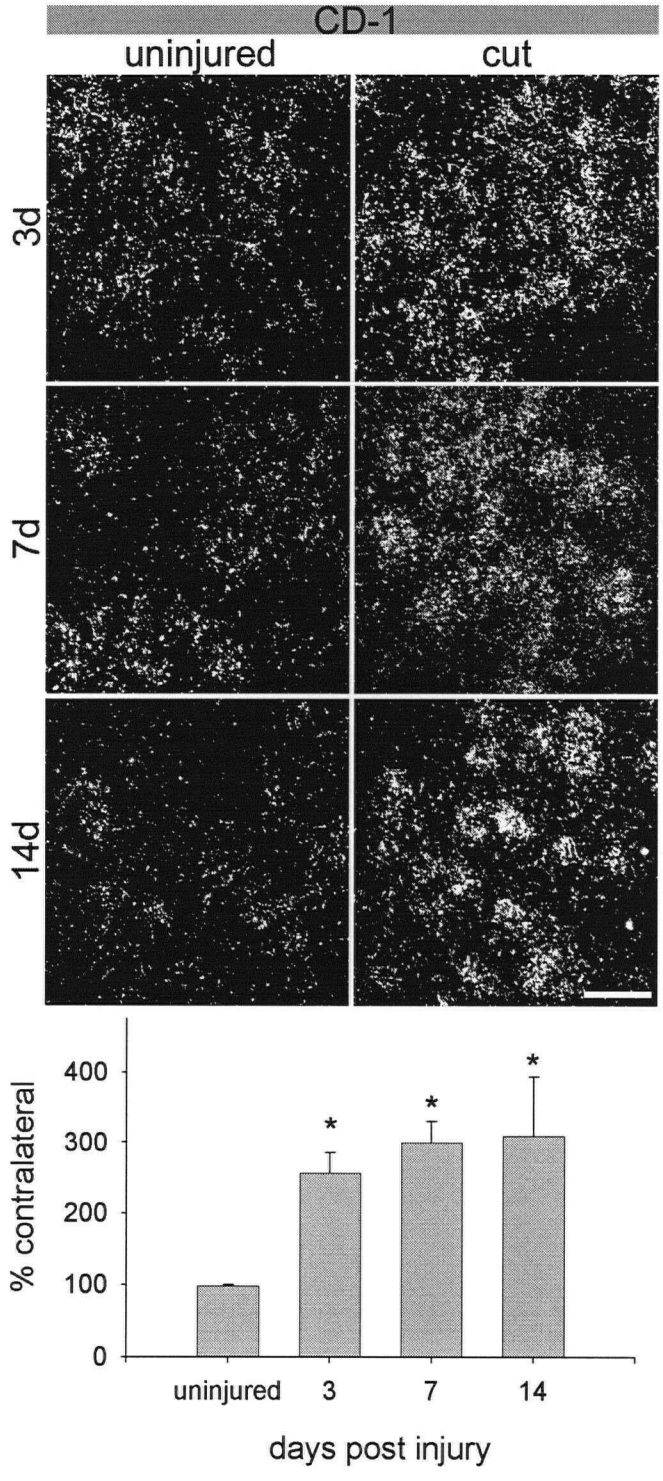

CD-1
uninjured
crush
3d
7d
14d
% contralateral
400
300
200
100
0
*
*
uninjured
3
7
14
days post injury

**Figure 5.4 Gal1 ISH in CD-1 mice after a nerve resection**

Gal1 mRNA is persistently upregulated by facial nerve transection in outbred CD1 mice. At 3 days post lesion (top panel), the average area occupied by silver grains over axotomized facial motoneurons was 257.3 ± 26.6% (mean ± SEM) of the contralateral side and 299.6 ± 29.1% at 7 days (middle panel). Compared to the levels in uninjured motoneurons these ISH signals were significantly different with $p<0.01$. At 14 days following axotomy (bottom panel), there was an increase ISH signal of 309.8 ± 83.2% of contralateral which was significantly greater than uninjured control animals ($p<0.015$, bottom histogram). * indicates $p<0.05$ compared to uninjured animals, scale bar = 50 microns.


CD-1
uninjured
cut
3d
7d
14d
% contralateral
400
300
200
100
0
*
*
*
uninjured
3
7
14
days post injury

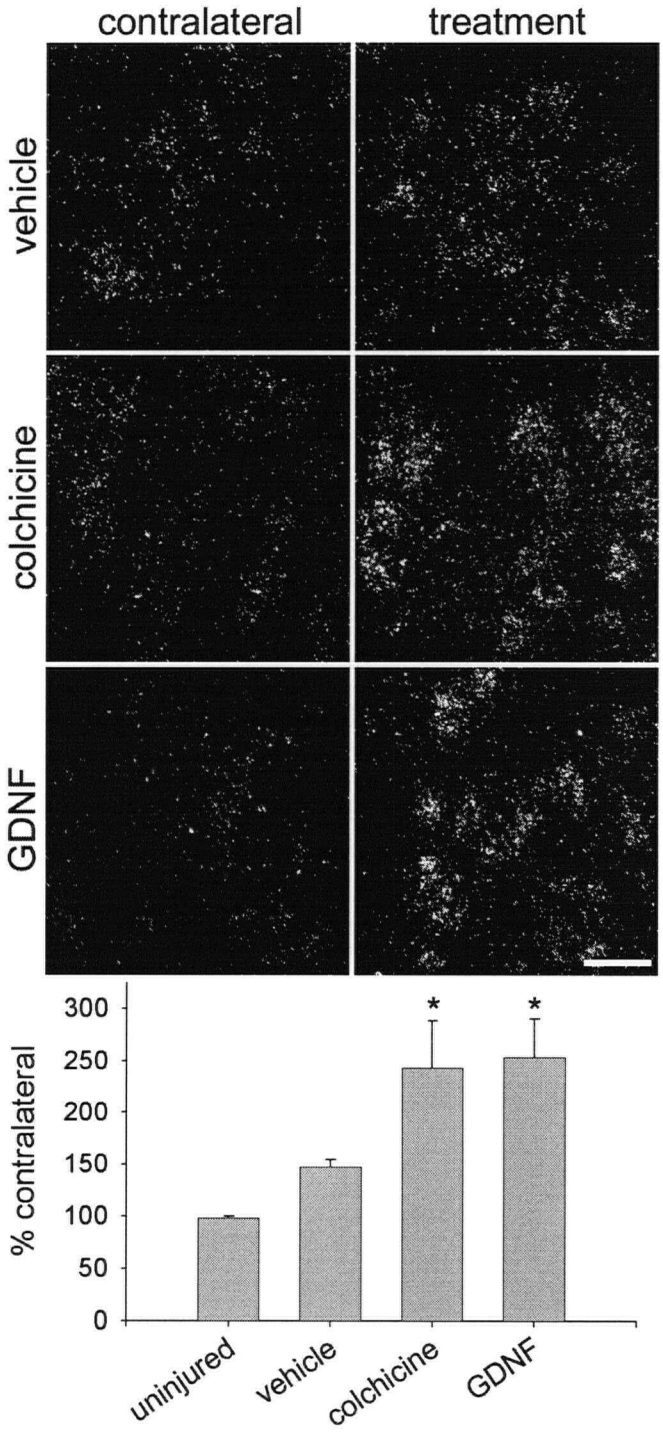
*Signals increasing Gal1 mRNA expression*

Since the experiments above suggested a regulation of Gal1 by target-derived factors I investigated the potential role of retrograde transport on Gal1 mRNA regulation. The cessation of axonal transport can be achieved through the application of the plant alkaloid colchicine, which halts axonal transport by disassembling neuronal microtubules. Colchicine or vehicle solution (n=3 per group) was injected into the facial nerve of CD-1 mice. Colchicine increased the Gal1 ISH signal to 243.9 ± 45.2% of contralateral by 3 days, rendering it significantly different from uninjured ($p<0.01$) or saline ($p<0.02$) injected motoneurons (Figure 5.5). Saline injection alone increased the average ISH signal to 147.3 ± 7.0% of the untreated contralateral side (Figure 5.5) but this level of expression was not significantly different from untreated animals. The non-significant increased Gal1 mRNA observed with saline injection is likely due to the neuronal damage caused by the nerve manipulation and injury induced by the injection.

In addition to the depletion of target derived factors playing a role in regulating gene expression after axonal injury, the release of trophic factors and cytokines at the site of injury may serve as a positive signal driving the neuronal gene expression response (Fu and Gordon, 1997). Activated peri-lesion Schwann cells increase the expression of glial cell line-derived neurotrophic factor (GDNF) (Hoke et al., 2000) and here GDNF was injected into the intact nerve of CD-1 mice (n=3 per group). Like colchicine GDNF significantly increased the ISH signal to 253.5 ± 36.8% of the contralateral side, a level significantly higher than the level in uninjured ($p<0.01$) or saline injected ($p<0.02$) motoneurons (Figure 5.5).

**Figure 5.5 Positive and negative signals regulate Gal1 mRNA**

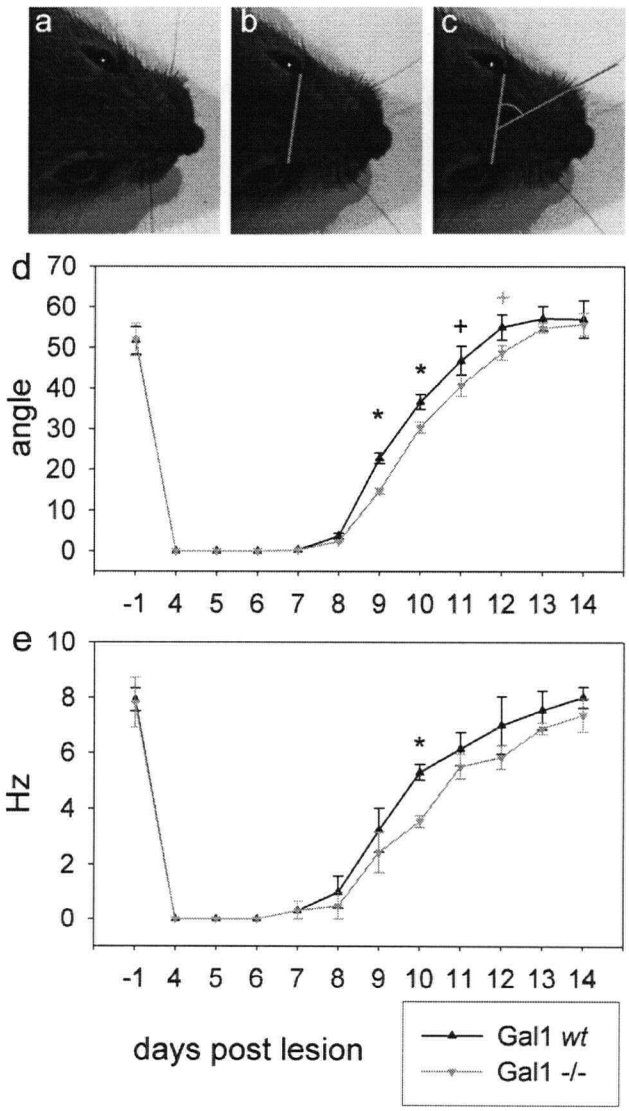
Saline treatment (top right panel) increases the average area occupied by silver grains on the treated side to 147.3 ± 7.0% (mean ± SEM) of the untreated contralateral side. This level of expression was not significantly increased above the untreated group alone ($p>0.05$, top left panel and bottom histogram). Colchicine injection (middle right panel) results in a 243.9 ± 45.2% increase (middle left panel) that was significantly greater than the uninjured animal ($p<0.01$) or vehicle injection ($p<0.02$). GDNF injection (bottom right panel) also leads to a 253.5 ± 36.8% increase (bottom left panel and bottom histogram) compared to the uninjured animal ($p<0.01$) or saline injected animal ($p<0.02$). * indicates $p<0.05$ compared to uninjured and saline treated animals, scale bar = 50 microns.


contralateral
treatment
vehicle
colchicine
GDNF
% contralateral
300
250
200
150
100
50
0
*
*
uninjured
vehicle
colchicine
GDNF

*Functional analysis of FMN injury*

Previous reports have demonstrated that exogenously applied recombinant Gal1-Ox increases the rate of transected sensory and motor axonal regeneration (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). Conversely, function-blocking Gal1 antibodies applied to the transection site reduce the rate of axonal regrowth (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). Here I wanted to examine the whether endogenous expression affects functional recovery. To accomplish this, the facial nerve was crushed in Gal1 *wt* and -/- and then assessed functional recovery by recording and measuring whisking movements. Except for two whiskers in the C-row of the whisker pad all whiskers were trimmed close to the skin (Figure 5.6A). Using video analysis (see materials and methods) the maximum angle and frequency of whisker movement was measured before and after a nerve crush. When compared to the maximum angle achieved before injury, Gal1 -/- mice required 12 days to return to pre-injury levels (Figure 5.6D, grey +) as compared to the 11 days required for the Gal1 *wt* mice (Figure 5.6D, black +). The duration of 11 days in the wt mice is very similar to other mouse strains (Ferri et al., 1998; Serpe et al., 2002; Kamijo et al., 2003). Also, Gal1 *wt* had achieved a significantly larger maximum angle when compared to Gal1 -/- mice at 9 and 10 days post injury (Figure 5.6D, $p<0.05$). When the frequency of movement was measured, there was no difference between Gal1 -/- or wt animals prior to or after a nerve crush except at 10 days post- lesion (Figure 5.6E). At this time point Gal1 wt mice had a significantly higher whisking frequency than Gal1 -/- mice. These data show that both Gal1 -/- and wt motor axons successfully regenerate to their targets after a nerve crush but that the rate of full functional recovery is slower in Gal1 -/- mice.

**Figure 5.6 Behavioural recovery following a facial nerve crush in Gal1 *wt* and -/- mice**
Using video analysis, mouse whisker movement (whisking) was recorded. A horizontal line was drawn between the inner orbits of each eye (6B). This line became the 0 degree angle from which the maximum whisking angle and frequency were measured (Figure 5.6C). Seven days after a nerve crush, whisking movements were observed. There was a significant difference in the total angle moved on day 9 and 10 between Gal1 -/- and *wt* animals (* indicates $p<0.05$, ANOVA $F=138.5$). The maximum angle of movement returns to uninjured levels one day later in Gal1 -/- mice (+ indicates when maximum angle movement is not significant different prior to crush lesion). No significant difference in the frequency of movement was observed between Gal1 -/- or *wt* animal prior to or after a nerve crush except 10 days after the lesion (* indicates $p<0.05$, 6E, ANOVA $F=40.9$).


a
b
c
d
angle
70
60
50
40
30
20
10
0
-1 4 5 6 7 8 9 10 11 12 13 14
*
*
+
e
Hz
10
8
6
4
2
0
-1 4 5 6 7 8 9 10 11 12 13 14
*
days post lesion
Gal1 wt
Gal1 -/-

***Discussion***

Both crush and resection of the mouse facial nerve resulted in a significant increase in Gal1 mRNA expression by 7 days post injury. Furthermore, I demonstrated that both interneural injections of the axonal transport inhibitor colchicine or the trophic factor GDNF induced increased Gal1 mRNA expression in uninjured facial motoneurons. These results are summarized in Table 4. The absence of Gal1 in Gal -/- mice attenuated the rate of recovery of whisker movement after a facial nerve crush.

*Gal1 mRNA expression is regulated by target-derived as well as injury-derived signals*

In the crush model, Gal1 mRNA expression was not significantly different from control levels by 14 days after injury. At this time complete functional whisker movement was restored indicating target reinnervation. This observation indicates that the increase of Gal1 mRNA after axonal injury is in part due to the loss of target-derived factor(s). A number of putative signals exist to regulate gene expression after axotomy, but to date they are poorly understood. These are postulated to be either positive signals, such factors originating at the injury site to initiate neuronal changes, or negative signals, such as the interruption of target-derived factors (Cragg, 1970; Fernandes and Tetzlaff, 2000; McGraw et al., 2002). Colchicine application to the uninjured nerve halts axonal transport through microtubule disassembly, resulting in the loss of retrograde transport. The loss of a retrograde signal derived from a target has long been thought of as one of the injury-signaling mechanisms (Cragg, 1970). For example, in the uninjured nerve, when transport is interrupted through colchicine or cold block, gene expression increases significantly for specific genes such as Tα1 tubulin and GAP-43 (Woolf et al., 1990; Wu et al., 1993; Bormann et al., 1998). In the present study, the increased Gal1 expression observed after axonal transport inhibition suggests that Gal1 expression is partly suppressed by (a) target derived factor(s). After axotomy or transport blockade, the putative repressor(s) would be absent, thus increasing Gal1 mRNA expression.

| | galectin –1 mRNA changes | | |
|---|---|---|---|
| **treatment** | **3 days** | **7 days** | **14 days** |
| nerve resection | ↑ | ↑ | ↑ |
| nerve crush | ↑ | ↑ | ↔ |
| saline injection | ↔ | | |
| colchicine injection | ↑ | | |
| BDNF injection | ↑ | | |

**Table 4. A summary of Gal1 mRNA changes in Facial motoneurons.**

Changes of Gal1 mRNA expression in facial motoneurons as observed in Chapter 5 following facial nerve treatment in CD-1 or Gal1 *wt* mice.

Neuronal gene expression changes also occur due to positive injury signals produced and/or released at the injury site. For example, the neurotrophin GDNF has potent effects on motoneuronal regeneration (Henderson et al., 1994; Blesch and Tuszynski, 2001; Boyd and Gordon, 2003). After a nerve injury, GDNF expression increases in Schwann cells distal to the lesion site where it is taken up by injured axons and retrogradely transported to promote axonal regrowth (Yan et al., 1995; Naveilhan et al., 1997; Burazin and Gundlach, 1998; Hoke et al., 2000; Blesch and Tuszynski, 2001). Intrathecal GDNF application also results in an increase of neuronal proteins such as calcitonin gene-related peptide in uninjured motoneurons (Ramer et al., 2003). Here, I report that intraneural GDNF injection increases Gal1 mRNA expression within uninjured motoneurons and illustrates its potential role as a positive regulator of Gal1.

Taken together, these data indicate that both injury site- and target-derived factors regulate Gal1 mRNA expression after axotomy.

*Endogenous Gal1 facilitates functional recovery*

Through the use of Gal1 -/- mice I have attempted to ascertain the role of Gal1 in neuronal injury and repair. This null mutant mouse does not have any known compensatory changes in the expression of other galectins or in immune cell numbers (Poirier and Robertson, 1993). To determine differences in regenerative responses between animals a facial crush was performed on both Gal1 *wt* and Gal1 -/- mice. In the adult mouse, a facial nerve crush results in regeneration and functionally complete whisking behavior within 11 days in both CD-1 and ICR mice which is comparable to the behavioural recovery time of Gal wt mice (Ferri et al., 1998; Serpe et al., 2002). Using video analysis, I quantified the rate of facial nerve functional recovery by recording the angle and frequency of vibrissae movement (Guntinas-Lichius et al., 2002). In agreement with other whisker movement data (Guntinas-Lichius et al., 2002), I observed that the measured average maximum angle was 49.9 ± 3.6 with a frequency of 7.2 ± 0.6 Hz (Figure 5.6). After a facial nerve crush, the motoneuronal regenerative response, as indicated by whisker movement, proceeded through three distinct phases. The first stage, occurring 0-7 days post injury, resulted in a complete paralysis of moment (Figure 5.6). During this time high levels of Gal1 mRNA were expressed within the injured facial somata (Figures 5.3 and 5.4). The second stage, characterized as minimal

whisker movement, occurs from 8-10 days post injury (Figure 5.6). Here Gal1 mRNA expression presumably remained elevated. The third stage is characterized by complete functional recovery of whisker movement, which is correlated with a decrease in Gal1 mRNA expression in the injured cell bodies (Figure 5.2). The behavioural recovery usually occurs after the $10^{th}$ day post injury. Although little difference in the frequency movement of whiskers was detected, whisking returned to its pre-injury state one day earlier in the wt mice than in the Gal1 -/- mice. Since recovery of whisking involves both axonal regeneration rate and synaptogenesis, (which have not been separated in this analysis), it is unclear as to which of these processes involves Gal1. However, both gain and loss of function experiments have ascribed Gal1 with a role in both regeneration rate and synaptogenesis (see below).

*Gal1's role in axonal repair*

The data presented here supports other studies demonstrating that Gal1 increases the rate of regeneration through its involvement in the initiation of the regeneration process (Horie et al., 1999; Horie and Kadoya, 2000; Fukaya et al., 2003). Exogenous Gal1 infusion into an acellular bridge at the site of a sciatic nerve injury increased both the rate of regeneration and Schwann cell migration (Horie et al., 1999; Fukaya et al., 2003). Conversely, in this model, application of Gal1 function-blocking antibodies significantly reduced the rate of axonal regeneration compared to vehicle-treated animals (Horie et al., 1999; Fukaya et al., 2003). The endogenous neuronal expression of Gal1 has been reported in neuroblastoma cells (Avellana-Adalid et al., 1994), sensory neurons (Imbe et al., 2003; Sango et al., 2004) and motoneurons (Hynes et al., 1990; Fukaya et al., 2003). Gal1 immunoreactivity is also detected within distal axons of motor and sensory neurons (Horie et al., 1999; Fukaya et al., 2003). Externalization of the Gal1 protein can occur across the growth cone plasma membrane via the non-classical pathway, despite lacking the recognizable acetylated N terminus secretion sequence (Cooper and Barondes, 1990; Inagaki et al., 2000; Schafer et al., 2003; Sango et al., 2004). Once in the extracellular space, Gal1 becomes oxidized, changes conformation and exhibits neuronal growth-promoting abilities (Horie and Kadoya, 2000; Horie et al., 2004). In the oxidized form, Gal1 acts as a cytokine by binding to and activating macrophages, which then release an unidentified growth-promoting factor that is larger than 10 kDa (Horie et al., 2004). Interestingly, the macrophage-stimulating factor zymosan also causes the release of a 14 kDa factor from macrophages that increases neuronal regrowth of

axotomized retinal ganglion cells (Yin et al., 2003). Therefore after injury the neuronal-expressed Gal1 is likely to be secreted into the extracellular space at the injury site thereby inducing macrophages to release both growth-promoting and Schwann cell-activating factors (Cooper and Barondes, 1990; Horie et al., 1999; Fukaya et al., 2003; Horie et al., 2004; Sango et al., 2004). Both macrophages and Schwann cells are known to promote successful regeneration through a variety of mechanisms such as removing myelin debris and providing trophic support (reviewed in Fu and Gordon, 1997). Experiments that alter the responses of macrophages and Schwann cells demonstrate their importance in the regenerative process. For example, acellular nerve grafts do not support peripheral nerve growth when Schwann cell migration or proliferation is inhibited (Chong et al., 1994a; Enver and Hall, 1994). In addition, inhibiting the macrophage response also reduces regenerative success (Calcutt et al., 1994; Dailey et al., 1998). In the C57BL/Wld$^s$ mutant mouse, delayed Wallerian degeneration occurs due to mutation in an ubiquitination factor (Conforti et al., 2000). In these mutant mice, functional recovery after a facial nerve crush is also delayed due to the failure of macrophages to clear axonal and myelin debris and inhibitory proteins (Perry et al., 1990; Chen and Bisby, 1993; Glass et al., 1993). Conversely, activating macrophages promotes sensory neuronal outgrowth (Lu and Richardson, 1991; Luk et al., 2003). In the present study the absence of Gal1 in mutant mice may have acted to delay the initial degenerative response following a facial nerve crush, thus reducing the rate of functional recovery.

The results presented in this study demonstrate that endogenous neuronal Gal1 mRNA expression increases after a nerve injury. This elevated expression may be a result of both neuronal target loss as well as expression of factors at the injury site. Furthermore this data suggests that endogenous Gal1 expression may contribute to the rate of functional recovery after a nerve crush.

## CHAPTER 6: GENERAL DISCUSSION

*Summary of Results*

This thesis set out to address the hypothesis that the ***endogenous neuronal Gal1 expression influences the growth potential of axotomized neurons***. The results presented in the previous chapters and summarized in Table 5 support this hypothesis. In chapters 2 and 5, I demonstrated increased Gal1 expression within regenerating axotomized motoneurons and reduced Gal1 expression in non-regenerating axotomized rubrospinal neurons. After the infusion of the growth-promoting trophic factor BDNF into the vicinity of the red nucleus, the axotomy-induced reduction of Gal1 was reversed, resulting in an increase in Gal1 expression. In the facial nucleus (Chapter 5), Gal1 expression remained elevated until the axotomized nerve re-innervated its target. Further evidence that Gal1 plays an important role in functional recovery was observed in the Gal1 -/- mouse. The absence of Gal1 in Gal -/- mice attenuated the rate of whisker movement recovery after a facial nerve crush (Chapter 5). These data suggest that Gal1 contributes to the regenerative success of the injured motoneurons.

Sensory neurons, which are able to regenerate successfully after a peripheral injury, were also examined for Gal1 expression. In Chapter 3, Gal1 expression within the DRG was examined after both a peripheral axotomy and a rhizotomy. Only after a peripheral axotomy and not rhizotomy was Gal1 expression significantly increased within the sensory neurons. Intrathecal rhGal1-Ox application to the rhizotomized DRG did promote limited regeneration of small diameter fibers into the CNS. These data further support the observation in Chapters 2 and 5 that Gal1 expression correlates with regenerative ability and that Gal1-Ox promotes limited growth into the CNS.

| neuronal population | change in Gal1 expression following axotomy |
|---|---|
| spinal motoneurons | ↑ |
| | |
| red nucleus | ↓ |
| red nucleus + vehicle | ↔ |
| red nucleus + BDNF | ↑ |
| | |
| DRG – central axotomy | ↔ |
| DRG – peripheral axotomy | ↑ |
| | |
| facial nucleus | ↑ |

**Table 5. A summary of changes observed in Gal1 expression following injury.**

Summary of chapters 2-5 of the changes observed in Gal1 expression in neurons following an axotomy.

The sensory behavior and anatomy of both Gal1 wt and Gal1 -/- mice was examined to investigate potential importance of Gal1 in sensory function. Unexpectedly, normal nociception was impaired in Gal1 -/- mice when compared to Gal1 *wt* mice. The data presented in Chapter 4 demonstrate that the attenuated nocifensive responses correspond to differences in primary afferent distribution and termination within the dorsal horn of Gal1 -/- mice. These data suggest that endogenous Gal1 expression may contribute to normal sensory function.

Overall, the four data-containing chapters of this thesis examined a variety of neuronal populations and their Gal1 responses to injury. In every neuronal system examined, neuronal Gal1 expression was found to correlate with the regenerative propensity of injured neurons. Furthermore, the absence of Gal1 affected both normal sensory functions and functional recovery following injury.

*Limitations and future remedies*

In Chapters 2 through 5 of this thesis I examined the intrinsic expression of Gal1. Previous work demonstrated that Gal1 exists in two conformations, each of which causes a different effect (see Introduction, Gal1: 2 redox forms). The inability to distinguish between Gal1-Ox and Gal1-Red within (or outside) of neurons is a major limitation of the experiments within this thesis. To overcome this issue, Dr. Horie is attempting to create monoclonal antibodies specific to Gal1-Ox or Gal1-Red (Dr. Horie, personal communication). Once created, these Gal1 redox specific antibodies are expected to give further insight into the location of Gal1-Ox activity.

Although Gal1 protein has been observed in Schwann cells as well as neurons (Horie et al., 1999; Sango et al., 2004) most of the results presented in this thesis focuses on the neuronal expression of Gal1.. Chapter 3, Figure 3.9 shows an increase in Gal1 mRNA expression within the peripheral side of the DREZ after a rhizotomy. This increased Gal1 mRNA is associated with an increased number of Nissl stained cells. *In vitro,* both Schwann cells and neurons but not macrophages express Gal1, therefore the cells central to the rhizotomy site are most likely to be Schwann cells (Sango et al., 2004). Unfortunately for

many of the experiments in this thesis the properties of radioactive decay and photographic emulsion do not allow for fine spatial resolution of silver grains to small glial cells.

Although previous studies have demonstrated the increase in Gal1 expression at the peripheral nerve lesion site, in Chapters 2, 3 or 5 the lesion site was not examined for Gal1 mRNA or protein expression. Furthermore, Gal1 protein is transported from the neuronal cell body to the axon terminals (Chapter 3, Figure 7 and Sango et al., 2004). The amount of Gal1 transport to the terminals remains uncertain. In light of this, Gal1 expression at the injury site, as well as the potential Gal1 transport to the injury site should examined.

The peripheral expression of Gal1 protein is not only limited to neurons and possibly Schwann cells; smooth and skeletal muscle cells also express Gal1 (Gu et al., 1994; Moiseeva et al., 2000). In muscle cells, Gal1-Red is involved in proliferation, differentiation and adhesion (Gu et al., 1994; Moiseeva et al., 1999; Moiseeva et al., 2000). Since expression also occurs in the muscle and possibly Schwann cells, differences in skin and muscle cytology should be examined between Gal1 *wt* and -/- mice. In particular, differences in primary sensory endings within the periphery and their distribution relative to sensory transducers or specific receptor channels should be considered. Temperature-sensitive channels called temperature-activated transient receptor potential ion channels (thermo TRPs) are expressed in DRGs and the spinal cord (reviewed in Patapoutian et al., 2003). In Chapter 4, thermal nociceptive tests revealed increased nocifensive response times in Gal1 -/- mice when compared to Gal1 *wt*. Changes were observed in both sensory fiber termination within the dorsal horn and an alteration in the proportion of sensory neuronal phenotype that may have led to difference of nocifensive responses between *wt* and mutant mice. Differences in sensory transducer expression between these mice may also lead to the observed behavioral differences. Specifically the expression of thermoTRPs associated with high heat (>42 °C) and low cold (<12 °C) temperatures, called Trpv1 and Anktm1 respectively, should be examined in both the Gal1 -/- and *wt* mice (Tominaga et al., 1998; Patapoutian et al., 2003; Story et al., 2003). A family of two-pore $K^+$ channel controls some of these thermoTRPs (Fink et al., 1998; Maingret et al., 2000). Gal1-Ox is suggested to bind an unspecified $K^+$ channel (Dr. Horie, personal communication). The peripheral distribution

of receptors and thermo TRPs should be examined considering Gal1 expression may also occur in Schwann and muscle cells in the periphery.

In Chapter 5, the absence of Gal1 in Gal1 -/- mice attenuated the restoration of function. A number of different factors that were not examined could lead to these observed changes. Since Gal1-Ox increases the rate of neuronal regeneration in rats it seems likely that the regenerative rate would be decreased in Gal1 -/- mice compared to Gal1 wt mice. However examination of the rate of growth within the axotomized nerve was not undertaken. The differences in functional recovery could also be attributed to alterations in restoring functional synaptic connections. In uninjured adult Gal1 -/- mice both olfactory and DRG neurons are in the correct vicinity of their appropriate targets but they appear to have made inappropriate terminations. This suggests Gal1's involvement in either target finding or maintenance of the axon. In the periphery, muscles cells also express Gal1 (Watt et al., 2004). *In vitro,* Gal1 promotes olfactory neuron growth through cell adhesion mechanisms (Mahanthappa et al., 1994). It is unknown whether Gal1 expressed in target muscle of growing axons can facilitate axonal growth via attractant or adhesive mechanisms. Accordingly I can only demonstrate that functional recovery is attenuated in Gal1 -/- mice but the underlying mechanism by which this happens remains unanswered.

In Chapters 4 and 5, the 129P3/J Gal1 -/- was compared to the 129P3/J Gal1 wt mouse strain for both functional and anatomical assessments. Gal1 wt strain was obtained from a commercial supplier and used for both behavioral and anatomical comparisons whereas the Gal1 -/- was obtained from Dr. Poirier. Even though the Gal1 -/- and wt mice are both inbred mice strains that reduce genetic variability, a limited amount of generational genetic change could occur. These possible genetic changes could lead to differences, other than those attributed to the Gal1 null mutation, between the Gal1 -/- and Gal1 wt mouse strains. However, the heritability of nociceptive response of inbred mouse strains is considered high compared to those of outbred mouse strains (Mogil et al., 1999b). In particular, thermal nociceptive traits of 129 mice have little variation (Mogil and Adhikari, 1999; Mogil et al., 1999a, b). When testing thermal nociception in mice, there is a greater variability between experimenters than between differing inbred and outbred mouse strains (Chesler et al., 2002a, b). Great care was taken to minimize testing variability by age

matching mice and having the same experimenter perform the behavioral tests (see methods Chapter 4 and 5).

***How does Gal1 promote axonal growth?***

This thesis examined neuronal Gal1 expression, its putative involvement in regeneration and the effect of the absence of Gal1 expression. These experiments do not address the putative growth-promoting mechanisms of Gal1. Evidence from both *in vitro* and *in vivo* research suggests that Gal1 can promote axonal growth through both extracellular and intracellular mechanisms. In light of the results presented in this thesis, the mechanisms by which this might occur are discussed below.

*Redox State*

Redox modulation by covalent modification of sulfhydryl groups on cysteine residues can regulate protein function (Lipton, 1999). Gal1 is an example of the biological activity being dependant on the redox state. As previously stated, direct application of Gal1-Ox promotes axonal growth *in vitro* and *in vivo* whereas Gal1-Red does not promote neurite outgrowth (Horie et al., 1999). In the extracellular space of neurons or Schwann cells, Gal1 exists in both the reduced and oxidized forms but within the cell Gal1 is believed to exist mainly in the reduced form (Sango et al., 2004). The protein's redox environment is determined by equilibrium between oxidants, such as reactive oxygen species, and antioxidant mechanisms. Contributing to an oxidizing environment are reactive nitrogen intermediates produced by nitric oxide synthase (NOS). Modification of critical cysteine residues by reactive nitrogen intermediates has been shown to regulate various ion channels, G-proteins, growth factors, enzymes and transcription factors (reviewed in Stamler et al., 1997). NOS may also regulate Gal1's redox state. The expression of NOS increases after a peripheral nerve injury site and mediates thermal hypersensitivity (Zochodne et al., 1999; Levy et al., 2000). Gal1 binding to macrophages regulates nitric oxide production by NOS and the nitric oxide may also promote neuronal hyperexcitability in neuropathic pain models of injury (Wiesenfeld-Hallin et al., 1993; Michaelis et al., 1995; Correa et al., 2003). Interestingly, Gal1 -/- mice have decreased responses to thermal noxious stimuli that may be, in part, related to changes in nitric oxide levels. The mechanisms regulating Gal1's redox state are not understood. We are gaining a greater insight into roles of both Gal1-Ox and Gal1-Red within and outside the cell.

*Extracellular actions*

THE EXTRA CELLULAR MATRIX (ECM)

The ECM plays an important role in successful axonal regeneration of sensory neurons in both peripheral and central nervous systems. Condroitin proteoglycan (CSPG), an ECM molecule, has specifically been shown to prevent DRG growth in the central nervous system (McKeon et al., 1991; Davies et al., 1997) and also limits successful regrowth after a peripheral axotomy (Zuo et al., 2002). The ability of axotomized CNS or PNS to successfully regrow increases when CSPGs are enzymatically reduced with chondroitinase ABC at the lesion site (McKeon et al., 1991; Moon et al., 2001; Bradbury et al., 2002; Morgenstern et al., 2002; Zuo et al., 2002; Yick et al., 2003). Recent *in vitro* evidence suggests that Gal1red binds to CSPG, thus preventing its incorporation into the ECM (Moiseeva et al., 2003a). This implies that high Gal1 expression reduces CSPG incorporation, creating an environment more permissive to growth.

MACROPHAGES

Macrophages are an important component of successful peripheral nerve regeneration. By removing myelin debris, modulating the Schwann cell proteases, promoting Schwann cell proliferation and providing trophic support of neurons the macrophages create a more permissive environment for peripheral regeneration (Fu and Gordon, 1997). For example, during the peripheral regeneration process macrophages secret the anti-inflammatory cytokine IL-10 and the trophic factor transforming growth factor – β serving to limit secondary damage while providing a permissive growth environment. After a CNS injury, peripheral macrophages enter they injury site where they can exacerbate the injury (Popovich and Hickey, 2001). This is demonstrated when peripheral macrophages are depleted, reducing the number that would normally enter the CNS, and thus reducing CNS tissue damage (Popovich et al., 1999). Simulating macrophages and/or microglia with either Zymosan or lysophophatidylcholine within the CNS leads to further tissue damage (Fitch and Silver, 1997b; Ousman and David, 2000; Popovich et al., 2002). These results suggest that peripheral activated macrophages support axonal peripheral regeneration whereas if activation occurs within the CNS these cells inhibit repair and exacerbate the injury. This notion is further demonstrated using macrophage transplant techniques. Macrophages can reduce secondary tissue damage and promote limited axonal growth within the injured CNS

if these macrophages are cultured on degenerating peripheral myelin first and then transplanted into the injured CNS (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998). This notion was illustrated when macrophages that were stimulated with either Zymosan injected directly into the DRG (Steinmetz et al., 2003) or by an intraperitoneal injection of Corynebacterium parvum (Lu and Richardson, 1991) led to increase neuronal growth. To find any putative factors that these peripherally activated macrophages might release, Yin et al (2003) injected Zymosan into the vitrius of the eye to simulate peripheral macrophages. Not only did they observe increased growth of injured CNS rentinal ganglion cells compared to saline injections but also they discovered that Zymosan stimulated macrophages *in vitro* led to the release of an unidentified 14-kDa protein that increased axonal growth. These results suggest that peripherally-primed macrophages release a specific factor that promotes axonal regrowth.

Recently, Gal1ox has been shown to specifically bind peripheral macrophages *in vitro* (Horie et al., 2004). Although the macrophage receptor remains unidentified, this binding led to both kinase phosphorylation and the release of an unidentified factor that was larger than 10 kDa (Horie et al., 2004). The Gal1/macrophage-conditioned media stimulated both axonal growth and Schwann cell migration. Both Zymosan and Gal1 stimulate peripheral macrophages to promote axonal growth *in vitro* and *in vivo* (Horie et al., 1999; Yin et al., 2003; Horie et al., 2004). It would be interesting to know if the 14 kDa molecule released from Zymosan stimulated macrophages is the same (or similar) molecule released from Gal1 stimulated macrophages. Also peripherally derived macrophages could be stimulated with Gal1 and then transplanted into the injured CNS which may reduce secondary damage and/or induce regeneration. Another approach would be through the use of the well-established peripheral nerve graft paradigm. Specifically, degenerated peripheral nerve grafts from either Gal1 -/- or *wt* mice could be transplanted into either CNS tissue (rubrospinal tract) or onto another peripheral nerve of Gal1 *wt* mice to assess the growth permissiveness of Gal1 -/- tissue. Currently, the search for the Gal1 macrophage receptor and the unidentified compound(s) released from Gal1-stimulated macrophages is currently underway (Dr. Horie, personal communication). Once these two pieces of information are revealed, further

investigation into the mechanisms into Gal1 macrophage activation can be explored and exploited for possible CNS repair.

MICROGLIA

The full complement of cells that bind Gal1 is unknown. Specifically, no *in vitro* Gal1 binding assays have been undertaken with CNS cells. Astrocytes and olfactory neurons are the only known cell within the CNS to bind Gal1-Red (Sasaki et al., 2004). Once bound to astrocytes, Gal1 causes BDNF release and astrocyte differentiation (Sasaki et al., 2004). In chapters 2 and 5 of thesis, I demonstrate the increased mRNA expression in the axotomized facial nucleus and the decrease Gal1 expression in the axotomized rubrospinal nucleus. The glial response is also quite different in these two injury paradigms: after a facial nerve axotomy, there is an increase both astrocyte and microglial reactivity around the neuronal cell bodies (Graeber et al., 1988; Tetzlaff et al., 1988; Barron et al., 1990; Tseng et al., 1996). After a rubrospinal tract injury, a muted or non-existent glial response occurs around the red nucleus (Tseng et al., 1996; Liu et al., 2003). Although the gliotic response following injury creates an inhibitory barrier to growing axons, these inflammatory reactive cells also can produce trophic factors that support growth (Streit et al., 1998; Fitch and Silver, 1999). For example, a significant glial response occurs around the facial nucleus following a facial nerve axotomy. These reactive glial cells are thought to be growth-promoting since they are able to release trophic factors around the cell body (Ridet et al., 1997; Batchelor et al., 2002). Since Gal1 can be released from cell bodies (Sango et al., 2004) and I demonstrated increased Gal1 expression in both facial and spinal motoneuronal cell bodies following axotomy, it seems plausible that Gal1 released from neurons may beneficially stimulate glial cells around the neuronal cell bodies. Therefore the decease in Gal1 expression observed in the red nucleus after axotomy may contribute to the observed regenerative failure by not initiating a glial response. Gal1 infusions into the red nucleus after a rubrospinal tract axotomy might further aid in determining whether this postulated hypothesis is accurate.

*Intracellular actions*

The early reports that Gal1 promoted axonal growth *in vitro* and *in vivo* focused on the exogenous application of this lectin. In chapters 2-5 of this thesis, I have reported either mRNA or protein expression within the neuronal nucleus or cytoplasm. These observations

of the intrinsic Gal1 expression are not new. The first paper describing Gal1 expression in the DRG demonstrated nuclear and/or cytoplasmic Gal1 immunoreactivity in the nervous system was in 1986 (Dodd and Jessell, 1986). In recent reports, only application of Gal1-Ox at the injury site was examined even though neurons express Gal1. Although the exact role this lectin plays in the uninjured neuron remains unclear, there are indications that it serves important basic neuronal functions. A greater understanding of the intracellular processes in which Gal1 is involved will lead to some indication of its overall role both uninjured and axotomized neurons.

SURVIVAL OF MOTOR NEURON BINDING

Gal1 has been identified as a component of the Survival of Motor Neuron complex (Park et al.,.2001). More specifically, Gal1-Red binds to Gemin4 within this 12-protein structure. This Survival of Motor Neuron complex, found within both the nuclear and cytoplasmic compartments of motoneurons, associates indirectly or directly with RNA, RNA polymerase II and zinc-finger binding proteins and is required for cell viability (Gangwani et al., 2001; Pellizzoni et al., 2001; Frugier et al., 2002). *Cre*-mediated deletion of the SMA gene (which disrupts the Survival of Motor Neuron complex) instigated within a *Cre-LoxP* SMA mutant mouse leads to death of the mouse four weeks later due to severe motoneuronal deficits. Specifically, there was a 73% reduction of motor axons and a 23% reduction in cell body size with neurofilaments accumulating in synaptic terminals (Frugier et al., 2000; Cifuentes-Diaz et al., 2001). Conversely, over-expression of SMN within motoneurons *in vitro* enhances neuronal outgrowth (Rossoll et al., 2003). Gal1 binding to the SMN complex is not required for mouse survival since the Gal1 -/- mice are completely viable. This may be in part due to the ability of galectin-3 to also bind Gemin4 (Park et al., 2001). The interaction of Gal1 with neuronal survival and growth proteins within the neuron may contribute to neuronal maintenance and growth.

In the fatal neurodegenerative disease familial amyotrophic lateral sclerosis (ALS), abnormal accumulations of neurofilaments within axons and perikarya of motor neurons occur. These neurofilamentous lesions have a high accumulation of Gal1 (Kato et al., 2001). The mechanisms and potential significance of the close association between the

neurofilaments and Gal1 remain uncertain, but evidence from *in vitro* and pathological conditions offers some insight.

### SIGNALING CASCADES

Within the neuron, the regenerative machinery required to maintain the active extension of the axon and axonal growth cone (e.g. cytoskeletal proteins), as well as factors which are involved in maintaining neuronal phenotype (such as neurotrophins and their receptors) are required for successful regeneration. Included in the first category is GAP-43, which acts as a link between membrane rafts and the actin cytoskeleton, and mediates growth cone motility (Frey et al., 2000; Laux et al., 2000). GAP-43 is massively and persistently upregulated in spinal motoneurons, but is less robustly and only transiently upregulated in cervically axotomized RSNs (Fernandes et al., 1999). In the second category are neurotrophins and their receptors such as BDNF and trkB, respectively. Receptor binding by the appropriate neurotrophin leads to various signal cascades. Depending upon whether the Raf-1/MEK/ERK or PI3K signaling cascade is activated the outcome can effect neuronal growth and/or survival respectively (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Within cells, Gal1 may act in an intermediate role by altering second messenger cascades of this neurotrophin signaling. Gal1-Red recently has been shown to stabilize the GTPase H-ras to non-raft microdomains resulting in stabilization of the signaling domains of the second messenger system (Prior et al., 2003). This stabilization by Gal1-Red increases the potential for the Raf-1/MEK/ERK activation, and not the PI3K signaling cascade (Paz et al., 2001; Elad-Sfadia et al., 2002). The Raf-1/MEK/ERK signaling pathway is involved in axon extension (Markus et al., 2002). Thus Gal1 may facilitate intracellular signaling cascades and alter the growth capacity of neurons.

### ACTIN CYTOSKELETON

Cellular motility and growth require the cytoskeletal rearrangement of actin. In astrocytes, motility is dependant on F-actin (Abd-el-Basset et al., 1991). In astrocytic tumours (glioblastoma), for example, malignancy is a direct result of the cell's ability to move and diffusely infiltrate normal brain tissue. In these glioblastomas, malignancy is directly associated with Gal1 expression (Kopitz et al., 1998; Yamaoka et al., 2000; Camby et al., 2001). *In vitro*, Gal1 antisense injuected into gliomas results in arrested growth (Yamaoka et

al., 2000). After Gal1 application to glioblastoma cells *in vitro*, at relatively low concentrations (0.1 ng/ml), there is a 30% increase in cell motility and F-actin polymerization, and up to a 75% increase in RhoA expression. Unfortunately, the authors of this study did not report whether Gal1 induced changes in RhoA activation. The regulation of actin polymerization/depolymerization involves the small GTPases of the Rho family (Maekawa et al., 1999). In growing neurons, F-actin polymerization is required for neurite extension and outgrowth (Mackay et al., 1995). Changes in RhoA activation leads to changes in a neuron's outgrowth ability *in vitro* and *in vivo* (Lehmann et al., 1999; Borisoff et al., 2003). Although no published study exists demonstrating Gal1-induced changes in neuronal RhoA expression, many of the original studies examining signaling to the cytoskeleton were first carried out in fibroblasts and later repeated in cultured neurons. Since Gal1 promotes neurite outgrowth and Schwann cell migration *in vitro* and *in vivo* (Mahanthappa et al., 1994; Horie et al., 1999; Fukaya et al., 2003; Horie et al., 2003), and high expression is associated with growing neurons (Chapters 2, 3 and 5) it might be fruitful to examine whether Gal1 also has direct effects on either RhoA expression or activation within neurons.

Schwann cells have a highly regulated mechanism to establish myelin sheaths around axons in either development or regeneration. These cells express laminin receptors, one of which, $\alpha_6\beta_1$ integrin, is directly linked to F-actin. Function blocking $\beta_1$ integrin receptor antibodies prevents both proper Schwann cell myelination and basal laminal formation. The $\beta_1$ integrin null mutant mouse is embryonically non-viable but the conditional mutant mouse demonstrates that this receptor is required for proper myelination. *In vitro*, Gal1 binds to the $\beta_1$ integrin of smooth muscle cells and increases cell proliferation and migration (Moiseeva et al., 2000; Moiseeva et al., 2003b). In Schwann cells, $\beta_1$ integrin activation causes dimerization of the receptor that then leads to the autophosphorylation of the focal adhesion kinase (FAK), promoting myelination and migration (Siciliano et al., 1996; Chen et al., 2000; Taylor et al., 2003). In both astrocytes and Schwann cells, Gal1 promotes changes in F-actin to increase migration. In the CNS, astrocyte migration or movement is usually associated with malignancy whereas in the PNS, Schwann cell migration is viewed as beneficial for peripheral axonal repair.

***Consequences of an enhanced neuronal growth mode***

*Axonal Regeneration*

As outlined in the General Introduction, there are many obstacles the damaged neuron must overcome to achieve successful regeneration in both peripheral can central nervous systems. After peripheral nerve injury, Gal1 may increase regenerative success by facilitating some of these repair mechanisms. After injury, neurons have a reduced ability to regulate membrane permeability leading to in an increased Gal1 release into the extracellular space (Povlishock and Pettus, 1996). Once in the extracellular space, Gal1 promotes Schwann cell migration and macrophage release of neuronal growth-inducing factors to promote axonal repair and functional recovery (Horie et al., 1999; Horie et al., 2004; Sango et al., 2004). Within the injured neuron, Gal1 expression increases possibly leading to more Gal1 release and increased Gal1 within the cell body. Neuronal Gal1 is involved in signal cascade modulation, neuronal survival protein binding and possibly actin cytoskeleton interactions, all of which contribute to an increased capability of injured neurons to survive and grow both extra and intracellular processes. Obviously these cellular processes are not dependant on Gal1 since injured neurons in Gal1 -/- mice (Chapter 5) are able to make functional connections (albeit at a slower rate). This illustrates that Gal1 is involved but is not essential for successful neuronal repair.

*Neuropathic pain*

Partial nerve injury is associated with hyperalgesia (increased pain sensitivity), allodynia (pain from non-noxious stimuli), spontaneous and general ongoing pain. These conditions are due in part to axotomy-induced changes within the peripheral neuron. Large-diameter axotomized afferents begin to discharge spontaneously and increase their expression of BDNF (Michael et al., 1999; Boucher et al., 2000; Liu et al., 2000a; Liu et al., 2000b). This increase in spontaneous activity is thought to sensitize spared fibers by reducing the dorsal horn neurons threshold for activation in a process called central sensitization (Woolf, 2000). Furthermore, the spared fibers have greater access to target-derived trophic factors, such as NGF, due to the loss of axonal transport in injured axons. NGF has been shown to regulate behavioural sensitivity to pain (Koltzenburg et al., 1999). These injury-induced changes increase the expression of the excitatory neuropeptide substance P as well as BDNF

(Noguchi et al., 1995; Michael et al., 1997; Pezet et al., 2002a). In the dorsal horn, BDNF increases hyperalgesia, whereas reduction in BDNF attenuates hyperalgesia in a neuropathic pain model (Groth and Aanonsen, 2002; Pezet et al., 2002b). In Chapter 3 of this thesis, I demonstrated that large-diameter fibers also increase their Gal1 expression in the DRG and dorsal horn after axotomy. In a neuropathic pain rat model, a similar change in Gal1 protein distribution is observed within the dorsal horn (Imbe et al., 2003). At these sensory neuron terminals, Gal1 release can then occur (Sango et al., 2004). Once released, Gal1 can bind to astrocytes causing these glial cells to release BDNF into the extracellular environment, thereby further increasing hyperalgesia (Sasaki et al., 2004). Interestingly, the infusion of Gal1 function blocking antibodies to the intrathecal space around the spinal cord in a neuropathic pain model reduces mechanical allodynia as well as substance P receptor expression (NK-1) in the dorsal horn (Imbe et al., 2003). In Gal -/- mice, I demonstrated reduced thermal nociceptive responses when compared to either Gal1 *wt* or CD-1 mice strains. The increased expression of Gal1 in neurons that have increased activity may contribute to axotomy-induced pain.

Further experiments to determine Gal1's role in neuropathic pain seem warranted. Using established mouse models for neuropathic pain, differences in thermal allodynia and hyperalgesia between Gal1 *wt* and -/- mice should be examined. It would be useful to test whether exogenous Gal1 application in either Gal1 wt or -/- mice increases thermal nociception in either naïve mice or in mice with neuropathic pain. Furthermore, differences in the expression of substance P, NK-1 and BDNF within the dorsal horn in these mice (Gal1 *wt* and -/-) in the uninjured and neuropathic pain model should be investigated.

*Axotomy induced sprouting and plasticity*

In the ECM, there are other molecules that limit axonal plasticity. For example, the ECM molecule that inhibits axonal and neurite outgrowth, CSPG, also inhibits plasticity in the spinal cord and ocular dominance columns (McKeon et al., 1991; Davies et al., 1997; Bradbury et al., 2002; Pizzorusso et al., 2002). When the CSPGs are enzymatically cleaved using chondroitinase ABC, plasticity terminal sprouting increases in the ocular dominance columns (Pizzorusso et al., 2002). After injury to ascending sensory fibers in the dorsal column, chondroitinase ABC increases plasticity and not growth, to increase some sensory

function (Bradbury et al., 2002). Recent *in vitro* evidence suggests that Gal1-Red binds to CSPG, thus preventing its incorporation into the ECM (Moiseeva et al., 2003a). It is interesting to speculate whether this process occurs within the nervous system as well. If this does occur after injury then high Gal1 expression could potentially attenuate CSPG incorporation in the ECM, creating an environment more permissive to growth.

The maintenance and refinement of the hypersensitivity associated with acute and chronic pain is due in part to neuronal plasticity. In particular nociceptive stimuli instigate a variety of intracellular signaling cascades including the ERK 1/2 pathway (Aley et al., 2001; Dai et al., 2002). ERKs have been implicated in neuronal plasticity associated with learning and memory as well as plasticity associated with nociceptor sensitization (Bailey et al., 1997; Martin et al., 1997; Aley et al., 2001). The hyperalgesia associated with ERK1/2 activation depends on cytoskeletal changes (Dina et al., 2003). Accordingly, modifications to cytoskeletal signaling may modify nociceptive neuronal activity and contribute to chronic pain.

After partial peripheral nerve injuries, activation of injury nociceptive pathways leads to central sensitization of intact pathways in turn, leading to long-lasting changes in a sensory neuron's receptive field (expansion or contraction). These changes may also be elicited in the absence of injury by the increased activity of nociceptive afferents (McMahon and Wall, 1984; Cook et al., 1987). This synaptic re-organization requires molecular links to the neuronal cytoskeleton (Dina et al., 2003). Intracellular Gal1 interacts with both signaling and cytoskeletal elements. As previously described (see SIGNALING CASCADES), Gal1 increases ERK activation over the PI3K cascades (Paz et al., 2001; Elad-Sfadia et al., 2002) and Gal1 expression can lead to cytoskeletal re-arrangement (Maekawa et al., 1999). This may occur with Rho GTPases that also have a prominent role in affecting axonal and dendritic structure (reviewed in Hall and Nobes, 2000). Gal1 has been shown to effect F-actin polymerization in astrocytes and has been associated with neurofilaments in the lesions of ALS patients. Furthermore, the involvement of Gal1 in Raf-1/MEK/ERK signaling cascades suggests Gal1's involvement in cytoskeletal re-arrangements and outgrowth. In neuropathic pain, in which synaptic remodeling occurs, function blocking antibodies to Gal1 reduces mechanical hyperalgesia (Imbe et al., 2003). Also, sensory neurons, specifically DRG and olfactory

system as well as spinal motoneurons, are all neuronal systems with activity-dependent induced synaptic plasticity (Wong and Ghosh, 2002). In these systems, we observe inappropriate neuronal termination in Gal1 -/- mice when compared to *wt* mice (Chapter 4)(Puche et al., 1996). It is uncertain whether these changes are a result of inappropriate outgrowth during development or changes in neuronal maintenance in the adult. After a facial nerve injury there is a delay in restoring functional recovery in Gal1 -/- mice compared to Gal1 wt mice. This delay could be due to a reduced initiation rate as suggested by Gal1 function blocking antibodies in the rat (Horie et al., 1999; Fukaya et al., 2003), or by a change in the rate or success of synapse formation at the target. Furthermore, examination of Gal1-Ox-induced growth of axotomized sciatic nerves shows that Gal1 not only increases growth but also the branching of injured neurons (Horie et al., 1999; Fukaya et al., 2003). In Chapter 5, Gal1-Ox application to rhizotomized DRGs neurons did not stimulate robust regeneration into the CNS but promoted a limited growth both in the number and distance of injured neurons (Chapter 3). These Gal1-Ox effects on both the limited CNS growth after rhizotomy and increased branching after peripheral nerve lesion could be attributed to an increase sprouting response. All of this *circumstantial* evidence suggests that endogenously expressed Gal1 is involved in the neuronal maintenance as well as the remodeling of functional connections in pathological conditions. However, more direct evidence clearly is required to substantiate this hypothesis.

Further assessment of the differences between the Gal1 -/- and *wt* mice will provide insight into Gal1's involvement in axonal sprouting and/or growth. In particular, interactions between Gal1 and the cytoskeleton could be examined using cultured cells from Gal1 -/- and *wt* animals. Changes in CSPG incorporation in the ECM, of both Gal1 -/- and *wt* mice should be examined, both before and after injury.

***Other Future directions***

*Galectin-3 and 8*

This thesis focused exclusively on Gal1 but there are other galectins in the CNS that bear further investigation. These are Galectin-3 and 8. As discussed in the Chapter 1 of this thesis, each of these galectins belongs to a separate subfamily based on structural similarities. Of this group, Gal3 is the second most studied galectin due to its early discovery and immune

system involvement. In the CNS, Gal3 immunoreactivity occurs in a subset of DRG small diameter neurons that slightly overlaps with Gal1 immunoreactivity (Regan et al., 1986). Gal3 appears to be expressed and regulated by TrkA expressing sensory neurons since NGF maintains Gal3 expression within DRG *in vitro* and Gal3 expression is observed in laminae I and II (Cameron et al., 1997; Pesheva et al., 2000). *In vitro,* Gal3 also promotes adhesion and growth of DRG and cerebellar neurons (Pesheva et al., 1998a; Mahoney et al., 2000), and after a partial peripheral nerve lesion Gal3 immunoreactivity increases in the dorsal horn (Cameron et al., 1997). Within the CNS, Gal3 expression occurs in cultured microglia (Pesheva et al., 1998b). After a facial nerve axotomy, Gal3 expression appears to increase within the motoneurons but not in microglia around the somata (Walther et al., 2000) suggesting that like Gal1, Gal3 also may be involved in neuronal maintenance and possibly repair. These injury-induced changes of Gal3 protein in motoneurons remains to be properly assessed. Interestingly, the Gal3 -/- and the double null mutant Gal1-/- Gal3-/- mice are viable suggesting that neither of these lectins are essential for development but they would be useful for examining both neuronal injury and neuropathic pain (Colnot et al., 1998).

The recently discovered galectin, Gal8, is also expressed in CNS tissue, but the precise location of such expression is not known (Hadari et al., 1995). Although Gal8 is not in the same family as Gal1, the two share quite a few similarities. Gal8 has four cysteine residues that could possibly alter redox states, as has been observed in Gal1 (Hadari et al., 1995). Furthermore, elevated levels of Gal8 expression also occur in malignant astrocytomas (Colnot et al., 1998). Gal8 affects the ERK 1/2 signal cascade that then promotes cellular motility via F-actin (Levy et al., 2003). These data suggest that like Gal1, Gal8 may also be involved in signaling and outgrowth mechanisms.

Only recently has the significance of Gal1 expression within the nervous system begun to be understood. With evidence now indicating that both Gal3 and 8 also may serve important roles in neuronal development and in neuropathological states, further investigation appears to be warranted.

*Overall Significance*

Although neuronal Galectin (Gal)-1 protein expression has been known for a considerable amount of time, only recently has the role of this protein in both sensory and spinal motor nerve regeneration been investigated. To date, only the developmental and adult expression of Gal1 mRNA and protein has been reported in cranial nerves, primary sensory afferents and spinal motor nerves and that exogenous Gal1ox application increases neuronal regeneration. The studies in this thesis demonstrate four findings. First I show increases in Gal1 mRNA and protein expression in DRGs and spinal motor neurons after injury. Second, this is the first report of Gal1 expression in the red nucleus, and in contrast to injured spinal motor neurons, this expression decreases after injury. Third, this is the first report of a neurotrophic factor (BDNF), effecting Gal1 mRNA expression within any neuronal population. Fourth, the absence of Gal1 in Gal1 -/- mice leads to functional and anatomical nociceptive deficits. Collectively, these findings further demonstrate the importance of Gal1 within the nervous system and as a result pose many more questions with each finding.

*Concluding remarks*

We are only beginning to elucidate galectin neurobiology. The potential involvement of galectin proteins in such wide-ranging conditions such as ALS, neuropathic pain, axonal injury and possibly synaptic plasticity further underscores the wide-ranging role galectins may have within the nervous system. Hopefully, by further understanding galectin neurobiology, better treatment or management of neuronal injury or dysfunction can be achieved.

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