Chapter 5: General Discussion

5.1 Overview

Trauma to the CNS, especially after SCI normally results in paralysis and loss of neural function below the site of injury. In an effort to understand the pathological manifestations of this injury and promote regeneration in the adult CNS, many animal species including rodents and even non-human primates have been used as models of injury (reviewed in (Moon and Bunge, 2005). In recent years, progress in SCI research have shed some light into the underlying mechanism following the initial CNS trauma, as well as the effects behind secondary injury; which have led to the development of clinical therapies, such as the current use of methylprednisolone (reviewed in (Lammertse, 2004). Consequently, paradigms alike have generated a considerable expectation that translational animal work will soon provide new clinical therapies for SCI patients (reviewed in (Amador and Guest, 2005).

In this final discussion chapter, we provide a brief overview into the dichotomy of macrophage activation, analysis of experimental findings, and future directions for SCI in relation to our results.

5.1.1 Macrophage Activation: A Double, Split Personality

As aforementioned in the main introduction, inflammation by the activation of macrophages has been previously regarded as injurious to the system,

especially in chronic conditions where inflammation is non-regulated, such as in MS (reviewed in (Correale and Villa, 2004). However, based on recent evidence this preconceived notion is constantly being modified from evidence suggesting that under the right conditions, an immune response can be favourable to the injured system (Hauben and Schwartz, 2003; Hendrix and Nitsch, 2007; Moalem et al., 1999; Rapalino et al., 1998; Yin et al., 2003). This evidence has identified inflammatory cells as a 'double edge sword' (Bethea, 2000; Wyss-Coray and Mucke, 2002) given their dual ability to cause neuronal damage and concomitantly exert neuroprotection (Stoll et al., 2002).

It is clear that inflammation, whether 'classically (pro-inflammatory)' or 'alternatively (anti-inflammatory)' will remain controversial in CNS repair and/or regeneration for quite some time. This is based on the emerging disparity in experimental evidence suggesting a dichotomous role for inflammation after CNS injury. For instance, the use of pharmacological anti-inflammatory agents (i.e. clinically approved FDA drug) for SCI, methylprednisolone (MP), has been reported to improve neurological recovery in patients as long as this drug is administered within the first 8 hours after injury (Bracken et al., 1990) and for at least 48 hours (Bracken et al., 1997). However, based on experimental data it has been argued that the use of MP has resulted in other complications with no neurological improvement (reviewed in (Sipski and Pearse, 2006), along with debatable effectiveness (Hurlbert, 2000). For example, in spinal cord injured rats, the use of this agent in the acute phase post lesion did not lead to tissue

sparing and/or recovery of locomotor function (Rabchevsky et al., 2002). Yet, in another rodent study MP was described to be neuroprotective to the injured spinal cord by reducing the extent of gliosis formed after trauma (Liu et al., 2008). It is noteworthy that a limiting factor in the efficacy of MP as a clinical treatment is the required timing for which this drug needs to be administered to the patient, given that some SCI cases might not be able to receive this treatment within the specified therapeutic window of 8 hours post injury. In addition, the use of MP has been described as ineffective in the treatment of penetrating spinal cord injuries (Levy et al., 1996).

Inflammation might be beneficial under certain circumstances, however, the use of anti-inflammatory drugs in demyelinating CNS lesions, has been shown to reduce oligodendrocyte remyelination, although, this effect was reversed after termination of treatment (Chari et al., 2006). Moreover, the selective depletion of these inflammatory cells in the acute remyelination phase, impairs oligodendrocyte functionality (Kotter et al., 2001). In contrast, decreased infiltration of inflammatory cells following SCI has reportedly attenuated vascular permeability, reduced tissue damage and improved locomotor recovery (Noble et al., 2002). Given that the amount of anatomical sparing by the demyelination process after axonal injury has been attributed to the influx of inflammatory cells (Bruck et al., 1995). Similarly, their depletion has also been demonstrated to decrease post-traumatic tissue loss and improve locomotion (Popovich et al., 1999).

At this time, it is difficult to control the split personality of inflammatory cells and hence the inability to use them as clinical therapies for the repair and regeneration of injured neurons in SCI patients. Experimental work aiming to understand the concept 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) is likely to remain an area of interest for quite some time as scientist decipher the true functionality underlying these cells. This balance in cellular activation, as illustrated in a hypothetical model in response to CNS injury, is likely to be mediated by: (i) genetic influences (i.e. immunity and vulnerability of the organism); (ii) cytokine and trophic factor exposure; (iii) local glial reaction; as well as, (iv) the predominance of immune cells in the CNS (reviewed in (Olsson et al., 2003).

5.2 Analysis of Experiments

The work described in this thesis has examined the mechanism/s underlying the pre-conditioned lesion model. Here, we report that the beneficial regenerative response of this model is mediated by the immunological changes triggered by the pre-conditioning peripheral nerve lesion. According to our findings, these changes are driven by a systemic increase in serum BDNF concentration and a favourable macrophage functionality at the SCI epicentre. In addition, we have provided further evidence into the immune and nervous system interaction, which we believe is contributing to the early presence of inflammatory cells within

the CNS compartment and therefore supporting regeneration of injured primary afferent fibres.

5.2.1 Pre-Conditioned Lesion Model

Axotomy of the peripheral DRG branch initiates a multi-phasic cellular and molecular response within the DRG cell body that appears to be synergistically coupled to a regenerative response of injured sensory axons. For instance, this peripheral lesion has been associated with triggering a cascade of events within the DRG such as: (i) generation of an increased immune cell activation (Lu and Richardson, 1991) observed in the ipsi- and contra-lateral DRG (Dubovy et al., 2007); (ii) increased glial expression of nerve growth factor receptor in bilateral DRG (Zhou et al., 1996); (iii) formation of peri-cellular baskets around DRG cell bodies (McLachlan et al., 1993); (iv) increased synthesis and secretion of NTs like BDNF from the DRG (Deng et al., 2000); and (v) increased expression of regeneration-associated genes (RAGs) within the DRG, for example GAP-43 (Schreyer and Skene, 1993). These factors are most likely interacting with and contributing to the observed regeneration of the DRG branch extending within the CNS (Richardson and Issa, 1984). This is particularly true given that if only the CNS part is lesioned, there is no axonal regeneration, as normally demonstrated in other mature CNS neurons (reviewed in (Filbin, 2003).

Hereafter, we will briefly discuss possible factors contributing to and/or underlying the observed CNS regeneration in the pre-conditioned lesion model and how these changes relate to our findings. Some of these

factors include: cyclic adenosine monophosphate, BDNF and macrophage activation, infiltration and depletion.

5.2.1.1 Molecular Level

5.2.1.1.1 Implications of DRG Cell Body Activation

As mentioned above, several changes occur within the DRG in response to peripheral nerve axotomy, some of which occur at the molecular level. For instance, one of two studies (published simultaneously in the same journal) indicated that the *in vivo* intraganglionic microinjection of a cAMP (cyclic adenosine monophosphate) analog (i.e. db-cAMP), effectively mimicked the regenerative effect of the DRG CNS branch, normally attributed to peripheral axotomy (Neumann et al., 2002). In fact, *in vitro* culture of DRG neurons already exposed to cAMP rendered these neurons non-responsive to the inhibitory properties of CNS myelin, consequently resulting in significant neurite outgrowth (Neumann et al., 2002). In another study, Qiu and colleagues demonstrated that cAMP expression is increased 3-fold 1-day post peripheral lesion compared to normal levels and by 1-week post lesion even though cAMP levels returned to baseline, the growth of DRG neurons in the presence of inhibitory cues improved (Qiu et al., 2002). Interestingly, in RGC elevation in cAMP levels concomitantly increases the responsiveness to trophic factors such as BDNF, thus proposing that cell death might be counteracted by a combination treatment of cAMP and trophic factors (Goldberg and Barres, 2000; Shen et al., 1999). Certainly, the effects of BDNF on CNS regeneration has been demonstrated to be beneficial to

axonal growth (Bouhy et al., 2006; Kobayashi et al., 1997; Mamounas et al., 2000; Xu et al., 1995), especially in chronically injured spinal cord neurons, which were shown to regenerate and remain responsive to BDNF even 1-year after CNS trauma (Kwon et al., 2002). Certainly, earlier observations by Hammond et al, indicated that *in vivo* BDNF treatment of axotomised CNS neurons within the acute injury phase appeared to prolong neuronal survival for at least 42-days post lesion (Hammond et al., 1999). These findings have proven to be extremely valuable in understanding the underlying mechanism behind the regeneration of injured CNS neurons, given the clinical impracticality of performing a peripheral conditioning lesion to treat SCI in humans.

Interestingly, based on findings from our study (Chapter 2) we have provided evidence indicating that axotomy of the peripheral DRG branch, just prior to CNS injury, concurs with elevated BDNF levels, not found after CNS injury only. Mechanistically, although not tested here, we propose that based on the increased levels in BDNF prior to SCI, and the beneficial effect of trophic factors on CNS regeneration, these events are most likely integrating with cAMP and consequently reducing the responsiveness of injured neurons to myelin and its associated inhibitors (Neumann et al., 2002; Qiu et al., 2002). Which is in accordance to what is observed early in development. Therefore, it is conceivable that some type of recapitulation of environmental cues from adulthood to early in development might be the key in the complete regeneration of adult spinal cord injured neurons (reviewed in (Harel and Strittmatter, 2006). Given

that, cAMP levels have been shown to be higher in younger neurons, which are not inhibited by myelin during development, however, as the system matures levels of cAMP are reduced and this coincides with the onset of inhibition in the CNS (Cai et al., 2001).

As discussed here, there are several molecular factors that can mimic the regenerative effects of the primary afferent CNS fibres after a pre-conditioning lesion to the peripheral DRG branch. Also of interest, is to investigate the relationship between this conditioning lesion and the cellular events triggered by these factors in order to understand and design strategic approaches suitable for SCI patients.

5.2.1.2 Cellular Level

5.2.1.2.1 Macrophage Cells

The intrinsic ability of adult DRG neurons to regeneration in the mature CNS has been attributed to the events resulting from the peripheral nerve lesion (Richardson and Issa, 1984) and in addition to the molecular factors described above, there is also a cellular contribution to this regenerative competence. The cellular component participating in the regeneration of the central DRG branch has been ascribed to the inflammatory response within the DRG cell body following peripheral axotomy (Lu and Richardson, 1991). Which has been found to extend to both ipsi- and contra-lateral DRGs after unilateral nerve injury (Dubovy et al., 2007). Interestingly, intraganglionic injections with either macrophage cells or an

inflammatory agent consequently promoted axonal regeneration, hence indicating that inflammation within the DRG cell body is important for regeneration (Lu and Richardson, 1991).

Here, consistent with other studies, we report that there are cellular changes originating from the peripheral nerve axotomy that extend to both ipsi- and contra-lateral DRGs, which include infiltration of inflammatory cells (i.e. macrophages) (Dubovy et al., 2007; Hu and McLachlan, 2003a; McLachlan et al., 1993), as well as an up-regulation in satellite glia expression (Zhou et al., 1999). Pathologically, after PNS injury, both of these cells types form perineuronal baskets enclosing DRG cell bodies, a factor, which might also be contributing to the regenerative trigger by the release/synthesis of trophic factors (Zhou et al., 1999; Zhou et al., 1996), cytokines (reviewed in (Ramer et al., 1999) and chemokines from the injured neuron and/or the satellite glia (Hu and McLachlan, 2003a).

Macrophages are multi-functional, inflammatory cells involved in angiogenesis (Kodelja et al., 1997), development (Gouon-Evans et al., 2000; Lang and Bishop, 1993), axonal sprouting (Batchelor et al., 2002), secretion of trophic factors (Batchelor et al., 1999; Bouhy et al., 2006), phagocytosis of myelin debris (Bruck and Friede, 1990; David et al., 1990) and regeneration (Lazarov-Spiegler et al., 1996; Luk et al., 2003; Perry et al., 1987; Rapalino et al., 1998; Yin et al., 2003). Moreover, in accordance to our results, we can confirm that in the pre-conditioned lesion model, macrophage cells play a beneficial role in the axonal regeneration of the

adult DRG branch extending into the CNS. On several occasions at the SCI epicentre, we found a positive relationship between macrophage presence and CNS regeneration. This relationship was strengthened by the active/direct vaccination of pre-conditioned animals with a PNS homogenate (sciatic nerve), which resulted in enhanced macrophage infiltration and improved CNS regeneration (Chapter 2). Which indirectly suggests the involvement of the animal's immune system (Ellezam et al., 2003; Huang et al., 1999), as a tool for ameliorating the limited amount of CNS regeneration observed in the pre-conditioned lesion model.

The relationship between macrophage cells and CNS regeneration was further examined by the depletion of these cells during the conditioning phase of DRG neurons (i.e. axotomy of peripheral DRG branch). Interestingly, here we report that the systemic depletion of these cells via a liposome treatment delivery abolished the regenerative trigger characteristic of the pre-conditioned lesion model (Chapter 4). In addition, as a result of the temporal macrophage depletion during the conditioning phase, we found an increased astrocytic expression within the vicinity of the CNS lesion, as well as a reduction in BDNF serum levels. We propose several possibilities for the lack of axonal regeneration in this model after macrophage depletion and these might due to a reduction in: (i) trophic support to injured neurons; (ii) phagocytosis of myelin debris; (iii) cAMP levels; (iv) regeneration-associated genes; (v) macrophage numbers; and/or (vi) cellular interaction/communication with other immune/neuronal cells.

In relation to the regenerative trigger observed in the pre-conditioned model, we propose that based on our findings peripheral nerve lesion allows for a rapid/early macrophage infiltration into the CNS compartment prior to the disruption of the BBB by the CNS injury (i.e. dorsal column cut). Mechanistically, we believe this key event provides a considerable advantage in the regrowth of injured CNS neurons with early macrophage activation prior to the full expression/activation of inhibitory molecules in the degenerating CNS (Fig. 36A-B). We believe this is an advantageous attribute in the regeneration of injured CNS neurons, given the proposal that: (i) the limited communication between the immune and nervous system is responsible for the inadequate ability of the CNS to mount a suitable immune response required for regeneration to occur (reviewed in (Schwartz et al., 1999b); (ii) the intrinsic capacity for PNS neurons to regenerate is likely to be mediated by the efficient clearance of myelin debris in the PNS, compared to the delayed removal in the CNS (reviewed in (Vargas and Barres, 2007) attributed to the kinetics of macrophage cells (reviewed in (Trivedi et al., 2006); and (iii) macrophages have the ability to change the non-growth inhibitory property of the CNS environment into a growth permissive (David et al., 1990) as well as contribute to oligodendrocyte precursor differentiation important for remyelination (Kotter et al., 2006).

Currently, the only direct explanation as to the differential effects exerted by macrophages (mentioned above) is that these cells might be

expressing different phenotypes (Duffield, 2003; Gordon, 2003; Ma et al., 2003; Mosser, 2003). To exploit the beneficial effects of inflammatory cells, like macrophages, different factors such as the site, timing and intensity of cellular activation need to be considered (Schwartz et al., 2006a; Schwartz and Yoles, 2006). Therefore, based on our results, we believe that the complex cascade of events originating from the peripheral nerve lesion might be beneficially activating macrophages, which is in turn permitting immune cell access into the CNS compartment by priming it *per se*, prior to SCI. Thus providing the system with the required ingredients for successful regeneration within the CNS. In addition, we reiterate that axotomy of the peripheral DRG branch did not trigger the regeneration of injured neurons in other parts of the CNS (optic nerve lesion – Chapter 3), suggesting that the beneficial effects of macrophage functionality, at least after peripheral axotomy, were limited only to the central DRG branch. Concomitantly, we propose that the somatic DRG response as well as the proximity to the lesioned neurons is critical for the regeneration of the central DRG branch to occur, due to an increased support in trophic factors and up-regulation in RAGs. Which is consistent with the regeneration of injured CNS neurons as a result of increased RAG expression, shown to be dependant on the proximity of these neurons to their cell bodies (Fernandes et al., 1999).

The reality that macrophages are known for their controversial role in CNS repair and regeneration, together with the fact that we are only just beginning to understand some of their basic functionality, ultimately do not

provide a risk to benefit ratio suitable for the use of these cells as a therapeutic agent in a clinical setting. This is mainly due to the probability that these cells can potentially exacerbate the existing injury (Donelly and Popovich, 2008). Nonetheless, although the use of these cells seems like a challenging task at hand, we have provided some interesting evidence for possible avenues to be further investigated, such as the development of strategies to induce early macrophage infiltration and rapid phagocytosis of myelin debris, perhaps through a vaccination regimen, to provide a growth permissive CNS environment for axonal regeneration.

5.3 Future Direction

Given the conflicting view of macrophage cells in CNS regeneration it is clear that more work needs to be undertaken to understand the dichotomy of macrophage activation and how these changes can be modulated to favour CNS repair and regeneration. Based on our findings, we propose that the following experiments be conducted:

A) Macrophage isolation *ex vivo* from pre-conditioned lesion animals to study these cells under what could be regarded as “appropriate phenotypic activation”. This could provide direct insight into the beneficial functionality of these cells in CNS regeneration following axotomy of the peripheral DRG branch and in relation to their cytokine profile and/or phagocytotic properties.

B) Characterisation of the neuro-immune relationship (i.e. after peripheral nerve axotomy), which appears to be allowing for the infiltration of activated macrophage cells into the CNS compartment under minimal BBB disruption. The mechanisms underlying this inflammatory cell response would be of great interest in other pathological conditions, like in neuropathic pain (Zhang et al., 2007).

C) Investigation of the extent of plasticity in this pre-conditioned lesion model, such as the formation of new intraspinal circuitry (Bareyre et al., 2004). Which could provide useful information regarding the regenerative potential and extent of functionality that could be gained from this type of conditioning injury and consequent neural reorganisation within the CNS. Further work should also incorporate synaptic and electrical assessments to determine possible synaptic connectivity to appropriate targets.

5.4 Final Word

Of utmost importance to human SCI patients is the development of suitable clinical therapies, one that could ideally accommodate for acute, chronic and penetrating spinal injuries. It is widely understood by the scientific community that an efficacious SCI treatment will most likely be a combinatorial approach, probably utilizing the best effective therapies to treat this type of injury (Harel and Strittmatter, 2006). Furthermore, the finding that only a small percentage of spared fibres (5-10%) is sufficient for effective locomotion (Blight, 1983), certainly seems promising. Ultimately, findings such as the ones presented here could help redefine

methods for selective immunomodulation, which may preserve or promote regeneration after SCI. In principle, what is needed, is a therapy designed to maximise the beneficial inflammatory effects and at the same time reduce factors that create injury; a therapy like this currently does not exist (reviewed in (Donelly and Popovich, 2008).

5.5 Reference List

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