Chapter 3: Optic Nerve Paradigm

3.1 Introduction

Under normal conditions injured optic neurons, like any other neurons in the mammalian CNS, do not successfully regenerate after injury. In the CNS, there are many different inhibitory molecules that have been described to limit optic nerve growth (reviewed in (Domeniconi and Filbin, 2005). Clinically, optic nerve (ON) neuropathies start with optic nerve degeneration and end with retinal ganglion cell (RGC) death (Jonas and Budde, 2000). Experimentally after ON injury similar events have been described, although it was suggested that RGC death might be determined by the proximity of the lesion to the ON globe and by the availability of trophic support (Berkelaar et al., 1994). The regeneration and functional connectivity of injured ON has been successfully demonstrated with the inhibition of the inflammatory response within the vitreous body, concomitant with the use of PNS graft connected to the superior colliculus (Thanos et al., 1997).

Certainly, the use of PNS grafts to bypass the CNS environment has proven a successful approach to stimulate optic nerve regeneration (Vidal-Sanz et al., 1987) and formation of functionally synapses (Keirstead et al., 1989; Whiteley et al., 1998). Concomitantly, these results supported findings within other areas of the CNS, such as in the spinal cord, where remarkable axonal growth has been observed with the implementation of PNS grafts (Richardson and Issa, 1984). These models of regeneration have provided

further evidence that mature CNS neurons have an intrinsic ability to regrow their axons given the appropriate environment (David and Aguayo, 1981). A major limitation to these aforementioned studies is that although these are successful in providing evidence for the regenerative properties of injured CNS axons, they do not show the capacity of growing axons to extend through the degenerating myelin and associated glial inhibitors. Ideally, a therapeutic approach to stimulate regeneration of injured CNS neurons that is clinically relevant, should demonstrate growth to occur through the CNS environment utilizing minimally invasive strategies.

Several studies have suggested that the key for optic nerve regeneration lies within the eye, that is, at the RGC level (Berry et al., 1996; Berry et al., 1999; Fischer et al., 2000; Lazarov-Spiegler et al., 1996; Leon et al., 2000). Consequently, this concept steered regenerative therapies away from grafting experiments to interventions within the eye compartment. Such strategies have allowed regeneration to occur within the optic nerve and have relied on different measures to stimulate RGC survival and optic nerve regeneration by changing the environment within the vitreous body. For instance, by injuring the optic lens (Fischer et al., 2001; Leon et al., 2000) or by implanting peripheral nerve fragments into the vitreous body (Berry et al., 1996; Berry et al., 1999), both of these approaches stimulated RGCs to regenerate through the injury site and into the distal optic nerve. Recently, Fischer and colleagues (2001), demonstrated that after optic nerve transection, RGCs

stimulated by lens injury were reported to unprecedentedly extend axons as far back as to the superior colliculus with a direct beneficial role attributed to inflammatory cells, such as macrophages (Fischer et al., 2001).

In addition, there are other measures that have been implemented to promote optic nerve regeneration and these include: macrophage transplantation to speed up debris removal (Lazarov-Spiegler et al., 1998b), antibody treatment against myelin inhibitors (Weibel et al., 1994) and/or the manipulation of oncomodulin, a macrophage secreted factor, to effectively promote optic nerve generation has also been successfully reported (Yin et al., 2003). These therapies although methodologically different, have one thing in common; all involve macrophage activation. Directly activating macrophages with intravitreal injections of Zymosan, a potent macrophage activator, was found to produce even stronger axon regeneration than lens injury (Yin et al., 2003). Similarly, macrophage activation, induced by intraocular inflammation supported the survival of axotomised RGCs and concomitantly these macrophage cells have been reported to modify the non-permissive regenerative properties of the injured ON into a growth promoting environment (David et al., 1990). The survival and neurite outgrowth of RGCs has been associated with the release of NTs such as BDNF and NT4/5, which might be acting through a shared signalling pathway (Cohen et al., 1994).

Interestingly, BDNF can be retrogradely transported by RGCs and intravitreal administration of BDNF to axotomised RGCs has been shown to result in an increase in growth associated protein (GAP-43), which correlated with the stimulation of sprouting of injured RGCs (Fournier et al., 1997). Similarly, the exogenous application of trophic factors like BDNF into the superior colliculus demonstrated the survival of RGCs (Ma et al., 1998), an intravitreal injections of BDNF has led to the suppression of lesion-induced degeneration of RGCs (Mey and Thanos, 1993). Furthermore, the prolonged administration of BDNF stimulated the lengthening of RGC axons after ON transection (Sawai et al., 1996). The relationship between macrophage cells and BDNF, as aforementioned has been regarded as beneficial in regeneration (Bouhy et al., 2006) and paradoxically this relationship within the eye is an interesting concept given that normally the vitreous body is suppressive to inflammation, however, after lens injury these inflammatory cells become up-regulated and their presence is correlated with RGC axonal regeneration (Leon et al., 2000).

However, as aforementioned in the main introduction, inflammation has also been categorised as a ‘double edge sword’ with both protective and detrimental effects being reported (Bethea, 2000; Filbin, 2006). In fact macrophages are often associated with neurodegenerative diseases (Hendriks et al., 2005), but as mentioned here these cells can also be neuroprotective (Fischer et al., 2001; Leon et al., 2000; Streit, 2002). There is no direct explanation as to the differential effects exerted by macrophages,

although it has been proposed that under different conditions these cells may be expressing different phenotypes (Schwartz et al., 2006a; van Rossum et al., 2008) and this might be dependant on the timing of macrophage activation (Yin et al., 2003).

Further evidence of immune cell involvement in CNS regeneration was provided in our previous chapter, where we discussed a study by Ellezam et al., indicating that vaccination with a spinal cord homogenate promoted axonal regeneration in the adult optic nerve, however, this type of vaccination did not promote RGC survival (Ellezam et al., 2003). This study raised two key issues: firstly, was the synergistic involvement of the immune system in CNS regeneration and finally, was the importance of cell body involvement in CNS regeneration, which was demonstrated to play a role in the relationship between DRG and spinal cord regeneration (Lu and Richardson, 1991). It is noteworthy that while both RGC and DRG structures anatomically lie in the peripheral part of the nervous system, they both have nerve branches projecting inward into the CNS environment, perhaps a minor anatomical observation that could prove significant when exploring CNS regeneration.

3.1.1 Project Rationale

The optic nerve has long been used as a model for understanding the regenerative success or failure within the CNS and the fact that it has never been considered a part of the pre-conditioned peripheral nerve lesion

complex, makes this an interesting combination. The main purpose for conducting this optic nerve project relied heavily on observations from previously learned at our laboratory, where three important findings were noted. Briefly, a peripheral pre-conditioning lesion led not only to the CNS regeneration of ascending but also descending fibres through the SCI epicentre. Secondly, this type of CNS regeneration was enhanced by vaccination with sciatic nerve homogenate in animals vaccinated when neonates or adults but suppressed through passive, pre-conception vaccination. Finally, macrophage quantification in the SCI epicentre was found to be positively correlated with the amount of CNS regeneration. Taken together these results indirectly outlined the possibility that the pre-conditioned peripheral nerve lesion could potentially contribute to a systemic effect in CNS regeneration via the involvement of the immune system.

3.1.2 Hypothesis

Axotomy of peripheral DRG neurons normally leads to enhanced afferent CNS regeneration, it is then possible that the effects of the pre-conditioning peripheral lesion may also enhance regeneration of other lesioned neurons in different locations within the CNS; whereby no contact between the two lesions is made. A positive finding here would suggest the involvement of a systemic mechanism in CNS regeneration (Fig. 17).

3.1.3 Proposed Model of Injury

Given that DRG neurons project centrally to the brainstem and peripherally to the sciatic nerve (Richardson and Issa, 1984), this provides an excellent model for studying some aspects of axonal regeneration in the CNS after a pre-conditioned peripheral lesion. However, to assess the possibility of systemic CNS regeneration after peripheral axotomy, the location of the CNS injury needs to be changed from the spinal cord to the optic nerve. In this proposed model of injury, a peripheral lesion will be carried out as per original model (Richardson and Issa, 1984) at day 0 followed by an optic nerve crush (ONC) at day 7 (Fig. 18).

3.2 Method and Materials

3.2.1 Animals

Adult female Sprague Dawley (SD) rats (10-12 weeks) were used 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.

3.2.2 Surgical Procedures

Animals were divided into 2 experimental groups: *Sham control group* (n=20) received a sciatic nerve exposure only (day 0) followed by an optic nerve

crush (ONC) on day 7; *Test group* (n=20) received a sciatic nerve ligation and cut (day 0) followed by ONC on day 7 (Fig. 18). Both of these groups received neuronal tracer injection of Fluororuby (FR, 0.1 μl) into the optic nerve immediately after ONC. Therefore, the only variable between these groups was the peripheral nerve injury received by the test group animals. Note that for retrograde tracing studies, these experimental groups (n=5/group) were replicated with exactly the same surgical procedures, except for the omission of the FR tracer injection into the optic nerve (refer to section 3.3.1).

For all peripheral nerve injuries, animals were anaesthetised by halothane inhalation through a mouthpiece, whereas, for optic nerve injuries animals were anaesthetised with a mixture of ketamine (100 mg/kg) and xylazine (100 mg/kg) delivered intraperitoneally (i.p.). This change in anaesthetic was due to an inherent inability to manoeuvre around the animal's eye and exposed optic nerve with their inhaler mouthpiece on. 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.

3.2.2.1 Sciatic Nerve Ligation and Cut

Briefly, a longitudinal cut was made on the skin adjacent to the femur followed by a blunt dissection through the leg muscles, the sciatic nerve was then exposed, ligated just proximal to its trifurcation and cut below the ligation site

with fine surgical scissors (refer to chapter 2 section 2.2.3.1 for more information).

3.2.2.2 Optic Nerve Crush (ONC)

Briefly, a longitudinal incision was made on the skin close to the superior orbital rim, the superior extraocular muscles and lacrimal glands were spread with a retractor and the optic nerve was crushed for 30 seconds, 3-4 mm distal from the globe (Leon et al., 2000). Nerve injury was verified by the appearance of a clear band at the crush site. Care was taken not to interfere with retinal blood supply.

3.2.2.3 Fluororuby Tracer Injection into Optic Nerve

To investigate the presence of any regenerated fibres post CNS lesion, a tracer injection of Fluororuby (FR, 10% in distilled water, Molecular Probes) was given 1-2mm proximal to ONC site to label fibres distal to crush. This tracer is highly sensitive, quick and reliable as an axonal tracer and has the advantage of labelling axons retrogradely but particularly anterogradely (Fritzsch and Sonntag, 1991; Schmued et al., 1990).

Briefly, sterile cotton balls were placed underneath the exposed optic nerve prior to tracer injection to provide optic nerve stability during injection and to allow visibility of any tracer leakage at the injection site. To deliver the tracer

injections, we used a 50 ml syringe connected to a three-way stop-cock, as well as to capillary tubing line connected to a fine glass micropipette, which was safely secured to the injection arm of the stereotaxic frame. At the completion of tracer delivery, the needle was left in place for an additional 2 minutes to avoid any tracer drawback into the micropipette. Care taken not to further damage the optic nerve.

Note that animals were excluded from the study if any of the injections punctured directly through the optic nerve and/or if there was any interference with retinal blood supply resulting in haemorrhage or blindness in the animals soon after surgery.

3.2.2.4 Stereotaxic Coordinates from Brain Aspiration

With an overdose of ketamine (100 mg/kg) and xylazine (100 mg/kg) delivered intraperitoneally (i.p.) two animals were sacrificed to obtain our own stereotaxic coordinates of the superior colliculus. Using a stereotaxic frame, the top of the animal's head was fixed, shaved and the scalp was incised in an area overlying the midline of the skull. Two sets of eight burr holes were drilled in parallel in a caudal direction from the bregma to approximately 5mm passed the lambda. This allowed the careful removal of a portion of the skull and exposed the brain tissue through a small bony window in the skull. Using a 1.5mm needle connected to a vacuum pump (Vela et al., 2002), the top of the cortex overlaying the superior colliculus (SC) was aspirated. The tissue

was sufficiently removed to fully expose the area of interest and enabled the acquisition of accurate stereotaxic coordinates to retrograde label RGC bodies (Fig. 21).

The main reason for obtaining our own personalised stereotaxic values and not resorting to those given in a published rat atlas (Paxinos and Watson, 1998), was to due to variability in tracer delivery resulting in unsuccessful RGC labelling. This inconsistency could be attributed to the fact that this atlas was specifically designed for adult male Wistar rats weighting 270-310gm, as opposed to female SD rats (250-300gm) used in this study. These differences in animal strains made a significant difference when targeting small, localised areas within the rat brain.

3.2.2.5 Stereotaxic Coordinates of Superior Colliculus

The coordinates obtained represented perimeter values for the boundaries of the SC that enabled the proper delivery of the retrograde tracer within those boundaries. In all, when using stereotaxic coordinates three important measures need to be considered: A) the ‘bregma’ refers to the point of origin with an assigned value of zero (in millimetres), preceded by a minus sign if located posterior to it, in a caudal direction; B) the ‘depth’ refers to the intracerebral trajectory from the top of the skull to the surface of the SC; and C) the ‘midline’ refers to the distance measured away from the midline in a tempo-lateral direction.

3.2.2.6 Retrograde Labelling of RGCs from Superior Colliculus

To determine the presence of regenerated fibres extending from the ONC site to the SC, an injection of the retrograde tracer, fast blue (FB, 5% in saline, Sigma), was used to label RGCs ipsilateral to lesion. Briefly, anaesthetized rats were held in a stereotaxic frame, a 1cm midline linear incision was made on the skin overlaying the skull and the dura was removed. Burr holes were drilled in the skull at the appropriate co-ordinates (Fig. 21) and a volume of 0.1µl of FB was injected bilaterally into the SC using a pulled glass micropipette needle at two different locations and at two different depths. Once the tracer had been delivered, the needle was left in place for an additional 2 minutes to avoid any fluid drawback into the needle. The incision was closed with sutures (6.0 silk sutures), and animals were allowed to survive 7 days post injection to allow FB to be transported back to RGC bodies (Ahmed et al., 2001). Note that depth values here were greater (i.e. deeper) than those specified in the total SC perimeter to allow for sufficient needle penetration within the CNS tissue.

3.2.3 Perfusion

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 also dissolved in PB (0.1 M, pH 7.4). Perfusions were performed either at 2, 4, 7 or 14 weeks post CNS lesion 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 (refer to chapter 2 section 2.2.4 for more details).

3.2.4 Retina Dissection

To examine RGC labelling, both eyes were enucleated within 24 hours post fixation and processed accordingly (see below).

3.2.4.1 Retinal Flat-Mount

RGC survival after ONC was examined in whole mount retinas by the presence of FB^+ cell bodies retrogradely labelled from SC. At the end of the experimental period, rats were euthanised and their eyes were enucleated and fixed with 4% PFA in PB (0.1 M, pH 7.4). The retinas were then removed and whole-mounted as previously described (van Wijngaarden et al., 2007).

Briefly, dissections were performed under an operating microscope and with the use of chilled phosphate buffered saline (PBS 0.1 M, pH 7.4) to avoid retinas from drying. Fat and connective tissue were dissociated away to expose the sclera and a short stump of optic nerve was left protruding from the globe to allow manipulation of the eye without damaging the RGCs. An incision was made immediately anterior to the corneoscleral limbus with a scalpel blade and fine-toothed forceps were used to grasp the cornea (van Wijngaarden et al., 2007). The incision was continued circumferentially and

the cornea, iris and crystalline lens were discarded.

Four equally spaced radial incisions, extending from the retinal periphery to the optic nerve head, were made with a scalpel to flatten the eyecup. The flattened eyecup was turned face down and carefully transferred to a 2% gelatine-coated slide. For convenience, the remainder of the dissection was performed on a microscope slide.

A drop of chilled PBS was added to the tissue and a blunt probe was inserted between the sclera and retina (sub-retinal space) via the cut edges of the eyecup. The choroid and sclera were carefully separated from each quadrant of the retina and removed with micro-dissection scissors. The optic nerve stump and the surrounding rim of choroid and sclera were cut away and the retina was carefully turned. Two pairs of fine forceps were used simultaneously to pull away vitreous gel from retinal surface (van Wijngaarden et al., 2007). A drop of chilled PBS was added to prevent dehydration of the retina. The microscope slide was viewed using fluorescent microscope to locate any RGC labelled by the tracer.

3.2.5 Cryosectioning

All dissected tissues were post-fixed and cryoprotected accordingly prior to cryosectioning (refer to section 3.2.3 for more information). Cryosectioning was performed on a cryostat microtome at -20^{0} Celsius and due to

morphological variation of dissected tissues, these were cryosectioned at different thicknesses. Optic nerves were cut at 15μm (longitudinal sections) and brains cut at 40μm (cross-section) through the SC to ensure maximal visibility of any FR^+ fibres at respective locations. All specimens were mounted on 2% gelatine-coated glass slides.

3.2.6 Immunohistochemistry

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

3.2.6.1 Fluorescence Staining

The primary antibodies used in this study for single, double and/or triple labelling were: mouse-anti-rat cluster differentiation 68 (CD68, macrophage, 1:400, Serotec), rabbit-anti-glial fibrillary acidic protein (GFAP, astrocyte, 1:500, Dako), mouse-anti-neurofilament 200 (NF-200, 1:300, Chemicon). A combination of the following secondary antibodies for single, double and/or triple labelling were: sheep-anti-mouse-cy3-IgG (Jackson, 1:500), donkey-anti-mouse-cy2-IgG (Jackson, 1:500), donkey-anti-rabbit-488-IgG (Jackson, 1:500), sheep-anti-mouse-cy5-IgG (Jackson, 1:500).

Note that a similar general IHC protocol was followed throughout this thesis (refer to chapter 2 section 2.2.6 for more information).

3.2.7 Cell Quantification Method

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) (refer to chapter 2 section 2.2.9 for more details).

3.2.7.1 Statistics

In all graphs, columns represent an averaged mean (n=4 or 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 $P<0.05$.

3.3 Results

3.3.1 No Regenerated Fibres Found in Optic Nerve

Results from FR tracer injection in both experimental groups, 4 weeks post CNS lesion did not reveal any regenerated FR^+ fibres 3-4mm distal to crush site (white arrows, Fig. 19A-B). Specifically, only FR^+ granules were found distal from crush site. These FR^+ granules as previously described by Lu and colleagues (2001), most likely represent disrupted profiles of injured axons with irregular thickness, as well as swollen or discontinuous fragments belonging to degenerated fibres (Lu et al., 2001). This finding failed to support our stated hypothesis and our proposed model of injury predicting substantial CNS regeneration after a pre-conditioned lesion. In addition, there were no regenerated and/or intact axons identifiable by a continuos band of similar thickness with even fluorescence (Lu et al., 2001), seen distally in either of these treatment groups.

The possibility that the FR tracer injection into the optic nerve further damaged potentially surviving and/or regenerated fibres, was considered firstly by closer examination of macrophage cells present at the injection site (Fig. 19A, 19C-E) and secondly by retrograde labelling of axonal fibres from the SC without tracer injection administration into the optic nerve (refer to section 3.3.5). It was clear from the immunostaining of macrophage cells ($CD68^+$) that the FR tracer injection (white asterisks, Fig. 19D-E) did damage the optic nerve tissue and this was evident by the macrophage cell density

present at the injection site and from the colocalisation of FR^+ fragments phagocytosed by macrophage cells (green coloured, Fig. 19D). There are two possibilities for this colocalisation, either by: direct macrophage ingestion of the tracer itself or by indirect debris clearance of degenerated FR^+ labelled neurons, a well-recognised function of these macrophage cells (Lombard et al., 1994).

Identification of the ONC site, as determined by astrocyte immunostaining of the scar formation ($GFAP^+$, Fig. 19F) revealed small FR^+ fibres found at or immediately proximal to crush site 4 weeks post CNS lesion (Fig. 19A, 19G) in close proximity to macrophage cells ($CD68^+$, Fig. 19H-I). Despite this, within 5-8 weeks post CNS lesion, these remaining fibres completed degenerated with only FR^+ granules remaining *in situ*.

3.3.2 No Regenerated Fibres Found in Optic Tract and Superior Colliculus

Further observation 6 weeks post CNS lesion in both experimental groups did not reveal any FR^+ fibres extending as far as the optic tract (Fig. 20F, 20J) and/or the SC (Fig. 20B) through the retinocollicular pathway, into the region of RGC axonal synapse (Fischer et al., 2001). Here, the presence of FR^+ granules and the lack of continuos FR^+ fibres confirmed our previous results of these being fragmented degenerated fibres (Fig. 20F, 20J). In the optic tract, these granules were easily visualised by a surrounding trail of activated

macrophage cells ($CD68^+$, Fig. 20A, 20E, 20I) and astrocytes ($GFAP^+$, Fig. 20C-D, 20G-H, 20K-L) most likely corresponding to effects from Wallerian degeneration (Fig. 20E, 20I). It should be noted that due to the anatomical nature of the SC in relation to the optic tract, the number of macrophage cells presented in this SC image did not represent the quantified number of macrophage cells found within this region (Fig. 20A). These cells were already present in the SC within 2 weeks post CNS lesion in both groups (Fig. 26).

Thus far our FR anterograde tracer studies have resulted in a lack of regenerated FR^+ fibres in both experimental groups, yet the likelihood that the optic nerve tracer injection led to further neuronal degeneration remained to be explored (refer to section 3.3.5).

3.3.3 Superior Colliculus Stereotaxic Coordinates

The brain aspiration procedure (refer to section 3.2.2.4) allowed the complete acquisition of SC stereotaxic coordinates to be established (Fig. 21A-B) due to several unsuccessful attempts to retrograde label RGCs from the specified coordinates provided by Paxinos and Watson (1998). These newly described coordinates assigned numerically from 1-7 outlined the entire perimeter of the SC and facilitated the efficient delivery of 2 bilateral FB tracer injections each at 2 different depths within these boundaries (red asterisks); that proved accurate and efficacious in retrograde labelling RGCs (Fig. 21A-C).

3.3.4 Superior Colliculus Cell Body Labelling

Confirmed success of FB tracer delivery was obtained from cross sections of the SC (Fig. 22A). These sections revealed FB staining of cell bodies in the SC region with minimal intracerebral damage resulting from the tracer microinjections, depicted by the low astrocytic ($GFAP^+$) and almost absent inflammatory cell ($CD68^+$) expression, activation and/or infiltration (Fig. 22A-I), as compared to normal uninjured brain tissue (data not shown).

3.3.5 Retrograde Labelling of Retinal Ganglion Cells

Here, we addressed the possibility of whether FR tracer injections into the optic nerve caused more damage to potentially surviving and/or regenerated optic nerves. Within their respective experimental groups animals were lesioned as previous, however, on this occasion no FR anterograde tracer injections were administered into the crushed optic nerves. This allowed us to retrograde trace RGCs from the SC without jeopardising the integrity of spared and/or regenerated optic nerve fibres.

Retrograde FB tracer delivery from SC (Fig. 23A) resulted in successful labelling of RGCs using our newly acquired SC target coordinates (Fig. 21). Yet, upon closer observation of retinal flat mounts, no FB^+ labelled RGCs were found ipsilateral to ONC on either of the experimental groups (Fig. 23B, 23E), only RGCs in the contralateral eye (uninjured nerve) were labelled (Fig. 23B-D). The use of the contralateral RGCs as positive controls confirmed the

successful tracer delivery. However, inability to label ipsilateral RGCs revealed two important possibilities: 1) there are no long optic nerve fibres extending from the ONC site to the SC; and 2) this type of retrograde labelling does not account for the fact that there could be smaller regenerated fibres within the optic nerve. Therefore, the latter possibility was assessed next by immunostaining with the appropriate neuronal markers (see below).

3.3.6 Immunohistochemistry Staining of Neuronal Fibres

Within 2 weeks post CNS lesion, IHC staining using a neurofilament marker (NF-200) to stain major elements of the cytoskeleton supporting axons was performed. Both experimental groups displayed extensive optic nerve fibre retraction at the ONC site, moving away from the globe in a distal direction ($NF\text{-}200^{+}$, Fig. 24A, 24C-D, 24F-G, 24I). The amount of axonal retraction was correlated to the extent of scar formation ($GFAP^{+}$) developed at the crush site as a result of the mechanical injury (Fig. 24B-C, 24E-F, 24H-I). Closer examination at the crush site revealed significant axonal collapse and degeneration (white arrows, Fig. 24G, 24I).

Chronologically, 14 weeks post CNS lesion, in both experimental groups no $NF\text{-}200^{+}$ fibres were found 3-4mm distal from crush site (Fig. 24J, 24L), only astrocytic processes being found throughout ($GFAP^{+}$, Fig. 24K-L, 24N-O). Optic nerve fibres had retracted as far as the optic chiasm (yellow arrows, Fig. 24M, 24O), with the assumption of further retraction to be expected with

longer survival time points. Within these optic nerves, during the entire course of this study, there was no other positive immunostaining for neurite growth or axonal elongation observed at any time, as tested with other sensitive neuronal growth markers (GAP-43, beta-III tubulin - data not shown) (Benowitz and Yin, 2007; Berry et al., 1996). The lack of neuronal staining using these markers confirmed the absence of systemic contribution of the pre-conditioned peripheral nerve lesion to CNS regeneration.

3.3.7 Macrophage Quantification at CRUSH SITE

At the crush site macrophage quantification during the 14-week period post CNS injury, demonstrated statistically significant differences in macrophage numbers ($CD68^+$) between groups but only at 4 weeks post injury (Fig. 25A). This difference was significantly higher in the pre-conditioned lesion group, as compared to the control group ($P<0.05$), although it is important to note that macrophage numbers in both groups peaked at 2 weeks post ONC and decreased 5-fold by 4 weeks. In addition, compared to normal uninjured tissue, macrophage values represented a 25-fold increase in the acute phase post CNS injury, progressively decreasing after 2 weeks and plateauing thereafter, as these cells probably migrated deeper into subcortical regions (Fig. 26). There was some increase in macrophage numbers observed in the contralateral (uninjured) optic nerve within 2 weeks post CNS lesion, but these numbers were relatively low and not statistically significant ($P>0.05$;

data not shown). Note that, macrophage numbers 1-week post CNS lesion was not examined in any of the experimental groups.

3.3.8 Macrophage Quantification 3-4mm DISTAL to Crush Site

In addition, 3-4mm distally there was also a statistically significant difference in macrophage numbers in the pre-conditioned group 2 weeks post CNS lesion, as compared to the control group (Fig. 25B, $P<0.05$). This variation in significance in macrophage numbers quantified at the crush site (Fig. 25A) versus 3-4mm distally might be attributed to the migratory pattern of these cells from the crush site in a distal direction along the examined tissue. This difference was not seen again for the remaining 12 weeks during the course of the study, as macrophage numbers between groups did not reach statistical significance thereafter.

3.3.9 Macrophage Quantification in OPTIC TRACT and SUPERIOR COLLICULUS

Observed macrophage numbers in the ipsilateral optic tract and SC had a completely different migratory pattern to that observed at and distal from the ONC site. Here, macrophage cells in the pre-conditioned lesion were significantly higher from 7-14 weeks in subcortical regions (Fig. 26), as compared to macrophage numbers in close proximity to ONC at 2-4 weeks post CNS lesion (Fig. 26A-B). Within the optic tract and SC these

macrophage cells were detected within 2 weeks post CNS lesion but were not significantly different between groups until 7-14 weeks post CNS lesion, with increased macrophage numbers observed in pre-conditioned group, as compared to control group ($P<0.05$ & $P<0.01$ respectively; Fig. 26). These cell numbers progressively increased from 7 to 14 weeks in the pre-conditioned lesion group, whereas they remained constant in the control group (Fig. 26). The macrophage numbers were slightly higher at 7-14 weeks post CNS lesion in the optic tract (Fig. 26A) as compared to the SC (Fig. 26B) which, as previously mentioned, most likely reflects Wallerian degeneration and the migratory pattern of these cells.

3.4 Limitations

Previous studies on susceptibility to experimental autoimmune encephalomyelitis (EAE), an animal model of MS, lists Sprague Dawley rats as one of the more resistant strains to inflammatory stimuli including myelin basic proteins (Levine and Wenk, 1961; Luo et al., 2007). This resistance to inflammatory conditions creates an added variable of uncertainty, which could have suppressed the beneficial extent of the pre-conditioned peripheral lesion in optic nerve regeneration. This is of importance when interpreting these results and if possible, this experiment should be replicated in rodents more susceptible to inflammation, such as Fischer and/or Lewis rats (Levine and Wenk, 1961; Luo et al., 2007).

3.5 Discussion

In this study, the potential for optic nerve regeneration following a pre-conditioned peripheral nerve lesion was explored. The combination of these two lesions had never been previously assessed and warranted testing based on our previous findings. Here, we discuss the effect of a pre-conditioning peripheral nerve lesion in the regeneration of optic nerve fibres and macrophage activity.

3.5.1 Effects on Regeneration

Our results indicated that while a pre-conditioned peripheral lesion normally leads to afferent nerve regeneration in the adult spinal cord (Richardson and Issa, 1984), it did not promote regeneration of crushed optic nerves nor did it support RGC survival. This was evident from the absence of FR labelled fibres and the presence of FR^{+} fragments of degenerated neurons observed throughout the retinocollicular pathway. Originally, anterograde tracer injections into the optic nerve were thought to have exacerbated the extent of the mechanical injury by incrementing the damage to the optic nerve, resulting in more axonal degeneration. This aspect was examined by retrograde labelling of RGCs from the SC, thereby avoiding the injection of the anterograde tracer into the already injured optic nerve. This, however, required the obtainment of our own SC stereotaxic coordinates given that earlier attempts to retrograde label RGCs using specified coordinates by Paxinos and Watson (1998) failed to yield positive results on consecutive

occasions. Fortunately, our stereotaxic coordinates successfully labelled RGCs but only in the contralateral uninjured eye and not ipsilateral to ONC site. By treating the contralateral eye as a positive control, our results confirmed the efficacy of the retrograde tracer delivery and further highlighted three possibilities for this result: A) spared or regenerated nerves did not extend as far as the SC; B) only short fibres remained in the optic nerve in close proximity to the ONC site; and/or C) no fibres were present *in situ*.

These possibilities were assessed by IHC using a combination of neuronal markers detecting for neurofilaments (NF-200), tubulin (βIII-Tubulin) and growth associated protein (GAP-43) and demonstrated that there were no labelled fibres proximal or 3-4mm distal from ONC site. Thorough examination of the optic nerve tissue revealed extensive fibre retraction and collapse by 2 weeks post CNS lesion in both experimental groups, withdrawing as far as the optic chiasm by 14 weeks. These immunohistochemical studies therefore confirmed the notion that a pre-conditioned peripheral nerve lesion did not result in optic nerve regeneration. Therefore, we were able to reject our proposed hypothesis outlining the potential for systemic contribution to CNS regeneration.

3.5.2 Effects on Macrophage Activity

The introduction of a mechanical lesion such as an optic nerve crush resulted in a 25-fold increase in macrophage cells by 2 weeks post CNS lesion as

compared to what is seen in normal uninjured tissue. Although elevated, this result was not significant between experimental groups at the ONC site. Chronologically, during a 14-week observation period post CNS lesion, there were significant differences between experimental groups by 4 weeks at crush site, by 2 weeks 3-4mm distally from ONC and by 7-14 weeks at the optic tract and SC, with greater macrophage numbers detected in pre-conditioned lesion animals, as compared to controls. This phasic variation in cell numbers may denote a Wallerian degeneration time course and infiltration of $CD68^+$ cells throughout the retinocollicular pathway similar to those briefly described by Battisti and colleagues in 1995. As expected in the injured optic nerve close to the crush site, macrophage activation occurred early in the process, however, in the optic tract and SC (i.e. deeper subcortical regions), cell activation, migration and/or infiltration, even though it occurred early, was maximal and significantly greater than controls at a later phase, specifically 7-14 weeks post CNS lesion.

Interestingly, even though the effects of the pre-conditioned peripheral lesion did not trigger optic nerve regeneration, they did promote a greater influx of $CD68^+$ inflammatory cells (macrophages) into the CNS as compared to our control group. This phenomenon was recently reported by Zhang and colleagues (2007), where they described a significant increase in macrophage infiltration into the spinal parenchyma after a single peripheral nerve lesion (Zhang et al., 2007). Our results support Zhang et al's findings

and we extend their descriptive views from local macrophage infiltration into the CNS compartment, to a more robust macrophage influx post peripheral nerve lesion throughout the CNS. Intriguingly, we also report that the combination of the pre-conditioning peripheral nerve axotomy and optic nerve lesion, resulted in low numbers of macrophage cells ($CD68^{+}$) observed throughout the spinal cord, as compared to controls (data not shown). This provides further evidence for the contribution of peripheral nerve lesion in macrophage infiltration into the CNS compartment. Our data also suggest that the infiltration of macrophages in this case, was not sufficient to trigger the regeneration of the optic nerve. It is possible that other factors may play a role in the enhanced optic nerve regeneration described following optic lens injury and sciatic nerve implantation into the vitreous body outlined in previous studies (Berry et al., 1996; Fischer et al., 2001).

According to Ransohoff and colleagues (2003), infiltration of macrophage cells under normal CNS physiological conditions details essential functions of immunosurveillance and as explained by others, it offers an extensive host defence range through regions of the CNS previously assumed to be immunoprivileged sites (Carson et al., 2006; Ransohoff et al., 2003). In the past decade different views, including ours, have redefined the CNS from a state of immune privilege to immunologically ready whenever necessary. This state can dramatically be altered after CNS trauma due to an overwhelming invasion of circulating immune cells and activation of astrocytes. Interestingly,

a dual role for astrocytes has been described, where they are detrimental to growing axons due the glial scar formation but beneficial in BBB repair and attenuation in leukocyte infiltration as demonstrated in mutant mice depleted of GFAP-positive astrocytes (Bush et al., 1999). Therefore, it is important that we recognised that the CNS, has the ability to mount a highly organised response through the induction of an innate and adaptive immune response (Nguyen et al., 2002).

It is noteworthy that from our observations macrophage cells were also detected in close proximity to the uninjured contralateral eye as early as 7 days post CNS lesion, with close examination of retinal flat-mount revealing the presence and/or activation of $CD68^{+}$ cells inside the uninjured eye (refer to Appendix A Fig. A3). This was an unexpected observation since the vitreous body is normally suppressive to inflammation (Leon et al., 2000). However, we interpret this as a form of an autoimmune eye disease known as sympathetic ophthalmia, where injury in one eye leads to the progressive loss of vision in the contralateral, uninjured eye (Bom et al., 2002). Although, the incidence of this condition is low, even when the injured eye is retained (Gurdal et al., 2002).

Even though not explored here, it can be assumed that local inhibitory molecules affected both experimental groups in this study by limiting optic nerve growth. This could have been due to the absence of beneficial factors

mediated by the effects of macrophage activation following lens injury (Fischer et al., 2001; Leon et al., 2000) and peripheral nerve implantation in the vitreous body (Berry et al., 1996; Berry et al., 1999). It was noted by Berry and colleagues (1996) that peripheral nerve fragment implantation into the vitreous body stimulated more RGCs to regenerate their axons into the optic nerve, especially when the implant was derived from an injured peripheral nerve, allowed to degenerate *in vivo* for several days prior to use (Berry et al., 1996). Authors credited their results to a beneficial local environment created within the vitreous body and to the fact that injured nerve implants stimulated considerable macrophage infiltration into the eye, which contributed to the observed local regenerative response. Conversely, a study by Ellezam and colleagues (2001) reported that the effects of optic nerve regeneration observed following spinal cord homogenate vaccination were attributed to the formation of systemic antibodies that enabled CNS regeneration (Ellezam et al., 2003). It is therefore, clear that different mechanisms might be playing a role here. Based on our results, the beneficial effects of adult pre-conditioned DRG neurons only extends locally and in this case these do not appear to be mediated by primed circulating antibodies. Furthermore, we propose that the cell body DRG response to injury and the proximity to the centrally lesioned neurons is critical for the regeneration to occur. Which is likely to be correlated with elevated levels in trophic support and/or regeneration-associated genes (RAGs), as proven in a study investigating the effects of successful CNS regeneration shown to be dependant on the proximity of

these neurons to their cell bodies (Fernandes et al., 1999).

3.6 Conclusion

The combination of a pre-conditioned peripheral nerve axotomy and optic nerve lesion did not result in optic nerve regeneration. However, even though there was no regeneration observed in this model, our results revealed that the pre-conditioning peripheral nerve lesion did trigger a robust macrophage infiltration into the CNS compartment. In this model of injury, macrophage infiltration was not sufficient to promote the regeneration of injured optic nerves but at the same time these cells cannot be ruled out as contributing factors to CNS regeneration. Since it is evident that in the conditioning of adult DRG neurons, these cells might be playing a crucial role (Lu and Richardson, 1991).

3.7 Future Studies

As per original injury model described by Richardson and Issa (1984), to maximise the intrinsic capability for CNS neurons to regenerate, our optic nerve lesion was performed 7 days post peripheral nerve lesion. If the infiltration of macrophage cells is responsible for the intrinsic capability of adult DRG neurons to regenerate, then it is possible that these cells did not migrate the optic nerve within the specified time. In a follow up project, the 7 days post CNS injury should be extended and/or the location of peripheral

nerve injury should be relocated to accommodate for the distances between lesions. For instance, if the local environment plays a crucial role in CNS regeneration then a pre-conditioned peripheral lesion (e.g. trigeminal nerve injury) within a closer proximity to the CNS lesion (e.g. optic nerve crush) could result in improved CNS regeneration. In addition, the notion that the presence of macrophage cells is critical for the regeneration of the CNS DRG branch is absolutely necessary should be assessed by the depletion of these cells from the system (explored in Chapter 4).

3.8 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.

Benowitz, L., Yin, Y., 2007. Combinatorial treatments for promoting axon regeneration in the CNS: Strategies for overcoming inhibitory signals and activating neurons' intrinsic growth state. Develop. Neurobiol. 67, 1148-1165.

Berkelaar, M., Clarke, D., Wang, Y., Bray, G., Aguayo, A., 1994. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J Neurosci. 14, 4368-4374.

Berry, M., Carlile, J., Hunter, A., 1996. Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. J Neurocytol. 25, 147-170.

Berry, M., Carlile, J., Hunter, A., Tsang, W., Rosustrel, P., Sievers, J., 1999. Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts. J Neurocytol. 28, 721-741.

Bethea, J., 2000. Spinal cord injury-induced inflammation: a dual-edged sword. Prog. Brain Res. 128, 33-42.

Bom, S., Young, S., Gregor, Z., Lightman, S., 2002. Surgery for Choroidal Neovascularization in Sympathetic Opthalmia. Retina 22, 109-111.

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.

Bush, T., Puvanachandra, N., Horner, C., Polito, A., Ostenfeld, T., Svendsen, C., Mucke, L., Johnson, M., Sofroniew, M., 1999. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scarforming, reactive astrocytes in adult transgenic mice. Neuron 23, 297-308.

Carson, M., Doose, J., Melchior, B., Schmid, C., Ploix, C., 2006. CNS immune privilege: hiding in plain sight. Immunol. Rev. 213, 48-65.

Cohen, A., Bray, G., Aguayo, A., 1994. Neurotrophin-4/5 (NT 4/5) increases adult rat retinal ganglion cell survival and neurite outgrowth in vitro. J Neurobiol. 25, 953-959.

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

David, S., Bouchard, C., Tsatas, O., Giftochristos, N., 1990. Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. Neuron 5, 463-469.

Domeniconi, M., Filbin, M., 2005. Overcoming inhibitors in myelin to promote axonal regeneration. J Neurol. Sci. 233, 43-47.

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.

Fernandes, K., Fan, D., Tsui, B., Cassar, S., Tetzlaff, W., 1999. Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. J Comp. Neurol. 414, 495-510.

Filbin, M., 2006. How inflammation promotes regeneration. Nat. Neurosci. 9, 715-717.
Fischer, D., Heiduschka, P., Thanos, S., 2001. Lens-injury stimulated axonal regeneration throughout the optic pathway of adult rats. Exp. Neurol. 172, 257-272.
Fischer, D., Pavlidis, M., Thanos, S., 2000. Cataractogenic lens injury prevents traumatic ganglion cell death and promotes axonal regeneration both in vivo and in culture. Invest. Ophthalmol. Vis. Sci. 41, 3943-3954.
Fournier, A., Beer, J., Arregui, C., Essagian, C., Aguayo, A., Mckerracher, L., 1997. Brain-derived neurotrophic factor modulates GAP-43 but not T alpha1 expression in injured retinal ganglion cells of adult rats. J Neurosci. Res. 47, 561-572.
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.
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.
Gurdal, C., Erdener, U., Irkec, M., Orhan, M., 2002. Incidence of sympathetic ophthalmia after penetrating eye injury and choice of treatment. Ocul. Immunol. Inflamm. 10, 223-227.
Hendriks, J., Teunissen, C., de Vries, H., Dijkstra, C., 2005. Macrophages and neurodegeneration. Brain Res. Rev. 48, 185-195.
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.
Jonas, J., Budde, W., 2000. Diagnosis and pathogenesis of glaucomatous optic neuropathy: morphological aspects. Prog. Retin. Eye Res. 19, 1-40.
Keirstead, S., Rasminsky, M., Fukada, Y., Carter, D., Aguayo, A., Vidal-Sanz, M., 1989. Electrophysiologic responses in hamster superior colliculus evoked by regenerating retinal axons. Science 246, 255-257.
Lazarov-Spiegler, O., Solomon, A., Schwartz, M., 1998b. Peripheral Nerve-Stimulated Macrophages Simulate a Peripheral Nerve-Like Regenerative Response in Rat Transected Optic Nerve. Glia 24, 329-337.
Lazarov-Spiegler, O., Solomon, A., Zeev-Brann, A., Hirschberg, D., Lavie, V., Schwartz, M., 1996. Transplantation of activated macrophages overcomes central nervous system regrowth failure. FASEB J 10, 1296-1302.
Leon, S., Yin, Y., Nguyen, J., Irwin, N., Benowitz, L., 2000. Lens injury stimulates axon regeneration in the mature rat optic nerve. J Neurosci. 20, 4615-4626.
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.
Lombard, Y., Giaimis, J., Makaya-Kumba, M., Fonteneau, P., Poindron, P., 1994. A new method for studying the binding and ingestion of Zymosan particles by macrophages. J Immunol. Meth. 174, 155-165.

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, Y., Hsieh, T., Forbes, M., Johnson, J., Frost, D., 1998. BDNF injected into the superior colliculus reduces developmental retinal ganglion cell death. J Neurosci. 18, 2097-2107.
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.
Mey, J., Thanos, S., 1993. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res. 602, 304-317.
Nguyen, M., Julien, J., Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? . Nat. Rev. Neurosci. 3, 216-227.
Paxinos, G., Watson, C., 1998. The rat brain. Academic Press, California.
Ransohoff, R., Kivisakk, P., Kidd, G., 2003. Three or more routes for leukocyte migration into the central nervous system. Nat. Rev. Immunol. 3.
Richardson, P., Issa, V., 1984. Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309, 791-793.
Sawai, H., Clarke, D., Kittlerova, P., Bray, G., Aguayo, A., 1996. Brain-derived neurotrophic factor and neurotrophin 4//5 stimulate growth of axonal branches from regenerating retinal ganglion cells. J Neurosci. 16, 3887-3894.
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.
Schwartz, M., Butovsky, O., Bruck, W., Hanisch, U., 2006a. Microglial phenotype: is the commitment reversible? TINS 29, 68-74.
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.
Streit, W., 2002. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40, 133-139.
Thanos, S., Naskar, R., Heiduschka, P., 1997. Regenerating ganglion cell axons in the adult rat establish retinofugal topography and restore visual function. Exp. Brain Res. 114, 483-491.
van Rossum, D., Hilbert, S., Strabenburg, S., Hanisch, U., Bruck, W., 2008. Myelin-phagocytosing macrophages in isolated sciatic and optic nerves reveal a unique reactive phenotype. Glia 56, 271-283.

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.

Vela, J., Yanez, A., Gonzalez, B., Castellano, B., 2002. Time course of proliferation and elimination of microglia/macrophages in different neurodegenerative conditions. J Neurotrauma 19, 1503-1520.

Vidal-Sanz, M., Bray, G., Villegas-Perez, M., Thanos, S., Aguayo, A., 1987. Axonal regeneration and synapse formation in the superior colliculus by retinal ganglion cells in the adult rat. J Neurosci. 7, 2894-2909.

Weibel, D., Cadelli, D., Schwab, M., 1994. Regeneration of lesioned rat optic nerve fibres is improved after neutralization of myelin-associated neurite growth inhibitors. Brain Res. 642, 259-266.

Whiteley, S., Sauve, Y., Aviles-Trigueros, M., Vidal-Sanz, M., Lund, R., 1998. Extent and duration of recovered pupillary light reflex following retinal ganglion cell axon regeneration through peripheral nerve grafts directed to the pretectum in adult rats. Exp. Neurol. 154, 560-572.

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.

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.