Chapter 2: Sciatic Nerve Vaccination

2.1 *Introduction*

Unlike neurons of the mature peripheral nervous system (PNS), injured neurons in the adult mammalian central nervous system (CNS) usually do not regenerate. This regenerative failure was first described in 1928 by Ramón y Cajal, as he reported that after injury, the ends of lesioned axons distended into 'dystrophic end balls', which he believed were no longer capable of regeneration (Ramón y Cajal, 1928). However, his views were modified several decades later in an important study by David and Aguayo (1981), where the regenerative potential of mature CNS neurons was demonstrated using a peripheral nerve bridge (growth supporting) to allow axons to bypass the CNS terrain. This approach resulted in the successful regeneration of injured CNS axons and the researchers attributed their findings to the growth-promoting properties of the PNS transplant and to the inhibitory properties of the CNS (David and Aguayo, 1981).

Many studies *in vivo* and *in vitro* since then have confirmed that the components of the CNS environment, in particular myelin and myelin-associated proteins, inhibit axonal regeneration of adult CNS neurons (Bahr and Przyrembel, 1995; Caroni and Schwab, 1988b; Crutcher, 1989; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Schnell and Schwab, 1990). After the identification of the inhibitory properties of myelin and its associated molecules, what appeared to be 'dystrophic end balls' to Ramon y Cajal, were actually signs of axonal collapsed and retraction.

Ramon y Cajal's study, although perceived negatively by the scientific community due to the implication that injured CNS neurons do not regenerate, it only told half of the story. The remaining half of the story was discovered with the finding that injured CNS neurons had the capacity to regenerate but as aforementioned, these were merely inhibited by their immediate, degenerating CNS environment.

Important exceptions to this lack of regeneration by mature CNS neurons have been demonstrated in two distinct *in vivo* models, where axonal regeneration can occur through the CNS environment. The first was described in cat spinal cords, whereby injury as neonates results in CNS regeneration, however, if injury occurs as adults then spinal cord regeneration does not take effect, proposing the notion of CNS plasticity (Bregman and Goldberger, 1982). The second was observed by the regeneration of the CNS branch of adult dorsal root ganglion (DRG) neurons after a pre-conditioned, peripheral nerve lesion (Richardson and Issa, 1984). It should be noted that in the latter paradigm, if only the CNS branch is lesioned, axonal regeneration is halted, which consistent with other studies investigating injury to adult CNS neurons. These two examples discussed here provide fundamental evidence for the intrinsic capacity of injured neurons to regenerate through the adult CNS terrain.

Intriguingly, in the case of young neurons regeneration is possible (Bregman and Goldberger, 1982) and this might be due to developmental changes that modify the response of young neurons to the changing

environment. For example, it has been postulated that differences between CNS and PNS neuronal regeneration lies in their response to myelin and its associated inhibitors, a factor that might be related to the timely neuronal expression of receptors for inhibitory molecules (Shewan et al., 1995). In fact, this developmental regenerative ability has been correlated to elevated levels of cyclic AMP (cAMP) in younger neurons, given that when these cAMP levels are increased in adult neurons extension of neurites occurs (Cai et al., 2001). It is reasonable to state that indeed myelin results in the inhibition of adult but not younger neurons, however, adult neurons still possess the intrinsic ability to regenerate under the appropriate conditions.

For instance, in adult DRG neurons, CNS regeneration can be significantly improved by a pre-conditioning peripheral nerve lesion, which has been shown to be beneficial for axonal growth of afferent CNS fibres (Richardson and Issa, 1984). Currently, the exact mechanism mediating this regenerative response remains largely unknown, providing only limited regeneration of some CNS fibres. In addition, from a clinical perspective the lesioning of peripheral nerve branch as a means to stimulate the regeneration of CNS fibres in humans is obviously impractical. Nonetheless, this model of injury represent an interesting mechanistic component underlying CNS regeneration, which warrants more attention and will form the basis of this thesis.

Certainly, recent studies in an effort to promote regeneration by adult CNS

neurons have taken direct approaches against the inhibitory molecules of the CNS. These have focused on the elimination of myelin inhibitory signals by degrading inhibitory molecules found at the glial scar (Bradbury et al., 2002; Domeniconi et al., 2002; Wang et al., 2002) or by deletion of the associated inhibitory molecules using knockout animals specific for these molecules such as in Nogo-deficient mice (Simonen et al., 2003; Zheng et al., 2003). These studies date back to 1988 with earlier attempts at CNS regeneration using a monoclonal antibody (IN-1) to neutralise the effects of myelin (Caroni and Schwab, 1988b). The *in vivo* use of the IN-1 antibody after intracerebral administration in complete transected cut spinal cords, resulted in the robust sprouting and fibre elongation at the lesion site, thus confirming the capacity for CNS neurons to regenerate after neutralisation of myelin inhibitors (Schnell and Schwab, 1990). This approach although successful in providing evidence for the regenerative properties of adult CNS neurons, reported a small number of axonal regeneration. It is clear that to achieve a greater amount of regeneration in the CNS, a better neutralization of CNS inhibitor would be needed. To circumvent this, Huang and colleagues (1999) attempted to simultaneously block all inhibitory CNS factors by immunising animals with a myelin rich-spinal cord homogenate (SCH), resulting in a robust regeneration of corticospinal tract (CST) axons (Huang et al., 1999).

This prominent study by Huang and colleagues (1999), introduced the importance of the immune system in axonal CNS regeneration and conceptualised the possibility that an immunotherapy approach to

modulate the CNS environment might be a possible avenue for CNS repair. In this study it was shown that: (A) SCH immunised animals produced antibodies with higher levels of IgG and IgM, which were able to cross the blood brain barrier (BBB), bind to inhibitors associated with myelin and consequently stimulate axon regeneration over long distances; and (B) serum derived from SCH immunised animals was able to sustain neurite growth on myelin substrates by blocking its inhibitory effects (Huang et al., 1999). The idea that antibodies blocked myelin-derived growth inhibition was further investigated by Ellezam and colleagues in 2003, whereby the possibility that SCH vaccination of adult rats could promote regeneration of retinal ganglion cells (RGCs) was evaluated. Their findings indicated that axonal regeneration in SCH vaccinated animals significantly improved as a result of the SCH treatment (Ellezam et al., 2003). In addition, these authors suggested that the beneficial effects on regeneration were not mediated by antibodies against growth-inhibitory proteins but instead were directed against neuronal epitopes, such as receptors for growth-inhibitory molecules (Ellezam et al., 2003). In both studies, it was concluded that further investigation was needed to understand the pro-regenerative properties of CNS homogenate vaccinations.

Thus far, here we have briefly provided evidence supporting: A) the inhibitory properties of the CNS environment; B) the use of peripheral nerve grafts to stimulate regeneration of CNS neurons; C) the use of a pre-conditioning lesion of adult DRG neurons as a model to investigate

CNS regeneration; D) the use of the animal's own immune system as a tool for simultaneously blocking the inhibitory molecules of the CNS; and E) the use of CNS homogenate vaccination to promote CNS regeneration.

2.1.1 Project Rationale

In vivo immunisation with SCH results in CNS regeneration of some but not all CST fibres in adult neurons (Huang et al., 1999). Hypothetically, the limited amount of CNS axonal regeneration could have been due to the presence of inhibitory molecules in the homogenised CNS tissue used, however, this was not tested by the respective authors (Ellezam et al., 2003; Huang et al., 1999). Furthermore, immunisations with CNS constituents have also been associated with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and could potentially create adverse immunological effects (Kim et al., 1998).

Conversely, PNS axons have a remarkable ability for regeneration (Guth, 1956), have long been used as grafts for CNS neurons to grow (David and Aguayo, 1981; Richardson et al., 1980), have been demonstrated to be useful in the pre-conditioning of DRG neurons with reported improvements in CNS regeneration (Lu and Richardson, 1991; Richardson and Issa, 1984), and have even been administered minced into the spinal lesion to further encourage CNS regeneration (Feng et al., 2008). At present, there are no reports describing *in vivo* vaccinations using peripheral nerve homogenates (PNH) to target CNS regeneration.

2.1.2 Proposed Testing

Based on the aforementioned factors, here we propose that if CNS constituents from SCH can lead to axonal regeneration of some but not all nerves, then it is possible that vaccination with a PNH (i.e. sciatic nerve), characteristic for its regenerative ability, could trigger an immune response with an enhanced CNS regeneration.

In the pre-conditioned model, axotomy of the peripheral DRG branch leads to regeneration of the CNS branch (Richardson and Issa, 1984). This provided a clear indication that the peripheral nerve injury is crucial for this type of CNS regeneration, given that CNS injury alone does not result in regeneration of mature neurons. Consequently, using the pre-conditioned lesion as a successful mechanistic model to investigate the regenerative properties of adult CNS neurons, vaccination of spinal cord injured rats with a PNH could result in a more robust regeneration of the injured CNS branch. Furthermore, as suggested by previous studies (Ellezam et al., 2003; Huang et al., 1999), to test whether immune system differences affect this type of regeneration, vaccinations will be conducted in animals with varying immunological repertoires depending on the maturity of the organism, such as adults (i.e. mature system) versus neonates (i.e. immature system) (Waddington et al., 2004). In addition, to explore the possibility that serum specific antibodies such as IgGs contribute to CNS regeneration, we will test this by examining the effects of PNH immune exposure through maternal vaccination, since maternal IgG antibodies can

enter foetal circulation through the placenta and be passively transferred to the offspring (Zinkernagel, 2001).

2.1.3 Hypothesis

Given that peripheral nerves are capable of regeneration, then *in vivo* vaccination with a PNS constituent could potentially enhance CNS regeneration of pre-conditioned lesion adult neurons. In addition, to determine the extent of the immune system involvement in neuronal CNS regeneration, immunisation at different developmental stages will be assessed.

2.1.4 Model of Injury

Consistent with original model described by Richardson and Issa (1984), the peripheral DRG branch will be injured (day 0) and this will be followed by injury to the DRG branch extending in the CNS (day 7). The timing of the CNS injury relates to the optimal conditioning of DRG neurons, which occurs at seven days post peripheral nerve axotomy, resulting in the maximum amount of CNS regeneration possible (Richardson and Issa, 1984) (Fig. 3).

2.2 Method and Materials

2.2.1 Animals

Female Sprague Dawley (SD) rats were used in this study, under the guidelines of the National Health and Medical Research Council of

Australia and approved by the Animal Welfare Committee of Flinders University of South Australia.

This study was divided into two major parts: A) a time course study to investigate the mechanisms underlying the pre-conditioned peripheral lesion (section 2.2.1.1); and B) a vaccination study to examine the effects of PNH treatment administered at different developmental stages (section 2.2.1.2). These two groups were treated separately and their results were not interchanged between studies. Note that time course study animals did not receive any type of vaccination and were only surgically operated as specified below.

2.2.1.1 Time Course Study (Part One)

This part of the study was designed to investigate acute changes following peripheral and/or CNS injury. Animals were randomly assigned into three subgroups. Those that received a single injury and were perfused 7 days later: I) sciatic nerve injury alone (SNI, n=5), II) spinal cord injury alone (SCI, n=5) or III) those that received a combination of the aforementioned surgeries (SNI + SCI) and were perfused at 7 days post CNS injury (7D, n=5). Normal uninjured animals (n=5) were used as controls where appropriate.

2.2.1.2 Treatment Groups (Part Two)

Animals were randomly allocated into three treatment subgroups depending on the timing of their exposure to the PNH: A) neonatal, B) adults, and C) maternal (see below). In addition, regardless of their grouping, all animals received a pre-conditioned lesion as an adult, as described per injury model. Note that these animals were only vaccinated twice, on separate occasions as specified in each treatment subgroup.

2.2.1.2.1 Neonatal-Treated Group

These animals were immunised as neonates on two separate occasions. Once on day 1 and once on day 7 post partum with either PNH (Test, n=5) or sterile saline (Control, n=5) emulsified in Freund's adjuvant (refer to Table 3 for immunisation details).

Note that no further treatment was given to these animals after days 1 and 7. As adults (week 8 post partum), animals received a pre-conditioned lesion and were perfused 50 days post CNS lesion (refer to section 2.2.4 for perfusion details).

2.2.1.2.2 Adult-Treated Group

These animals were immunised as adults on two separate occasions, once on day 56 and once 7 days later on day 63, with either PNH (Test, n=5) or sterile saline (Control, n=5) emulsified in adjuvant (refer to Table

3). These animals were then received a pre-conditioned lesion and were perfused 50 days post CNS lesion.

2.2.1.2.3 Maternal-Treated Group

Adult female rats were immunised on two separate occasions (as per previous adult-treated group). Once on day 56 and once on day 63 with either PNH (Test, n=3) or sterile saline (Control, n=3) emulsified in adjuvant (refer to Table 3). Subsequently, these adult animals were mated and their respective neonates were collected and assigned into two separate subgroups (i.e. Test or Control) depending on whether they were born to a parent treated with PNH (Test, n=5) or saline (Control, n=5).

Note that these offspring did not receive any further treatment post partum or during development. As adults, these animals received a pre-conditioned lesion as per previous groups and were perfused 50 days post CNS lesion. These animals formed the passively vaccinated treatment group.

2.2.2 PNH Preparation and Animal Vaccination

To prepare the PNH, a freshly dissected sciatic nerve from a donor normal uninjured SD rat was used. Immediately following dissection, the sciatic nerve was snapped frozen in liquid nitrogen (to preserved its integrity), and transferred to a pre-cooled pestle and mortar bowl where it was crushed using small quantities of liquid nitrogen. The collected crushed nerve was

resuspended in 3ml of sterile saline water and centrifuged at 700g for 15 minutes at 4^0 Celsius. The supernatant was then carefully removed (without disturbing the pellet), freeze-thawed 3 times by alternate immersions in liquid nitrogen and in a warm water bath and centrifuged as previous. The remaining supernatant was used for subcutaneous injections with the respective adjuvant used in a 1:1 dilution (Table 3).

2.2.2.1 Protein Concentration

The total protein concentration of our PNH sample was calculated using a commercially available bicinchoninic acid (BCA) protein assay kit (Pierce, USA). This kit allowed a colorimetric detection and quantification of the total protein content at an optical density of 562 nm, based on the standard protein colour response curve (for protein kit instructions refer to www.piercenet.com). The total protein concentration of our PNH sample was adjusted to 1.00mg/ml.

Table 3. Vaccination Protocol for All Three Experimental Groups

Group	**Time of Vaccination**		**Type of Immunisation**		**Total Dosage/ Injection (s.c.)**	
Neonatal (Test/Control)	Day 1	Day 7	PNH or SS + CFA	PNH or SS + IFA	200 μl	200 μl
Adult (Test/Control)	Day 56	Day 63	PNH or SS + CFA	PNH or SS + IFA	200 μl	200 μl

Maternal (Test/Control)	Day 56	Day 63	PNH or SS + CFA	PNH or SS + IFA	200 μl	200 μl

Abbreviations: PNH=peripheral nerve homogenate; SS=sterile saline; CFA=complete Freund's adjuvant; IFA=incomplete Freund's adjuvant; S.C.=subcutaneous

2.2.3 Surgical Procedures

All surgical procedures were performed under aseptic conditions using an operating microscope, during which animals were anaesthetised by halothane inhalation through a mouthpiece. The pinch-reflex test was used to determine effectiveness of the anaesthetic prior to surgery, until there was no response from the animal after pinching their plantar foot surface. Note that all procedures were carried out in sterile conditions and animals were returned to their cages until anaesthesia effects had already subsided. These conditions were also applicable to experiments conducted in chapters 3 and 4 of this thesis.

2.2.3.1 Sciatic Nerve Injury (SNI)

Briefly, a primary longitudinal cut was made on the skin overlaying the femur of the left hind limb. The incision was extended proximally and distally exposing the thigh muscle. Sharp surgical scissors were inserted and opened into the muscle through the first layer to the level at which the sciatic nerve runs. After locating the sciatic nerve, the surrounding

connective tissue was severed to free the nerve and allowing easy access to it. The branching of the sciatic nerve was the area of interest, where it was ligated just proximal to its trifurcation and cut below the ligation site with fine surgical scissors. The wound was sutured closed using a 6/0 surgical silk suture and animals were placed into individual cages.

2.2.3.2 Spinal Cord Injury (SCI)

Briefly, spinal cords were exposed by a laminectomy between vertebrae thoracic 9-10 followed by a small incision in the dura mater, thus exposing the dorsal side of the spinal cord. Injury to the spinal cord (i.e. dorsal column cut) was performed by positioning the tips of open-blade, fine microsurgical scissors on either side of the exposed spinal cord and subsequently closing them for about 10 seconds. This was carried out at a depth of approximately 1.5-2mm (marked by a black line on the scissors' tip) and resulted in a bilateral cut through the dorsal columns (Qiu et al., 2005). A small piece of gelfoam was temporarily placed over the lesion site to encourage blood clotting and the overlaying muscles were sewn together with a 6/0 surgical silk suture. The skin was stapled and the animals were returned to their cages.

Note that after each surgery, animals were housed separately and received subcutaneous injections of the analgesic drug buprenorphine (0.03 mg/kg) for a period of up to 5 days to alleviate postoperative pain. For all spinal cord injured animals manual bladder expression was performed two to three times per day and the animals were treated with

antibiotics if required (Brambilla et al., 2005). It is important to mention that the type of SCI procedure used here is restricted to the dorsalmost aspect of the spinal cord (corticospinal tract specific, Fig. 3).

2.2.3.2.1 Retrograde Tracer Injection

To investigate the presence of ascending regenerated fibres in the injured dorsal column of the spinal cord (i.e. across the injury epicentre), a somatic retrograde tracer known as fast blue (FB, 5% in saline, Sigma) was injected into the proximal stump of the spinal cord. Ideally, ascending afferent fibres originating from the distal stump, if regenerated, elongate across the SCI site, take up the FB tracer from the opposite proximal stump, retrogradely transport it and consequently label their cell bodies back to their respective DRG (Ahmed et al., 2001; Hu and McLachlan, 2002; Song et al., 2008).

2.2.3.2.2 Anterograde Tracer Injection

To anterograde label ascending fibres, a tracer known as Fluororuby dextran tetramethylrhodamine (FR, 10% in distilled water, Molecular Probes) was used due to its fast, reliable, and histochemically simple properties facilitated by its immunofluorescent tag (Fritzsch and Sonntag, 1991; Schmued et al., 1990). Briefly, using a stereotaxic frame and a pulled glass micropipette needle, FR was directly injected into the dorsal column in the lumbar region of the spinal cord (total volume delivered 0.1μl), ipsilateral to the SNI at a depth of 1-1.2mm (Song et al., 2008). As previously mentioned, the possibility of tracer leakage was minimised by

placing sterile cotton balls around the injection site. This type of tracing facilitated labelling of ascending afferent fibres within the dorsal column of the spinal cord (Lu et al., 2001).

2.2.4 Perfusion

A primary incision was made along the floating ribs above the diaphragm, made possible by the removal of the skin on the ventral side of the animal. The thoracic cavity was opened by an incision along either side of the rib cage until reaching the clavicle, followed by the piercing of the diaphragm. Once the rib cage was completely pulled back and the heart was exposed, two solutions were individually delivered via an injection to the left ventricle of the heart.

All animals were injected i.p. with 5% chloral hydrate in distilled water and perfused transcardially with 1% sodium nitrite ($NaNO_2$) in phosphate buffer (PB, 0.1 M, pH 7.4) followed by a 4% paraformaldehyde (PFA) fixative flush in PB (0.1 M, pH 7.4). Perfusions were performed as per specified time points (refer to section 2.2.1), with all dissected tissues post-fixed in 4% PFA and cryoprotected in 30% sucrose solution in PB (0.1 M, pH 7.4), each for 48 hours at 4^0 Celsius.

2.2.5 Cryosectioning

All dissected tissues were post-fixed and cryoprotected accordingly prior to cryosectioning (refer to section 2.2.4). Cryosectioning was performed on a cryostat microtome at -20^0 Celsius and due to the morphological variation

of dissected tissues, these were cryosectioned at different thicknesses. DRGs were cut at 20μm (coronal sections) and spinal cords were cut at 40μm (longitudinal sections). All specimens were mounted on 2% gelatine-coated glass slides.

2.2.6 Immunohistochemistry (IHC)

All IHC procedures were performed as per specified protocols using single or double labelling immunoreactivity where appropriate (Li et al., 2002; Song et al., 2008).

2.2.6.1 Fluorescence Staining

For IHC, DRG and spinal cord sections were washed in 0.5% hydrogen peroxide (H_2O_2) in 50% ethanol at room temperature for 30 minutes to quench endogenous peroxide activity, washed thoroughly in phosphate buffered saline (PBS, pH 7.4), followed by three rinses in phosphate buffered saline containing 1% Tween detergent (PBST). These sections were blocked in 20% normal horse serum (NHS) for 2 hours before incubated with primary antibodies at 4^0 Celsius for 48 hours: mouse-anti-rat cluster differentiation 68 (CD68, macrophage, 1:400, Serotec), rabbit-anti-glial fibrillary acidic protein (GFAP, astrocyte/satellite cells, 1:500, Dako). Combinations of the following secondary antibodies for single and/or double labelling were purchased from Jackson ImmunoResearch: sheep-anti-mouse-cy3-IgG (1:500), donkey-anti-mouse-cy2-IgG (1:500), donkey-anti-rabbit-488-IgG (1:500), sheep-anti-rabbit-cy3-IgG (1:500). The

specificity of the observed IHC procedure was validated by omitting the primary antibody or by using a non-immune serum instead of the primary antibody (Song et al., 2008).

2.2.7 Neuronal Basket Quantification in DRGs

The relative number of perineuronal baskets formed by $GFAP^+$ and $CD68^+$ cells was calculated by serially counting every fifth section of the ipsilateral and contralateral L4 DRG (total of 4 sections/animal, n=4). This allowed for the relative estimation of the number of $GFAP^+$ and $CD68^+$ rings formed around neuronal DRG cell bodies. The obtained value was then averaged and divided by the total number of neuronal DRG cell bodies with visible nuclei (Zhou et al., 1991). Note that immunoreactive $GFAP^+$ or $CD68^+$ cells in close proximity to but not enclosing DRG cell bodies were not counted.

2.2.8 Relative DRG Somatic Count

The relative number of retrograde labelled FB^+ DRG neurons was obtained by serially counting every fourth section of the ipsilateral and contralateral L4 DRG (total of 5 sections/animal, n=5). This method of counting allowed for the relative estimation of FB labelled cell bodies and to avoid the possibility of double counting, only those neurons where the nucleus was visible were counted (Li et al., 2002; Zhou et al., 1991). Note that in all instances, FB^+ neurons were considered regenerated neurons (Song et al., 2008).

2.2.9 Cell Quantification Method

Due to the complexity in identifying individual populations of macrophage cells ($CD68^+$) and astrocytes ($GFAP^+$) present in the spinal cord after injury, we employed a quantification technique, which determined the percentage area fraction of the section occupied by these stained structures (Hu et al., 2007; Piantino et al., 2006; Popovich et al., 1999). Briefly, using ImageJ (image processing program, NIH version 1.37) 20x magnification images stained for the aforementioned antibodies were converted to binary contrast images (black and white). This provided a threshold by subtracting background levels from the immunoreactive stained areas and allowed the determination of the percentage area fraction per image to be collected, tabulated and statistically analysed (Gray et al., 2007; Hu et al., 2007; Maruyama et al., 2005; van Wijngaarden et al., 2007).

The areas of interest quantified in this thesis varied between the different cell markers used due to the spread of damage, for instance at the SCI epicentre there was a predominant macrophage presence ($CD68^+$) with minimal astrocyte ($GFAP^+$) immunoreactivity, whereas, astrocyte presence was found around the periphery of the SCI epicentre where macrophage presence was reduced (Appendix A Fig. A1). For these conditions, we chose to examine macrophage presence 1-2mm rostral, 1-2mm distal and the injury epicentre, and in contrast for astrocytes we examined areas 3mm rostral and 3mm distal from the SCI epicentre. This provided us with a more logical approach to study these cell populations.

2.2.9.1 Statistics

In all graphs, columns represent an averaged mean (n=5 as specified per figure) and error bars indicate standard error of mean (+/- S.E.). Comparisons between groups were made by ANOVA and/or independent samples t-test where appropriate. Results were considered significant if $P<0.05$. Note that no statistical comparisons were preformed between different regions (i.e. rostral, caudal or epicentre), among and/or between any of the groups.

2.2.10 Sandwich BDNF ELISA Test

Brain derived neurotrophic factor (BDNF) expression is known for its favourable action on regeneration (Kipnis et al., 2000; Mamounas et al., 2000; Plunet et al., 2002), its neuroprotective effects (Carson, 2002; Donelly and Popovich, 2008) and its secretion from macrophage cells (Bouhy et al., 2006; Moalem et al., 2000; Schulte-Herbruggen et al., 2005) after peripheral and/or CNS injury. Therefore, here we examined BDNF concentrations in relation to the timing of the SNI and/or SCI, in an effort to understand the beneficial synergistic effect behind the pre-conditioning peripheral lesion.

At the end of the experimental period serum samples were collected from animals in the time course study (Part I). These samples were tested using a commercially available ELISA kit pre-coated with BDNF (Millipore, USA,

www.millipore.com). Which provided an estimate of the BDNF serum concentration level that was measured and analysed in all animals.

Briefly, our serum samples were incubated overnight at 2-4^{0}Celsius in BDNF ELISA plates pre-coated with rabbit anti-human BDNF polyclonal antibody, reactive to both human and rat samples. After incubation, plates were thoroughly washed and incubated at RT for 2-3 hours with a biotinylated mouse anti-human BDNF monoclonal antibody (1:1000). Plates were then washed and incubated at RT for 1 hour with a streptavidin-HRP conjugate (1:1000), washed again, developed with a TMB/E substrate at RT for 15 minutes and then stopped with a HCl solution. The optical density (OD) for each plate was measured at 450nm and plotted on a standard curve (see Appendix A Fig. A2).

2.3 Results

2.3.1 Part One: Mechanisms of Action

2.3.1.1 DRG Inflammatory Response Post SNI

To develop an understanding underlying the beneficial regenerative CNS response ascribed to the pre-conditioned lesion model, we examined its peripheral and CNS effects according to the optimal timing determined by Richardson and Issa (1984), at 7 days post SNI.

As previously described in other studies examining the inflammatory response post SNI (Hu and McLachlan, 2002, 2003a, 2004), we confirmed

their findings and report that peripheral axotomy of the sciatic nerve resulted in an increased formation of $GFAP^+$ baskets around DRG cell bodies (Fig. 4-5). These $GFAP^+$ baskets formed by satellite glial cells, were clearly visible in both ipsilateral and contralateral DRG, but were upregulated in numbers ipsilateral to the peripheral injury (Fig. 4-5A, 4-5C-D, 4-5F-G). Similarly, SNI also resulted in an increased infiltration of macrophage cells ($CD68^+$) into the ipsilateral DRG, as compared to the contralateral DRG. Interestingly, high magnification images revealed macrophage cells enclosing DRG cell bodies, both ipsilateral and contralateral to SNI (Fig. 4-5B-C, 4-5E-F, 4-5H-I). This infiltration of macrophage cells in both ipsilateral and contralateral DRG is consistent with previous observations after unilateral injuries (Dubovy et al., 2007). It is clear that infiltration of macrophage cells and upregulation of GFAP by satellite glia cells is representative of the severity of the mechanical insult and these changes not only extended ipsilateral but also contralateral to injury, although no other types of sciatic nerve injuries were investigated in this study.

As expected due to the proximity of the nerve injury, quantification of macrophage cells surrounding DRG cell bodies was significantly higher in the ipsilateral DRG as compared to the contralateral DRG (Fig. 6A-B, $P<0.0001$). Similarly, although to a lesser extent, quantified $GFAP^+$ forming baskets around DRG cell bodies were also significantly higher in the ipsilateral DRG, as compared to contralaterally (Fig. 6A-B, $P<0.01$). In the DRG, the functional response of these cells to injury and consequent

formation of cellular baskets enclosing DRG cell bodies is not completely understood. Although, previous studies have suggested this response might serve a neuronal sprouting role before degeneration begins (McLachlan et al., 1993). Note that in the normal uninjured DRG, some $GFAP^+$ and $CD68^+$ cells were present in low numbers within the DRG compartment, however, under normal conditions these cells did not form baskets around neuronal cell bodies, as these were only seen after SNI and were therefore not counted (Fig. 6).

2.3.1.2 BDNF ELISA Serum Levels

To further our understanding into the mechanism of action following mature CNS neuronal regeneration in pre-conditioned lesion animals, we measured the neurotrophic serum levels of BDNF, since its expression is known for its beneficial action on regeneration and neuroprotection (refer to section 2.2.10). Our results indicated that the greatest level of BDNF serum concentration was observed 7 days post SNI, as compared to normal levels (Fig. 7, $P<0.001$) and SCI 7D alone ($P<0.001$). In the pre-conditioned lesion model, this elevated trophic level following SNI alone, coincided with the timing of the CNS spinal injury but was not observed after SCI 7D alone. In fact, SCI alone did not significantly change BDNF concentration 7 days post CNS injury, as compared to normal trophic levels (Fig. 7).

2.3.1.3 Early Macrophage and Astrocyte Expression

To further investigate the mechanism of action in the pre-conditioned lesion, we examined the acute inflammatory response in the spinal cord 7 days post CNS injury, using animals that received a SCI only as controls. Overall, our results indicated that, 7 days post CNS lesion the number of macrophage cells ($CD68^+$) in pre-conditioned lesion animals was significantly higher, as compared to macrophage numbers in the 7 day SCI only group (Fig. 8B, $P<0.05$). Specifically, this difference was observed rostrally and at the epicentre (Fig. 8A, $P<0.05$), while no significant differences were observed caudally. Interestingly, after SNI alone, macrophage cells ($CD68^+$) were also consistently observed inside the CNS compartment extending throughout the length of the spinal cord (data not shown); a cellular change that under non-pathological conditions is not normally seen in the uninjured spinal cord (Zhang et al., 2007).

Conversely, astrocyte expression ($GFAP^+$) 7 days post CNS injury between pre-conditioned lesion and SCI only animals, did not yield any significant differences rostral or caudal to the SCI epicentre with both groups displaying similar $GFAP^+$ expression levels (Fig. 9B). However, it is important to note that astrocyte expression in the lumbar region between normal uninjured animals and SNI only animals resulted in a significant difference, with a higher astrocyte expression observed after SNI only (Fig. 9A, $P<0.0001$).

It is then clear that in the pre-conditioned lesion, the timing of the SCI 7 days post SNI (Richardson and Issa, 1984), occurs when the CNS environment has already been modified by an increased macrophage infiltration and astrocyte expression, which differ from what is normally observed in the CNS compartment.

2.3.2 Part Two: Effects of PNH Vaccination

2.3.2.1 Retrograde Tracing in the DRG (FB)

PNH vaccination resulted in some interesting findings depending on the timing of treatment administration among all treated groups. Qualitatively, we observed differences in FB^+ labelled DRG neurons between PNH-treated and controls animals, with a higher number of FB^+ neurons in the ipsilateral DRG of adult and neonatal PNH-treated groups (Fig. 10-11, respectively). Quantitatively, PNH vaccination in adults revealed a significantly greater number of regenerated FB^+ DRG neurons, as compared to saline-treated control animals (Fig. 10, $P<0.01$). Similarly, PNH vaccination in neonates also resulted in significantly more regenerated FB^+ DRG neurons, as compared to saline-treated animals (Fig. 11, $P<0.01$).

Unexpectedly, however qualitative observations of FB^+ DRG neurons between animals passively exposed to PNH or saline through maternal vaccination, revealed a higher number of regenerated FB^+ DRG neurons in the latter control group, as compared to passively PNH-treated animals.

Indeed, PNH exposure through maternal vaccination, resulted in a significant reduction of FB^+ DRG neurons in offspring passively exposed to PNH, as compared to control animals passively exposed to saline (Fig.12, $P<0.0001$). This difference in FB labelling was attributed to a suppressed regenerative response in animals passively exposed to PNH. Note that for all experimental groups, low numbers of FB^+ neurons were also observed in the contralateral DRG, although to a lesser extent as originally described by Richardson and Issa (1984; data not shown). Comparatively, the contralateral DRG was used as a control to determine the extent of neuronal regeneration in the ipsilateral DRG of each group.

2.3.2.2 Anterograde Tracing of Ascending Spinal Cord Fibres (FR)

Qualitatively, here we demonstrated the presence of regenerated ascending FR^+ neurons in the injured spinal cord of an adult PNH-treated animal. This was possible after a direct FR tracer injection into the dorsal column of the spinal cord. These ascending nerves were not quantitatively assessed given that these have been extensively described in earlier studies (Lu and Richardson, 1991; Neumann and Woolf, 1999; Richardson and Issa, 1984; Richardson and Verge, 1987; Song et al., 2008), and here we consistently demonstrate the presence of these regenerated fibres extending only a few millimetres from the SCI epicentre in a rostral direction (Fig. 13).

2.3.2.3 Macrophage and Astrocyte Quantification

In animals PNH vaccinated as adults, macrophage quantification revealed an increased macrophage presence ($CD68^+$), as compared to saline-treated control animals. This difference was observed throughout the examined area, rostrally, caudally and in the SCI epicentre (Fig. 14A, $P<0.05$, $P<0.001$, $P<0.01$, respectively). In addition, a similar pattern was observed in neonatal PNH-treated animals with greater macrophage presence in all aforementioned regions, as compared to control animals (Fig. 15A, $P<0.001$, $P<0.05$, $P<0.05$, respectively). Interestingly however, macrophage presence in animals passively exposed to PNH was significantly reduced, as compared to passively exposed saline-treated animals. This difference was specifically observed caudal to SCI (Fig. 16A, $P<0.05$), with no other differences noted between groups rostrally or at the SCI epicentre. It should be mentioned that in all instances macrophage presence was higher caudal to the lesion, as opposed to rostral. This variation in macrophage numbers most likely reflects Wallerian degeneration effects caused by the mechanical CNS injury (Vargas and Barres, 2007).

Astrocyte expression ($GFAP^+$) in both adult and/or neonatal PNH vaccinated animals did not differ from their respective control group, rostral or caudal to the SCI epicentre (Fig. 14-15B). Similarly, there were no differences observed rostrally in animals passively exposed to either PNH or saline (Fig. 16B). Yet caudally, there was a significantly greater astrocyte upregulation in animals passively exposed to PNH, as compared

to control animals passively exposed to saline (Fig. 16B, $P<0.05$). Hypothetically, this increase in astrocyte upregulation could have been a contributing factor to the reduced number of regenerated FB^+ neurons found in the ipsilateral DRG of PNH-passively treated animals, since astrocyte reactivity is known to represent the magnitude of glial scarring formed after SCIs (Faulkner et al., 2004; Sofroniew et al., 2001). It should also be mentioned that for all vaccinated groups including controls, astrocyte expression was always qualitatively higher in the caudal segment. Again, although not examined further, this difference could possibly reflect Wallerian degeneration effects.

2.4 Limitations

In earlier attempts to stimulate CNS regeneration of mature neurons, researchers used CNS tissue homogenates emulsified in incomplete Freund adjuvant as part of their vaccination scheme (Ellezam et al., 2003; Huang et al., 1999). This adjuvant, as opposed to the CFA used here, does not contain mycobacteria, a product known for inducing inflammatory responses. However, mycobacteria has also been mechanistically proposed as an important component in the beneficial effects observed on MS (Shahabi et al., 2006). In any case, we did not observe any type of abnormalities throughout the course of the experiments and more importantly, the animal strain used here (Sprague Dawley) have previously been demonstrated to be one of the more resistant strains to inflammation (Levine and Wenk, 1961; Luo et al., 2007).

In addition, treatment injections with our PNS constituents emulsified in mycobacteria-containing adjuvant not only allowed a better animal immune response to the treatment, as compared to the one without mycobacteria (Stills, 2005) but also reduced the need for repeated treatment injections as conducted in earlier studies (Ellezam et al., 2003; Huang et al., 1999).

2.5 Discussion

2.5.1 Part One: Mechanisms of Action

2.5.1.1 DRG Changes

In the pre-conditioned lesion, mature injured CNS neurons are able to regenerate (Richardson and Issa, 1984). This regenerative ability might be, partly mediated by the inflammatory response in the ipsilateral DRG following SNI (Lu and Richardson, 1991). Consistent with previous studies investigating various types of peripheral nerve injuries (Dubovy et al., 2007; Hu and McLachlan, 2002; Li and Zhou, 2001; Lu and Richardson, 1991), our results similarly revealed the strong presence of inflammatory cells, namely macrophages ($CD68^+$), as well as an increased upregulation of satellite glial cells ($GFAP^+$), in the ipsilateral DRG following peripheral nerve axotomy. Pathologically, after PNS injury, these cells form perineuronal baskets or rings enclosing DRG cell bodies (Dubovy et al., 2007; Hu and McLachlan, 2003a). Which might be mediated by the release of neurotrophins (Zhou et al., 1996), cytokines from the injured neuron (Ramer et al., 1999), and/or from macrophage cells (Mueller et al., 2001). The functionality of these perineuronal rings is not completely

understood, although previous studies suggest this might be an attempt at neuronal sprouting before degeneration begins (McLachlan et al., 1993). In a later study, it was demonstrated that BDNF synthesized and secreted from DRGs was involved in the sprouting of DRG neurons after peripheral nerve injury (Deng et al., 2000).

In parallel to the timely formation of perineuronal rings, BDNF serum levels were also examined. Our results indicated that BDNF levels were greatest at 7 days post SNI, as compared to BDNF levels in normal uninjured animals. Interestingly, CNS injury alone did not significantly elevate BDNF serum concentration above normal levels. It is important to note that immediately prior to SCI, 7 days post SNI, resulted in the greatest amount of BDNF concentration in the serum. This is the period responsible for the optimum conditioning of DRG neurons, triggering the greatest amount of CNS regeneration possible (Richardson and Issa, 1984). In addition, as aforementioned, neurotrophins such as BDNF, have been reported to have a beneficial role in regeneration (Kipnis et al., 2000; Mamounas et al., 2000; Plunet et al., 2002) and neuroprotection (Carson, 2002).

2.5.1.2 Spinal Cord Changes

Here, we compared the cellular expression of macrophages ($CD68^+$) and astrocytes ($GFAP^+$) 7 days post CNS injury in the spinal cord of pre-conditioned lesion animals (SNI + SCI) and those that received only a SCI (i.e. no conditioning lesion). Our results indicated that pre-conditioned lesion animals had a greater number of macrophage cells present 7 days

post SCI, as compared to SCI only animals. Specifically, this difference was observed at the SCI epicentre and rostral to the injury, while no differences were found caudally. Our pooled data revealed that the pre-conditioning peripheral nerve lesion enhanced the number of macrophage cells present in the injured spinal cord, as compared to animals that received only a SCI. Conversely, no differences in astrocyte expression were found rostral or caudal from the SCI epicentre between animals pre-conditioned lesion and/or those with a SCI alone at 7 days post CNS injury, although the astrocyte expression was higher caudal to the injury, as opposed to rostrally. Closer examination of the spinal cord following 7 days post SNI alone revealed a significant astrocyte upregulation in the lumbar spinal cord region as compared to normal uninjured tissue. Similarly, SNI alone also led to an increased presence of macrophage cells in the uninjured spinal cord (data not shown). This, as well as an upregulation in astrocyte expression provided evidence for the cellular changes that occur in the spinal cord tissue prior to SCI.

This observed upregulation in macrophage cells and astrocytes in the DRG and spinal cord of pre-conditioned lesion animals might be associated with the observed elevation in BDNF serum concentration since, BDNF can be secreted by immune cells such as, macrophages (Bouhy et al., 2006; Schulte-Herbruggen et al., 2005) and activated T cells (Hammarberg et al., 2000; Moalem et al., 2000; Serpe et al., 2005). In addition, BDNF secretion has also been described to enhance phagocytic ability of macrophage cells (Zeev-Brann et al., 1998), consequently

allowing the fast removal of debris for the regeneration process to begin (Vargas and Barres, 2007). Hypothetically, in relation to the pre-conditioned lesion, this offers a plausible explanation for the observed axonal growth of CNS fibres following peripheral injury, during which systemic BDNF concentration levels are high and more than one immune cell might be involved in the secretion and/or neuroprotection of spared and/or injured CNS fibres. It is clear that this requires further investigation.

Interestingly, inflammation namely by the activation of macrophages is often considered detrimental, especially when it is prolonged and/or non-regulated, such as in neuroinflammatory conditions like MS (reviewed in (Correale and Villa, 2004). However, this negative view has been progressively modified from experimental evidence indicating that a well-controlled immune response can be beneficial to the repair of the injured organism (Hauben and Schwartz, 2003; Hendrix and Nitsch, 2007; Moalem et al., 1999; Rapalino et al., 1998; Yin et al., 2003). In addition, it has been proposed that the beneficial and detrimental properties of these cells might be mediated by specific phenotypic activation (reviewed in (Duffield, 2003; Gordon, 2003; Ma et al., 2003) yet this will require further work to completely understand and characterize. Based on the evidence provided here, we attribute a beneficial role to macrophage cells in the regeneration of injured CNS fibres.

Overall, in an effort to further decipher the mechanism of the pre-conditioned lesion, here we described: A) perineuronal ring formation

consisting of $GFAP^+$ and $CD68^+$ cells enclosing neuronal DRG cell bodies after SNI (as described by others); B) the highest BDNF serum concentration level was found 7 days post SNI alone; C) animals with a pre-conditioned lesion resulted in greater numbers of macrophage cells infiltrating into the CNS compartment; D) SNI alone resulted in a high astrocyte expression and macrophage presence in the uninjured spinal cord; E) both ascending and descending CNS fibres are capable of regenerating following a pre-conditioning SNI.

2.5.2 Part Two: Effects of PNH Vaccination

2.5.2.1 Effects of Adult and Neonatal PNH Vaccination

Normally a pre-conditioned lesion results in the successful regeneration of CNS fibres, however, its beneficial effects extend only to a limited number of afferent CNS fibres (Richardson and Issa, 1984). Here, we have provided evidence that the number of regenerating injured CNS neurons can be enhanced following PNH vaccination, to greater numbers than those originally described by Richardson and Issa (1984). Our results indicated that PNH vaccination effectively enhanced the number of regenerated afferent fibres in animals vaccinated as adults or as neonates, and that their respective immunological repertoire at the time of vaccination (i.e. mature or immature) did not result in significant differences between these two treatment groups. Yet, comparatively against their respective controls, the number of regenerated FB^+ DRG neurons in both PNH vaccinated groups was significantly higher. This

indicated that the use and timing of PNH vaccination, regardless of the animal's immunological predisposition had a positive effect on CNS regeneration, evident by the enhanced number of regenerated FB^+ DRG neurons. Given that, PNS axons have a remarkable regenerative ability (Guth, 1956) has led to the investigation of several approaches to stimulate CNS neurons to regrow and this demonstrated not only the intrinsic growth capabilities of injured CNS neurons but also revealed that the CNS environment is not conducive for regeneration (Cheng et al., 1996; Crutcher, 1989; David and Aguayo, 1981; Richardson et al., 1980; Schnell and Schwab, 1990; Schwab et al., 1993). Similarly, here we provide an effective use of the regenerative properties of a PNS constituent to stimulate the growth state of injured neurons in the CNS.

Furthermore, besides from the enhancement in CNS regeneration demonstrated through PNH vaccination, varying numbers of quantified inflammatory cells were also found at the SCI site between groups. The relative number of macrophage cells ($CD68^+$) found in animals actively vaccinated with the PNH was significantly higher than those in their respective controls. This difference was observed in all animals PNH vaccinated as adults or as neonates, with both demonstrating similar macrophage distribution patterns. This resulted in the highest macrophage numbers at the caudal end, progressively decreasing through the SCI epicentre and through the rostral end, as compared to their respective controls. Note that, quantified astrocyte expression ($GFAP^+$) between PNH vaccinated and control animals did not differ, although astrocyte

expression was highest caudal to SCI epicentre. The increased presence of macrophage cells and astrocyte expression caudal to the SCI could possibly be interacting with Wallerian degeneration, yet this was not further investigated.

2.5.2.2 Effects of Maternal PNH Vaccination

Conversely, animals passively exposed to PNH through maternal vaccination revealed contradictory findings to those resulting from direct PNH vaccination (i.e. adult and neonatal), both qualitatively and quantitatively. Interestingly, given that specific antibodies, such as IgGs have been implicated as possible contributors to CNS regeneration (Ellezam et al., 2003; Huang et al., 1999), then exposure to these antibodies through maternal transfer should have theoretically resulted in enhanced CNS regeneration. However, here we demonstrated that in offspring passively exposed to PNH through maternal vaccination, this capability for enhanced CNS regeneration was not observed. In fact, our results indicated that rather, a reduced number of regenerated FB^+ DRG neurons were found in the offspring of PNH vaccinated animals. Indeed, animals passively exposed to PNH appeared to develop tolerance to the regenerative trigger characteristic of the pre-conditioned lesion, as compared to successfully regenerated CNS neurons in control animals, which were passively exposed to saline through maternal vaccination. At present, the underlying mechanism mediating this type of suppressed response in CNS regeneration remains unclear. Yet, closer examination in

the injured spinal cord, at the cellular level has provided some evidence into the factors that might have contributed to this suppression.

Macrophage quantification in passively exposed animals to PNH resulted in a reduced number of macrophage cells found caudal to the SCI epicentre with no other differences found at the SCI epicentre or rostral to the injury, as compared to control animals. Comparatively, among all actively and passively PNH vaccinated groups, this represented an overall reduction in macrophage numbers in animals passively exposed to PNH. Interestingly, these passively PNH vaccinated animals also displayed a significantly higher astrocyte expression ($GFAP^+$) caudal to the injury, as compared to their respective controls. Therefore, it is likely that the reduced presence of macrophages at the caudal end interrupted important repair mechanisms such as, myelin debris clearance (Vargas and Barres, 2007) resulting in reduced sprouting of regenerated fibres, which were also halted by an increased glial scar formation (Faulkner et al., 2004; Silver and Miller, 2004; Sofroniew et al., 2001).

Overall, it is conceivable that the observed enhancement in CNS regeneration through PNH vaccination might be attributed to the direct effects of the PNS constituents, which indicative from our results might be influencing the role of macrophage cells. Given that, the presence of these cells appears to be associated with the regenerative properties of the pre-conditioned lesion, not only by allowing their infiltration into the CNS compartment before and after SCI but also following SNI, thereby

contributing to the conditioning DRG neurons to regenerate. This would then suggest a positive role for inflammatory cells in CNS regeneration that needs to be further investigated.

2.6 Conclusion

This study underscores the importance of PNH vaccination on axonal CNS regeneration beneficial for the regrowth of adult injured CNS neurons. The enhanced CNS regeneration observed here following direct PNH vaccination most likely reflects a suppression of the inhibitory molecules present in the CNS. This, as proposed and tested by Huang and colleagues (1999), could be due to the production of increased levels of circulating myelin-reactive antibodies, able to bind to myelin and/or glial scar inhibitors at the injury site (Ellezam et al., 2003; Huang et al., 1999), most likely in the early stages after trauma and/or before these molecules were fully activated. Furthermore, early macrophage recruitment into the CNS due to the properties of the pre-conditioned lesion and to the presence of PNS constituents expressed at the lesion site, could both have contributed to axonal regeneration. Conversely, the beneficial effects of the PNS constituents did not apply to developing neurons, normally not inhibited by environmental CNS cues during development (Shewan et al., 1995), resulting in a suppressed regenerative response. The exact mechanism mediating this response remains to be elucidated.

In addition, it is clear that the immunological response induced by the peripheral injury plays a crucial role in facilitating mature CNS

regeneration and this conditioning injury might also contribute to immune cell infiltration into the CNS terrain, resulting in a systemic immune response prior to SCI, beneficial to all CNS neurons. It is worth mentioning that based on these results, especially in regards to vaccination with a PNS homogenerate, we do not recommend this as a clinical treatment due to unpredicted side effects from the use of impure materials within the homogenate. Instead, this experimental approach highlights the potential interaction between the immune and nervous system in CNS regeneration. Certainly, a process not well understood.

2.7 Future Studies

Theoretically, if a systemic immune response, namely from macrophages, is responsible for the observed CNS regeneration following a pre-conditioned lesion, then changing the location of the CNS injury to another region within the CNS (whereby no direct contact between the two lesions is made) could result assist axonal regeneration. Hypothetically, if axonal regeneration of other injured adult neurons in the CNS were to occur as a result of a peripheral DRG injury, this would indicate a systemic immune contribution to CNS regeneration triggered by the initial peripheral lesion (explored in Chapter 3).

2.8 Reference List

Ahmed, F., Ingoglia, N., Sharma, S., 2001. Axon resealing following transection takes longer in central axons than in peripheral axons: implications for axonal regeneration. Exp. Neurol. 167, 451-455.

Bahr, M., Przyrembel, C., 1995. Myelin from Peripheral and Central Nervous System is a Non-Permissive Substrate for Retinal Ganglion Cell Axons. Exp. Neurol. 134, 87-93.

Bouhy, D., Malgrange, B., Multon, S., Poirrier, A., Scholtes, F., Schoenen, J., Franzen, R., 2006. Delayed GM-CSF treatment stimulates axonal regeneration and functional recovery in paraplegic rats via an increased BDNF expression by endogenous macrophages. FASEB J 20, E493-E502.

Bradbury, E., Moon, L., Popat, R., King, V., Bennett, G., Patel, P., Fawcett, J., McMahon, S., 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416, 634-640.

Brambilla, R., Bracchi-Ricard, V., Hu, W., Frydel, B., Bramwell, A., Karmally, S., Green, J., Bethea, J., 2005. Inhibition of astroglial nuclear factor kappa-beta reduces inflammation and improves functional recovery after spinal cord injury. J Exp. Med. 202, 145-156.

Bregman, B., Goldberger, M., 1982. Anatomical plasticity and sparing of function after spinal cord damage in neonatal cats. Science 217, 553-555.

Cai, D., Qiu, J., Cao, Z., McAtee, M., Bregma, B., Filbin, M., 2001. Neuronal cyclic AMP controls the developmental loss of ability of axons to regenerate. J Neurosci. 21, 4731-4739.

Caroni, P., Schwab, M., 1988b. Antibody Against Myelin-Associated Inhibitor of Neurite Growth Neutralizes NonPermissive Substrate Properties of CNS White Matter. Neuron 1, 85-96.

Carson, M., 2002. Microglia as liaisons between the immune and central nervous system: functinal implications for multiple sclerosis. Glia 40, 218-231.

Cheng, H., Cao, Y., Olson, L., 1996. Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function. Science 273, 510-513.

Correale, J., Villa, A., 2004. The neuroprotective role of inflammation in nervous system injuries. J Neurol. 251, 1304-1316.

Crutcher, K., 1989. Tissue sections from the mature rat brain and spinal cord as substrates for neurite outgrowth in vitro: extensive growth on gray matter but little growth on white matter. Exp. Neurol. 104, 39-54.

David, S., Aguayo, A., 1981. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214, 931-933.

Deng, Y., Zhong, J., Zhou, X., 2000. BDNF is involved in sympathetic sprouting in the dorsal root ganglia following peripheral nerve injury in rats. Neurotox Res 1, 311-322.

Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, R., Nikulina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., He, Z., Filbin, M., 2002. Myelin associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. Neuron 35.

Donelly, D., Popovich, P., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 209, 378-388.

Dubovy, P., Tuckova, L., Jancalek, R., Svizenska, I., Klusakova, I., 2007. Increased invasion of ED-1 positive macrophages in both ipsi- and contralateral dorsal root ganglia following unilateral nerve injuries. Neurosci. Lett. 427, 88-93.

Duffield, J., 2003. The inflammatory macrophage: a story of Jekyll and Hyde. Clin. Sci. 104, 27-38.
Ellezam, B., Bertrand, J., Dergham, P., Mckerracher, L., 2003. Vaccination stimulates retinal ganglion cell regeneration in the adult optic nerve. Neurobiol. Dis. 12, 1-10.
Faulkner, J., Herrmann, J., Woo, M., Tansey, K., Doan, N., Sofroniew, M., 2004. Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci. 24, 2143-2155.
Feng, S., Zhou, X., Rush, R., Ferguson, I., 2008. Graft of pre-injured sural nerve promotes regeneration of corticospinal tract and functional recovery in rats with chronic spinal cord injury. Brain Res. 1209, 40-48.
Fritzsch, B., Sonntag, R., 1991. Sequential double labelling with different fluorescent dyes coupled to dextran amines as a tool to estimate the accuracy of tracer application and of regeneration. J Neurosci. Methods 39, 9-17.
Gordon, S., 2003. Alternative activation of macrophages. Nat. Rev. 3, 23-35.
Gray, M., Palispis, W., Popovich, P., Van Rooijen, N., Gupta, R., 2007. Macrophage depletion alters the blood-nerve barrier without affecting Schwann cell function after neural injury. J Neurosci. Res. 85, 766-777.
Guth, L., 1956. Regeneration in the mammalian peripheral nervous system. Physiol. Rev. 36, 441-478.
Hammarberg, H., Lidman, O., Lundberg, C., Eltayeb, S., Gielen, A., Muhallab, S., Svenningsson, A., Lindå, H., van der Meide, P., Culheim, S., Olsson, T., Piehl, F., 2000. Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. J Neurosci. 20, 5283-5291.
Hauben, E., Schwartz, M., 2003. Therapeutic Vaccination for Spinal Cord Injury: Helping the Body to Cure Itself. TRENDS in Pharmacol Sci 24, 7-12.
Hendrix, S., Nitsch, R., 2007. The role of T helper cells in neuroprotection and regeneration. J Neuroimmunol. 184, 100-112.
Hu, P., Bembrick, A., Keay, K., McLachlan, E., 2007. Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. Brain, Behav. and Immunity 21, 599-616.
Hu, P., McLachlan, E., 2002. Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. Neurosci. 112, 23-38.
Hu, P., McLachlan, E., 2003a. Distinct functional types of macrophage in dorsal root ganglia and spinal nerves proximal to sciatic and spinal nerve transections in the rat. Exp. Neurol. 184, 590-605.
Hu, P., McLachlan, E., 2004. Inflammation in sympathetic ganglia proximal to sciatic nerve transection in rats. Neurosci. Lett. 365, 39-42.
Huang, W., Mckerracher, L., Braun, P., David, S., 1999. A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. Neuron 24, 639-647.
Kim, G., Kohyama, K., Tanuma, N., Arimito, H., Matsumuto, Y., 1998. Persistent expression of experimental autoimmune encephalomyelitis (EAE)-specific Vb8.2 TCR spectratype in the central nervous system of rats with chronic relapsing EAE. J Immunol. 161, 6993-6998.
Kipnis, J., Yoles, E., Porat, Z., Cohen, A., Mor, F., Sela, M., Cohen, I., Schwartz, M., 2000. T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. PNAS 97, 7446-7451.

Levine, S., Wenk, E., 1961. Studies on the mechanism of altered susceptibility to experimental allergic encephalomyelitis. Am. J Pathol. 39, 419-441.
Li, L., Xian, C., Zhong, J., Zhou, X., 2002. Effect of Lumbar 5 Ventral Root Transection on Pain Behaviors: A Novel Rat Model for Neuropathic Pain without Axotomy of Primary Sensory Neurons. Exp. Neurol. 175, 23-34.
Li, L., Zhou, X., 2001. Pericellular Griffonia simplicifolia I isolectin B4-binding ring structures in the dorsal root ganglia following peripheral nerve injury in rats. J Comp. Neurol. 439, 259-274.
Lu, J., Ashwell, K., Hayek, R., Waite, P., 2001. Fluororuby as a marker for detection of acute axonal injury in rat spinal cord. Brain Res. 915, 118-123.
Lu, X., Richardson, P., 1991. Inflammation near the nerve cell body enhances axonal regeneration. J Neurosci. 11, 972-978.
Luo, J., Zhi, Y., Chen, Q., Cen, L., Zhang, C., Lam, D., Harvey, A., Cui, Q., 2007. Influence of macrophages and lymphocytes on the survival and axon regeneration of injured retinal ganglion cells in rats from different autoimmune backgrounds. Eur. J Neurosci. 26, 3475-3485.
Ma, J., Chen, T., Mandelin, J., Ceponis, A., Miller, N., Hukkanen, M., Ma, G., Konttinen, Y., 2003. Regulation of macrophage activation. Cell. Mol. Life Sci. 60, 2334-2346.
Mamounas, L., Altar, C., Blue, M., Kaplan, D., Tessarollo, L., Lyons, W., 2000. BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. J Neurosci. 20, 771-782.
Maruyama, K., Li, M., Cursiefen, C., Jackson, D., Keino, H., Tomita, M., Van Rooijen, N., Takenaka, H., D'Amore, P., Stein-Streilein, J., Losordo, D., Streilein, J., 2005. Inflammation-induced lymphoangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin. Invest. 115, 2363-2372.
McKerracher, L., David, S., Jackson, D., Kottis, V., Dunn, R., Braun, P., 1994. Identification of Myelin-Associated Glycoprotein as a Major Myelin-Derived Inhibitor of Neurite Growth. Neuron 13, 805-811.
McLachlan, E., Janig, W., Devor, M., Michaelis, M., 1993. Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. Nature 363, 543-546.
Moalem, G., Gdalyahu, A., Shani, Y., Otten, U., Lazarovici, P., Cohen, I., Schwartz, M., 2000. Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. J Autoimm. 15, 331-345.
Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I., Schwartz, M., 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. Nat. Med. 5, 49-55.
Mueller, M., Wacker, K., Ringelstein, E., Hickey, W., Imai, Y., Kiefer, R., 2001. Rapid response of identified resident endoneurial macrophages to nerve injury. Am. J Pathol. 159, 2187-2197.
Mukhopadhyay, G., Doherty, P., Walsh, F., Crocker, P., Filbin, M., 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13, 757-767.
Neumann, S., Woolf, C., 1999. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23, 83-91.
Piantino, J., Burdick, J., Goldberg, D., Langer, R., Benowitz, L., 2006. An injectable, biodegradable hydrogel for trophic factor delivery enhances axonal rewiring and improves performance after spinal cord injury. Exp. Neurol. 201, 359-367.

Plunet, W., Kwon, B., Tetzlaff, W., 2002. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. J Neurosci. Res. 68, 1-6.
Popovich, P., Guan, Z., Wei, P., Huitinga, I., Van Rooijen, N., Stokes, B., 1999. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp. Neurol. 158, 351-365.
Qiu, J., Cafferty, W., McMahon, S., Thompson, S., 2005. Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. J Neurosci. 25, 1645-1653.
Ramer, M., Thompson, S., McMahon, S., 1999. Causes and consequences of sympathetic basket formation in dorsal root ganglia. Pain 6, S111-120.
Ramón y Cajal, S., 1928. Degeneration and Regeneration of the Nervous System. Oxford University Press, London.
Rapalino, O., Lazarov-Spiegler, O., Agranov, E., Velan, G., Yoles, E., Fraidakis, M., Solomon, A., Gepstein, R., Katz, A., Belkin, M., Hadani, M., Schwartz, M., 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat. Med. 4, 814-821.
Richardson, P., Issa, V., 1984. Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309, 791-793.
Richardson, P., McGuinness, U., Aguayo, A., 1980. Axons from CNS neurones regenerate into PNS grafts. Nature 284, 264-265.
Richardson, P., Verge, V., 1987. Axonal regeneration in dorsal spinal roots in accelerated by peripheral axonal transection. Brain Res. 411, 406-408.
Schmued, L., Kyriakidis, K., Heimer, L., 1990. In vivo anterograde and retrograde axonal transport of the fluorescent rhodamine-dextran-amine, fluoro-ruby, within the CNS. Brain Res. 526, 127-134.
Schnell, L., Schwab, M., 1990. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343, 269-272.
Schulte-Herbruggen, O., Nassenstein, C., Lommatzsch, M., Quarcoo, D., Renz, H., Braun, A., 2005. Tumor necrosis factor-alpha and interleukin-6 regulate secretion of brain-derived neurotrophic factor in human monocytes. J Neuroimmunol. 160, 204-209.
Schwab, M., Kapfhammer, J., Bandtlow, C., 1993. Inhibitors of neurite growth. Annu. Rev. Neurosci. 16, 565-595.
Serpe, C., Byram, S., Sanders, V., Jones, K., 2005. Brain derived neurotrophic factor supports facial motoneuron survival after facial nerve transection in immunodeficient mice. Brain, Behav. and Immunity 19, 173-180.
Shahabi, S., Hassan, Z., Jazani, N., 2006. Any beneficial effects of mycobacteria on multiple sclerosis and experimental autoimmune encephalitis may include stimulation of the sympathetic nervous system. Med. Hypotheses 67, 164-168.
Shewan, D., Berry, M., Cohen, J., 1995. Extensive regeneration in vitro by early embryonic neurons on immature and adult CNS tissue
. J Neurosci. 15, 2057-2062.
Silver, J., Miller, J., 2004. Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5, 146-156.
Simonen, M., Pedersen, V., Weinmann, O., Schnell, L., Buss, A., Ledermann, B., Christ, F., Sansig, G., van der Putten, H., Schwab, M., 2003. Systemic deletion of

the myelin-associated outgrowth inhibitor nogo-a improves regenerative and plastic responses after spinal cord injury. Neuron 38, 201-211.
Sofroniew, M., Howe, C., Mobley, W., 2001. Nerve growth factor signalling, neuroprotection, and neural repair. Annu. Rev. Neurosci. 24, 1217-1281.
Song, X., Li, F., Zhang, F., Zhong, J., Zhou, X., 2008. Peripherally-Derived BDNF Promotes Regeneration of Ascending Sensory Neurons after Spinal Cord Injury. PLoS One 3, e1707.
Stills, H., 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J 46, 280-293.
van Wijngaarden, P., Brereton, H., Coster, D., Williams, K., 2007. Genetic influences on susceptibility to oxygen-induced retinopathy. Invest. Ophthalmol. Vis. Sci. 48, 1761-1766.
Vargas, M., Barres, B., 2007. Why is Wallerian degeneration in the CNS so slow? Annu. Rev. Neurosci. 30, 153-179.
Waddington, S., Kennea, N., Buckley, S., Gregory, L., Themis, M., Coutelle, C., 2004. Fetal and neonatal gene therapy: benefits and pitfalls. Gene Ther. 11, S92-97.
Wang, K., Koprivica, V., Kim, J., Sivasankaran, R., Guo, Y., Neve, R., He, Z., 2002. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417, 941-944.
Yin, Y., Cui, Q., Irwin, N., Fischer, D., Harvey, A., Benowitz, L., 2003. Macrophage-derived factors stimulate optic nerve regeneration. J Neurosci. 23, 2284-2293.
Zeev-Brann, A., Lazarov-Spiegler, O., Brenner, T., Schwartz, M., 1998. Differential effects of central and peripheral nerves on macrophages and microglia. Glia 23, 181-190.
Zhang, J., Shi, X., Echeverry, S., Mogil, J., de Koninck, Y., Rivest, S., 2007. Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. J Neurosci. 27, 12396-12406.
Zheng, B., Ho, C., Li, S., Keirstead, H., Steward, O., Tessier-Lavigne, M., 2003. Lack of enhanced spinal regeneration in nogo-deficient mice. Neuron 38, 213-224.
Zhou, X., Oldfield, B., Livett, B., 1991. Substance P-containing sensory neurons in the rar dorsal root ganglia innervate the adrenal medulla. J Autonomic Nerv. Syst. 33, 247-254.
Zhou, X., Rush, R., McLachlan, E., 1996. Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. J Neurosci. 16, 2901-2911.
Zinkernagel, R., 2001. Maternal antibodies, childhood infections, and autoimmune diseases. Adv. Immunol. 345, 1331-1334.