DETERMINING IF PRE-EXISTING INFLAMMATION AFFECTS FACIAL MOTONEURONAL SURVIVAL

A thesis submitted for the Degree of Doctor of Philosophy

THE UNIVERSITY of ADELAIDE

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Bachelor of Health Science (Hons)

Discipline of Anatomy and Pathology

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Adelaide, Australia

October, 2016

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Abstract

Motor Neuron Disease (MND) is a debilitating condition affecting ageing individuals that is characterised by the death of motoneurones. Changes in the neurotrophic support of motoneurones, altered glial and synaptic activity and levels of inflammation have been proposed as mechanisms underlying MND, but these have not been considered in the light of advancing age, which is the single biggest risk factor for MND. This thesis investigates the roles of age, neurotrophic factors, perineuronal responses through glial and synaptic activity, and levels of inflammation in experimental models of rat facial motoneuronal death and rescue. It pays particular attention to an isoform of IGF-1 derived from active muscle, known as Mechano Growth Factor (MGF) and its mechanism of action. It investigates if perineuronal responses can predict the fate of injured motoneurones. It also correlates age-related changes in levels of inflammatory mediators with motoneuronal survival after injury. It reports that: (i) MGF rescues mature motoneurones by a mechanism that is independent of the IGF-1 receptor and Protein Kinase C; (ii) perineuronal responses such as glial and synaptic activity may still predict the fate of injured motoneurones; (iii) the survival of avulsed facial motoneurones increases with age and that this is associated with an increase in levels of inflammation in the CNS but not in the blood; (iv) immune-priming does not affect the survival of mature motoneurones and (v) the immune response to challenge changes with age. Results are considered in the context of current ideas on the roles of ageing, perineuronal activity, neurotrophic factors and inflammation in neuronal death and survival. It is proposed that immature and mature motoneurones respond differently to motoneurone rescue factors and changes in levels of inflammation. This calls into question some commonly held views on the mechanisms underlying age-related neuronal degeneration such as MND.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Dedication

To Komalam Perumal and Narasamy Katharesan.

For all that you sacrificed and continue to do…

For always encouraging me to learn…

For all your love…

Acknowledgements

First and foremost, I would like to thank my supervisor Associate Professor Ian Johnson and his complimenting wife Carole, for their invaluable support academically and otherwise. Excellent supervisors leave a huge imprint on a student's life and Ian was no exception to this with his constant guidance and high level of patience. This thesis and my minor academic achievements would not have been possible without his trust, support and motivation.

Secondly, I must thank the numerous technical and academic staff that have been just as pivotal to my research: Professor Robert Vink as a secondary supervisor and Team Neuro; Staff from the University of Adelaide Histology Laboratory; Adelaide Microscopy; Laboratory of Animal Services within the Medical School and Waite Campus.

To the friends that have motivated me endlessly at work: Chris Leigh for being the versatile realist, advisor and motivator; My girls, namely Kimberley Mander, for sharing the many peaks and troughs within our cosy PhD office; Danijela and Mario for the last big bout of push that I needed.

To my small family – Ma, Pa, Bro and Leny – my 4 pillars of strength. I want to thank them for helping me to realise and celebrate even the smallest of accomplishments along the way. Last, but in no way least, to my best friend and love - Leny. I want to thank him for putting up with me throughout my tantrums, early hour drop-offs, late-night pickups, donut surprises on long days, for providing a listening ear to my endless woes and for his supportive shoulder.

1 Literature Review

1.1 Motoneurone anatomy and pathology

Motoneurones are the final common pathway for transferring neural activity in the Central Nervous System (CNS) to muscle (Sherrington 1906). Upper motoneurones (UMNs) originate in the cerebral cortex and extend their axons out to synapse on Lower Motoneurones (LMNs), which reside within either cranial nerve nuclei or the spinal cord. LMNs then extend their axons to innervate peripheral target muscle via neuromuscular junctions, to elicit muscular contractions. Sufficient motoneuronal death results in muscle paralysis and as muscles supplying vital life functions fail, eventual death of the organism ensues (Paez-Colasante, Figueroa-Romero et al. 2015).

Motor Neuron Disease/Amyotrophic Lateral Sclerosis (MND/ALS) is a debilitating condition, predominantly affecting ageing individuals and is characterised by the death of motoneurones. Sporadic MND affects 90 – 95% of the population while 5 – 10% can be attributed to familial MND (fMND) (Van Den Bosch 2011, Philips and Rothstein 2015). Muscular atrophy and eventual fatal respiratory failure are clinical symptoms of MND, among others (Ekestern 2004). The disease drastically decreases quality of life and kills approximately 2 people every day in Australia, as 2 more are diagnosed with it (Talbot 2002). Its cause is unknown, there is no cure and therapeutic development for MND is severely hampered because the pathogenesis remains unknown. There are also multiple subsets of MND as different motoneurone populations can be affected (Kumar, Islam et al. 2016).

The degeneration of motoneurones can be limited to specifically the UMNs or the LMNs, or alternatively a combination of both UMNs and LMNs. Primary lateral

sclerosis for example, involves the degeneration of UMNs within the corticospinal tract. Whereas progressive bulbar palsy predominantly affects the corticobulbar tracts, involving cranial motoneurones, such as the facial motoneurones that supply facial muscles. Corticobulbar symptoms present more in middle to late age women and has a poor prognosis (McCombe and Henderson 2010). However, the overall clinical course of MND is highly variable. This suggests that pathogenesis could be multifactorial although pathogenetic mechanisms are often researched as separate entities using common experimental models (Bruijn, Miller et al. 2004).

1.2 Experimental Models and translational discrepancies

Currently available experimental models of motoneuronal degeneration predominantly take form as genetic MND experimental models. Such models have questionable applicability to human MND, as these experimental models utilise the immature motor system with familial MND genetic mutations. Data from these immature experimental models of fMND (which accounts for only approximately 5% of all human MND) are then extrapolated to the mature human system where 95% of MND occurs sporadically. One of the most commonly used experimental models to test hypotheses of MND mechanisms and potential therapies is the mutant Cu/Zn Superoxide Dismutase (SOD1) mouse model (Philips and Rothstein 2015). This model has arguable limitations in paralleling the most common form of MND. Sporadic MND accounts for 90-95% of cases, has no clear genetic component and affects individuals aged 50-60 years old (Wijesekera and Leigh 2009). In contrast, the SOD-1 mouse model has a genetic mutation present in less than 2% of cases of MND, its symptoms present within a few months of birth and 50% of these mice die by the time they are 4 months old (Bruijn, Miller et al. 2004). These discrepancies have often been neglected since familial and

sporadic MND are thought to be clinically similar (Vucic, Rothstein et al. 2014). That this view may not be entirely appropriate, however, is highlighted by the translational discrepancy between experimental studies and clinical trials. Thus, Mitsumoto et al (2014) reported that positive studies in SOD1 mice led to 67% of misled negative studies and up to 78% of negative randomised controlled trials (Van Den Bosch 2011, Philips and Rothstein 2015). Many preclinical models use in-vitro methods or the immature motor system and these models have important limitations. Cell culture models are often devoid of non-neuronal cells, afferent to motoneurones or peripheral target contact by the motoneurones, all of which are important in MND, as they could contribute to disease onset and progression (Haidet-Phillips, Hester et al. 2011). Likewise, the immature motor system is intrinsically different to the mature motor system, both in the normal state and its reaction to injury (Greensmith and Vrbova 1996, Johnson 2016). Given that MND predominates as a condition of later life, efforts should be made to take into account age-related variations in experimental models, if we ever aim to hasten their translation to clinical situations.

There are limited studies inherently looking at motoneuronal death and survival in aging animals. We suggest two possible reasons for this. Firstly, sustaining geriatric animals for long periods is difficult, as a previous study, without experimentation, had to humanely kill approximately 50% of ageing rats (Johnson and Duberley 1998). Secondly, unlike their immature counterparts, mature animals can be resistant to losing significant numbers of motoneurones following injury (Johnson, Sears et al. 1991, Aperghis, Johnson et al. 2003). Thus, researchers may prefer working on immature animals which are easier to obtain and maintain, and in which it is easier to provoke rapid and substantial motoneuronal death (Hamburger and Levi-Montalcini 1949,

Oppenheim 1991, Aperghis, Johnson et al. 2003) to then test the efficacy of therapeutics.

Taking all of this into consideration, our chosen injury model is the facial nerve axotomy model. When studying motoneuronal death (Chapters 2, 3, 4, 5 and 6), the facial nerve avulsion technique was employed and when studying motoneuronal survival (Chapter 3), the facial nerve transection technique was employed. The facial nerve axotomy model lends itself as an ideal test system to evaluate age-related and injury-associated differences because different types of nerve injury cause markedly different degrees of motoneuronal loss, ranging from lesions that are associated with the survival of most motoneurones and others that are associated with the death of most motoneurones (Moran and Graeber 2004). Numerous studies have shown massive motoneuronal death following nerve avulsion in mature adult rats (Aperghis, Johnson et al. 2003, Sakamoto, Kawazoe et al. 2003, Aperghis, Johnson et al. 2004). Nerve avulsion has been documented in spinal nerves to ultra-structurally cause axotomy at the nerve-rootlet-CNS interface (Fraher 1999) and this appears to be no different for cranial nerves such as the facial nerve (Guntinas-Lichius, Neiss et al. 1994, Moran and Graeber 2004, Sanagi, Nakamura et al. 2012). Thus, the facial nerve axotomy model will enable the study of motoneuronal death and rescue in mature animals, without any clear genetic component contributing to the degeneration of motoneurones. This may more accurately represent sporadic MND than the use of immature and/or genetically manipulated motoneurones. In this way, Chapters 2 to 6 use the facial nerve injury model to study the survival and death of injured facial motoneurones and the relationship of this to changes in their surrounding environments.

1.3 Current treatments and therapeutic strategies

Currently, the only treatment licensed for use in MND (Riluzole) is a glutamate antagonist that extends survival by merely three months, with very little functional improvement (Miller, Mitchell et al. 2007, Paez-Colasante, Figueroa-Romero et al. 2015). Thus, current interventions are primarily palliative to attempt to maintain patient quality of life. More recent investigations of microRNA (miRNA) based therapeutics are looking at: (i) antagonists to inhibit and (ii) antisense RNA to reduce, toxic-gain of function. This is to knockdown miRNA that has been dysregulated as a cause or consequence of cytotoxicity by diminished neurotrophic support, detrimental activity surrounding motoneurones and/or inflammation (Paez-Colasante, Figueroa-Romero et al. 2015, Kumar, Islam et al. 2016). However, an ongoing phase 1 clinical trial is only targeting SOD1 mRNA in fMND, so the applicability to sporadic MND remains unclear. Other therapeutics that have been trialled on the basis of encouraging animal studies are neurotrophic factors and immunomodulatory drugs (Talbot 2002). Insulin-like growth factor 1 (IGF-1) is one such neurotrophic factor subjected to scrutiny after displaying positive results in pre-clinical experimental models but not translating to the clinical situation (Kaspar, Llado et al. 2003, Dodge, Haidet et al. 2008, Kumar, Islam et al. 2016). The inefficacy of neurotrophic factors have been largely attributed to their varying mechanisms of neuroprotection, which can alter with age (Papanikolaou and Ellerby 2009).

Other therapeutic strategies for MND have looked at the suppression of glial activity. Glia are presumed to determine whether the motoneurones survive or die by modifying perineuronal synapses, altering neuroglial trophic support or by mediating direct neuronal damage (Strong 2003). Most glial modifying strategies (e.g. celecoxib,

cannabinoids, thalidomide and tocilizumab) also incorporate an anti-inflammatory element as these two pathogenetic mechanisms are thought to be interrelated in how they propagate disease (Kumar, Islam et al. 2016). The inefficiency of these drugs can be put down to the fact that there is still debate over whether glial activation and inflammation could be neuroprotective rather than neurodegenerative as noted in SOD1 mice models (Henkel, Beers et al. 2009, Lewis, Rasmussen et al. 2014, Jeyachandran, Mertens et al. 2015).

Given that neurotrophic factors, perineuronal synaptic- and glial- activity and also inflammation may play a role in determining motoneuronal survival, it is likely that therapeutic interventions will need to be based on a multifactorial approach towards the pathogenesis of MND (Zhou, Liu et al. 2016).

1.4 Neurotrophic factors and rescue mechanisms

One mechanism underlying motoneuronal rescue that has been largely researched is the role of neurotrophic support (Henderson, Yamamoto et al. 1998, Tovar-y-Romo, Ramírez-Jarquín et al. 2014). Neurotrophic factors promote repair and prevent cell loss in post-mitotic tissues such as motoneurones in the postnatal animal (Beck, Karch et al. 2001). However, caution needs to be taken when extrapolating data from studies of the role of neuroprotective agents in immature animals, to ageing animals and then to ageing humans in clinical trials. The immature system is often in “development mode”, thus predicting the “survival-promoting-effects” of neurotrophic factors in a developing system that is already largely dependent on neurotrophic signals may yield false positive results.

In keeping with this, numerous neurotrophic factors are known to act on motoneurones (Oppenheim, Haverkamp et al. 1988, Bennet, Gibson et al. 2002, Gould and Enomoto

2009, Casella, Almeida et al. 2010, Ohta, Arai et al. 2011). However, Glial cell line-derived neurotrophic factor (GDNF) (Hottinger, Azzouz et al. 2000, Sakamoto, Kawazoe et al. 2003), the liver-type isoform of Insulin-like growth factor 1 (IGF-1Ea) (Aperghis, Johnson et al. 2004, Goldspink and Harridge 2004) and more recently, an isoform of IGF-1 (IGF-1Eb) known as Mechano-Growth factor (MGF) (Dluzniewska, Sarnowska et al. 2005, Kandalla, Goldspink et al. 2011, Quesada, Ogi et al. 2011) have been found to be potent rescue factors for mature adult motoneurones. GDNF is produced by astrocytes and viral gene transfer to motoneurones has illustrated its neuroprotective effect on injured adult motoneurones (Sakamoto, Kawazoe et al. 2003, Kuno, Yoshida et al. 2006). IGF-1Ea is derived from the liver and promotes cell proliferation, differentiation and survival in non-neurodegenerative situations (Thimmaiah, Easton et al. 2003) and its homologues can shorten or prolong lifespan markedly in a variety of species (Shimokawa, Higami et al. 2002, Barbieri, Bonafe et al. 2003, Bonafe, Barbieri et al. 2003, Kaletsky and Murphy 2010). The isoform of IGF-1 known as MGF is expressed in the peripheral targets of motoneurones – i.e. muscle, in response to stretch and injury. Alternative splicing of the IGF-1 gene at the three prime end yields a transcript containing exon 4 spliced to exon 5 and 6 which creates this MGF isoform (Matheny, Nindl et al. 2010, Spangenburg 2010). More importantly, we and others have noted MGF to be a more potent rescue factor for adult motoneurones than the commonly used liver-type isoform IGF-1 (Yang and Goldspink 2002, Gorecki, Beresewicz et al. 2007). MGF is also acknowledged as a potential neuroprotectant in other models of neurodegeneration such as Parkinson's disease (Quesada, Micevych et al. 2009, Quesada, Ogi et al. 2011) and experimental stroke (Dluzniewska, Sarnowska et al. 2005). It is able to act in a paracrine and autocrine manner rather than an endocrine manner, due to its local synthesis (Aperghis, Johnson

et al. 2004). MGF’s short half-life can also be increased experimentally to increase efficacy as a therapeutic (Niu, Chen et al. 2014). As a result, MGF stands out as having the potential to play a role in both motoneuronal survival and ageing, thus there is a need to understand the mechanism by which it mediates protection of injured motoneurones.

In general, cellular effects of neurotrophic factors are mediated by primary receptors or secondary messengers as part of downstream signalling cascade systems. Endogenous IGF-1 has a high binding affinity to the IGF-1 receptor (IGF1r) which mediates its regulatory effects on neuronal populations (Dai, Wu et al. 2010, Werner and LeRoith 2014). While IGF-1r is reported to be largely expressed in the immature rat brain, during maturation, some regional concentrations have been noted where motor functions are coordinated (Bondy, Werner et al. 1992). Circulating IGF-1 is able to cross the blood-brain barrier via the IGF-1r and may be one reason why it comes across as a potent neuroprotectant (Werner and LeRoith 2014). IGF-1r gene mutations, however, have been shown to be present among centenarians, suggesting reduced IGF-1 signalling results in a longer lifespan, even though gene levels have not always correlated with expressed protein levels (Suh, Atzmon et al. 2008, Vogel and Marcotte 2012).

IGF-1 also has anti-apoptotic functions through multiple signalling pathways including the Protein Kinase C (PKC) and the Phosphotidylinositide 3 kinase-Protein Kinase B-mechanistic target of rapamycin (PI3k-Akt-mTOR) pathways (Zheng and Quirion 2006, Ohta, Arai et al. 2011). These pathways have also been linked to aging, neuroprotection and human SOD1 motoneurone survival (Li, Xu et al. 2003, Kirby, Ning et al. 2011, Ohta, Arai et al. 2011, O'Neill 2013). Quesada, Ogi et al. (2011) suggested that MGF elicited broad-spectrum neuroprotection by activating PKC to induce gene transcripts of

heme oxygenase-1, also known as heat shock protein 43 (HO-1). HO-1 acts as a fundamental defence for neurons exposed to oxidative stress challenges (e.g. reactive oxygen species).

MGF mediated neuroprotection of neurones in experimental Parkinson's disease appears to involve PKC along with other kinases and/or antioxidants. Yet, PKC knockdown alone completely blocked MGF-induced neuroprotection against 6OHDA cytotoxicity (Quesada, Ogi et al. 2011). Thus, it may be that MGF can act via different mechanisms to protect different subsets of neurones. Ohta, Arai et al. (2011) have demonstrated neurotrophic effects of a photosensitising dye through the activation of the PI3k-Akt survival signalling pathway. This in turn prevented the degeneration of cerebellar neurones and resulted in attenuation of motor discoordination in an animal model of cerebellar ataxia. Evolving research into Akt highlights it as a counteractant to apoptosis by the inhibition of caspase-activated enzymes. As a result, cell growth abilities and repair mechanisms are thought to be preserved (Martelli, Tabellini et al. 2012, Dajas-Bailador, Bantounas et al. 2014, Asati, Mahapatra et al. 2016). Successful ageing has also been attributed to optimal regulation of the PI3k-Akt pathway with reduced, but not abolished, activation of the pathway, resulting in healthy lifespan of mammals. In contrast, aberrant activation of the same pathway leads to neurodegeneration (O'Neill 2013). With an extensive range of results within just three main mechanisms, Chapter 2 therefore hypothesizes that MGF as an isoform of IGF-1, may confer neuroprotection using IGF-1r, PKC or the Akt pathway, as outlined above. Identifying mechanisms underlying the protection of motoneurones by MGF could lead to the development of therapeutics that slow- or halt- disease progression. However, while research into neuroprotective mechanisms tend to focus on the direct

motoneuronal effects, the perineuronal responses are often neglected. This is considered below.

1.5 Perineuronal responses: glial involvement and synapses

Changes in the activity of glial cells and synapses following injury of nearby neurones, are collectively referred to as perineuronal responses. These perineuronal responses are thought to reflect the fate of injured motoneurones and have been studied in the context of varied models of nerve injuries, namely nerve crushes and nerve transections. Early studies discovered that the increased presence of microglia and astrocytes (gliosis) around injured motoneurones orchestrated the displacement of synaptic terminals (Blinzinger and Kreutzberg 1968). This deafferentation of injured motoneurones was said to be a protective response to enable a metabolic change to occur, and allow cessation of neurotransmission for the cell to focus efforts on regeneration (Soreide 1981). These were possibly the earliest theories supporting that microglial upregulation post-injury may be protective in their ability to assess the damage and provide instructions to uninjured motoneurones, to compensate for the loss of overall function. Kerns and Hinsman (1973) put forth this argument when they noted that a sciatic neurectomy injured motoneurones but some synaptic boutons were selectively being displaced while others were phagocytosed. More recently, this theory was echoed by Bessis, Bechade et al. (2007) who documented that a protein involved in microglial activation, consequently altered synaptic protein content. The early theory then evolved to become a cross-talk between neurones and microglia, to determine the intensity and duration of perineuronal responses based on the severity of motoneuronal injury (Torvik and Skjörten 1971). Damaged motoneurones were thought to activate microglia

which then instructed uninjured motoneurones to form compensatory synapses, if the injury had a drastic effect on overall function. This was initially subjected to debate by Soreide (1981) who noted no difference in increased glial activity and synaptic stripping, between facial nerve crush lesions (no motoneuronal death) and avulsion lesions (marked motoneuronal death). However upon further analysis, Soreide (1981) noted that long-term, the crush lesions showed reversible perineuronal changes whereas nerve avulsion was associated with phagocytosis of degenerating motoneurones by microglial cells. Other studies have also shown milder perineuronal responses in the way of gliosis and synaptic loss to be associated with regenerating/surviving motoneurones whereas increased perineuronal changes to be associated with motoneuronal degeneration (Guntinas-Lichius, Neiss et al. 1994). This was mirrored by increased astrocytic protein expression seen in lethal neuronal injury (by injection of ricin toxin) compared to sub-lethal insults (in crush lesions) (Streit and Kreutzberg 1988).

Pehar, Cassina et al. (2004) have suggested that gliosis is destructive since astrocytes that produced nerve-growth factor were found to be destructive to SOD1 motoneurones. In contrast, there are studies that indicate that perineuronal reactive astrocytes are anti-apoptotic and help to maintain neuronal integrity. Thus, increased contact that astrocytic processes make with injured motoneurones, is thought to assist in the uptake of excessive glutamate to prevent excitotoxicity (Vargas and Johnson 2010, Yamada, Nakanishi et al. 2011, Tyzack, Sitnikov et al. 2014), Overall, changes in neuroglia and synaptic terminals have the potential to be indicators of the fate of injured motoneurones. Chapter 3 explores the perineuronal response using facial nerve transection (where motoneurones survive) and compared this with facial nerve avulsion (where motoneurones die) (Moran and Graeber 2004). The role of perineuronal

responses in MND is unclear. Some authors maintain that they may be both cause and effect of the condition (Valori, Brambilla et al. 2014), while others believe that glial cells contribute to motoneuronal degeneration by inflammatory mechanisms (Sargsyan, Monk et al. 2005, Staszewski and Prinz 2014, Xavier, Menezes et al. 2014, Radford, Morsch et al. 2015).

1.6 Inflammaging

Advancing age is associated with a chronic, but low-grade inflammatory state known as “inflammaging” which largely involves the innate immune system (Krabbe, Pedersen et al. 2004). This used to be attributed to increased adipose tissue, decreased production of sex steroids along with dormant infections and other chronic disorders. Thus, inflammaging was once simplistically considered directly pathogenetic and as a strong predictor of mortality. However, longevity-associated degeneration is yet to be concretely associated with a low-grade inflammatory state. In contrast, the *in vivo* inflammatory response is often related to successful ageing. These paradoxical effects of age-related changes in immune status might be reconciled if inflammaging was the result of efforts of the immune system, to evolve to suit optimal functioning of ageing cells which may have different metabolic requirements.

For example, in advancing age, reliability on the periphery to provide motoneuronal support seems to be reduced (Kandalla, Goldspink et al. 2011). This could be a result of processes such as sarcopenia in target muscle (Hameed, Harridge et al. 2002, Goldspink 2012). With reduced peripheral support, motoneurones could switch their dependence to central support, potentiated by glial cells such as microglia and astrocytes which are known to mediate central immune effects (Sargsyan, Monk et al. 2005). In line with

this, ageing animals have been found to have increased neuroglial activation compared to adults (Johnson, Sears et al. 1991, Hains, Loram et al. 2010).

Activated glia are known to produce inflammatory cytokines. When tissue damage is present, microglia switch from a "resting" to an "activated" phenotype and promote the inflammatory response. This further engages the immune system and tissue repair mechanisms, in a self-limiting manner. Under normal conditions, genes involved in inflammatory responses are thought to be repressed and only induced when cells are injured. Injured cells, releasing ATP for example, are detected by glial cells through utilisation of pattern-recognition receptors known as Toll-Like Receptors (TLRs) (Balistreri, Colonna-Romano et al. 2009, Di Virgilio, Ceruti et al. 2009, Doi, Mizuno et al. 2009, Takeuchi and Akira 2010). Microglia are the major initial sensors of TLR4 signals, which occur due to their binding of bacterial endotoxin ligands such as Lipopolysaccharide (Saijo, Winner et al. 2009). Upon receptor-binding, downstream signalling pathways are activated to control transcription factors that regulate the expression of numerous inflammatory cytokines. Further amplification of the inflammatory response is then achieved by involving astrocytes which can also secrete inflammatory cytokines (Saijo, Winner et al. 2009). Whether the secretion of these inflammatory cytokines results in neurodegeneration or neuroprotection, has been the subject of multiple primary research and review articles; a few of which are discussed below (Bareyre and Schwab 2003, Qin, Wu et al. 2007, Glass, Saijo et al. 2010, Xavier, Menezes et al. 2014).

Van Dyke, Smit-Oistad et al. (2016) studied microglial markers such as CD11b along with the protein expression of two cytokines – IL-1β and TNFα. This was assayed in muscle homogenates of both symptomatic and end-stage SOD1 rats. GDNF is a neurotrophic factor secreted primarily by glial cells under normal conditions (Glass,

Saijo et al. 2010). Van Dyke, Smit-Oistad et al. (2016) modified stem cells to release GDNF and grafted these into limb muscles of SOD1 rats to protect neuromuscular junctions and motoneurones, along with extended survival of rats. The intramuscular GDNF administration was also supported by reduced levels of inflammatory markers compared to exogenous GDNF delivery, that did not protect motoneurones. From this, the authors concluded that inflammation assayed near denervated neuromuscular junction, was detrimental to motoneuronal survival. A caveat to this study is that the immunosuppressant cyclosporine was administered to rats that received intramuscular stem cells to prevent rejection of the grafts. The immunosuppressant, as an anti-inflammatory agent, may well have confounded the study by influencing the general inflammatory state of the animals that received the grafts. This calls into question: (i) the distinction of inflammatory states and (ii) the inflammatory cytokine milieu and age-related motoneuronal death that contributes to the overall inflammatory state.

1.6.1 Distinction of inflammatory states

Inflammatory states can be differentiated as peripheral/systemic inflammation and central/neuroinflammation, both of which are correlated with neuroprotective- and neurodegenerative- effects.

1.6.1.1 Peripheral/Systemic Inflammation

Few studies have shown the effect of peripheral inflammation on motoneurone function. Van Dyke, Smit-Oistad et al. (2016) correlated high levels of peripheral inflammation with the disassociation of terminal Schwann cells from neuromuscular junctions of SOD1 mutant rats. This suggests a neurodegenerative effect of systemic inflammation. Studies of the presence of more macrophages and higher levels of their activity have also been noted in the peripheral nerves of MND mouse models, as MND

progressed. This has been correlated with increased cellular debris as motor axons degenerated (Chiu, Phatnani et al. 2009, Dibaj, Steffens et al. 2011). This suggests that inflammatory responses are simply a consequence of motoneurone degeneration. Lu, Allen et al. (2016) echoed this in their study of ALS patients that had lower levels of plasma cytokines compared to healthy controls. They argued that disease progression could be due to loss of the systemic regulation of inflammatory markers on T cell responses. As a result, they claimed that peripheral inflammatory markers could be positively correlated with prognosis, in an attempt to categorise diseases to improve clinical trials. This ideology was further supported by claims that systemic inflammatory cells are neuroprotective in function (Schwartz and Shechter 2010). Overall, these studies highlight the failures of specific (Cudkowicz, Shefner et al. 2006, Gordon, Moore et al. 2007) and non-specific (Meininger, Asselain et al. 2006) systemic anti-inflammatories used in MND clinical trials and how they showed no or adverse effects. Whether these results could apply to anti-inflammatories targeting neuroinflammation is unknown. Much of this is due to the continuing debate about both neurodegenerative- and neuroprotective- effects of neuroinflammation on motoneurones and in MND models.

1.6.1.2 Central-/Neuro- Inflammation

Recent literature has shed light on the knowledge that the CNS is not immune-privileged, as once thought to be (Schwartz and Shechter 2010, McCombe and Henderson 2011, Zhao, Beers et al. 2013). Instead, leukocytes such as T lymphocytes are known to continuously monitor and interact with CNS tissue (Henkel, Beers et al. 2013). This is thought to occur via the newfound glymphatic system, partially made up by glial cells (Jessen, Munk et al. 2015, Radford, Morsch et al. 2015). Accordingly, Mantovani, Garbelli et al. (2009) noted the increased presence of T lymphocytes around

degenerating spinal motoneurones of SOD1 mice and in the brain parenchyma of MND patients. Th1 lymphocytes are capable of producing cytokines such as IFNy to then activate microglia to further amplify cytokine production while Th2 lymphocytes produce cytokines that are specific to neurodegenerative phenotypical responses (Glass, Saijo et al. 2010, Dibaj, Steffens et al. 2011). The role of infiltrating lymphocytes in MND pathogenesis is still unclear. However, some studies have suggested the infiltration of T cells to be neuroprotective (Banerjee, Mosley et al. 2008, Beers, Henkel et al. 2008, Beers, Henkel et al. 2011), for example through the production of IL-4 by Th2 cells, which in turn signals activated microglia to produce neurotrophic factors such as IGF-1 (Chiu, Chen et al. 2008). Some studies indicate that the acute neuroinflammation slows disease progression at early-stages, and also that chronic neuroinflammation accelerates end-stage disease (Beers, Zhao et al. 2011, Moser, Bigini et al. 2013). The studies pinpointing the neurodegenerative effects of neuroinflammation, approach their investigations in the context of microglia and astrocytes. Accordingly Brown and Neher (2010) argue that pathogens along with neuronal- and vascular damage lead to inflammatory cytokine-release that encourage microglia to phagocytose all damaged neurones. However, more recent studies illustrated that microglia-associated neurodegeneration was attributed to a reduction in their phagocytic capacity rather than their presence (Xavier, Menezes et al. 2014). This aligns with older studies that have shown microglia to be selective for the particular motoneurones they phagocytose to ensure that the CNS connectome is optimally reorganised, in the event of an injury (Kerns and Hinsman 1973). Accordingly, impaired phagocytosis that is seen in a form of fMND is associated with the mutation in a gene responsible for actin dynamics in phagocytosis. In this way, mutated Profilin 1

results in impaired phagosome formation and dysfunctional phagocytosis (Petkau and Leavitt 2014, Radford, Morsch et al. 2015).

In a similar manner, diseased or dysfunctional astrocytes have been implicated in MND progression via neuroinflammation. In Chen, Qian et al. (2015), glial progenitors were derived from fMND patients' induced pluripotent stem cells (iPSC) and grafted into mice. This resulted in motoneuronal degeneration and motor deficits compared to control mice. Similarly, iPSC-derived astrocytes from healthy individuals transplanted into the lumbar spinal cord prolonged the lifespan of SOD1 mice (Kondo, Funayama et al. 2014). Furthermore, post-mortem astrocytes from fMND and sporadic MND patients were neurotoxic when co-cultured with motoneurones, albeit the caveat in this study was the already necrotic post-mortem environment (Haidet-Phillips, Hester et al. 2011). Along the same lines, this result was also mirrored when astrocytes were generated from iPSCs derived from fMND and sporadic MND patients. Thus, astrocyte-induced neurotoxicity seems common to both familial and sporadic MND (Radford, Morsch et al. 2015). In a neuroimaging study of MND patients using positron emission tomography ligands to activated microglial receptors and astrocytic metabolites, gliosis was evident through various symptomatic stages of MND while absent in non-disease controls (Chio, Pagani et al. 2014, Turner and Verstraete 2015). Taken together with animal models, recent studies suggest a shift in the temporal inflammatory-gliosis response from end-stage events to symptomatic stages, and in the future, perhaps even pre-symptomatic stages (Beers, Zhao et al. 2011). It is therefore evident that we need to approach the use of anti-inflammatories in MND with caution, as we are still unsure if inflammatory responses are causes or effects of motoneuronal degeneration (Dahlke, Saberi et al. 2015). Moreover, we know little of the inflammatory cytokine environment in the CNS parenchyma, surrounding motoneurones in healthy ageing and MND. Such

analyses in humans are outside the scope of the present study, but new information on ageing rats is presented and correlated with age-related changes in the survival of injured motoneurones (Chapters 4 to 6), and this may provide a basis for formulation of testable hypotheses in, for example, human post mortem material.

1.6.2 The inflammatory cytokine milieu and age-related motoneuronal death

Cytokine upregulation has been shown to precede caspase activation and contribute to apoptotic neuronal death (Bar 2000, Mhatre, Floyd et al. 2004). While it is unknown if motoneuronal degeneration in MND occurs via apoptosis, this is likely as a myriad of cytokines have been implicated in advancing age and experimental models of motoneuronal degeneration and survival. While an exhaustive list of cytokines exists, there is evidence of the role of selective cytokines in age-related degeneration. This has also led scientific companies to develop multiplex panels to assay these specific cytokines. Thus, twelve selected cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, TNFα, IFNγ and GM-CSF) are discussed below in the context of advancing age and a few of these will be revisited in association with motoneuronal death and survival.

1.6.2.1 Cytokines associated with advancing age

IL-1α is produced by glial cells (Penkowa 2008). Sheng et al. (1998) showed that the aged human brain had three- to eleven- fold increases in the number of enlarged microglia. These microglia were IL-1α-immunoreactive and IL-1α mRNA levels were considerably higher in individuals over 60 than in younger age groups. While circulating levels of IL-1α were increased in aged rats, exogenous IL-1α has been shown to activate endogenous production of IL-1α to cross the blood-brain barrier

(BBB) (Banks, Farr et al. 2001, Krabbe, Pedersen et al. 2004). IL-1α is able to protect human cell cultures from oxidative stress (Carlson, Wieggel et al. 1999, Bissonnette, Klegeris et al. 2004) and IL-1α binding, to cell-surfaces, has been shown to be critical for secretion of IL-6 in senescence (Orjalo, Bhaumik et al. 2009). Given the function of IL-6 can be both pro- and anti-inflammatory, IL-1α could be considered a mediator for either neuroprotection or neurodegeneration.

IL-2 has been shown to be vital in regulating T-cell function and therefore immune homeostasis. This is evidenced by IL-2 knockout mice that elicit spontaneous autoimmunity (Meola, Huang et al. 2013). IL-2 is known to provide trophic support, enhance neurite branching and modulate acetylcholine release in the CNS. Increased immunoreactivity and protein levels of IL-2 and its respective receptor were found in the spinal cord of aged dogs compared to adult dogs (Lee, Ahn et al. 2013). Post-mortem brains from Alzheimer's disease patients show higher IL-2 levels compared to controls, indicating that it may help regulate inflammation during healthy ageing (Araujo and Lapchak 1994).

There are contrasting reports on IL-4 in ageing. IL-4 can act as a pro-inflammatory cytokine to induce microglial proliferation when stimulated with GM-CSF yet it also acts as an anti-inflammatory cytokine and down regulates IFNγ (Mizuno, Sawada et al. 1994, Suzumura, Sawada et al. 1994, Suzumura 2008). Increased IL-4 receptor immunoreactivity and protein levels, have been found with advancing age in the spinal cord of dogs (Lee, Ahn et al. 2013) yet decreased levels of IL-4 with advancing age have been found in the human brain (Loane, Deighan et al. 2009). This change was suggested to be neuroprotective by blocking microglial activation yet Maher et al. (2005) showed that downregulation of IL-4 caused memory deficits in the aging

hippocampus. This highlights an important general concept relevant to the present study: the potential for a single cytokine to interact with others and to have multiple effects dependent on other factors, such as age.

Age-related reductions of the anti-inflammatory IL-10 in mice have been proposed as a reason for increased levels of IL-6 (Ye and Johnson 2001). Interleukin-6 is a neuroinflammatory mediator that is often elevated with age as seen in glial cell cultures from the cortex, hippocampus and striatum of aged rats compared to younger rats (Terao, Apte-Deshpande et al. 2002, Xie, Morgan et al. 2003). IL-6 levels are also higher in senescence-accelerated mice (Shimada and Hasegawa-Ishii 2011). Gene expression profiling from fetal, adult and Alzheimer's disease hippocampus shows a 2.2- fold increase in IL-6 in adults compared to fetal tissue and 3.8- fold increase in Alzheimer's disease patients compared to adults (Lukiw 2004). However, in the absence of age-matched controls, it is unclear if the IL-6 increase in Alzheimer's patients is due to age or pathology.

Akin to the aforementioned IL-4, IL-5 and IL-10 are also anti-inflammatory in function.

IL-5 vitally regulates gene upregulation in cell survival and maturation of the adaptive immune system (Kouro and Takatsu 2009). IL-5 has been downregulated in both hemispheres of immature rats during early traumatic brain injury (Tajiri, Hernandez et al. 2014). Whereas, deficiencies of IL-10 in aged animals is associated with behavioural and cognitive impairment (Ye and Johnson 2001, Frank, Barrientos et al. 2006). IL-10 is known to downregulate pro-inflammatory cytokines such as IFNγ through feedback mechanisms (Mizuno, Sawada et al. 1994, Suzumura, Sawada et al. 1994). An early study done by Sheng et al. (1995) showed that LPS-induced increases in IL-6 and TNFα were followed by upregulation of IL-10 mRNA expression and protein production.

Thus, augmentation of IL-5 and IL-10 was thought to dampen the neurotoxic effects of pro-inflammatory cytokines. However, their neuroprotective or neurodegenerative responses are unclear in advancing age using the sample injury models.

Interleukin 12p70 is produced by macrophages. It is involved in T-cell differentiation to stimulate Th1 responses and consequently other pro-inflammatory cytokines such as IFNγ (Aloisi, Penna et al. 1997, Penkowa 2008). Intraperitoneal administration of recombinant IL-12 has been reported to induce IFNγ-mediated protection against parasitic challenges (Lyke, Burges et al. 2004). In neurodegenerative studies such as Tan, Yu et al. (2014), knock down of the IL-12 subunit in their model of senescence-accelerated mice showed significantly less synaptic- and neuronal loss and reversed cognitive impairments, compared to normal mice. The study showed that IL-12 levels in the brain increased due to aging however did not look at peripheral levels in a parallel study, using the same animals. In clinical studies, patients with non-inflammatory neurological disorders had higher levels of IL-12 in their cerebrospinal fluid compared to Alzheimer's and Frontotemporal dementia patients (Rentzos, Paraskevas et al. 2006). Taken together, it may be that higher peripheral levels and lower CNS levels of IL-12p70 are beneficial in the presence of immune challenges, without senescence as a contributing factor. However, with advancing age, perhaps lower peripheral levels and higher CNS levels of IL-12p70 may be most conducive to the surrounding environment.

IL-13 has been detected exclusively in microglia (Shin, Lee et al. 2004). No studies thus far have reported any significant effects of IL-13 on normally ageing animals or humans. IL-13 is thought to be similar to IL-5 and IL-10 in terms of being an anti-

inflammatory cytokine. However, recent evidence has shown that one of its functions is not mimicked by all other anti-inflammatory cytokines, except IL-4 which shares the same receptor. Recent discoveries suggest IL-13 to be involved in the resolution of brain inflammation. In the context of acute inflammation, Shin et al. (2004) showed that IL-13 neutralising antibodies increased the survival of microglia, that were activated in response to an inflammatory agent, when compared to control rats. Consequently, TNF-α levels were sustained and neuronal death increased. Further studies showed that IL-13 mediated the death of activated microglia by enhancing the expression of cyclooxygenase-2 (Yang, Ji et al. 2006).

Interferon-gamma is produced by both glia and helper T-cells and is thought to serve a dual role of tissue damage induction but also neuronal preservation (Penkowa 2008). Increased IFNy is found in the spinal cord of aged dogs compared to younger adults (Chung, Choi et al. 2010). IFNy levels are also higher in experimentally-aged mice models at 2, 5 and 10 months of age (Shimada and Hasegawa-Ishii 2011). Whether this applied to normally aged mice is unknown.

Campuzano et al. (2009) localised TNF-α expression to astrocytes but not microglia or neurones. However, in long-term cell cultures microglia take-over from astrocytes as the primary source of TNF-α (Mennini, Bigini et al. 2004). Macrophages recruited to the CNS also produce TNF-α, which acts on the TNF receptor 1, to transiently activate the Nuclear-Factor-Kappa-b pathway which upregulates AMPA receptors and thereby contributes to glutamate excitotoxicity (Stellwagen, Beattie et al. 2005, Tolosa, Caraballo-Miralles et al. 2011). In-vitro studies have shown macrophages to initiate cellular senescence that triggers the inflammatory response through pathways such as the aforementioned NF-κb and p38MAPK and JNK (Veglianese, Lo Coco et al. 2006, Salminen, Ojala et al. 2011). In contrast to this acute role in cell death, long-term

binding to the TNF receptor 2 can activate PI3 kinases and upregulate anti-apoptotic proteins such as Bcl2 (Terrado, Monnier et al. 2000), although in other circumstances TNF-α may trigger caspase activation within microglia (Streit, Sammons et al. 2004, Stellwagen, Beattie et al. 2005, Correale 2008). Both T-cell activation and TNF-α protein expression increases at 24m in mice (Terao, Apte-Deshpande et al. 2002) and plasma TNF-α increases in human octogenarians (Bruunsgaard, Andersen-Ranberg et al. 1999). Higher plasma levels of TNF-α and respective receptors have also been reported in MND patients and animal models of this disease, although no correlation is seen with disease duration or severity (Poloni, Facchetti et al. 2000, Hensley 2003).

GM-CSF, is a cytokine produced by neurones and astrocytes that stimulates microglial cell growth (Ridwan, Bauer et al. 2012). GM-CSF is increased in the CSF and plasma of dementia patients and it has been suggested that it induces programmed cell death in the brain tissue of these patients a (Tarkowski, Wallin et al. 2001). In contrast, other studies show decreased GM-CSF in the normally aging brain (Weindruch, Kayo et al. 2002). The GM-CSF receptor α (GM-CSF Rα) has been markedly reduced in the hippocampus with slight reductions also seen in various cortical regions and brainstem nuclei of Alzheimer's patients (Liu, Wu et al. 2012). Since these are target regions of neurodegenerative changes in Alzheimer's disease, the reduction of GM-CSF Rα and consecutive down-regulation of GM-CSF signalling may contribute to the progressive course of neurodegeneration. Thus, it may be concluded that the outcomes of this overall increase in inflammatory molecules varies depending on the nature of the initial stimulation and the current immune environment (Morrisette-Thomas, Cohen et al. 2014).

1.6.2.2 Cytokines associated with motoneuronal degeneration and survival

IL-1β and TNF-α are able to activate microglia synergistically (Elliott 2001, Yoshihara, Ishigaki et al. 2002). IL1β is secreted by activated microglia and has been shown to speed motoneurone pathology in SOD-1 mouse model of MND (Yoshihara, Ishigaki et al. 2002). In SOD1 mice, antagonising the IL-1 receptor systemically slows disease progression (Meissner, Molawi et al. 2010). Likewise, TNF-α has been correlated with motoneuronal loss in fMND models and is present in higher concentrations in the serum of MND patients (Poloni, Facchetti et al. 2000, Elliott 2001). The neutralisation of circulating TNF-α has shown to protect immature motoneurones from death, but there is no evidence to suggest the same for mature motoneurones (Terrado, Monnier et al. 2000). TNF-α is also secreted by activated microglia and is responsible for the imbalance of excitatory and inhibitory receptors in *in vitro* cell cultures of motoneurones (Mir, Asensio et al. 2009, Tolosa, Caraballo-Miralles et al. 2011, Olmos, Llad et al. 2014). It has been assumed that TNF-α mediated excitotoxicity is the cause of motoneuronal death in MND (Yoshihara, Ishigaki et al. 2002, Olmos, Llad et al. 2014). However if this is the case, one would expect that "Riluzole", as a glutamate-antagonist would elicit more neuroprotection than it has clinically (Miller, Mitchell et al. 2007).

Lin, Pfluger et al. (2012) measured an alarmin cytokine called IL-33 that is typically released from damaged tissue along with its corresponding receptor ST2 which is found on glial cells. In MND patients, IL-33 levels were significantly lower but the respective receptor known as ST2 was significantly higher, compared to healthy controls (Lin, Pfluger et al. 2012). The study suggested that IL-33 was either being degraded by apoptotic cell-induced caspases, or alternatively, was the negative feedback effect of high receptor levels, as a result of gliosis, that was taken to indicate inflammation in

MND. From this, it was proposed that motoneuronal death was apoptotic in nature and thus, IL-33 was degraded by resultant caspases as ST2 increased in the resultant inflammatory response. In this case, therefore, inflammation was a consequence of motoneuronal degeneration in MND rather than cause. This was also echoed by Iłżecka (2012) who showed that serum caspase-9 levels were higher in MND patients compared to healthy controls. To further reinforce this, activated microglia in the wobbler mouse model of fMND, expressed TNF-α and this was associated with an astrocytic influx and caspase 3 activation, that was taken to drive motoneuronal degeneration (Dahlke, Saberi et al. 2015). While microglia are mostly viewed in a neurodegenerative light as secretors of pro-inflammatory cytokines, negative feedback mechanisms are also mediated by them, in the form of anti-inflammatory mediators such as IL-10 (Glass, Saijo et al. 2010).

While there is evidence linking IL-1β and TNF-α to motoneuronal degeneration *(vide supra)*, deletion of these genes have not altered disease progression in MND experimental models (Nguyen, Julien et al. 2001, Gowing, Dequen et al. 2006). It could be that depriving the entire inflammatory system of just two effector molecules does not alter the MND symptoms in experimental models. This calls into question the generalisations about changes in overall inflammatory status in the pathogenesis of MND, that are drawn from studies of alterations in only a few cytokines (Moreau, Devos et al. 2005).

Sustained inflammation is attributed to either the persistence of an inflammatory stimulus or failure in normal resolution mechanisms (Glass, Saijo et al. 2010). An alternative perspective is that inflammaging is an anticipatory protective response to neurodegeneration. This aligns with the view that inflammation is a highly regulated

biological program (Glass, Saijo et al. 2010). Perhaps the inflammatory response with advancing age does not self-limit, as in younger animals, but persists at a low-grade level due to age-related increases in the threshold of its responsiveness to cellular damage. It is therefore unknown if pre-existing inflammation affects the survival of immature and mature motoneurones, when faced with an injury. If so, the phenomenon could be paralleled with immune priming/conditioning within the PNS and the beneficial effects on axonal regeneration, could indicate that pre-existing inflammation may be neuroprotective.

1.6.3 Immune priming

As more studies investigate if inflammaging may be neuroprotective, theories are beginning to emerge about mechanisms by which repair and protection of neurones could be mediated by a chronic but low-grade, inflammatory response (Qiu, Cafferty et al. 2005, Donnelly and Popovich 2008, Salegio, Pollard et al. 2011, Blesch, Lu et al. 2012). Unlike the peripheral nervous system, the CNS is known to have its growth capacity suppressed for the development of an optimal CNS connectome (e.g. proper synaptic development), when transitioning from an immature to a mature system. Immune-conditioning or priming is thought to reactivate growth capacity in the PNS and multiple studies try to replicate this with the CNS, often in the hope of applying to spinal cord injury models (Abe and Cavalli 2008, Alexander and Popovich 2009, Salegio, Pollard et al. 2010).

Immature motoneurones show target-dependence for growth and survival (Oppenheim, Haverkamp et al. 1988) whereas mature motoneurones seem to rely less on the periphery (Oppenheim 1991, Goldspink 2012). In contrast to the developing neuromuscular system (Greensmith and Vrbova 1996), denervation of neuromuscular

junctions has been correlated with no loss of motoneurones in geriatric mice (Chai, Vukovic et al. 2011). Age-related processes such as sarcopenia (Goldspink and Harridge 2004) may be a primary reason motoneurones switch dependence to a more reliable central (CNS) support system. A chronic low-grade inflammatory environment with potentiated glial cells could serve as central support for normally ageing motoneurones.

Contrary to the above view that elevated immune status is protective for motoneurones, immune priming with Lipopolysaccharide (LPS) aggravated disease progression in pre-symptomatic fMND mice (Nguyen et al 2004). The study showed that chronic immune priming with LPS increased IL-12 levels in the CNS of SOD1 mice. IL-12 is a cytokine that is involved in the transition from innate to adaptive immune response and promotes differentiation of CD4 lymphocytes into IFNy-producing helper Th1 cells (Glass, Saijo et al. 2010). The functional significance of the increase in T cells and cytokines is still a point of controversy however, much of this has been attributed to efforts to augment microglial function at and distal to, the site of CNS injury (Alexander and Popovich 2009).

A protein that facilitates immune-primed-TLR4 responses is upregulated in the spinal cord of fMND mice (Nadeau and Rivest 2000, Nguyen et al 2001). However, results from these genetic experimental models (i.e. fMND mice) may not accurately illustrate the role of neuroinflammation in sporadic MND. The inherent genetic mutations of the animals to yield the MND phenotype, could alter the immune system, overwhelm repair mechanisms and consequently vary any responses from immune conditioning/priming lesions (Glass, Saijo et al. 2010). This would not reflect the environment seen around normal ageing motoneurones and their survival mechanisms. Accordingly, focal

injections of immune-priming ligands have accelerated microglia-mediated clearance of spinal cord injury sites and promoted axonal regeneration when combined with increased levels of neurotrophic factors (Perrin, Lacroix et al. 2005, Vallières, Berard et al. 2006, Chen, Smith et al. 2008). Other studies have also shown similar effects of regeneration mediated by the augmentation of CNS microglia, in injured optic nerves and spinal cord (Donnelly and Popovich 2008, Alexander and Popovich 2009). These studies challenge the popular concept that the presence of an inflammatory state impedes the survival of injured neurones. Indeed it is possible that pre-existing inflammation, in the form of immune conditioning/priming, may promote neuronal survival and repair.

Lipopolysaccharide with TLR4 activation, has been utilised as a widely established inflammatory response-inducer (Godbout, Berg et al. 2004, Wang, Rousset et al. 2006, Qin, Wu et al. 2007, Frank, Miguel et al. 2010, Hains, Loram et al. 2010, Gorina, Font-Nieves et al. 2011, Loram, Taylor et al. 2011). Thus, chapters 4 to 6 use LPS as an inflammatory agent to study the inflammatory signature across the lifespan of Sprague-Dawley rats and to then investigate whether pre-existing inflammation and immune priming (with LPS) neuroprotects injured mature and immature motoneurones. Facial motoneurones and the surrounding environment have been studied throughout the thesis. This was done to utilise the facial nerve axotomy model as an experimental model studying motoneuronal degeneration with no genetic manipulations associated with the motoneuronal injury. McCombe and Henderson (2010) highlighted that bulbar-onset MND (that affects facial motoneurones) are more common in female patients thus female rats are used across the studies in Chapters 2 to 6. This aligns with recent recommendations to generate data to balance gender in animal studies, with more experiments using females, as women tend to have longer lifespan and are therefore

more prone to neurodegenerative diseases (Clayton and Collins 2014, Wallace and Howlett 2016).

1.7 Aims of Manuscripts

Chapter II

To determine the mechanism of action of the neurotrophic factor known as Mechano Growth Factor (MGF) and is capable of rescuing mature motoneurones.

Chapter III

To investigate if perineuronal responses (through glial activity and synaptic presence) can determine the fate of injured motoneurones.

Chapter IV

To determine age-related differences in motoneuronal survival and the inflammatory milieu in CNS compared to the periphery.

Chapter V

To determine if immune-priming mature motoneurones affects their survival.

Chapter VI

To determine if immune-priming immature motoneurone affects their survival.

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CHAPTER II

Mechanisms of adult motoneuronal rescue by the c- terminal 24 amino acid sequence of Insulin-Like Growth Factor-1 Eb (Mechano Growth Factor).

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STATEMENT OF AUTHORSHIP

Title of Paper	Mechanisms of adult motoneuronal rescue by the c- terminal 24 amino acid sequence of Insulin-Like Growth Factor-1 Eb (Mechano Growth Factor).
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style

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Name of Principal Author (Candidate)	Viythia Katharesan		
Contribution to the Paper	Experimental design, data collection, analysis and interpretation, wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/09/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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2 Mechanisms of adult motoneuronal rescue by the c- terminal 24 amino acid sequence of Insulin-Like Growth Factor-1 Eb (Mechano Growth Factor).

2.1 Abstract

We have found that an isoform of insulin-like growth factor 1 (IGF1 Eb) termed Mechano Growth Factor (MGF) rescues adult motoneurones following transfer of functional copies of the gene and after delivery of the 24 amino acid E peptide (MGF24). MGF's mechanism of action, however, is unknown. Here we investigate whether MGF24-mediated neuroprotection of avulsed rat facial motoneurones involves the IGF-1 receptor and the neurotrophic factor downstream signalling molecules Protein Kinase C and Protein Kinase B/Akt. Immediately after facial nerve avulsion of groups of 4 - 6 rats, either MGF24, MGF24 plus an IGF-1 receptor antibody or MGF24 plus a blocker of Protein Kinase C (GF109203X) were injected into the stylomastoid foramen and surviving motoneurones estimated stereologically 1 month later. In other animals, the liver isoform of IGF-1 (IGF-1 Ea) or Glial Derived Neurotrophic Factor (GDNF) were injected alone or along with antibodies to their respective receptors. Limited analysis of the PI3k-Akt pathway's involvement in neuroprotection was also done by immunostaining for Akt 7d days after avulsion alone and avulsion + MGF24 tissue. Mean numbers of motoneurones surviving 1m after nerve avulsion plus MGF24 were unaffected by blocking the IGF-1 receptor or Protein Kinase C. No significant changes in the density of immunohistochemical staining for Akt was found 7d after avulsion alone or 7 d after avulsion +MGF24. While co-injection of IGF-1 and GDNF with antibodies to their receptors resulted in approximately 25% reduction of surviving

motoneurones at 1m, this was not statistically significant when stringent post hoc tests with corrected p values were applied. The data trends indicate that the present experimental model may, with larger sample sizes, be capable of showing significant reductions of neurotrophic rescue by IGF-1 and GDNF by blocking their respective receptors. We cannot, however, determine if MGF24 recue of avulsed motoneurones requires the IGF-1 receptor or PI3k/Akt as the present study appears underpowered.

2.2 Introduction

There is abundant information identifying neurotrophic factors that prevent the death of immature motoneurones, but most of these substances fail in MND which predominantly affects mature/ageing motoneurones. Many motoneuronal survival factors are now recognised, however one stands out as having the potential to play a role in both motoneuronal survival and ageing. This neurotrophic factor is Insulin-like growth factor 1 (IGF-1). IGF-1 promotes cell proliferation, differentiation and survival in non-neurodegenerative situations (Thimmaiah, Easton et al. 2003) and its homologues can shorten or prolong lifespan markedly in a variety of species (Shimokawa, Higami et al. 2002, Bonafe, Barbieri et al. 2003, Kaletsky and Murphy 2010). Although most IGF-1 is produced by the liver, an isoform of IGF-1 is produced by the peripheral target of motoneurones – muscle. Alternative splicing of the IGF-1 gene at the three prime end, to yield a transcript containing exon 4 spliced to exon 5 and 6 creates this isoform known as Mechano Growth Factor (MGF) (Matheny, Nindl et al. 2010). While definitions vary, the isoform of IGF-1 expressed by rat muscle is referred to as IGF-1 Eb, but the general term MGF will be used here for simplicity. The expression of MGF is upregulated in active muscle in response to stretch and injury and has been a much more potent rescue factor for adult motoneurones than the commonly-

used IGF-1 liver isoform (Musaro, McCullagh et al. 2001, Yang and Goldspink 2002, Aperghis, Johnson et al. 2004, Gorecki, Beresewicz et al. 2007, Quesada, Ogi et al. 2011). MGF has been shown to rescue motoneurones via gene-transfer, in non-genetic experimental injury models and in SOD1 mice and more recently the MGF peptide delivered as at time of injury has also shown some neuroprotection (Aperghis, Johnson et al. 2004, Evans, Harridge et al. 2010, Johnson, Goldspink et al. 2011). Most motoneurone rescue factors employ either primary receptors or secondary messengers to elicit neuroprotection. Quesada et al (2011) previously showed that MGF protected dopaminergic neurones by upregulating the heme oxygenase stress-response protein, in an oxidative stress-injury model. Our aim in this study was to determine the mechanisms involved in MGF rescue of motoneurones in a proximal axotomy model. Prior to that, we first tested our experimental model by administering two other trophic factors delivered into the stylomastoid foramen immediately after facial nerve avulsion. These were the liver-type isoform of IGF-1 and Glial cell line derived neurotrophic factor (GDNF). These were delivered with- or without- their respective inhibitors. In order to then investigate MGF's mechanism of eliciting neuroprotection, we experimented with one primary receptor (IGF-1 receptor) and one secondary messenger (Protein Kinase C), using inhibitors to these. MGF as an isoform of IGF-1, may utilise the IGF-1 receptor (IGF1r) or alternatively employ Protein Kinase C (PKC), as a typical downstream messenger involved in eliciting neuroprotection (Akita 2002, Quesada, Ogi et al. 2011). However, mature IGF-1 was also previously shown to activate another secondary messenger system known as the Phosphotidylinositide 3 kinase-Protein Kinase B-mechanistic target of rapamycin (PI3k-Akt-mTOR) pathway (Dai, Wu et al. 2010). This pathway has also been linked to aging, neuroprotection and human SOD1 motoneurone survival (Li, Xu et al. 2003, Kirby, Ning et al. 2011, Ohta, Arai et al.

2011, O'Neill 2013) Thus, using some archival tissue where MGF was administered post-avulsion, we also did some preliminary studies on the PI3kinase-Akt-mTOR pathway involvement by looking at immunohistochemical protein expression of a pan-Akt molecule.

2.3 Materials and Methods

2.3.1 Animals

Groups of 4 - 6 Sprague-Dawley rats were used at 3 months of age. Animals were housed under a standard 12-hour on/off lighting regime and given food and water *ad libitum*. The experimental study complied with the Australian code for the care and use of animals for scientific purposes (2016) and was approved by the University of Adelaide Animal Ethics Committee (M-29-2013).

2.3.2 Facial Nerve Avulsion

Under deep surgical anaesthesia with isoflurane, the right facial nerve was avulsed. Briefly, an incision was made behind the right ear to access the facial nerve between the sternocleidomastoid and masseter muscles. The facial nerve is then traced down to the exit point from the stylomastoid foramen and freed from its periosteal attachments and then avulsed. Detachment of the facial nerve rootlets as they emerge from the brainstem will result in a very proximal axotomy which results in extensive motoneuronal death.

2.3.3 Administration of trophic factors ± inhibitors

Facial nerve avulsion was followed by injection into the stylomastoid foramen of 10μl of saline or neurotrophic factors ± blockers. The foramen was then sealed with a Gelfoam plug. A previous pilot experiment verified that solutions injected into the stylomastoid foramen reached the site of nerve avulsion. Here, 10μl of Evans blue dye

was injected into a terminally-anaesthetised rat which was then decapitated. On opening the skull, the dye was clearly visible in the posterior cranial fossa around the area of the ipsilateral internal auditory meatus and a 1- 2 mm area of the adjacent brainstem, where the facial nucleus is located. The IGF-1 receptor antibody (IGF-1rAb) (Novus Biologicals, NBP1-19989) was used to inhibit IGF-1 receptor activity and Protein Kinase C activity was antagonised using the PKC inhibitor - GF109203X (AdipoGen Life Sciences, AG-CR1-0112).

2.3.4 Perfusions and tissue preparation

All rats were deeply anaesthetised (60mg/kg intraperitoneal sodium pentobarbital), 30 days after surgery and when limb and corneal reflexes disappeared, rats underwent intracardiac perfusion with fixative (4% paraformaldehyde in 0.1M sodium phosphate buffer with pH = 7.4). The brainstem was removed and left in fixative at 4ºC overnight. The brainstem was then trimmed 1mm above the rostral border and 2mm above the caudal border of the pons so that the 1.5mm long region containing the facial nuclei lay roughly midway in a piece of tissue 3-4mm long. A V-shaped nick was made in the posterolateral part of the left side of the brainstem to enable orientation of sections later on. The specimen was fixed to the stage of a vibratome using cyanoacrylate glue and serial 100µm sections were cut and transferred to phosphate buffer-filled wells. The specimen was sectioned until only 0.5mm of specimen remained on the stage. The section series containing the facial nucleus were identified using bright field microscopy.

2.3.5 Stereological counts of facial motoneurones

Numbers of motoneurones were estimated in every 5th section using an optical disector method modified for use in the confocal scanning laser microscope as described

previously (Johnson 2001). Briefly, 2 scans are taken in different colours 10μm apart and are merged. The total volume of the facial nucleus is determined by the Cavalieri method and taken into consideration to determine the final numbers of motoneurones.

2.3.6 Immunohistochemistry and semi-quantitative analysis

Cryoprotected archival Vibratome sections from Avulsion Only rats and Avulsion + MGF rats with 7 days survival were used. Randomly selected sections were defrosted using PBS. Sections were transferred to 5ml pots and rinsed in PBS twice for 5 mins each rinse. 0.3 % Hydrogen Peroxide (H_20_2) in 75% methanol was used to block endogenous peroxidase activity for 20 mins after which 0.1% Triton X-100 in PBS was used to permeabilise sections twice for 15 mins. 2.5% Normal Horse Serum (NHS) (Sigma) was applied to sections at room temperature for 20 mins to block non-specific antibody binding. Sections were then again permeabilised in 0.1% Triton X-100 in PBS as mentioned previously. Sections were incubated with 1:20 polyclonal rabbit anti-panAkt (Abcam, ab8805) primary antibody using 1% NHS in PBS as the diluent overnight at 4ºC. The primary antibody concentration was optimised for our tissue by pilot experiments. The following day, sections were taken out of the fridge and washed in PBS, twice for 10mins each time. Universal anti rabbit biotinylated secondary antibody (Vector Laboratories, USA) was applied for 30 mins. Sections were rinsed in PBS for 10mins each followed by Avidin-HRP-Biotin complex from the Vectastain ABC Kit Solution (Vector Laboratories, USA) which was applied for 30 mins. Sections were then washed in PBS twice for 10 mins before doing a pre-incubation in 3,3'-diaminobenzidine (DAB) (Vector Laboratories, USA) without the H_20_2 for 10 minutes and a further 5 mins with H_20_2 and Nickel. As soon as the desired colour was achieved, the DAB was quickly replaced with phosphate buffer. Sections were then placed on silane-coated slides and taken through graded ethanols (50%, 70% and 100%) followed

by a 50% methanol and 50% Xylene mixture and finally 100% Xylene to then be mounted using the depex mountant and coverslipped. As controls for the immunohistochemical procedure, either the primary or secondary antibodies were omitted with all other aspects of tissue processing, including blocking antisera, remaining the same. High quality 10x magnification images were taken using a bright field microscope to ensure the images retained characteristics from initial acquisition for analysis. Images were grayscaled using the batch manipulation function on Adobe Photoshop and analysed using Image J under blind conditions. Briefly, under Analyse > Set Measurements, "integrated pixel density" was selected and each operated and non-operated facial nucleus was measured individually. Raw data was then collected from the ROI Manager window. Mean pixel density per unit area (754 000μm^2) was calculated from three measurements within each nucleus, with at least three sections per rat (n = 4 – 6 rats per group). To control for staining variations between sections, pixel density was expressed as a ratio of the right (operated nucleus) over the left (non-operated nucleus).

2.3.7 Statistical Analysis

Datasets that did not meet the assumptions of normality (through Q-Q plots and Shapiro-Wilks tests) and homogeneity of variance (Levene's test) were analysed using the robust non-parametric Kruskal Wallis (result 3.1). Datasets that satisfied the aforementioned assumptions (results 3.2 and 3.3) were analysed using the parametric one-way Analysis of Variance (ANOVA) procedure and accordingly the unpaired t-test was performed on the semi-quantitative immunoperoxidase results. Post-hoc comparisons were done using the conservative Bonferroni procedure. Statistical significance is noted as $p<0.05$ unless otherwise stated.

2.4 Results

2.4.1 Co-injection of trophic factors with respective blockers

With current sample size n = 4 - 6, the Kruskal Wallis was statistically significant however post-hoc Mann-Whitney procedures were insignificant with the Bonferroni corrected p-value for multiple pair-wise comparisons. Nevertheless, a trend of increased loss of motoneurones post-injury, was noted when trophic factor activity was blocked by respective inhibitors (i.e. GDNF + GDNF receptor antibody, IGF1 + IGF1 receptor antibody) (Fig 1).

2.4.2 Co-injection of MGF with potential blockers

There were no significant differences in mean motoneuronal loss when MGF was administered post-injury compared to when MGF was co-injected with the IGF-1 receptor antibody (IGF-1rAb) and the Protein Kinase C inhibitor (PKC inhibitor) (Fig 2).

2.4.3 Immunohistochemical analysis of Akt

There was no statistically significant difference in pixel density staining in injured tissue compared to injured tissue with MGF although mean value is slightly higher in MGF treated tissue (Fig 3).

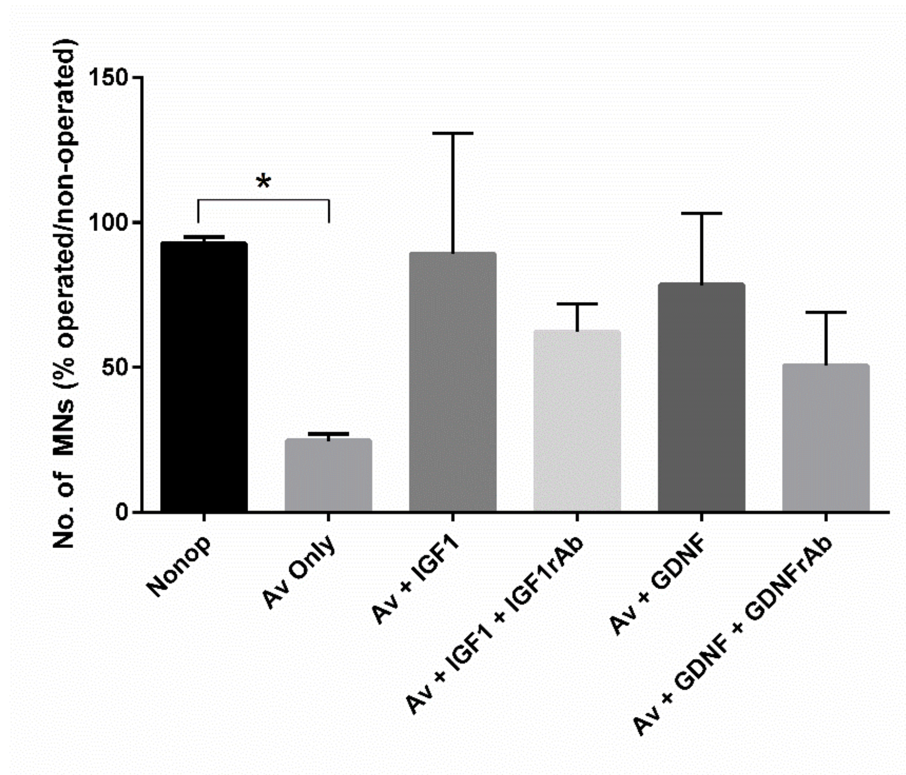

Figure 1: Co-injection of trophic factors with antisera to their respective receptors show reduction in number of motoneurones that survived post-injury. Asterisk denotes p<0.05

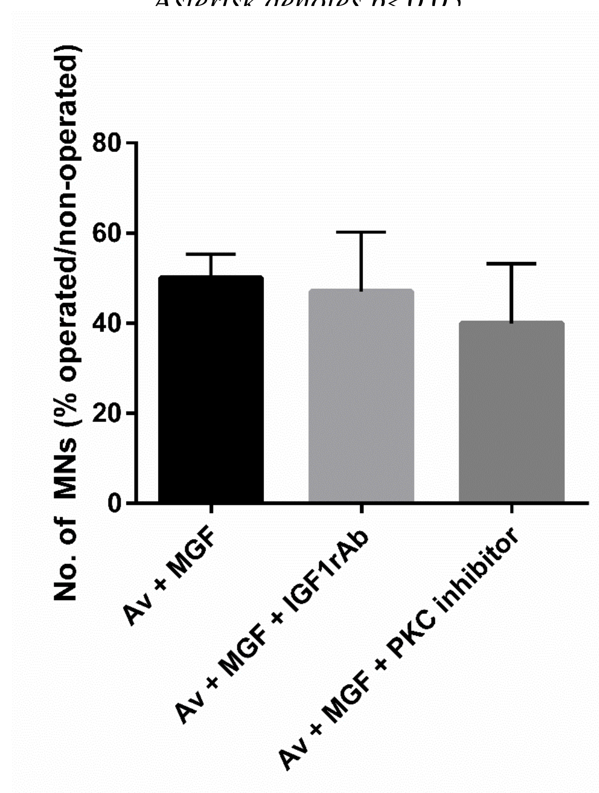

Figure 2: Post-injury co-injection of MGF with the IGF-1 receptor-antibody and Protein Kinase C inhibitor show no significant difference in mean number of motoneurones that survived..

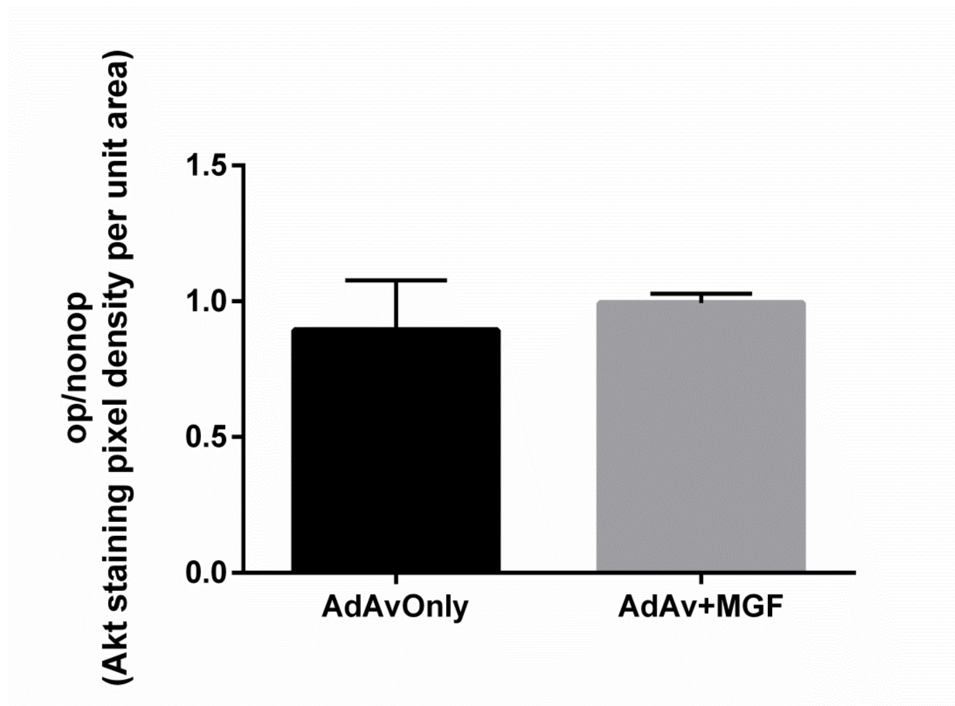

Figure 3: Mean pan-Akt staining pixel density in nerve-avulsed rats compared to counterparts with MGF.

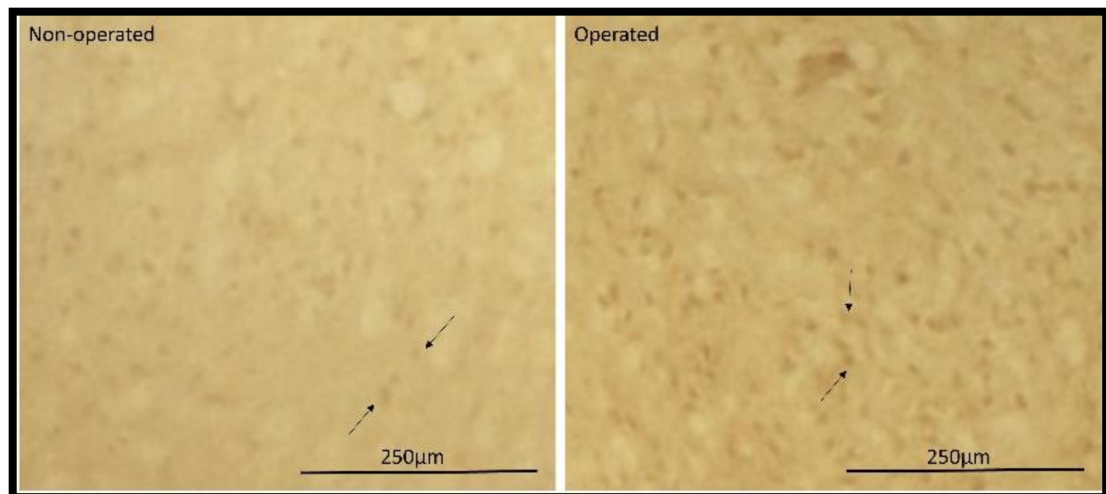

Figure 4: Representative images of non-op vs op nuclei from Avulsion+MGF rat (10x mag). Arrows point to panAkt staining localised to the cell membrane.

2.5 Discussion

We report that injuring motoneurones using nerve avulsion and then administering neurotrophic factors (IGF-1 and GDNF) with their known receptor blockers (IGF-1rAb and GDNFrAb) showed a trend of diminished neuroprotection by the trophic factors, that resulted in an approximate 25% reduction in mean motoneurone survival. This result was statistically significant with a Kruskal-Wallis test, however post-hoc Mann-Whitney procedures were insignificant with our limited sample size and stringent alpha value due to multiple pair-wise comparisons. The inability to completely suppress neuroprotection by IGF-1 and GDNF could be attributed to varying binding affinities of receptors to exogenous trophic factors even though endogenous IGF-1 has the highest affinity for the IGF-1 receptor (Varewijck and Janssen 2012). Alternatively, neuroprotection may have been viable through other secondary messengers even when direct pathways were antagonised as detailed by Clemmons et al. (2009) that IGF-1 receptor antagonists that work in the periphery often do not cross the blood-brain barrier leaving IGF-1 still able to elicit beneficial effects in the brain.

In contrast to the trend for 25% reduction when IGF-1 and GDNF were blocked, co-injection of MGF with potential inhibitors (IGF1rAb and PKC inhibitor) resulted in a negligible difference in mean motoneuronal loss. Thus, it is likely that MGF activity was not blocked by the IGF1r antibody nor by the Protein Kinase C inhibitor. This could be taken to indicate that MGF's activity may not be carried out via the usual mechanisms (typical receptors or downstream messengers) employed by most trophic factors such as the Protein Kinase C/NF-κB cascade used by IGF-1 (Werner and LeRoith 2014).

Immunohistochemical analysis of post-injury MGF treated tissue suggested a slight trend of increased general Akt protein expression in the operated/injured facial nuclei of rats (Fig 4). Akt staining was localised to the neuronal membranes. This suggests that Akt can then be phosphorylated by its activating kinases (e.g. Threonine 308 or serine 473) which then enables the activated Akt to translocate to the nucleus and either activate or deactivate substrates involved in neuronal survival and growth, cell cycle progression and apoptotic responses (Ascoli, Gastard et al. 2011, Dajas-Bailador, Bantounas et al. 2014). However, this data is only preliminary as the archival tissue analysed here was from rats assayed 7 days after either avulsion or avulsion + MGF. The qualitative staining of general Akt may be denser at other time-points when Akt cascade systems are more active and easier to be picked up immunohistochemically. Alternatively, immunofluorescence and sensitive multiphoton capture methods might lend themselves better to detecting subtle changes in Akt levels and distribution within facial motoneurones, or more specific phosphorylated-Akt proteins (e.g. Ser-473) could be studied using immunoperoxidase staining or assayed via homogenised tissue using multiplex kinase kits (Ries, Henchcliffe et al. 2006, Martelli, Tabellini et al. 2012). For further confirmation of the involvement of the PI3k-Akt-mTOR signalling pathway in MGF's neuroprotection of facial motoneurones, MGF could be co-injected with a PI3 kinase inhibitor or Rapamycin (mTOR inhibitor) after facial nerve injury with stereological numbers of motoneurones as an outcome.

The next potential mechanism to investigate would be the ERK signalling pathways which is claimed to be vital for neuronal differentiation and neuroprotection with- and without- Akt phosphorylation (Dai, Wu et al. 2010, Kim and Choi 2010, Tsao, Chiu et al. 2013, Wu, Wu et al. 2013, Asati, Mahapatra et al. 2016). Our result may just be specific to the experimental model that we have employed to evaluate MGF's

mechanism of action. However, other studies seem to corroborate our result. For example, Ates et al (2007) showed that MGF did not employ the IGF-1r to increase muscle progenitor cells in ALS and normal muscle. Another factor to consider would be that while MGF rescues motoneurones in SOD-1 mice, it has not been correlated with human MND (Evans, Harridge et al. 2010). Given that SOD-1 mutation, as a genetic model is dissimilar to sporadic MND, perhaps clinical development in later life suggests that different mechanisms eliciting neuroprotection need to be targeted.

Overall, the sample size limitations of the present study prevent firm conclusions being drawn about the mechanism of MGF24 neuroprotection of adult rat facial motoneurones after nerve avulsion. If the study is regarded as a pilot, however, the trend for approximately 25% reduced neuroprotection when IGF-1 or GDNF were co-administered with antibodies to their respective receptors points strongly towards the utility of the model. Taken together with the negligible effect of the IGF-1 receptor antibody and Protein Kinase C inhibitor on MGF-24 mediated motoneuronal survival here and on neuronal survival in other studies (Gorecki, Beresewicz et al. 2007), this points strongly towards MGF24 having a mechanism of neuroprotection that is different from other neurotrophic factors.

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CHAPTER III

Comparison of early central neuroglial and synaptic responses to peripheral nerve injuries leading to either motoneuronal survival or death

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STATEMENT OF AUTHORSHIP

Title of Paper	Comparison of early central neuroglial and synaptic responses to peripheral nerve injuries leading to either motoneuronal survival or death
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style

Principal Author

Name of Principal Author (Candidate)	Viythia Katharesan		
Contribution to the Paper	Experimental design, data collection, analysis and interpretation, wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/09/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Associate Professor Ian Johnson		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation		
Signature		Date	30/09/2016

3 Comparison of early central neuroglial and synaptic responses to peripheral nerve injuries leading to either motoneuronal survival or death

3.1 Abstract

Perineuronal neuroglia and synaptic terminals have been implicated in both the survival and death of neurones. These structures are very sensitive to injury of adjacent neurones and so could play a central role in determining whether neuronal responses to injury are followed by survival or death. To examine this relationship, we have utilised a facial nerve injury model that is associated with motoneuronal survival (nerve transection) or death (nerve avulsion). In groups of 4 - 6 rats, the facial nerve was either transected or avulsed. Seven day changes in astrocytes, microglial cells and synapses were assessed by quantifying changes in immunostaining at low magnification over 100µm thick sections of the whole facial nucleus for Glial Fibrillary Acidic Protein (GFAP), Ionized calcium binding adaptor molecule 1 (Iba-1) or Synaptophysin, respectively. Significantly higher levels of GFAP and Iba-1 immunostaining were noted after transection and avulsion injuries compared to non-operated rats, although there was no significant difference in immunostaining when transection and avulsion material was compared. No difference between any groups was found for synaptophysin immunostaining. The results suggest that any differences in neuroglial responses related to whether axotomised motoneurones are destined to live or die occur after 7 days. The failure to find any changes in synaptic staining is at odds with previous studies and may reflect technical problems of quantifying very small synaptic structures in a large volumes using large depth of focus microscopy.

3.2 Introduction

Abnormal glial activation in the spinal cord and peripheral nerve fibres, is thought to precede clinical signs of motor weakness in Motor Neuron Disease (MND) and thus assumed to be an early event (Kano, Beers et al. 2012). Enhanced excitatory synaptic transmission to motoneurones and abnormal astrocyte-mediated glutamate transport has also been implicated in the pathogenesis of MND (Rothstein, Van Kammen et al. 1995, Shaw 2005, Rothstein 2009). It is, however, unclear how perineuronal glial and synaptic responses to injury or disease correlate with motoneurone survival in MND. To investigate this relationship experimentally here, we have used the model of adult rat facial nerve avulsion and transection which are associated at 1m with 80% or 10% motoneuronal loss, respectively (unpublished data). These two models can therefore be considered representative of responses to injury that are characterised by either motoneuronal degeneration or motoneuronal survival. It has long been known that synapses contacting axotomised rat facial motoneurones are lost in the first week (Blinzinger and Kreutzberg 1968). It has also been reported that the ultrastructural response of synapses and neuroglia contacting adult rat facial motoneurones is qualitatively similar 4d after facial nerve avulsion, where motoneurones subsequently die, or following nerve crush where motoneurones survive (Soreide 1981). However, with only 2 rats examined at this time point and the additional sampling restrictions imposed by ultrastructural analyses, the generality of this observation can be called into question. More recent studies have shown that motoneurones depend on central nervous system support mediated by microglia and astrocytes (Cerbai, Lana et al. 2012, Valori, Brambilla et al. 2014). Moreover, much of the normal neurotransmission that motoneurones carry out depends on synaptic activity that is modulated by these glial cells (Sargsyan, Monk et al. 2005). Thus, *in-vitro* studies have shown that the presence

of mutant glia with the G93A allele of the superoxide dismutase gene (G93A) underlying both wild type and mutant G93A neurons can up or down regulate normal signalling pathways such as TGFβ and MAPK pathways that are involved in modulating neuronal damage in acute local injuries that involve transient inflammatory responses (Papadeas, Kraig et al. 2011, Phatnani, Guarnieri et al. 2013). When such neuroglial mutations are combined with oxidative stress, genes involved in signalling protein turnover are dysregulated. This affects the neuronal cytoskeleton and its transport and trafficking mechanisms, which in turn disrupts neuron-glia communication (Phatnani, Guarnieri et al. 2013). In the light of these results showing a complex interplay between synapses and neuroglia and repeated suggestions that changes in synapses and neuroglia may underlie degenerative changes in motoneurones in MND, we have re-examined the early perineuronal responses to nerve injuries destined to result in motoneuronal death or survival

3.3 Materials and Methods

3.3.1 Animals

Sprague-Dawley rats were used at 3 months of age. Groups of 4-6 rats were used. Animals were housed under a standard 12-hour on/off lighting regime and given food and water *ad libitum*. The experimental study complied with the Australian code for the care and use of animals for scientific purposes (2016) and was approved by the University of Adelaide Animal Ethics Committee (M-29-2013).

3.3.2 Facial Nerve Injuries (Transection or Avulsion)

Under deep surgical anaesthesia with isoflurane, the right facial nerve was either transected or avulsed. Briefly, an incision is made behind the right ear to access the

facial nerve covered by the sternocleidomastoid and masseter muscles. In transections, the nerve is cut to ensure the axons and nerve sheath are interrupted completely. Whereas in avulsions, the facial nerve is traced down to the exit point from the stylomastoid foramen and freed from its periosteal attachments and then avulsed. Detachment of the facial nerve rootlets as they emerge from the brainstem will result in a very proximal axotomy which results in motoneuronal death.

3.3.3 Perfusions and tissue preparation

All rats were deeply anaesthetised (60mg/kg intraperitoneal sodium pentobarbital), 7 days after surgery. When limb and corneal reflexes disappeared, rats underwent intracardiac perfusiuon with fixative (4% paraformaldehyde in 0.1M sodium phosphate buffer with pH = 7.4). The brainstem was removed and left in fixative at 4ºC overnight. The brainstem was then trimmed 1mm above the rostral border and 2mm above the caudal border of the pons so that the 1.5mm long region containing the facial nuclei lay roughly midway in a piece of tissue 3-4mm long. A V-shaped nick was made in the posterolateral part of the left side of the brainstem to enable orientation of sections later on. The specimen was fixed to the stage of a vibratome using cyanoacrylate glue and serial 100μm sections were cut and transferred to phosphate buffer-filled wells. The specimen was sectioned until only 0.5mm of specimen remained on the stage. The section series containing the facial nucleus were identified using bright field microscopy. A few sections were stained with 1% aqueous Toluidine Blue for light microscopy. Two rats were also perfused with 2% glutaraldehyde in 0.1M phosphate buffer 7 days after nerve avulsion and the brainstem was prepared for electron microscopy by the University of Adelaide Histology Laboratory.

3.3.4 Immunohistochemistry

Sections were selected randomly and systematically from the facial nucleus section series. These sections were defrosted by adding a few drops of room temperature PBS to the wells containing the selected sections. This procedure did not result in the defrosting of remaining sections. Sections were then transferred to 5ml pots and rinsed in PBS twice for 5 mins each rinse. 0.3 % Hydrogen Peroxide (H_20_2) in 75% methanol was used to block endogenous peroxidase activity for 20 mins after which 0.1% Triton X-100 in PBS was used to permeabilise sections twice for 15 mins. 2.5% Normal Horse Serum (NHS) (Sigma) was applied to sections at room temperature for 20 mins to block non-specific antibody binding. Sections were then again permeabilised in 0.1% Triton X-100 in PBS as mentioned previously. For Glial Fibrillary Acidic Protein (GFAP), Ionised calcium binding adaptor molecule 1 (Iba1) and Synaptophysin, sections were incubated with either 1:5000 of polyclonal rabbit anti-GFAP (Dako Agilent Pathology Solutions, ZO334), 1:5000 of polyclonal goat anti-Iba-1 (Abcam, ab5076) or 1:500 of polyclonal rabbit anti-Synaptophysin (Abcam, ab32594) primary antibodies respectively using 1% NHS in PBS as the diluent for GFAP and Iba-1 and 1% NGS in PBS for Synaptophysin, overnight at 4°C. These primary antibody dilutions were determined by pilot experiments. The following day, sections were taken out of the fridge and washed in PBS, twice for 10mins each time. Universal anti rabbit biotinylated secondary antibody (Vector Laboratories, USA) was applied to GFAP and Iba-1 sections and biotinylated anti-goat secondary was applied to Synaptophysin sections for 30 mins. Sections were rinsed in PBS for 10mins each followed by Avidin-HRP-Biotin complex from the Vectastain ABC Kit Solution (Vector Laboratories, USA) which was applied for 30 mins. Sections were then washed in PBS twice for 10 mins before doing a pre-incubation in 3,3'-diaminobenzidine (DAB) (Vector

Laboratories, USA) without the H_20_2 for 10 minutes and a further 5 mins with H_20_2 and Nickel. As soon as the desired colour was achieved, the DAB was quickly replaced with phosphate buffer. Sections were then placed on silane-coated slides and taken through graded ethanols (50%, 70% and 100%) followed by part methanol and part Xylene and finally 100% Xylene to then be mounted using the depex mountant and coverslipped. As controls for the immunohistochemical procedure, either the primary or secondary antibodies were omitted with all other aspects of tissue processing, including blocking antisera, remaining the same.

3.3.5 Preliminary Immunofluorescence

100µm sections were defrosted from 30% sucrose (used as cryoprotectant) and rinsed with PBS then permeabilised as mentioned in the Immunohistochemistry section. Non-specific binding was blocked using 2.5% Normal Goat Serum and following another permeabilisation, the primary antibody: rabbit polyclonal Synaptophysin (Abcam, ab32594) was applied to sections for an overnight incubation. The following day, a PBS rinse was done before the secondary antibody: goat anti-rabbit Alexa Fluor 568 (Abcam, ab175471) was applied at 1:250 concentration and coverslipped using the crystalmount aqueous mounting medium.

3.3.6 Image processing and semi-quantitative analysis

High quality 10x magnification images of the facial nucleus were exported from the NDPview2 software (Hamamatsu) to ensure the images retained characteristics from initial acquisition for analysis. Areas within each facial nucleus were randomly chosen. Images were grayscaled using the GIMP software (GNU Image Manipulation Program) and analysed using Image J under blind conditions. Briefly, under Analyse > Set Measurements, “integrated pixel density” was selected and each image was measured

individually. Raw data was then collected from the ROI Manager window. Mean pixel density per unit area (754 000μm^2) was calculated from 4 measurements within each nucleus, with at least 3 sections per rat (n= 4 - 6 rats per group). To control for staining variations between sections pixel density was expressed as a ratio of the right (operated) nucleus/left (non-operated nucleus).

3.3.7 Statistical Analysis

Datasets satisfied the assumptions of normality (through Q-Q plots and Shapiro-Wilks tests) and homogeneity of variance (Levene's test) and were analysed using the parametric one-way Analysis of Variance (ANOVA) procedure. Post-hoc comparisons were done using the Bonferroni procedure. Statistical significance is noted as $p<0.05$ unless otherwise stated.

3.4 Results

3.4.1 Astrocytes

Qualitatively, there was significantly more GFAP staining around the periphery of axotomised motoneurones at 7d regardless of type of nerve injury. When overall staining levels of the facial nucleus were compared quantitatively, there was significantly more staining following axotomy compared to non-operated rats (1.0 ± 0.04). No significant difference was found according to whether motoneurones had been injured by facial nerve transection (0.8 ± 0.02) or avulsion (0.7 ± 0.05) (Fig 1).

3.4.2 Microglia

Qualitatively, there was significantly more Iba-1 staining around the periphery of axotomised motoneurones at 7d regardless of type of nerve injury (Fig. 2). Many perineuronal microglia were observed around axotomised motoneurones with no

difference according to the type of nerve injury (Fig 3). When overall staining levels of the facial nucleus were compared quantitatively, there was significantly more staining following axotomy compared to non-operated rats (1.0 ± 0.01) (Fig 2). No significant difference was found according to whether motoneurones had been injured by facial nerve transection (0.77 ± 0.02) or avulsion (0.81 ± 0.02).

3.4.3 Synapses

No significant differences in mean Synaptophysin staining pixel density over the whole facial nucleus were found between the non-operated (1.03 ± 0.06), transection (1.04 ± 0.01) and avulsion (0.98 ± 0.03) groups (Fig 4).

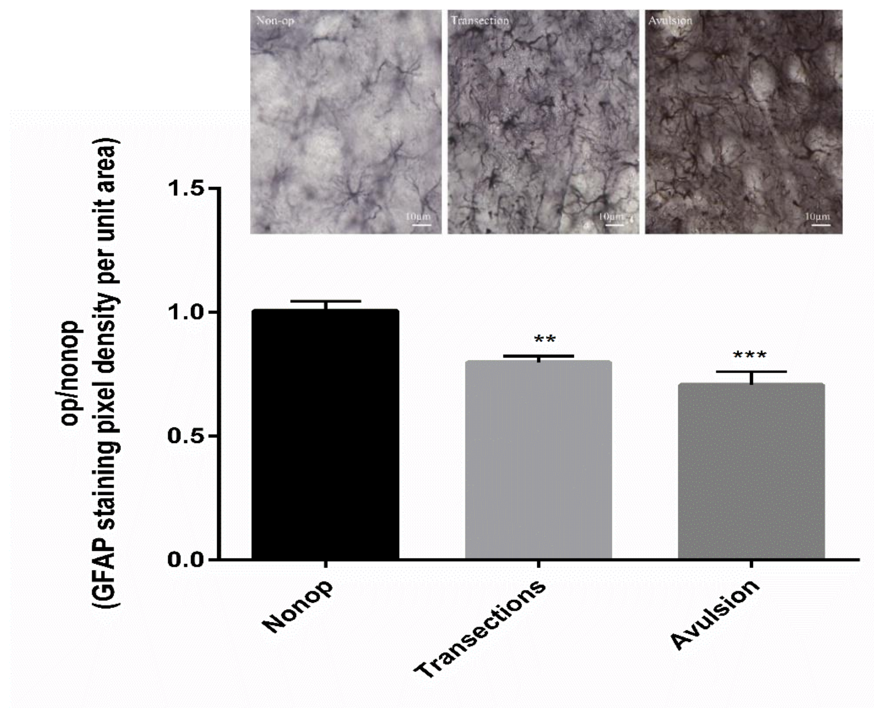

*Figure 1: Significantly increased GFAP staining pixel density in transection- and avulsion- injury rats compared to non-operated rats. ** denotes p<0.01 and *** denotes p<0.001. Corresponding representative 20x images above barchart.*

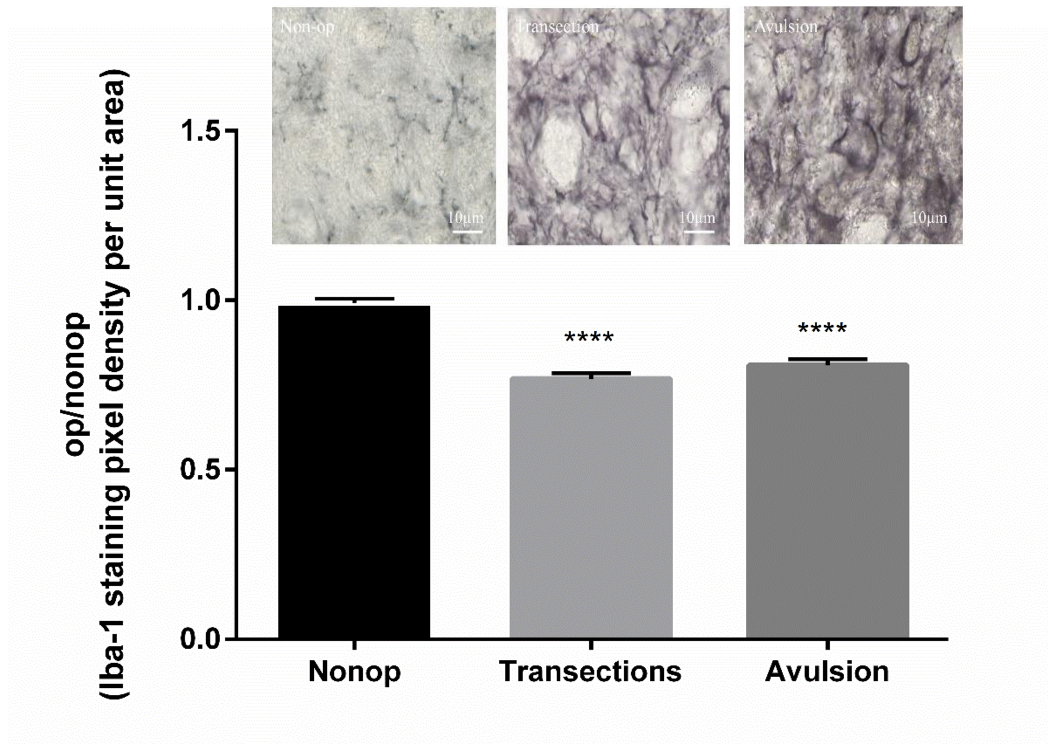

*Figure 2: Significantly increased Iba-1 staining pixel density in transection- and avulsion- injury rats compared to non-operated rats. **** denotes p<0.0001. Corresponding representative 20x images above barchart.*

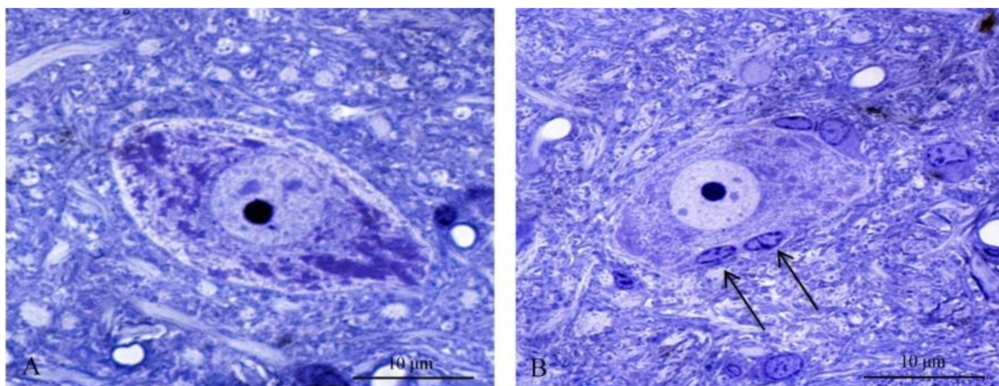

Figure 3: Light Microscopy images of 0.5µm Toluidine Blue stained sections from 7day avulsion tissue: (A) Motoneurone cell body from the non-operated nucleus, (B) motoneurone cell body from the operated nucleus with arrows indicating microglia on the borders of the soma.

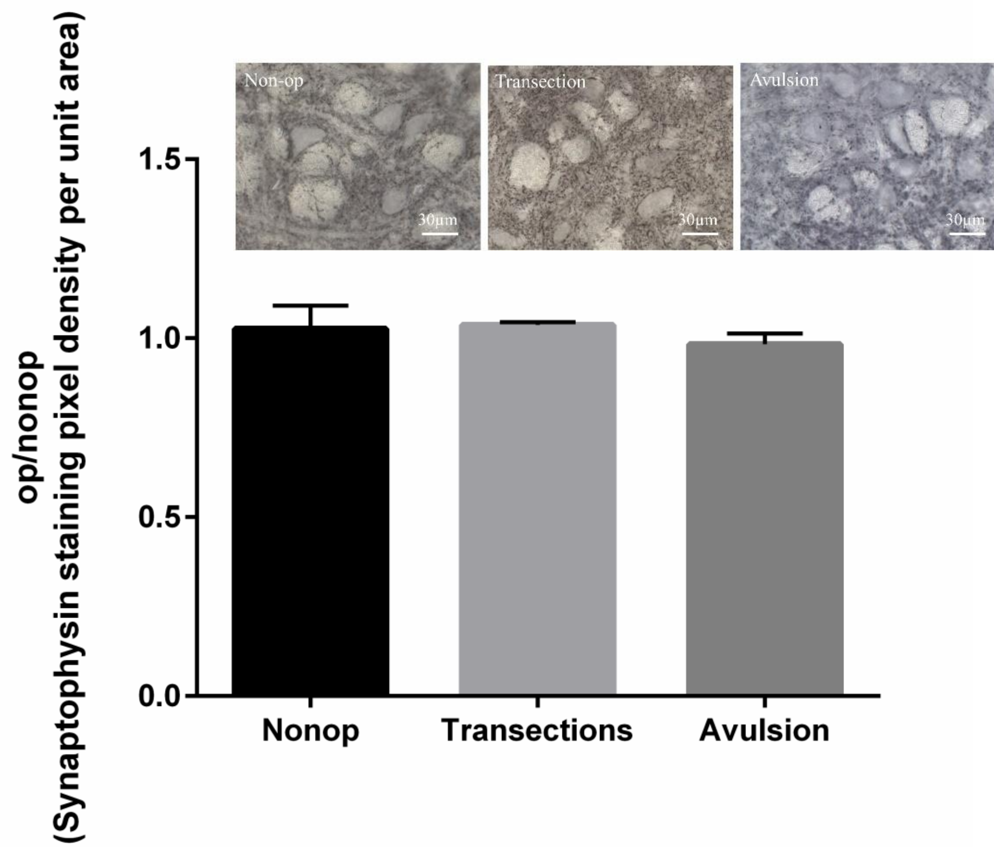

Figure 4: No significant differences in Synaptophysin staining pixel density across non-operated and transection- and avulsion- injury rats. Corresponding representative 20x images above bar chart.

3.5 Discussion

Using quantitative immunocytochemical methods, we report that the early (7d) perineuronal response of neuroglia and synapses to axotomy of adjacent facial motoneurones in adult rats does not differ according to whether motoneurones are destined to survive or die. This confirms a previous ultrastructural study at 3d post-facial axotomy by Soreide (1981), who also reported reversal of the neuroglial response by 2 months where motoneurones survived, but a persisting response seen where motoneurones died. Taken together with the recent observations, this indicates that differences in the perineuronal response according to whether injured motoneurones are destined to survive or die are likely to be seen at time points later than 7 days.

Astrocytes and microglia are activated under conditions where injured neurones repair and regenerate, but also under conditions where injured neurones die. Thus, it appears that these glial cells are involved in both neuroprotection and neurodegeneration (Kerns and Hinsman 1973, SÖReide 1981, Parisi, Arisi et al. 2013, Freitas-Andrade and Naus 2016). Van Dyke et al (2016) showed that ex-vivo GDNF delivery to limb muscles reduced microglia-mediated inflammation in a SOD-1 rat model of fALS. Since this gene therapy approach in the same model had also been associated with reduced spinal motoneurone loss, the result suggests that microglia-mediated inflammation may contribute actively to neuronal degeneration. In contrast, a review of literature primarily pertaining to the amyloid cascade neuroinflammation hypothesis in Alzheimer's Disease points towards loss of microglia-mediated neuroprotection resulting in age-related neuronal loss (Streit and Xue 2012). One possible reason for this dichotomy of opinion may be that the potential of microglia to become phagocytes and remove degenerating neurones is taken as evidence that they will kill neurones in a manner

similar to cytotoxic macrophages in the peripheral immune system. Our study provides no evidence that the microglia around neurones destined to live or die are any different at 7d. Further studies are needed to confirm this view, including measurement of neurotrophic factor and reactive oxygen species production by microglia at 7d and analysis of microglia during the phase of active motoneuronal loss, which in the rat facial nucleus occurs between 7-14d (unpublished observations).

The present study found significantly increased astrocytic and microglial activity around injured motoneurones, in both transection and avulsion injuries compared to non-operated animals. The close spatial relationship between both types of glia and the injured motoneurones as seen in Fig 1, 2 and 3, suggest ongoing interactions 7 days post-injury. More specifically, the microglia do not appear dystrophic in phenotype as they lack the typical cytorrhexis that is noted in degenerative conditions (Streit, Xue et al. 2014). This makes sense as facial nerve axotomies leave the blood-brain-barrier (BBB) fairly undisturbed and dystrophic microglia are usually only seen within the parenchyma when the BBB is breached (Xue, Yang et al. 2010). Instead, the microglia in the present study seem to be enveloping the injured motoneurones. At the stage (7d) examined, this microglial response could be presumed to be supportive and neuroprotective, possibly through the production of neurotrophic factors (Streit and Xue 2012). Whether the subsequent death of motoneurones following nerve avulsion is due to primary changes in the motoneurones or perineuronal elements remains to be determined.

For long, the involvement of astrocytes in motoneuronal degeneration was questioned as microglia were assumed to responsible for such degeneration and early facial nerve axotomy models showed that astrocytes did not undergo mitosis (Graeber, Tetzlaff et al. 1988). With the shift in opinion about microglia potentially being neuroprotective, more

recent literature has focussed on astrocytic responses in normal brain aging, neuroprotection and neurodegeneration. This has led to the notion that therapeutics for neurodegenerative diseases such as MND, need to look at the neuron-microglia-astrocyte triad as targets, as evidenced by the development of newer *in-vitro* models (Beaudet, Yang et al. 2015). In the present study, marked astrogliosis was seen following both nerve transection and avulsion but with no significant difference according to how the nerves had been injured. Astrocytes are thought to be the "housekeepers" that maintain the correct extracellular environment and excitatory-inhibitory balance around motoneurones by providing antioxidants to combat oxidative stress and prevent cell excitotoxicity by encouraging glutamate re-uptake (Benediktsson, Marrs et al. 2012). Injured neurones release soluble "SOS" signals which induce astroglial activation and recruitment to the site of injury (Xing, Wang et al. 2014). The degree of astrocytic response however, has been suggested to depend on the severity of the neuronal injury such as in traumatic brain injury with BBB disruption. Since we were unable to detect differences between transection and avulsion groups when overall staining for GFAP was quantified, counts of reactive vs. resting glia may show differences. Aged astrocytes have been reported to express more GFAP and to appear chronically activated compared to adult astrocytes (Hains, Loram et al. 2010, Devinsky, Vezzani et al. 2013). Such an approach may allow us to correlate our results with age and type of nerve injury.

We noted no differences in staining for synapses with synaptophysin across non-operated and injured rats. Our method of averaging staining at relative low power over the whole facial nucleus, however, may not be ideal for detecting small changes or detecting perineuronal changes. Western blots using synaptophysin may have allowed small changes to be detected, but would have required a separate group of animals to

obtain fresh tissue. Electron microscopy clearly shows synaptic stripping following axotomy (Fig.5), but this was not detected by immunostaining, possibly due to the thickness of the tissue used (100µm) causing immunostained profiles to superimpose on each other. Preliminary immunofluorescent studies have shown that Maximum Projections in the Confocal Laser Scanning Microscope could instead be used to study general synapses in a thick section or an alternative form of fluorescent image analysis on images such as the one seen in Fig 6, showing synapses around motoneurones in avulsed tissue stained with synaptophysin conjugated to AlexaFluor488. It is also possible that synapses displaced by microglia from injured motoneuronal cell bodies do not disappear but move slightly to contact dendrites in the neuropil. Such a subtle change would not be picked up by low magnification analysis of thick tissue slices. The effects of nerve transection and avulsion on excitatory and inhibitory synapses could also be assessed by immunostaining with antibodies to the vesicular glutamate transporter (vGLUT) antibody) and the vesicular GABA transporter (vGAT).

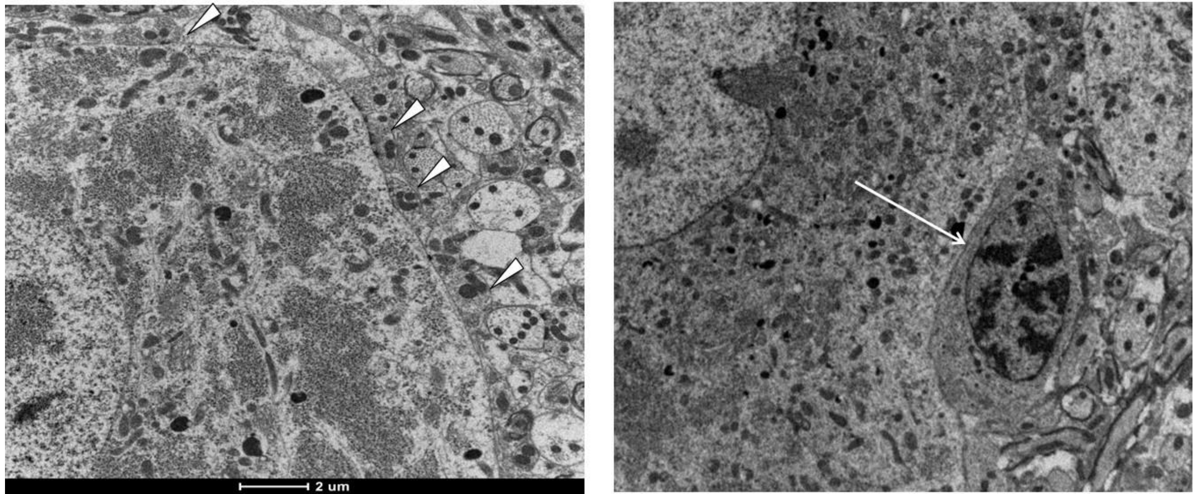

Figure 5: Electron Microscopy image of 7 day avulsion tissue: Left image showing non-operated motoneurone with synapses indicated by arrowheads. Right image showing operated motoneurone with microglial cell (arrow) displacing synapses.

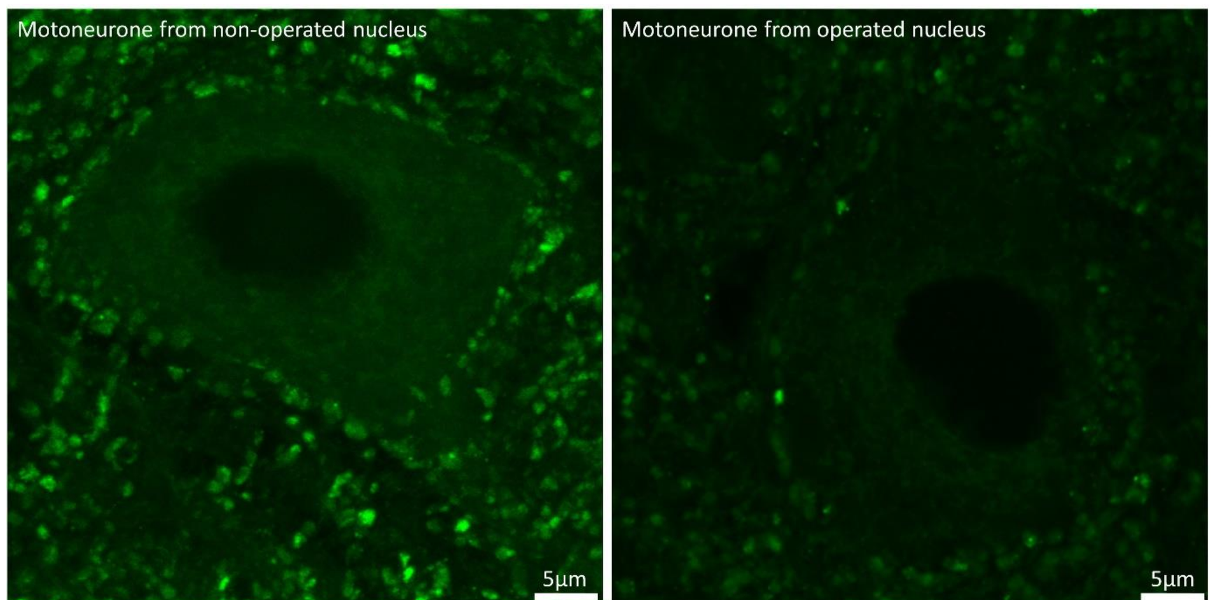

Figure 6: A motoneurone from the non-operated vs operated facial nucleus (315x magnification) of a nerve-avulsed rat. Preliminary images indicate that IF images may lend themselves to semi-quantification better than synaptic immunohistochemistry.

3.5.1 Conclusion

Taken together, persistent CNS reactions may be vain attempts to compensate for transient and/or permanent functional deficits of neurones. This could be attributed to the retraction or degeneration of synaptic terminals or be a secondary outcome to initial microglial activation (Streit 1993). In keeping with the theory of neuroprotective microglia, stripping or displacement of synapses (Fig. 5) from the surface of axotomised motoneurones may be intentional to prevent afferent excitation when the injured motoneurone has ceased neurotransmission and is attempting to regenerate itself (Blinzinger and Kreutzberg 1968).

This would also enable: (i) the astrocytes to insulate and better regulate excitatory influence and (ii) the microglia to be physically close to the cell body (as seen in our study), to either provide support to or rapidly phagocytose the injured motoneurone. Trauma and ischemia could be considered to be sterile injuries (Chen and Nunez 2010) that do not require cellular toxins to counteract the injury. This may well be the case for motoneuronal degenerations seen in MND where 95% of cases are sporadic and are not linked to any external toxins or internal genetic mutations. In such situations, the microglia and astrocytes may not be cytotoxic as their purpose could be limited to clearing out debris generated within the brain. However, a caveat to this hypothesis would be ageing motoneurones that sit within a pre-existing inflamed environment that is seen with increasing age and known as “inflammaging”. Perhaps when motoneurones are injured in these situations, the glia take on a destructive role in which could explain why therapeutics that work in experimental models to modify glial involvement struggle to be extrapolated to clinical trials.

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CHAPTER IV

Disparate changes in plasma and CNS cytokine levels in mature rats associated with age-related changes in motoneurone number, peripheral target morphology and exploratory behaviour.

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Title of Paper	Disparate changes in plasma and CNS cytokine levels in mature rats associated with age-related changes in motoneurone number, peripheral target morphology and exploratory behaviour.
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted to Frontiers of Neurology, Neurodegeneration section and undergoing review

Principal Author

Name of Principal Author (Candidate)	Viythia Katharesan		
Contribution to the Paper	Experimental design, data collection, analysis and interpretation, wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/09/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr. Martin Lewis		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation		
Signature		Date	30/09/2016

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Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation		
Signature		Date	28 September 2016

Name of Co-Author	Associate Professor Ian Johnson		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation, acted as corresponding author		
Signature		Date	30/09/2016

4 Disparate changes in plasma and CNS cytokine levels in mature rats associated with age-related changes in motoneurone number, peripheral target morphology and exploratory behaviour.

4.1 Abstract

An overall increase in inflammatory cytokines with age in both the blood and the central nervous system (CNS) has been proposed to explain many aspects of ageing, including decreased motor function and neurodegeneration. This study tests the hypothesis that age-related increases in inflammatory cytokines in the blood and CNS lead to facial motoneurone degeneration. Groups of 3-5 female Sprague-Dawley rats aged 3 months, 12-18 months and 24 months were used. Twelve cytokines (IL-1α, IL-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, TNFα, IFN-γ and GM-CSF) were measured in blood plasma and compared with those in the brainstem after first flushing blood from its vessels. The open-field test was used to measure exploratory behaviour and the morphology of the peripheral target-muscle of facial motoneurones was quantified. Total numbers of facial motoneurones were determined stereologically in separate groups of 3m and 24m rats. Ageing rats showed a significant 30-42% decrease in blood plasma (peripheral) concentrations of IL-12p70 and TNFα, and a significant 43-49% increase in brainstem (central) concentrations of IL-1α, IL-2, IL-4, IL-10 and TNFα. They also showed significant reductions in motoneurone numbers in the operated but not the non-operated facial nucleus, reduced exploratory behaviour and increased in peripheral target muscle size. Marginal age-related facial motoneuronal loss occurs in the ageing rat and is characterised by complex changes in the inflammatory signature, rather than a general increase in inflammatory cytokines.

4.2 Introduction

While there is abundant information from experimental studies on the survival requirements of young motoneurones, not much is known about adult and aged motoneurones, and there is reason to think that young motoneurones are inappropriate models for age-related neurodegeneration, such as MND (Johnson 2015). Using a nerve avulsion model and stereological analysis in the confocal microscope (Figs.1 and 2) we previously reported age-related differences in rat facial motoneurone survival (Johnson and Duberley 1998, Johnson 2001, Aperghis, Johnson et al. 2003, Aperghis, Johnson et al. 2004, Johnson, Goldspink et al. 2011). Here, we consider whether age-related increases in inflammation (i.e. “inflammaging”) (Amor, Peferoen et al. 2014) can affect facial motoneurone survival, by correlating age-related motoneuronal survival with changes in the Central Nervous System (CNS) parenchyma, in the brainstem at the level of the facial nucleus, and in the blood.

Inflammation and its regulation by cytokines is considered to play an important role in both healthy ageing of the nervous system and neurodegeneration (Streit and Xue 2010, Amor, Peferoen et al. 2014). Inflammatory cytokines have also been reported to affect motor functions (Pollmacher, Haack et al. 2002). The link between ageing and inflammation has led to the concept of ‘inflammaging’ (Franceschi and Campisi 2014) which is defined as a low-grade chronic inflammatory state associated with the ageing process. This concept centres on age-related inflammatory cytokine-driven innate immune responses in the peripheral immune system. Inflammaging has also been adduced to help explain age-related neuronal degeneration in the CNS (Chung, Kim et al. 2001, Amor, Peferoen et al. 2014). The extent to which information derived from studies of the peripheral immune system can be transposed to the CNS however is

unclear because there are both unique immunocompetent cells (microglia) in the CNS and there is evidence of significant cross talk between changes in the peripheral immune system and changes in the ageing CNS (Barrientos, Higgins et al. 2006). Almost all cytokines in the peripheral immune system are also produced in the CNS (Rothwell, Luheshi et al. 1996), and cytokines produced peripherally can act on the CNS through several mechanisms (Banks and Kastin 1997, Banks, Farr et al. 2001, Jurgens and Johnson 2012). Just how CNS and peripheral cytokines alter with age, however, is unclear as typical CNS samples also contain large amounts of peripheral blood (Loane, Deighan et al. 2009, Fu, Yang et al. 2014), raising the possibility that larger changes in systemic cytokines may mask smaller changes in CNS cytokines. In this study, we have sought to distinguish age-related changes in CNS cytokines from those occurring systemically, by analysing the brainstem at the level of the facial nucleus after flushing the blood from its vessels. Using a multivariate approach, we have studied 12 cytokine-proteins that are implicated in both ageing (Streit and Xue 2010, Njie, Boelen et al. 2012, Amor, Peferoen et al. 2014) and neurodegenerative conditions (Frank, Barrientos et al. 2006, Hopp, Royer et al. 2014) to identify the inflammatory signature characteristic of healthy ageing in rats. The cytokines studied were Interleukin (IL)-α, IL-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, Tumour Necrosis Factor-α (TNFα), Interferon (IFN)-γ and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). Of these cytokines, IL-1α, IL-β, IL-2, IL-6, IL-12p70, TNF-α, IFNγ and GM-CSF are pro-inflammatory, whereas IL-4, IL-5, IL-6, IL-10 and IL-13 are anti-inflammatory (Berger 2000, Shimada and Hasegawa-Ishii 2011). Age-related changes in cytokines have been compared with age-related changes in total numbers of facial motoneurones as well as age-related changes in the fibre density of the peripheral-target muscle and changes in exploratory behaviour.

4.3 Materials and Methods

4.3.1 Animals

For the cytokine analysis, groups of 3-5 female Sprague-Dawley rats aged 3 months, 12-18 months and 24 months were used. For morphometry of muscle, open field tests and motoneurone counts, groups of 4-12 rats aged 3m and 24m were used. Ageing rats are not available commercially in Australia. Rats were therefore obtained as adults and maintained until 24m old in the rodent facility of the University of Adelaide, before use. While the maximum reported lifespan of the *ad libitum*-fed Sprague Dawley rat is 36 months (Sengupta 2013), we found approximately 50% of the rats in our study had died by 24m, as reported previously for this strain (Vanden Noven, Seburn et al. 1996, Johnson and Duberley 1998). Animals were housed under a standard 12-hour on/off lighting regime and given food and water *ad libitum*. The experimental study complied with the Australian code for the care and use of animals for scientific purposes (2016) and was approved by the University of Adelaide Animal Ethics Committee (M-57-2013). The ageing animals analysed here did not have significant health issues although they did have age-related conditions such obesity, lipomas and arthritis that are typical of an ageing population.

4.3.2 Stereological counts of facial motoneurones

In rats terminally anaesthetised with sodium pentobarbitone, the brain was fixed by intracardiac perfusion with 4% phosphate-buffered paraformaldehyde following a saline rinse. 100µm vibratome sections were cut serially through the facial nucleus and numbers of motoneurones estimated in every 5th section using an optical disector method modified for use in the confocal scanning laser microscope as described previously (Fig. 1) (Johnson 2001).

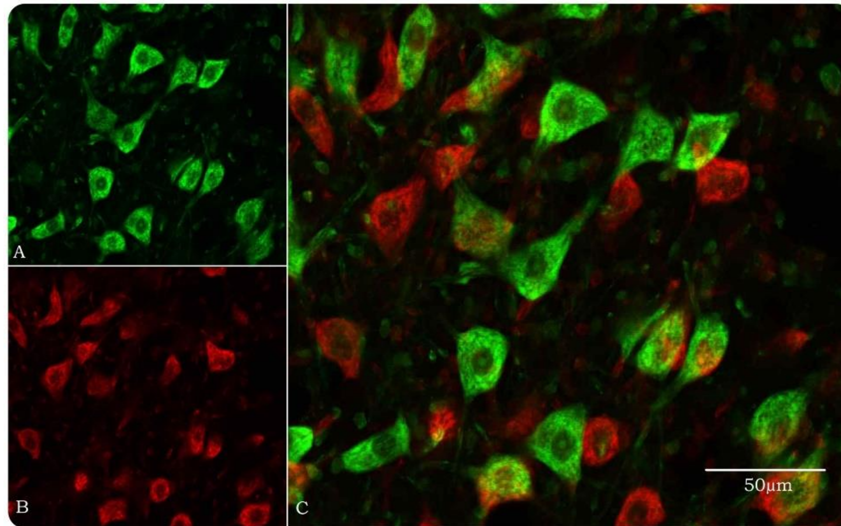


Figure 1: Stereological counts of motoneurone used an optical dissector method adapted for the confocal microscope. Scans A and B are 10μm apart. They are merged in C and only green neurones are counted. Total volume of the facial nucleus is determined by the Cavalieri method

4.3.3 Image processing and morphometric analysis of peripheral target-muscle

5 μm microtome sections of the paraffin-embedded peripheral muscle targets of facial motoneurones (i.e. snout muscle) was stained with Haematoxylin and Eosin for general muscle morphology measurements (Rosero-Salazar 2016) using the segmentation and analysis method (FIJI, Adelaide Microscopy). High quality 20x magnification images were collected via the NDPview software to ensure the images retained characteristics from initial acquisition for analysis and would not require enhancing in terms of brightness or contrast. Briefly, the scale on the images was set so that all subsequent measurements were correct (e.g. X number of pixels for a known distance). The threshold of images was then automatically adjusted by Image J before converting the coloured image to a binary image. The binary watershed function was used to ensure real muscle fibres were being segmented as “real particles”. Finally, particles were identified as the output type “maxima within tolerance” and noise tolerance was set to 200.00 before being analysed.

4.3.4 Functional test

An open field test was used as a measure of exploratory behaviour (Gould, Dao et al. 2009). A 100cm x 100cm square box acted as the “open field” and rats were placed in the centre of the open-field arena. Movement, in terms of total distance travelled, was then recorded for a period of 5 minutes. To ensure consistency between groups, the time at which the test was conducted, colour and texture of the open-field box, lighting, temperature, ambient noise and olfactory cues were all controlled for. The Stoelting “ANY-maze” software was used as the tracking system that automated this functional test (Prut and Belzung 2003).

4.3.5 Cardiac puncture, saline-perfusion, protein extraction and estimation

Rats were deeply anaesthetised by inhalation of 5% isofluorane in 2 litres oxygen/minute and while the anaesthetic nose cone was still attached, cardiac puncture was performed to withdraw blood into EDTA-coated blood tubes. Blood plasma was retrieved and stored at -80°C. Immediately following cardiac puncture, rats were perfused transcardially with approximately 200mL of sterile saline until the fluid flowing out of the right atrium was clear. The animals were then decapitated and the brainstem removed, trimmed at the mid pons level and approximately 1mm below the lower border of the pons to ensure it contained the facial nucleus, snap-frozen and stored at -80°C. Frozen brainstem samples were homogenised in lysis buffer made up with PBS, triton-X and protease inhibitors (Roche, cOmplete tablets). The supernatant was retrieved from homogenised samples and stored at -80°C. The BioRad DC Protein Assay (a modified Lowry method) was used to quantify the amount of protein in each sample as per the manufacturer’s instructions.

4.3.6 Multiplex Assay

Bio-Plex Pro Rat 12 plex cytokine assay kits (BioRad, New South Wales) were used to measure the concentration of twelve cytokines within each sample. Samples were loaded onto 96 well plates in duplicates (3m and 12-18m rats) and triplicates (24m rats). Plates were read using a Magpix Luminex multiplexing platform which uses a fluorescent imager (Abacus-ALS, Queensland) and data expressed as pg/ml of concentration. Experimental data was calibrated against standard curves of all 12 cytokines (BioRad, New South Wales). To validate the accuracy of the multiplex assay, a spike-recovery analysis was performed. This involved obtaining readings for cytokine standards serially diluted in buffer as per the manufacturer's instructions and comparing these with readings for cytokines diluted in brain homogenates ('spike recovery'). The latter represented the form in which the cytokines were measured in rats of different ages in this study. As seen in Table 1, slope differences of $\leq$ 30% were found. Using a Parallelism approach, this is generally taken to indicate that there are minimal effects of the matrix on the assays and resultant standard curves (Smolec, DeSilva et al. 2005).

4.3.7 Statistical Analysis

Mann-Whitney U tests were used for comparisons of motoneurone number, morphometric analysis of muscle and functional test results. A general linear model (SPSS statistics 22, IBM) was used to generate descriptive statistics for all three age groups and to check for interactions between cytokines. The 12 cytokines within the same sample were treated as "repeated measures" within each animal. Age categories were treated as between-subjects factors and the 12 cytokines were treated as within-subjects factors. Dunnett's post-hoc test was used to test for differences between

cytokines. The omnibus/homogeneity test confirmed that the spread of scores was roughly equal across the three age groups which meant that the comparisons were between populations with equal variances. A multivariate test was then run between age-categories to determine the significance of differences within cytokines in the different age groups. Statistical significance of $p<0.05$, $p<0.01$ and $p<0.001$ is reported.

4.4 Results

4.4.1 Age-related changes in facial motoneurones and their peripheral targets

Mean total numbers of motoneurones in the brainstems of 24m rats were 22% lower than those of 3m rats (Table 2). While this reduction was statistically significant for the right facial nucleus (24% reduction, $p=0.041$), it just failed to reach significance for the left (19% reduction, $p=0.052$). This probably reflects the small (n=6) sample size for the 24m rats. In contrast to the age-related reduction in number of facial motoneurones, analysis of the snout muscle, which represents the peripheral targets of these motoneurones, revealed an increase in muscle fibre size. Thus, mean pixel density measurements (pixels/21cm^2) from segmentation and analysis of 20x images showed that 3m old rats had 49% smaller ($p < 0.05$) peripheral-muscle fibre densities (22535 ± 822) compared to 24m old rats (43786 ± 7564) (Fig.2).

4.4.2 Open-field exploratory behaviour

In general, 3m rats were more active. This qualitative observation was confirmed using total distance travelled (m) during the open field test, where 3m old rats showed 44% more ($p<0.05$) exploratory behaviour than 24m old rats (Fig. 3).

Table 1: Slope differences (% difference) of 3m and 24m homogenate samples compared with standard curves

Cytokine Standards	Sample % Difference	
	3m	24m
IL-1α	0.59	0.09
IL-1β	8.78	4.07
IL-2	6.42	10.96
IL-4	4.07	-7.56
IL-5	30.51	3.82
IL-6	-7.22	11.56
IL-10	28.07	-2.24
IL-12(p70)	-8.32	3.57
IL-13	9.67	-1.61
TNF-α	-1.82	-2.46
IFN-γ	-1.01	4.22
GM-CSF	-21.79	-1.09

Table 2: Numbers of motoneurones in the left and right facial nuclei of 3m and 24m rats.

	3m rats		**24m rats**	
	left nucleus	**right nucleus**	**left nucleus**	**right nucleus**
	3404	3676	2518	2953
	3118	3622	2461	3063
	3385	3631	3872	3722
	3233	3666	1982	1842
	3090	3305	1866	1802
	3307	3222	2389	1998
	2958	2306		
	3501	4135		
	2821	3329		
	2232	2295		
	3127	3990		
	3105	3398		
n	12	12	6	6
Mean	3256.17	3520.33	2514.67	2563.33
SEM	38.36	58.23	292.43	324.73

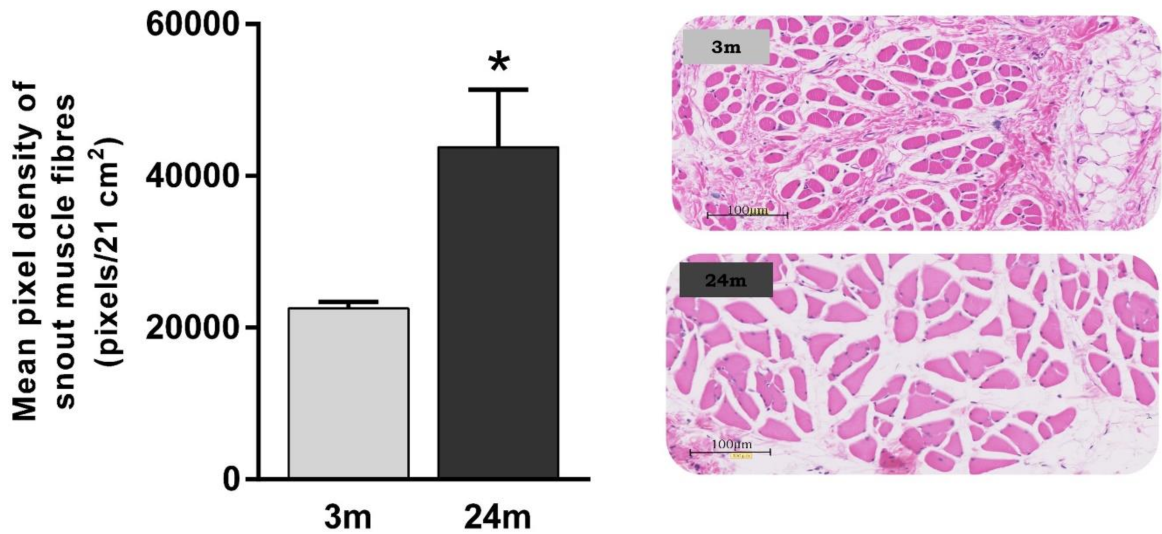


Figure 2: Significantly lower pixel density measurements (pixels per 21cm2) of 3m old rats' snout muscle fibres compared to 24m old rats (Mann-Whitney U test, $p<0.05$). Representative 20x H&E images of 3m (light grey label, n=5) and 24m (dark grey label, n=4) images.

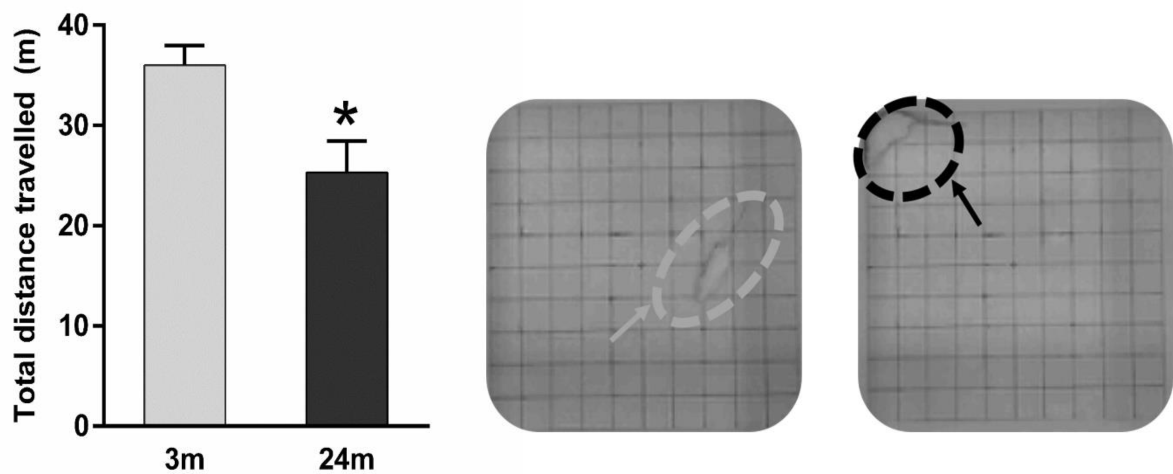


Figure 3: Mean ± SEM (m) total distance travelled in the open field. Asterisk denotes $p<0.05$ (Mann-Whitney U test) showing 24m old rats (n=5) travelling a significantly shorter distance vs 3m old rats (n=4). Example representative images of an adult rat exploring the open-field (grey arrow) and an aged rat crouched in the open-field corner (black arrow).

4.4.3 Age-related changes in brainstem and plasma cytokine levels

Overall, increasing age from 3m to 24m was associated with an increase in brainstem cytokine levels (Fig. 4) and a decrease in plasma cytokine levels (Fig. 5). Analysis of rats aged 12-18m revealed that this did not affect cytokines uniformly. Detailed comparisons across the 3 age groups (3m, 12-18m and 24m) are given below.

4.4.3.1 Comparison of cytokine levels in 3m and 12-18m old rats.

In the brainstem of 12-18m rats, the concentrations of 3 cytokines were significantly lowered by 17 - 65% compared to 3m rats ($p < 0.05$). The cytokines and their concentrations (pg/ml) were IL-5 (303 ± 12 vs 355 ± 13), IL-6 (203 ± 21 vs 335 ± 32) and IFNγ (353 ± 44 vs 519 ± 47) (Fig. 5). There were no significant differences in plasma samples of 3m and 12-18m old rats (Fig. 5).

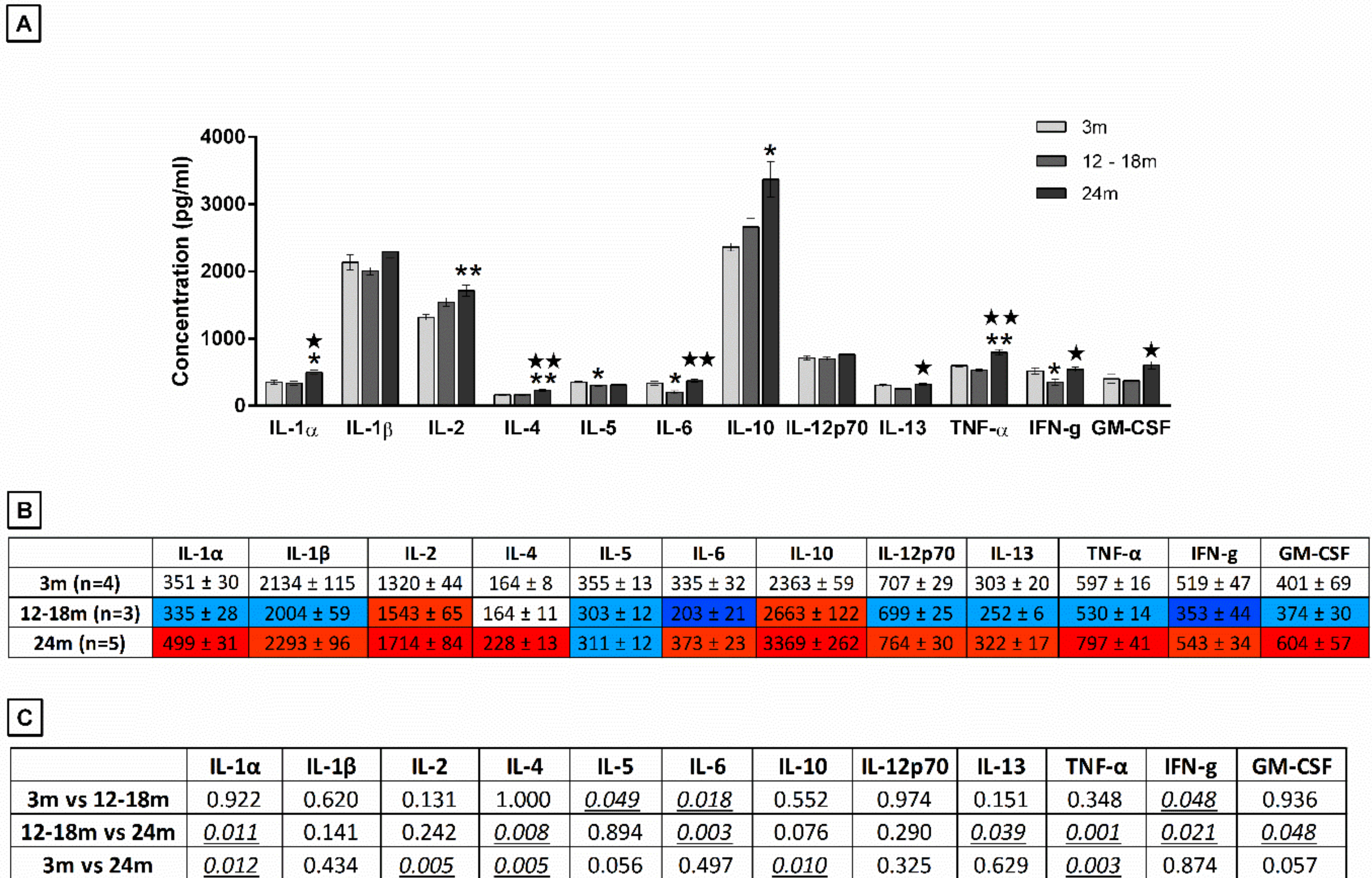
4.4.3.2 Comparison of cytokine levels in 3m and 24m old rats.

In the brainstem of 24m rats, the concentrations of 5 cytokines were significantly increased by 23-30% compared to 3m rats. The cytokines and their concentrations (pg/ml) were IL-1α (499± 31 vs 351 ± 30, $p < 0.05$), IL-2 (1714 ± 84 vs 1320 ± 44, $p < 0.01$), IL-4 (228 ± 13 vs 164 ± 8, $p < 0.01$), IL-10 (3369 ± 262 vs 2363 ± 59, $p < 0.05$) and TNFα (797 ± 41 vs 597 ± 16, $p < 0.01$) (Fig. 5). The three cytokines whose concentrations were decreased in the brainstems of 12-18m rats (section 4.4.3.1) were therefore no longer decreased, but they did not contribute to the increase at 24m. GM-CSF was also higher in the 24m group although this just failed to reach statistical significance (Fig. 4b). Compared to 3m rats, 24m old rats had significantly lower mean

plasma concentrations (pg/mL) of IL- 12p70 (1006 ± 255 vs 1778 ± 160) and TNF-α (370 ± 106 vs 720 ± 57) (Fig. 5). Interestingly, while TNF-α was decreased in the plasma of 24m rats, it was increased in the brainstem. Serum concentrations of IL-1β and IL-13 were also lower in 24m rats, although this just missed statistical significance (Figure 5b).

4.4.3.3 Comparison of cytokine levels in 12-18m and 24m old rats.

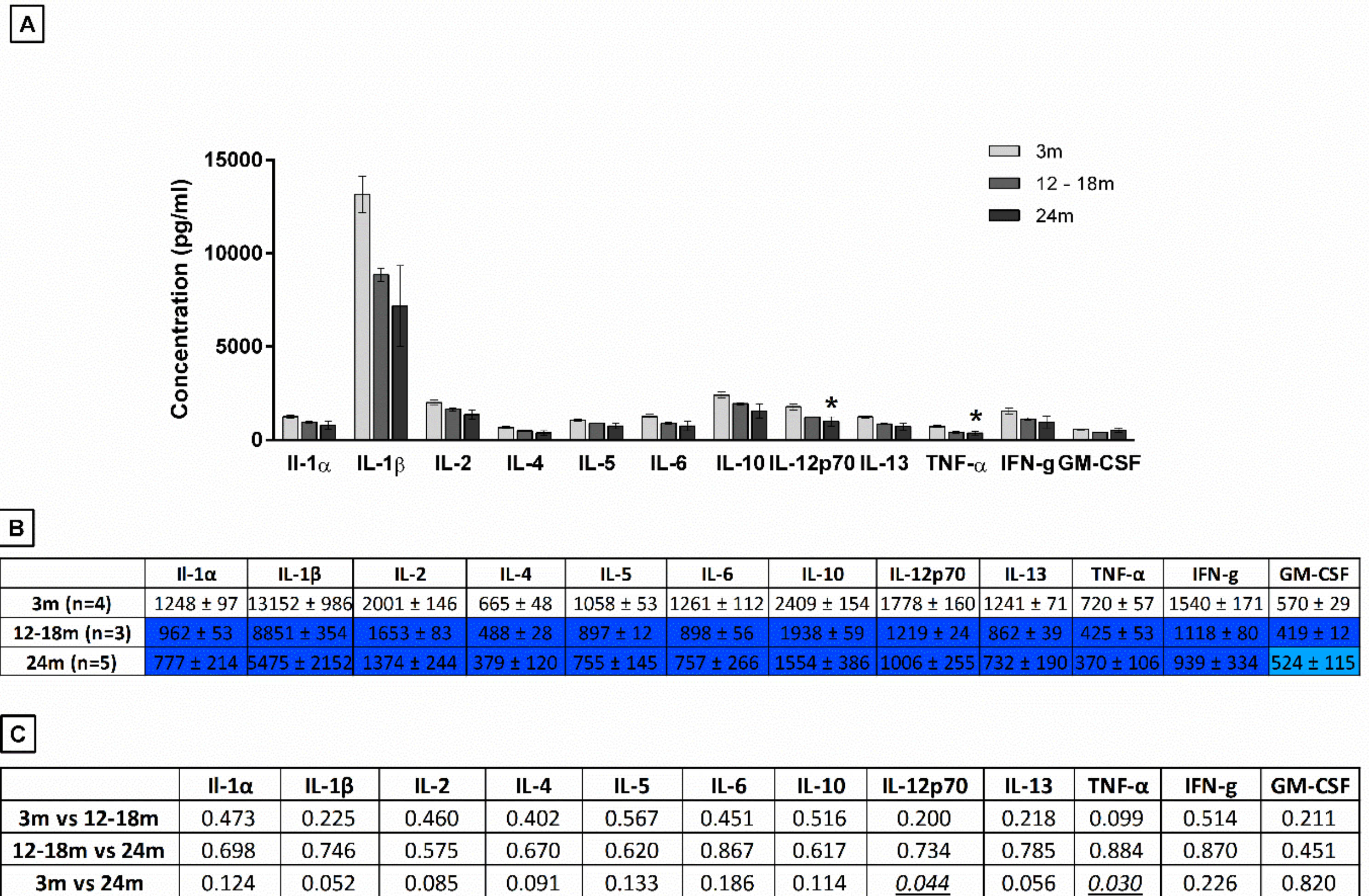
In the brainstem of 24m rats, the concentrations of 7 cytokines were significantly increased by 28-61% compared to 12-18m rats. The cytokines and their concentrations (pg/ml) were: IL-1α (335 ± 28 vs 499 ± 31, $p < 0.05$), IL-4 (164 ± 11 vs 228 ± 13, $p < 0.01$), IL-6 (203 ± 21 vs 373 ± 23, $p < 0.01$), IL-13 (252 ± 6 vs 322 ± 17, $p < 0.05$), TNF-α (530 ± 14 vs 797 ± 41, $p < 0.01$), IFNγ (353 ± 44 vs 543 ±34, $p < 0.05$) and GM-CSF (374 ± 30 vs 604 ± 57, $p < 0.05$) (Fig. 4). Three of these cytokines (IL-1α, IL-4 and TNF-α) were also elevated in 24m rats when compared with 3m rats, indicating that changes occur earlier and are longer lasting in these cytokines compared to other brainstem cytokines. No significant differences in plasma cytokine concentrations were found between 12-18m and 24m old rats (Fig. 5). Thus, changes in plasma cytokine concentrations appear to have stabilised by 12-18m, whereas changes in brainstem cytokine concentrations continue up to 24m.



	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70	IL-13	TNF-α	IFN-g	GM-CSF
3m (n=4)	351 ± 30	2134 ± 115	1320 ± 44	164 ± 8	355 ± 13	335 ± 32	2363 ± 59	707 ± 29	303 ± 20	597 ± 16	519 ± 47	401 ± 69
12-18m (n=3)	335 ± 28	2004 ± 59	1543 ± 65	164 ± 11	303 ± 12	203 ± 21	2663 ± 122	699 ± 25	252 ± 6	530 ± 14	353 ± 44	374 ± 30
24m (n=5)	499 ± 31	2293 ± 96	1714 ± 84	228 ± 13	311 ± 12	373 ± 23	3369 ± 262	764 ± 30	322 ± 17	797 ± 41	543 ± 34	604 ± 57

	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70	IL-13	TNF-α	IFN-g	GM-CSF
3m vs 12-18m	0.922	0.620	0.131	1.000	*0.049*	*0.018*	0.552	0.974	0.151	0.348	*0.048*	0.936
12-18m vs 24m	*0.011*	0.141	0.242	*0.008*	0.894	*0.003*	0.076	0.290	*0.039*	*0.001*	*0.021*	*0.048*
3m vs 24m	*0.012*	0.434	*0.005*	*0.005*	0.056	0.497	*0.010*	0.325	0.629	*0.003*	0.874	0.057

*Figure 4: A) Cytokine levels (pg/ml, mean ± SEM) in the brainstem of 3m, 12-18m and 24m rats. A single asterisk and double asterisks denote p<0.05 and p<0.01 vs 3m, respectively. * and ** denote p<0.05 and p<0.01 vs 12-18m, respectively. B) Colour-coded table showing changes in cytokine levels in the brainstem (mean ± SEM). Compared to 3m rats (no colour), >15% increase is represented by light red and >25% by dark red, >15% decrease is represented by light blue and >25% by dark blue. C) Summary of p-values of brainstem cytokine changes with age. Statistically significant differences are italicised and underlined. Note that changes in IL-5 and GM-CSF showed trends*



	Il-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70	IL-13	TNF-α	IFN-g	GM-CSF
3m (n=4)	1248 ± 97	13152 ± 986	2001 ± 146	665 ± 48	1058 ± 53	1261 ± 112	2409 ± 154	1778 ± 160	1241 ± 71	720 ± 57	1540 ± 171	570 ± 29
12-18m (n=3)	962 ± 53	8851 ± 354	1653 ± 83	488 ± 28	897 ± 12	898 ± 56	1938 ± 59	1219 ± 24	862 ± 39	425 ± 53	1118 ± 80	419 ± 12
24m (n=5)	777 ± 214	5475 ± 2152	1374 ± 244	379 ± 120	755 ± 145	757 ± 266	1554 ± 386	1006 ± 255	732 ± 190	370 ± 106	939 ± 334	524 ± 115

	Il-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70	IL-13	TNF-α	IFN-g	GM-CSF
3m vs 12-18m	0.473	0.225	0.460	0.402	0.567	0.451	0.516	0.200	0.218	0.099	0.514	0.211
12-18m vs 24m	0.698	0.746	0.575	0.670	0.620	0.867	0.617	0.734	0.785	0.884	0.870	0.451
3m vs 24m	0.124	0.052	0.085	0.091	0.133	0.186	0.114	*0.044*	0.056	*0.030*	0.226	0.820

*Figure 5: A) Cytokine levels (pg/ml, mean ± SEM) in the plasma of 3m, 12-18m and 24m rats. * denotes p<0.05 vs 3m. B) Colour-coded table showing changes in cytokine levels in the plasma (mean ± SEM). Compared to 3m rats (no colour), >15% decrease is represented by light blue and >25% by dark blue. C) Summary of p-values of plasma cytokine changes with age. Statistically significant differences are italicised and underlined. Note that changes in IL-1β and IL-13 showed trends that were not statistically significant.*

4.5 Discussion

We report that ageing (24m) rats, (i) have lower peripheral inflammatory markers, (ii) have higher central inflammatory markers, (iii) show reduced exploratory behaviour, (iv) have larger peripheral target muscle for facial motoneurones and (v) have fewer facial motoneurones than 3m rats. This points to complicated changes occurring in both the inflammatory signature and peripheral target interactions of ageing rats that are associated with age-related motoneurone loss.

The median lifespan of ad-libitum-fed Sprague-Dawley rats is 24-34m (Durbin, Williams et al. 1966, McDonald 1990) and in line with other studies, we found that 50% of our ad-libitum fed Sprague-Dawley rats died by the age of 24m (Vanden Noven, Seburn et al. 1996, Aperghis, Johnson et al. 2003). It is possible that the age-related health changes found in the rats analysed here such as obesity, lipomas and arthritis have contributed to the changes in inflammatory markers measured. However, these changes, often referred to as ‘frailties’ (Mohler, Fain et al. 2014, Whitehead, Hildebrand et al. 2014), are commonly found with age and so to have used rats where such age-related conditions are absent may not have been representative of normal ageing. It is also possible that rats showing reduced mortality at 24, such as Fischer 344 rats and diet-restricted rats (Johnson and Duberley 1998, Aperghis, Johnson et al. 2003), may show different cytokine changes.

We found statistically significant increases in IL-1α, IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, IFNγ and GM-CSF in the brainstem. Of these, IL-1α, IL-2, TNF-α, IFNγ and GM-CSF are pro-inflammatory, IL-4, IL-10 and IL-13 are considered anti-inflammatory and IL-6 falls under both categories. We also found statistically significant decreases of IL-12p70 and TNF- α in the plasma, both of which are pro-

inflammatory. While this is a complicated picture, our results could be taken to indicate that ageing is associated with a general decline in peripheral inflammatory cytokines and a general increase in central inflammatory cytokines. Age-related increases in CNS cytokines have been reported by others (Sheng, Mrak et al. 1998, Barrientos, Higgins et al. 2006, Sierra, Gottfried-Blackmore et al. 2007, Viviani and Boraso 2011), and associated with increased vulnerability of the CNS to injury (Soriano, Lipton et al. 1996, Boutin, LeFeuvre et al. 2001, Chen, Buchanan et al. 2008) as well as implicated in the development of age-related neurodegeneration (Allan and Rothwell 2001, Bodles and Barger 2004, Aiyaz, Lupton et al. 2012). In contrast to the brainstem region of the CNS, we find that peripheral cytokines, show a general decline with ageing, which is at odds with initial studies on "inflammaging" by others showing increased levels of individual inflammatory markers in the periphery, especially IL-6 (Wei, Xu et al. 1992, Bruunsgaard, Andersen-Ranberg et al. 1999, Forsey, Thompson et al. 2003). Notwithstanding the difficulty of extrapolating from rats to human lifespan, one possible reason for this difference could be that early studies employed individual ELISA kits/antibodies each with different sensitivities for single cytokines and used different samples with each kit. Also, the view that peripheral cytokines increase with advancing age has been largely based on measurements of IL-6 even though Franceschi et al (2007) showed that other cytokines, such as GM-CSF, were reduced in healthy aged humans. This highlights the need to study many inflammatory mediators within the same sample.

In adult mice, elevated levels of peripheral inflammatory cytokines after LPS challenge also decreases exploratory behaviour and is believed to facilitate recovery from acute infections (Dantzer 2004, Lim, Krajina et al. 2013). In our study, we found lower levels of exploratory behaviour in 24m rats, but this was associated instead with lower levels

of peripheral cytokines compared to 3m rats. Only in the brainstems of 24m rats did we find evidence of an increase in inflammatory cytokines. These results indicate that age, the site of inflammation (central or peripheral) and its time-course (acute or chronic) all likely contribute to behavioural changes.

Peripheral muscle wasting has been associated with increased cytokine signalling, especially with TNF-α largely implicated in the process (Remels, Gosker et al. 2010, Zhou, Liu et al. 2016) . The larger peripheral-target muscle morphometry of 24m rats observed in this study, compared to 3m animals could be associated with the reduced levels of peripheral inflammatory cytokines noted. A link between peripheral target size and motoneurone survival is well known from studies of the developing nervous system (Hamberger 1977, Greensmith and Vrbova 1996). This has generally been linked to the ability of the peripheral target to provide neurotrophic support to developing motoneurones (Oppenheim 1996). In our study of ageing rats, an increase in peripheral target size correlated with a decrease in motoneurones. Whether this reflects a decrease in peripheral neurotrophic support with age or is simply a result of the generally larger size of the ageing rats is unknown.

The initial concept of inflammaging revolved around the low-grade amplification of pro-inflammatory cytokines (Sparkman and Johnson 2008, Campuzano, Castillo-Ruiz et al. 2009). However, more recent studies show increases in both pro- and anti-inflammatory cytokines with advancing age (Morrisette-Thomas, Cohen et al. 2014). This more complicated pattern of change with ageing, is consistent with our current results, indicating that ageing is not a simple matter of increased inflammation in the whole animal. Acknowledging that only 50% of the rats in our study survived to 24m, and so must be considered ageing 'survivors', the general decrease in peripheral cytokines could be viewed beneficial, possibly ameliorating age-related increases in

other inflammatory mediators that were not measured here. Further studies, perhaps employing heterochronic parabiosis, involving the surgical attachment of young and old organisms so that they share a common vascular system (Conboy, Conboy et al. 2013, Murphy and Thuret 2015), could address this point. Neither do we know if the rats that reached 20m but failed to reach 24m of age, showed increased levels of peripheral cytokines, in keeping with the orthodox concept of inflammaging (Giunta, Fernandez et al. 2008), since we did not analyse the blood in these rats that died early. Our data shows no consistent increase in most of the cytokines measured from 3m to 12-18m to 24m, but we cannot discount changes occurring around the 24m mark in these cytokines. While we have only looked at 12 cytokines, this result forces the conclusion that changes in the total inflammatory-signature are likely to characterise ageing and that these changes are different in the CNS and periphery. Notwithstanding the possibility that our results on the brainstem may not generalise to the rest of the CNS, this view runs contrary to the concept of “inflammaging” for the whole animal and the various ways this concept has been adduced to explain age-related neuronal degeneration (Brown and Neher 2010, Lim, Krajina et al. 2013).

Conclusion

We show that the peripheral innate immune system of adult rats has higher levels of cytokines than the brainstem of the central nervous system and this balance is reversed in ageing rats. We also show that inflammatory changes in the ageing brainstem are different to those occurring in the blood. If our results for the brainstem at the level of the facial nucleus can be confirmed generally for the CNS, they may have implications for the design of therapeutic strategies for age-related diseases affecting the CNS or other parts of the body. The discrepancy between our results and others calls into question the importance of changes of cytokines with age. Our multi-analyte approach

leads us to suggest that changes in all inflammatory mediators, or the "inflammatory signature", may better link inflammation with regional ageing processes. The changes in the inflammatory signature may involve an intrinsic loss in competency of peripheral immunity with age or be the result of a regulatory system where increased cytokine levels in the CNS feedbacks to the periphery.

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CHAPTER V

The effect of pre-existing inflammation on the survival of facial motoneurones after nerve-avulsion injury

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STATEMENT OF AUTHORSHIP

Title of Paper	The effect of pre-existing inflammatory on the survival of facial motoneurones after nerve-avulsion injury
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style

Principal Author

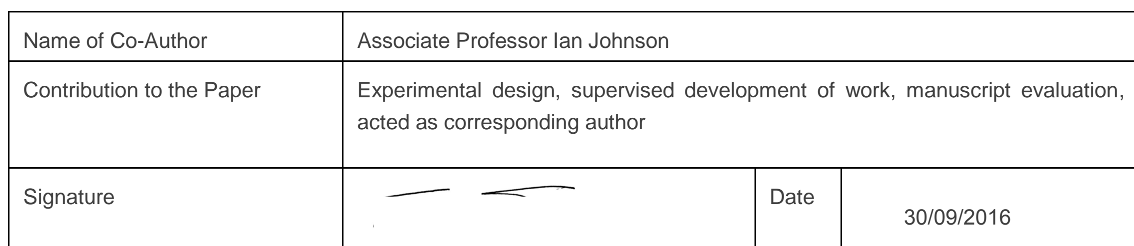
Name of Principal Author (Candidate)	Viythia Katharesan		
Contribution to the Paper	Experimental design, data collection, analysis and interpretation, wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/09/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Robert Vink		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation		
Signature		Date	28 September 2016



Name of Co-Author	Associate Professor Ian Johnson		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation, acted as corresponding author		
Signature		Date	30/09/2016

5 The effect of pre-existing inflammation on the survival of facial motoneurones after nerve-avulsion injury

5.1 Abstract

Inflammation has been known as a key feature of aging for long. Given the rise in neurodegenerative diseases such as Motor Neuron Disease in the aging demographic, it is important to elucidate the effects of pre-existing inflammation on the survival of motoneurones. This would enable future studies to fine-tune research into the causative mechanisms of such pathologies and better evaluate treatment strategies in more appropriate experimental models. In this study, groups of 4-8 Sprague Dawley rats aged 3 months were used to mimic the inflammatory cytokine milieu seen in aging rats, using the systemic inflammatory agent Lipopolysaccharide. In order to compare and parallel the low-grade inflammatory response seen in 24 month aged rats, open-field exploratory behaviour was studied as an assessor of general locomotion along with dose- and time- response studies. The facial nerve avulsion model was used as the injury model of motoneurones and stereological counts of surviving motoneurones are reported. The open-field tests along with the dose- and time- response studies in adults determined that the 0.1mg/kg of LPS dose and the 4 hour time-point best mimicked the inflammatory environment seen in aged rats. There were no significant differences in motoneuronal survival between adults with motoneuronal injury short-term (14 days) and their counterparts with pre-existing inflammation. However, a neurodegenerative trend was observed with adults with motoneuronal injury long-term (28 days) when compared to their counterparts with pre-existing inflammation. Our results are in line with recent studies that have shed light on how inflammation can be neuroprotective, neurodegenerative or have a null effect on different neuronal populations.

5.2 Introduction

The role of ageing in the development of neurodegenerative diseases such as Motor Neuron Disease (MND) has been largely ignored because of the difficulty in obtaining ageing experimental animals. One characteristic of ageing, thought to affect neuronal survival, is the presence of a low-grade but chronic inflammatory state. Genetic conformations (in the context of familial MND) and extraneous influences (in the context of sporadic MND) may well have different effects on aged vs. young individuals, given the disparity in their inflammatory states (Krabbe, Pedersen et al. 2004). It is therefore vital to first gauge the effects of inflammation on motoneuronal survival before attempting to evaluate treatment strategies in experimental models with a completely disparate *in-vivo* state. Such an understanding will not only contribute to improving the efficacy of treatment strategies in MND but possibly give an insight into the prevention of chronic diseases in the growing aging demographic. This raises the possibility that creating a similar inflammatory state in adult animals may allow us to determine the role of pre-existing inflammation on motoneuronal survival. The technique of facial nerve avulsion has been established as a means of provoking motoneuronal death across the lifespan of the rat (Moran and Graeber 2004). In parallel, this technique has been used to discover age-related differences in motoneuronal survival. Therefore, the aim of the current study is to mimic one of the current features of “inflammaging” (elevation of inflammatory cytokines) in adult animals to see if pre-existing inflammation affects how adult motoneurones survive and compare with aged motoneuronal survival. Intraperitoneal Lipopolysaccharide (LPS) is a widely used experimental inflammatory agent in mice and other strains of rats (Godbout, Berg et al. 2004, Qin, Wu et al. 2007). Dose- and time- response pilot studies were done along with a functional test studying general exploratory behaviour. The optimal dose

regimen and the optimal time to assay the rats post-LPS treatment, needed to closely mimic the inflammatory cytokine levels seen in aged rats. Both of these studies allowed us to determine when to induce nerve injury post-LPS treatment for the nerve-injury-response to occur in the “optimal inflammatory milieu” (i.e. that similar to aged rats). Upon optimisation of dose- and time- regimens, LPS was used to provoke systemic inflammation after which rats underwent the nerve injury surgery and were culled post-injury, at two time points (14 days and 28 days post-injury) where nerve injury response has been previously studied (unpublished data).

5.3 Materials and Methods

5.3.1 Animals

Sprague-Dawley rats were used at 3 months (adults) and 24 months (aged). For the cytokine analysis, groups of 4 – 8 female Sprague-Dawley rats were used. For open field tests and motoneurone counts, groups of 4-12 rats were used. Some adult groups received intraperitoneal Lipopolysaccharide (LPS) injections (from *Escherichia coli* 055:B5, Sigma-Aldrich, L2880). Ageing rats are not available commercially in Australia. Rats were therefore obtained as adults and aged in the rodent facility of the University of Adelaide. While the maximum reported lifespan of the *ad libitum*-fed Sprague Dawley rat is 36 months (Sengupta 2013), we found approximately 50% of the rats in our study had died by 24m, as reported previously (Johnson and Duberley 1998). Animals were housed under a standard 12-hour on/off lighting regime and given food and water *ad libitum*. The experimental study complied with the Australian code for the care and use of animals for scientific purposes (2016) and was approved by the University of Adelaide Animal Ethics Committee (M-57-2013). The ageing animals

analysed here did not have significant health issues although they did have age-related conditions such obesity, lipomas and arthritis that are typical of an ageing population.

5.3.2 Functional test

An open field test was used as a measure of exploratory behaviour (Gould, Dao et al. 2009). A 100cm x 100cm square box acted as the "open field" and rats were placed in the centre of the open-field arena. Movement, in terms of total distance travelled, was then recorded for a period of 5 minutes. To ensure consistency between groups, the time at which the test was conducted, colour and texture of the open-field box, lighting, temperature, ambient noise and olfactory cues were all controlled for. The Stoelting "ANY-maze" software was used as the tracking system that automated this functional test (Prut and Belzung 2003).

5.3.3 Saline-perfusion, protein extraction and estimation

Rats were deeply anaesthetised by inhalation of 5% isofluorane in 2 litres oxygen/minute perfused transcardially with approximately 200mL of sterile saline until the fluid flowing out of the right atrium was clear. The animals were then decapitated and the brainstem removed, trimmed at the mid pons level and approximately 1mm below the lower border of the pons to ensure it contained the facial nucleus, snap-frozen and stored at -80°C. Frozen brainstem samples were homogenised in lysis buffer made up with PBS, triton-X and protease inhibitors (Roche, cOmplete tablets). The supernatant was retrieved from homogenised samples and stored at -80°C. The BioRad DC Protein Assay (a modified Lowry method) was used to quantitate the amount of protein in each sample as per the manufacturer's instructions.

5.3.4 Multiplex Assay

Bio-Plex Pro Rat 12 plex cytokine assay kits (BioRad, New South Wales) were used to measure the concentration of twelve cytokines within each sample. Samples were loaded onto 96 well plates in duplicates (3m and 12-18m rats) and triplicates (24m rats). Plates were read using a Magpix Luminex multiplexing platform which uses a fluorescent imager (Abacus-ALS, Queensland) and data expressed as pg/ml of concentration. Experimental data was calibrated against standard curves of all 12 cytokines (BioRad, New South Wales). Spike-recovery analyses have been previously performed in-house to validate the accuracy of the multiplex for our sample types, showing minimal matrix difference as desired (Chapter 4).

5.3.5 Facial Nerve Avulsion Injury

Under deep surgical anaesthesia with isoflurane, the right facial nerve was avulsed by gentle traction (c.f. Fig. 1). Briefly, an incision is made behind the right ear to access the facial nerve covered by the sternocleidomastoid and masseter muscles. At the exit point from the stylomastoid foramen, the facial nerve is freed from its periosteal attachments and avulsed. Detachment of the facial nerve rootlets as they emerge from the brainstem will result in a very proximal axotomy which results in motoneuronal death.

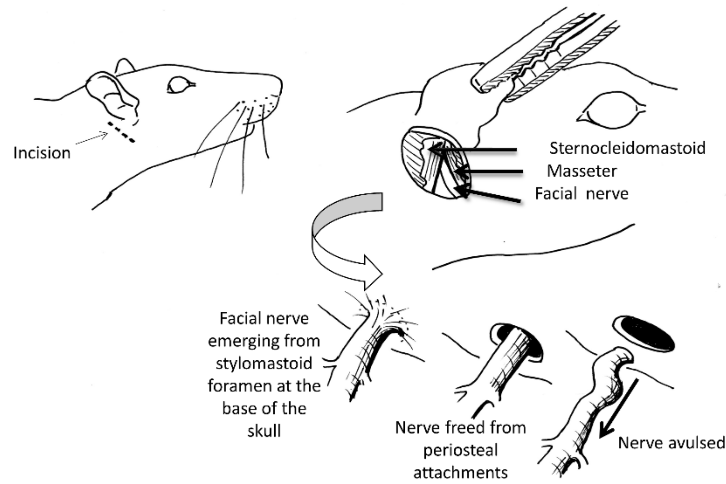


Figure 1: Schematic illustration of right facial nerve avulsion.

5.3.6 Stereological counts of facial motoneurones

In rats terminally anaesthetised with sodium pentobarbitone, the brain was fixed by intracardiac perfusion with 4% phosphate-buffered paraformaldehyde following a saline rinse. 100µm vibratome sections were cut serially through the facial nucleus and numbers of motoneurones estimated in every 5th section using an optical disector method modified for use in the confocal scanning laser microscope as described previously (Johnson 2001).

5.3.7 Statistical Analysis

Based on Q-Q plots, Skewness and Kurtosis values and Shapiro-Wilks tests (SPSS statistics 24, IBM), the multiplex data failed to meet normality assumptions for a parametric test. Thus, non-parametric Kruskal-Wallis results are reported. Additionally, Jonckheere-Terpstra tests were conducted for trends and post-hoc Mann-Whitney for pairwise comparisons with Bonferroni-corrected p-values (to counter the otherwise inflation of Type 1 error), where necessary. One-way Analysis of Variance (ANOVA) was used for comparisons of open-field exploratory behaviour and stereological counts of motoneurones as these datasets met the aforementioned normality and homogeneity of variance assumptions. Statistical significance of $p<0.05$ is reported unless otherwise stated.

5.4 Results

5.4.1 Open-field exploratory behaviour

Using total distance travelled (m) during the open-field test, rats with the 5mg/kg LPS dose showed significantly reduced exploratory behaviour compared to all other rats (Fig 2). In the time-response study, rats assayed after 8h and 24h showed significantly larger exploratory behaviour compared to all other rats, especially the aged rats.

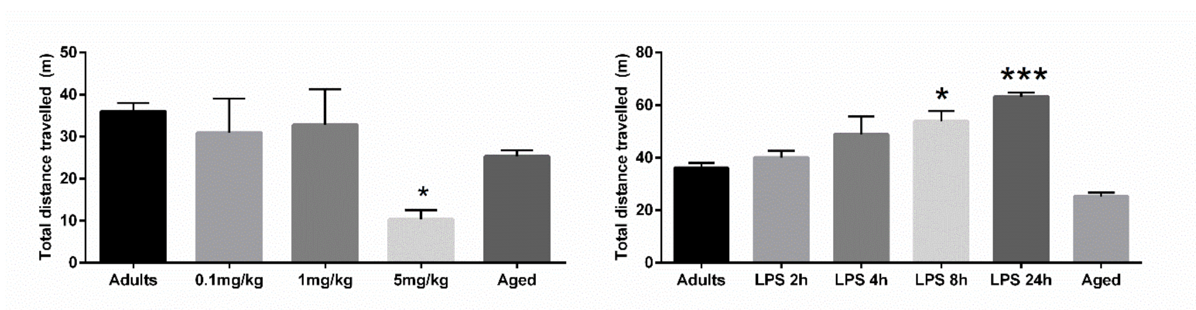


*Figure 2: Mean ± SEM (m) total distance travelled in the open field by dose- and time-response rats. (p<0.05 and p<0.001 denoted by * and *** respectively).*

5.4.2 Dose Response

All five datasets (adults, adults injected with 0.1mg/kg, 1 mg/kg, 5mg/kg and aged rats) were initially analysed together (5.4.2.1) (Fig 3). Two further analyses were conducted on selective groups: normal adults compared to dose-regimens (5.4.2.2) and aged group compared to dose-regimens using the same tests (5.4.2.3).

5.4.2.1 Collective comparison of all five groups (normal adults, adults injected with 0.1mg/kg, 1 mg/kg, 5mg/kg LPS and aged rats)

The Kruskal-Wallis showed that IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and TNFα were significantly affected by the 3 dose regimens ($H(4)$, $p<0.05$). However, with the present sample size and the stringent alpha value corrected for 9 pairwise comparisons, the robust non-parametric Mann-Whitney showed no significance with the Bonferroni corrected p-value of 0.005. Nevertheless, Jonckheere-Terpstra test revealed a significant trend in the data: as dose increased, the medians for IL-1α ($r = 4.15$) and TNF-α ($r = 0.49$) increased. The dose effects on IL-1α and TNF-α were considerable given the effect size value (r).

5.4.2.2 Comparison of normal adults with adults injected with 0.1mg/kg, 1 mg/kg and 5mg/kg of LPS

Concentrations of IL-1β, IL-6 and IL-12p70 were significantly affected by the 3 dose regimens ($H(3)$, $p<0.05$). However, post-hoc Mann-Whitney with a Bonferroni correction ($p<0.008$), showed no significant difference in the effect of the 3 dose-regimens on IL-1b, IL-6 and IL-12p70 levels. A significant trend was seen in the data: as dose increased, the medians for IL-1β ($r = 0.69$), IL-6 ($r = 0.12$) and IL-12p70 ($r = 0.6$) increased. Based on the effect size values (r) only dose effects on IL-1β and IL-

12p70 were fairly substantive findings. Given that we aimed to parallel a low-grade inflammatory state, based on the trend-effect on IL-1β and IL-12p70, 0.1mg/kg was decided as being the optimal dose for the purposes of this study.

5.4.2.3 Comparison of aged group with adults injected with 0.1mg/kg, 1 mg/kg and 5mg/kg of LPS

Concentrations of IL-1α, IL-1β IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and TNF-α were significantly affected by the 3 dose-regimens (H(3), $p<0.05$). However, post-hoc Mann-Whitney with a Bonferroni correction ($p<0.016$) showed no significant difference in the effect of the 3 dose regimens. However, Jonckheere-Terpstra test revealed significant trend effects in the data: as time after injection increased, the medians for IL-1α ($r = 0.81$), IL-2 ($r = 0.46$), IL-4 ($r = 0.58$), IL-6 ($r = 0.75$), IL-13 ($r = 0.48$) and TNF-α ($r = 0.52$) increased and that of IL-1β ($r = -0.19$), IL-10 ($r = -0.81$) and IL-12p70 ($r = -0.52$) decreased. The time-regimen effects considerably affected IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and TNF-α. This result supports that the dose regimens were able to roughly mimic the aged rats. Thus, further supporting section 5.4.2.2, 0.1mg/kg, as a minimal dose, was determined as the optimal dose for this study.

5.4.3 Time Response

All six datasets (adults, adults assayed 2h, 4h, 8h, 24h after LPS injection and aged rats) were initially analysed together (5.4.3.1) (Fig 4). Two further analyses were conducted on selective groups: normal adults compared to time-regimens (5.4.3.2) and aged group compared to time-regimens (5.4.3.3).

5.4.3.1 Collective comparison of all six groups

(normal adults, adults assayed 2h, 4h, 8h and 24h after LPS and aged rats)

IL-2, IL-4, IL-10 and TNF-a were significantly affected by the time-regimens (H(5), $p<0.05$). However, with the present sample size and the stringent alpha value corrected for 11 pairwise comparisons, the robust non-parametric Mann-Whitney showed no significance with the Bonferroni corrected p-value of 0.004. Again, Jonckheere-Terpstra test revealed a significant trend in the data: as time-regimens increased, the medians for IL-2 ($r = 0.56$), IL-4 ($r = 0.58$), IL-10 ($r = 0.6$) and TNFα ($r = 0.37$) increased. Based on the r values, the time regimens had substantial effects on IL-2, IL-4, IL-10 and TNFα.

5.4.3.2 Comparison of normal adults with adults assayed 2h, 4h, 8h and 24h after LPS

Concentrations of cytokines were not significantly affected by the 4 time-regimens (H(4), $p>0.05$) thus no post-hocs were conducted. However, Jonckheere-Terpstra test revealed significant trend effects in the data: as time after injection increased, the medians for IL-1α, IL-2, IL-4, IL-10 and GM-CSF increased whereas IL-5, IL-6, IL-12p70 and TNFα decreased.

5.4.3.3 Comparison of aged group with adults assayed 2h, 4h, 8h and 24h after LPS

Concentrations of IL-4, IL-10 and TNF-α were significantly affected by the 4 time-regimens (H(4), $p<0.05$). However, post-hoc Mann-Whitney with a Bonferroni correction ($p<0.0125$) showed no significant difference in the effect of the 4 time regimens. However, Jonckheere-Terpstra test revealed significant trend effects in the data: as time after injection increased, the medians for IL-2 ($r = 0.43$), IL-4 ($r = 0.67$), IL-10 ($r = 0.66$) and TNF-α ($r = 0$ .55) increased and these time-regimen effects were considerable based on r values. This result indicates that all the time regimens were able

to approximate the aged cytokine concentrations thus, LPS 4h was determined as the optimal dose for this study as supported by the functional test results seen above.

5.4.4 Stereological counts of motoneurones

There were no significant differences with the current sample size using one-way ANOVA, although some trends were noted (Fig 5). Adults injected with LPS and culled 14 days post-avulsion (Adults + LPS + Av 14d) were no different to adults that underwent the same nerve injury without pre-existing inflammation in the form of LPS (Adults Av 14d). However, adults injected with LPS and culled 28 days post-avulsion (Adults + LPS + Av 28d) illustrated a neurodegenerative trend with less surviving motoneurones compared to adults that underwent the same nerve injury without LPS injection (Adults Av 28d). It cannot be discounted that gender differences also exist in motoneurone numbers of adults that survive 28 days with just the avulsion injury such that females (seen here) have more surviving motoneurones compared to males (Johnson, Goldspink et al. 2011). It also seems noteworthy that adults injected with LPS and culled 28 days post-avulsion (Adults + LPS + Av 28d) had a similar mean to aged animals that underwent nerve injury and were culled 28 days later (Aged Av 28d).

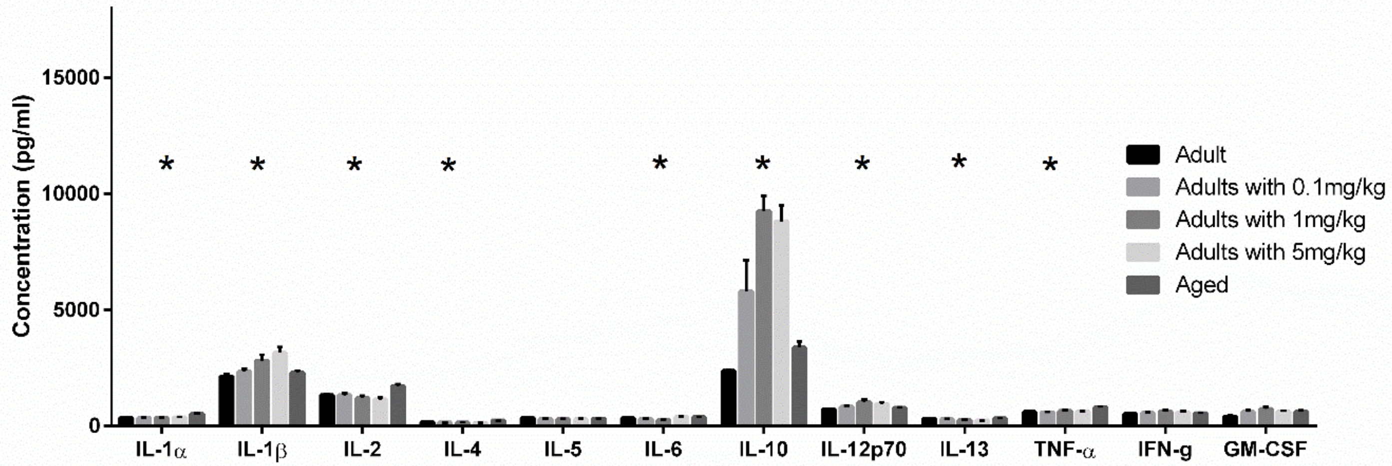


*Figure 3: Cytokine concentrations (pg/ml) around facial motoneurones in adult rats, adults with three different doses of LPS and aged rats. Kruskal-Wallis test used to determine significance at $p<0.05$ illustrated by *.*

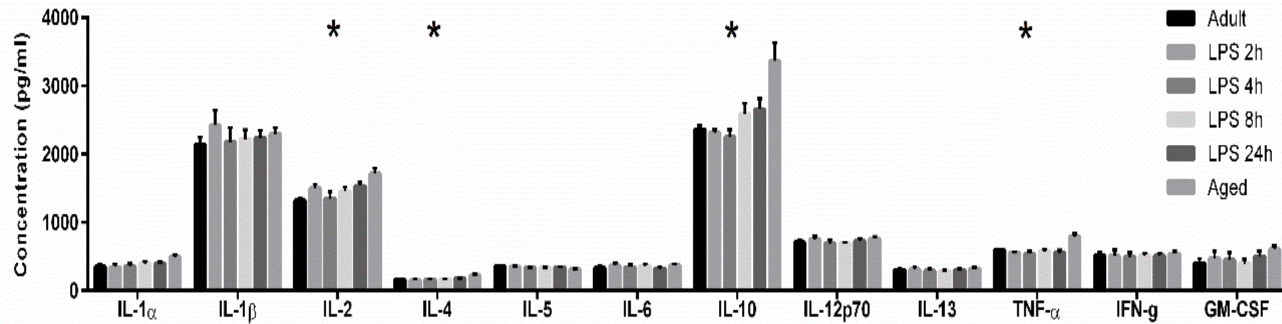


*Figure 4: Cytokine concentrations (pg/ml) around facial motoneurones in adult rats, adults assayed at four different time-points post-LPS injection and aged rats. Kruskal-Wallis test used to determine significance at $p<0.05$ illustrated by *.*

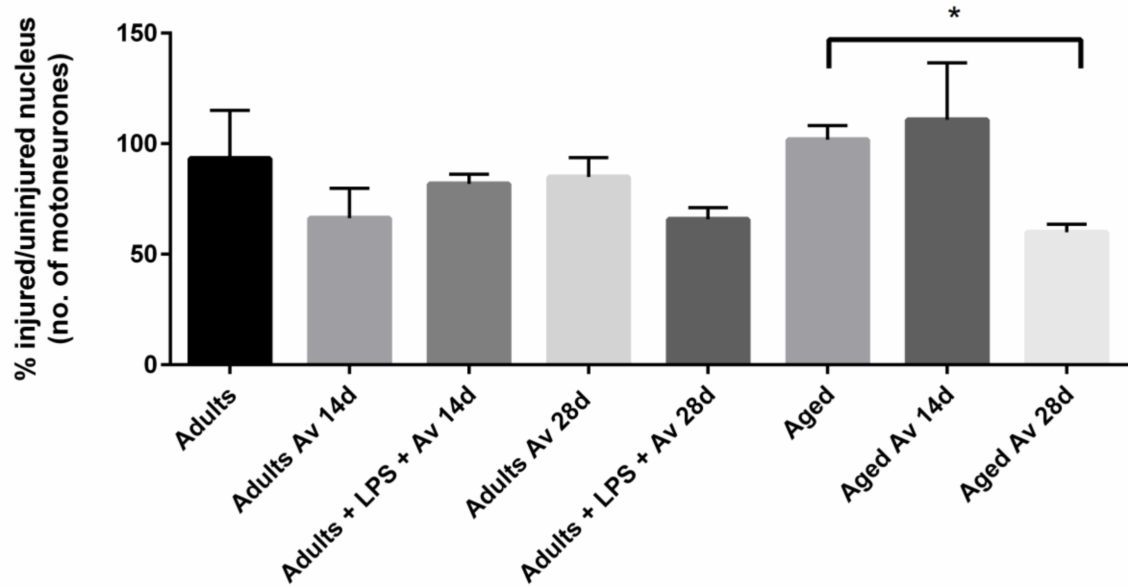


*Figure 5: Numbers of motoneurones from stereological counts expressed as percentage of motoneurones counted in the injured facial nucleus over uninjured facial nucleus. * denotes $p<0.05$. "Av" refers to nerve-avulsion injury while LPS refers to injection of the inflammatory agent Lipopolysaccharide.*

5.5 Discussion

We report that compared to aged rats, adults injected with 5mg/kg of LPS show significantly reduced exploratory behaviour whereas adults assayed 8- and 24 hours post-LPS show significantly larger exploratory behaviour. This was supportive of the decision to avoid the 5 mg/kg dosage and the 8- and 24-hour time points as we aimed to induce a low-grade inflammatory state, that would still mimic general behaviour seen in aged rats. This parallels the notion that aged humans do not become completely inactive with senescence but rather limit their general locomotion. A range of cytokines were affected in both the dose- (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and

TNFα) and time- response (IL-2, IL-4, IL-10 and TNF-a) studies. However, the use of robust non-parametric tests with our limited sample sizes yielded statistically insignificant differences in post-hoc tests with p-values corrected for multiple pairwise comparisons. Based on trend effects, the 0.1mg/kg dose and the 4h time-point was chosen as being optimal for this study.

Using this dose and time-point, there were no significant differences between adults that experienced the nerve injury and survived 14 days compared to their counterparts that were exposed to the inflammatory LPS prior to the injury. However, a slight trend, albeit statistically insignificant, of neurodegeneration was seen when adults had pre-existing inflammation and then underwent nerve injury and survived for 28 days compared to their counterparts who only had the nerve injury. We also found that the adult system, when primed with LPS and exposed to the nerve injury with a long-term survival period of 28 days, responded the same as the aged system with the nerve injury and 28 day-survival.

Our results in the context of nerve injury and motoneurones show an overall null effect of pre-existing inflammation. However, there seem to be indications that the presence of inflammation as at time of nerve-avulsion injury could be detrimental to the survival of motoneurones, perhaps by impairment of long-term repair processes (Vinoth Kumar, Oh et al. 2016). While inflammatory markers have always been thought to be neurodegenerative, as research has progressed over the years, there seem to be many accounts where elevated levels of inflammation has been protective in pathologies and experimental models (Vlad, Miller et al. 2008, Aebischer, Moumen et al. 2012, Tentillier, Etzerodt et al. 2016). Acute inflammation has been shown to stimulate neurogenesis and in some ways seen in our results, uncontrolled inflammation creates a detrimental environment around neurones (Whitney, Eidem et al. 2009). Perhaps this

concept also applies to motoneurones that are involved in the constant pruning of CNS circuitry. Furthermore, clinical trials using anti-inflammatories such as celecoxib and minocycline on adult and ageing patients with Motor Neuron Disease, have shown no protection or adverse effects (Cudkowicz, Shefner et al. 2006, Gordon, Moore et al. 2007). A study by Schwartz and Scheter (2010), suggests that destructive CNS inflammation, causing or arising from events such as the injury of motoneurones, need to be modified by immune cells recruited from the systemic peripheral environment. The failure of anti-inflammatory medications in clinical trials has been attributed to the suppression of the recruitment of such immune cells. This leads us back to our initial argument that inflammation and its effects on any neuronal population is complicated thus leaving most experimental studies, such as ours, with null effects (Krabbe, Pedersen et al. 2004). In order to tease out such effects, future studies could assess more outcomes with next generation RNA-sequencing or other epigenetic analyses to see if the results match up expressed protein levels (Paez-Colasante, Figueroa-Romero et al. 2015). Kinetics of cytokine production should be taken into consideration when studying the production of several cytokines, thus measuring at only one time-point is not always optimal (Krabbe, Pedersen et al. 2004). However, we were not concerned about absolute values or individual cytokines but rather were focussed on the "inflammatory milieu" in the form of cytokines concentrations relative to the groups, thus multiplexing seemed more suited for this purpose. This newer knowledge of neuroprotective inflammation cannot be discounted as inflammation as an evolutionary process, was designed as a repair response to injury. Notwithstanding such accounts however, in the context of pre-existing inflammation of the survival of motoneurones, neurodegeneration still seems just as tenable.

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CHAPTER VI

Age-related changes in cytokine levels correlated with motoneurone survival and the response to immune challenge in the blood and CNS of immature and mature rats

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STATEMENT OF AUTHORSHIP

Title of Paper	Age-related changes in cytokine levels correlated with motoneurone survival and the response to immune challenge in the blood and CNS of immature and mature rats
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style

Principal Author

Name of Principal Author (Candidate)	Viythia Katharesan		
Contribution to the Paper	Experimental design, data collection, analysis and interpretation, wrote manuscript		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/09/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Associate Professor Ian Johnson		
Contribution to the Paper	Experimental design, supervised development of work, manuscript evaluation		
Signature		Date	30/09/2016

6 Age-related changes in cytokine levels correlated with motoneurone survival and the response to immune challenge in the blood and CNS of immature and mature rats

6.1 Abstract

Inflammatory cytokines have been implicated in both the survival and death of neurones. To study the relationship between levels of inflammatory cytokines and neuronal survival, we have assayed 12 cytokine concentrations within the CNS parenchyma surrounding facial motoneurones and the peripheral blood, across the lifespan of the rat and correlated this with age-related differences in the survival of facial motoneurones following axotomy. We have also looked at immune priming or conditioning by challenging the immune systems of immature (young) rats and mature (adult) rats with the inflammatory agent, Lipopolysaccharide (LPS). Groups of 3 – 5 Sprague Dawley rats aged 7d, 3m and 24m were cardiac-punctured to obtain blood plasma and saline-perfused to retrieve brainstem devoid of peripheral blood. Other groups of rats received intraperitoneal LPS injections and blood and brainstem were assayed after 2, 4, 8 and 24 hours. Age-related increases were found in CNS cytokine concentrations, whereas plasma cytokine levels decreased in 24m rats. Inflamed rat pups elicited a different inflammatory profile in response to the LPS immune challenge, compared to inflamed adults. Injured facial motoneurones were most likely to die in 7d rats and least likely to die in 24m rats. Our results suggest that age-related increases in CNS cytokine concentrations may be neuroprotective and that data obtained on the role of cytokines in immature neuronal survival may not transpose to the ageing animal.

6.2 Introduction

Persistent inflammation with age has been implicated in age-related neurodegenerative diseases such as Motor Neuron Disease (MND), and experimentally increased inflammatory cytokine levels reduce motoneuronal survival (Godbout, Chen et al. 2005, Weydt and Moller 2005, Qin, Wu et al. 2007). The role of cytokine levels in motoneuronal survival, however, is unclear. Some studies suggest cytokine changes are responsible for motoneuronal loss in MND (Aebischer, Moumen et al. 2012, Dahlke, Saberi et al. 2015, Lu, Allen et al. 2016) while others indicate that changes in cytokines may be a consequence rather than a cause of the disease process (Chiu, Phatnani et al. 2009, Ehrhart, Smith et al. 2015). One reason for these divergent opinions may be that activation of microglia can be associated both with the removal of debris within the CNS and with neurotrophic support (Sargsyan, Monk et al. 2005). Thus, activation of microglia is associated with upregulation of inflammatory cytokines such as Interleukin 1α (IL-1α), Interleukin 1β (IL-1β) and Tumour Necrosis Factor-α (TNF- α) which can then amplify microgliosis to assess and respond to the injury (Elliott 2001, Yoshihara, Ishigaki et al. 2002). While a more direct neuroprotective function of microglia is evident in their ability to produce and secrete neurotrophic factors, in a targeted fashion, in response to the diverse needs of the neuron (Streit and Xue 2012, Gomes, Ferreira et al. 2013).

It is well established that abundant motoneuronal death generally follows axotomy in young animals and multiple mechanisms seem to be involved, including loss of peripheral target contact and neurotrophic support (Hamburger and Levi-Montalcini 1949, Bennet, Gibson et al. 2002). In adult animals, little or no motoneuronal death occurs after peripheral nerve crush or transection (Soreide 1981), although marked

motoneuronal death can still be produced by very proximal axotomies, such as nerve avulsion (Moran and Graeber 2004). In aged animals, however, we previously found that motoneurones were much less likely to die after nerve avulsion compared to adult and young animals (Chapter 3). We have also established that cytokines in the CNS parenchyma surrounding ageing rat facial motoneurones are higher than the periphery (Chapter 3). These results lead us to hypothesise that elevated levels of inflammatory cytokines in the CNS of ageing rats are neuroprotective and, by extension, that the decreased survival of injured motoneurones with decreasing age is the result of the decreased levels of CNS cytokines. Here, we have measured 12 cytokines in the CNS parenchyma and the peripheral blood, across the lifespan of the rat. We have also looked at immune priming or conditioning by challenging the immune systems of immature (young) rats and mature (adult) rats with the inflammatory agent, Lipopolysaccharide (LPS)

6.3 Materials and Methods

6.3.1 Animals and preparation of plasma and tissue.

For the cytokine analysis, groups of 3-5 Sprague-Dawley rats aged 7 days (gender unknown) 3 months (female) and 24 months (female)were used. Animals were housed under a standard 12-hour on/off lighting regime and given food and water *ad libitum*. The experimental study complied with the Australian code for the care and use of animals for scientific purposes (2016) and was approved by the University of Adelaide Animal Ethics Committee (M-57-2013). Rats were deeply anaesthetised by inhalation of 5% isofluorane in 2 litres oxygen/minute and while the anaesthetic nose cone was still attached, cardiac puncture was performed to withdraw blood into EDTA-coated blood tubes. Blood plasma was retrieved and stored at -80°C. Immediately following

cardiac puncture, rats were perfused transcardially with approximately 10ml (7d-old rats) or 200mL (3m and 24m rats) of sterile saline until the fluid flowing out of the right atrium was clear. The animals were then decapitated and the brainstem removed, trimmed at the mid pons level and approximately 1mm below the lower border of the pons to ensure it contained the facial nucleus, snap-frozen and stored at -80°C. Frozen brainstem samples were homogenised in lysis buffer made up with PBS, triton-X and protease inhibitors (Roche, cOmplete tablets). The supernatant was retrieved from homogenised samples and stored at -80°C. The BioRad DC Protein Assay (a modified Lowry method) was used to quantitate the amount of protein in each sample as per the manufacturer's instructions.

6.3.2 Multiplex Assay

Bio-Plex Pro Rat 12 plex cytokine assay kits (BioRad, New South Wales) were used to measure the concentration of twelve cytokines within each sample. Samples were loaded onto 96 well plates in duplicates (3m and 12-18m rats) and triplicates (24m rats). Plates were read using a Magpix Luminex multiplexing platform which uses a fluorescent imager (Abacus-ALS, Queensland) and data expressed as pg/ml of concentration. Experimental data was calibrated against standard curves of all 12 cytokines (BioRad, New South Wales). Spike-recovery analyses have been previously performed in-house to validate the accuracy of the multiplex for our sample types, showing minimal matrix difference as desired (Chapter 4).

6.3.3 Statistical Analysis

All data met parametric testing assumptions and so were analysed using either (i) mANOVA and follow-up univariate ANOVAS ± discriminant function analysis or (ii) two-way ANOVAs with Bonferroni-post hoc procedures.

6.4 Results

6.4.1 Plasma - Pups vs. adult and aged

A one-way mANOVA revealed an insignificant multivariate main effect of age groups on all cytokines using Pillai's Trace $F(18, 4) = 4.175$, $p=0.08$. However, when not considering the correlations of the cytokines, separate univariate ANOVAs with Bonferroni post-hoc procedures showed significant differences in plasma concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-12p70, IL-13 and GM-CSF when adults and aged rats were compared with pups (Fig 1). Differences between adult and aged rats have been described previously (Chapter 4).

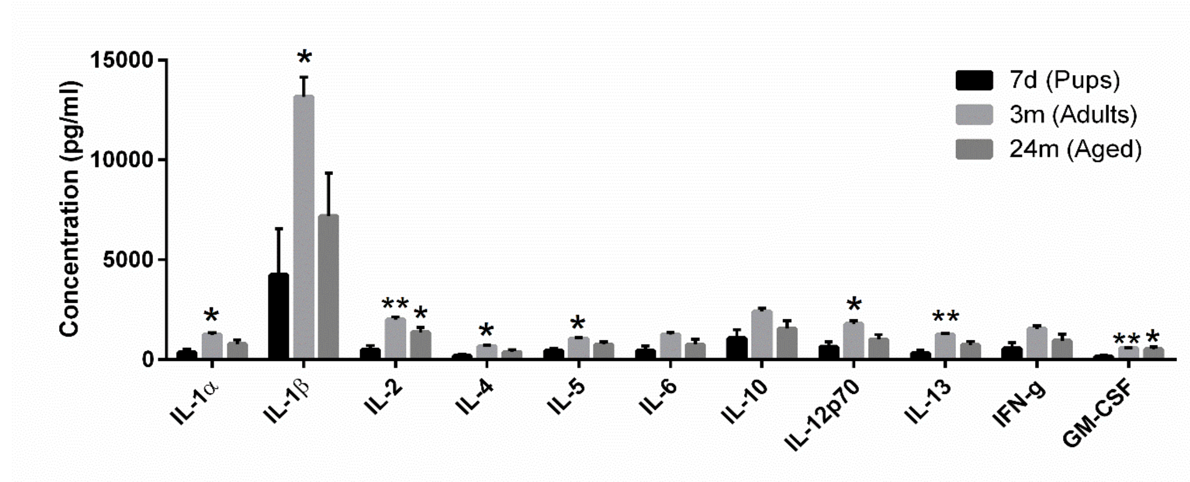

Figure 1: 8 cytokines with significantly higher plasma concentrations in adults, and 2 cytokines in aged rats compared to pups (, ** respectively indicate p<0.05 and p<0.01).*

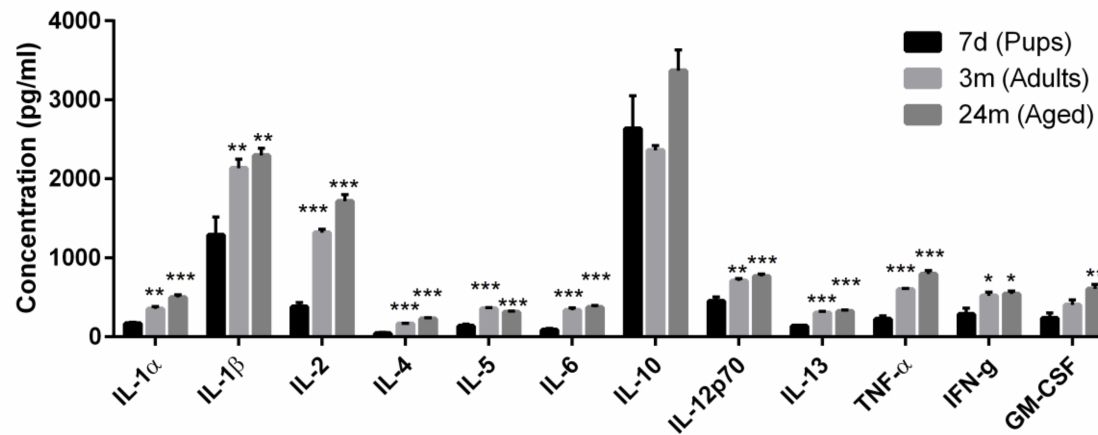

*Figure 2: 11 cytokine proteins expressed at higher concentrations in the CNS parenchyma surrounding facial motoneurones in adults and aged rats compared to pups. *, **, ***, **** denotes p<0.05, p<0.01, p< 0.001 and p<0.0001 respectively.*

6.4.2 Brainstem - Pups vs. adult vs. aged plasma

mANOVA using Pillai's Trace showed that there was a significant effect of age on the cytokines when considering their correlations $F(20,4) = 21.73$, $p<0.01$. Separate univariate ANOVAs with Bonferroni post-hoc procedures showed significant age effects on IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, TNF-α, IFN-γ and GM-CSF (Fig 2). The mANOVA was also followed up with a discriminant function analysis which revealed two discriminant functions. The first variate explained 93% of the variance ($R^2 = 0.99$) and the second only explained 7% ($R^2 = 0.98$). In combination, these discriminant functions significantly differentiated the age groups (1st variate $p<0.001$ and 2nd variate $p<0.01$). The correlations between outcomes and the discriminant functions revealed that GM-CSF loaded fairly highly onto Function 1 (r = -0.11) and IL-1α (-0.32), IL-1β (-0.13), IL-2 (-0.48), IL-4 (-0.4), IL-5 (-0.19), IL-6, (-0.25), IL-10 (-0.08), IL-12p70 (-0.17), IL-13 (-0.25), TNF-α (-0.43) and IFN-γ (-0.19) (i.e. all assayed cytokines but GM-CSF) loaded more highly on the second function. The discriminant function plot (Fig 3) showed that the first function discriminated the pups and aged rats from the adult group. Whereas, the second function discriminated the aged rats from the adult group.

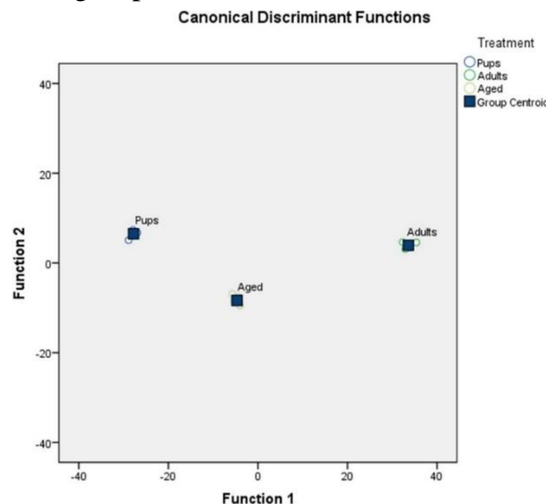

Figure 3: Discriminant Function Plot based on group centroids showing that Function 1 discriminated the pups and aged from the adults whereas Function 2 discriminated Aged from the Adults.

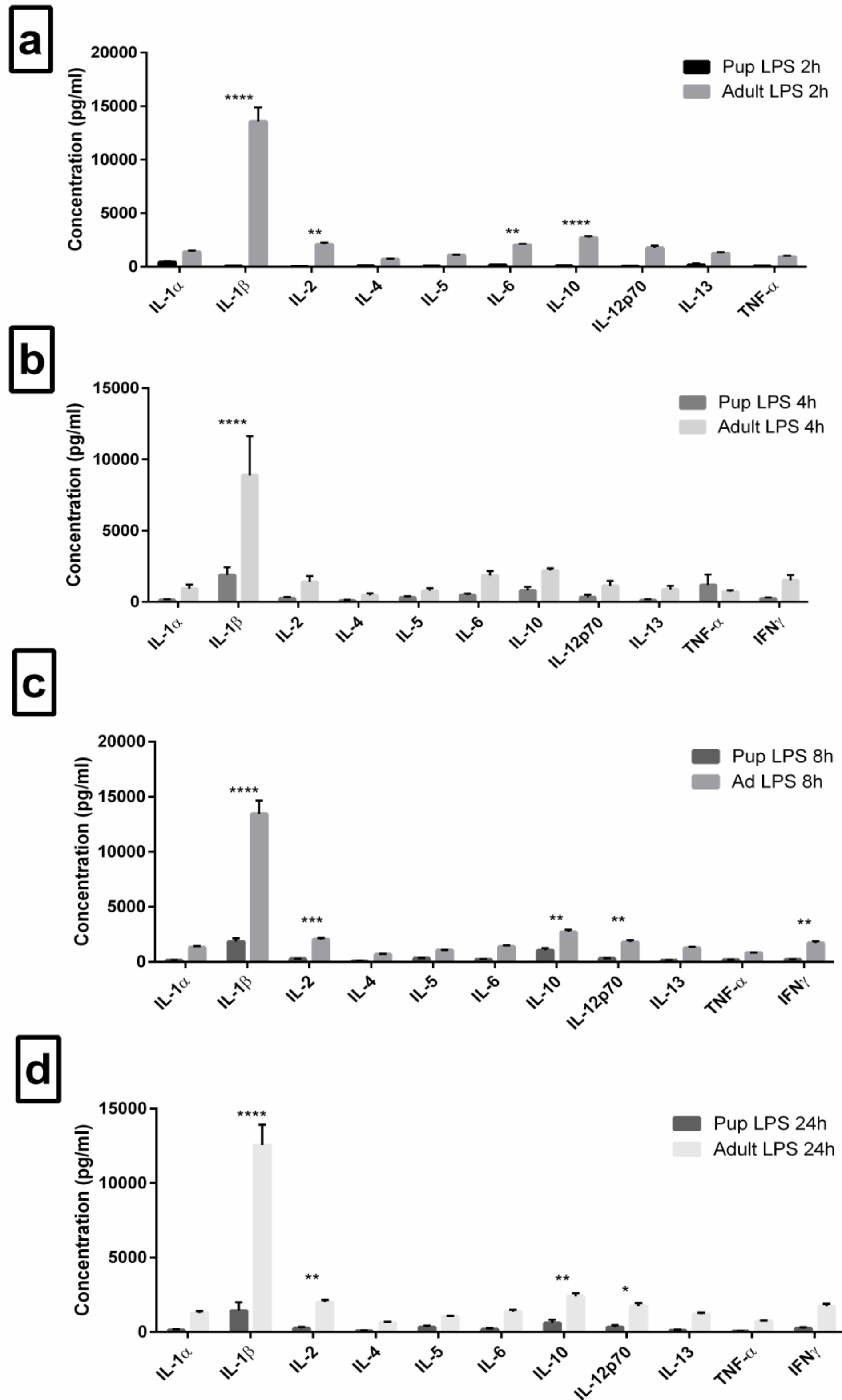

*Figure 4: Higher plasma concentrations of cytokines in adults with LPS compared to pups with LPS. *, **, ***, **** denotes p<0.05, p<0.01, p< 0.001 and p<0.0001 respectively.*

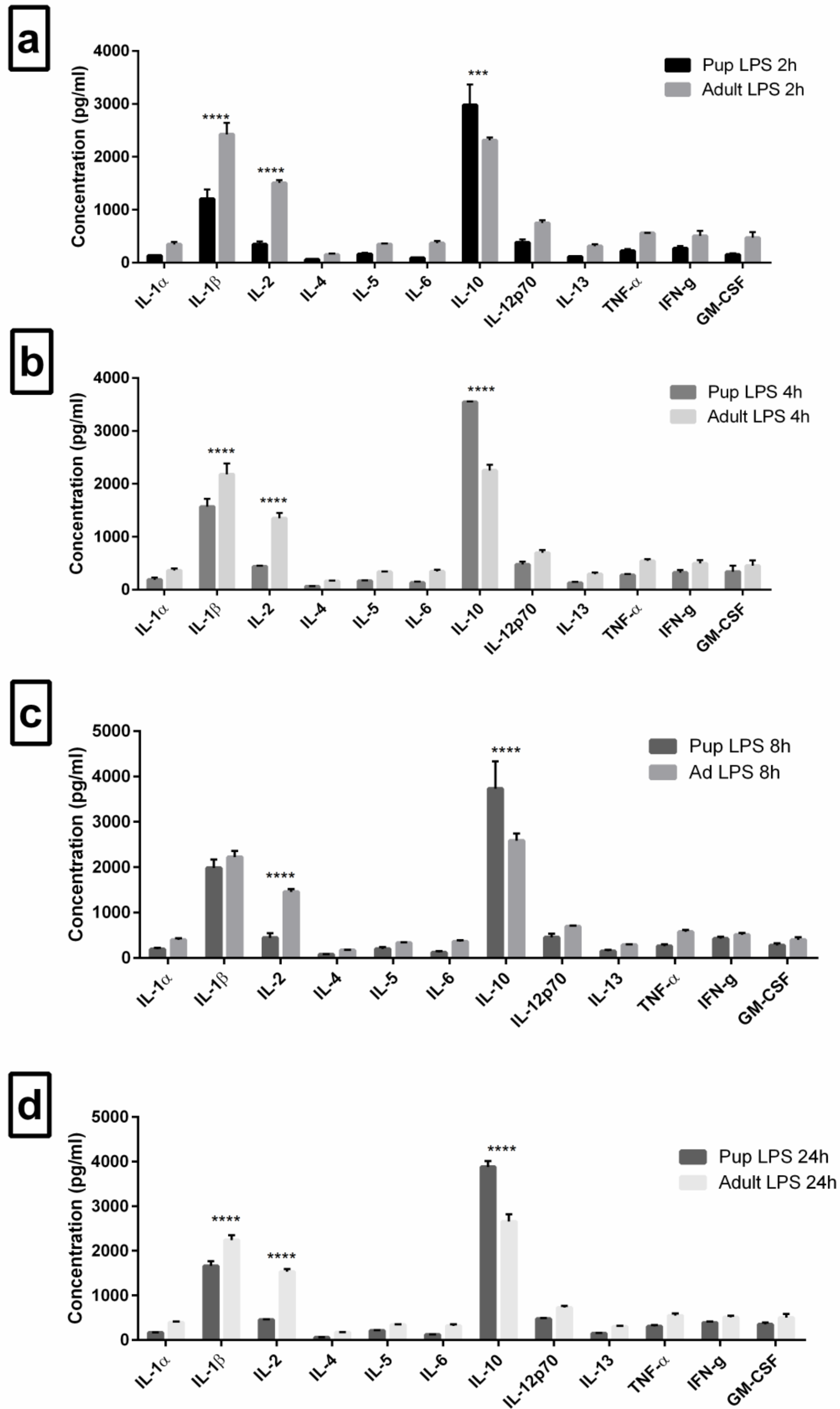

*Figure 5: Significantly higher brainstem concentrations in adults with LPS compared to their counterpart pups assayed at the same time-points (2h, 4h, 8h and 24h). *** and **** denotes p< 0.001 and p<0.0001 respectively.*

6.4.3 Plasma – Inflamed pups vs. inflamed adults

Significantly higher plasma cytokine concentrations (pg/mL) were seen in adults with LPS compared to pups with LPS, assayed at the same time points (Fig. 4). Significant differences at 2, 4, 8 and 24 hours post-LPS are noted in Table 1.

*Table 1: Mean plasma cytokine concentrations (pg/mL) that were significantly different between inflamed pups and inflamed adults across all four time-points (2h, 4h, 8h and 24h). *, **, ***, **** denote $p<0.05$, $p<0.01$, $p< 0.001$ and $p<0.0001$ respectively while "ns" indicates insignificance. Mean concentrations with n/a were not included in the analysis due to insufficient replicate values to derive accurate means.*

Plasma Cytokines		LPS 2h		LPS 4h		LPS 8h		LPS 24h	
		Pups	Adults	Pups	Adults	Pups	Adults	Pups	Adults
IL-1β	Mean	77	13573	1890	8892	1836	13437	1418	12551
	±	±	±	±	±	±	±	±	±
	SEM	25	1321	553	2735	298	1223	580	1369
	Significance	****		****		****		****	
IL-2	Mean	17	2091	251	1412	269	2037	245	1996
	±	±	±	±	±	±	±	±	±
	SEM	3	162	111	406	28	139	91	166
	Significance	**		ns		***		**	
IL-6	Mean	170	2042	472	1851	223	1393	176	1355
	±	±	±	±	±	±	±	±	±
	SEM	61	101	122	319	38	110	74	130
	Significance	**		ns		ns		ns	
IL-10	Mean	84	2715	803	2193	1044	2706	605	2397
	±	±	±	±	±	±	±	±	±
	SEM	32	149	256	181	210	209	209	201
	Significance	****		ns		**		**	
IL-12p70	Mean	n/a	1751	335	1128	312	1798	322	1746
	±		±	±	±	±	±	±	±
	SEM		201	167	349	34	180	136	194
	Significance	ns		ns		**		*	
IFNγ	Mean	n/a	1953	244	1501	216	1705	236	1718
	±		±	±	±	±	±	±	±
	SEM		119	75	393	33	165	83	162
	Significance	ns		ns		**		ns	

6.4.4 Brainstem – Inflamed pups vs. inflamed adults

Compared to inflamed adults, inflamed pups generally had reduced mean concentrations (pg/mL) of IL-1β and IL-2 whereas IL-10 was elevated (Fig. 5 and Table 2). However, 8 hours following LPS, there was no significant difference for IL-1β.

*Table 2: Mean CNS cytokine concentrations (pg/mL) that were significantly different between inflamed pups and inflamed adults across all four time-points (2h, 4h, 8h and 24h). ***, **** denote p< 0.001 and p<0.0001 respectively.*

CNS Cytokines		LPS 2h		LPS 4h		LPS 8h		LPS 24h	
		Pups	**Adults**	**Pups**	**Adults**	**Pups**	**Adults**	**Pups**	**Adults**
IL-1β	**Mean ± SEM**	1205 ± 179	2425 ± 217	1567 ± 152	2180 ± 207	1981 ± 187	2222 ± 132	1661 ± 102	2241 ± 111
	Significance	****		****		ns		****	
IL-2	**Mean ± SEM**	347 ± 55	1504 ± 58	437 ± 20	1350 ± 101	443 ± 102	1456 ± 63	457 ± 12	1533 ± 59
	Significance	****		****		****		****	
IL-10	**Mean ± SEM**	2977 ± 387	2311 ± 53	3545 ± 13	2252 ± 109	3728 ± 602	2585 ± 161	3882 ± 131	2658 ± 161
	Significance	***		****		****		****	

6.5 Discussion

We show a general increase in inflammatory cytokines in the CNS of rats aged 7d, 3m and 24m. Plasma cytokines also increased in 3m compared to 7d rats, but decreased in 24m rats (Chapter 4). The CNS cytokine changes correlate with an increase in the proportion of motoneurones surviving facial nerve avulsion (Chapter 4). This indicates that age-related increases in CNS inflammatory cytokines may be neuroprotective. This view runs contrary to the view that age-related increases in levels of inflammation are

detrimental to neuronal survival (Chen, Buchanan et al. 2008, Franceschi and Campisi 2014). We also report that the cytokine response to intraperitoneal LPS differs between 7 day (immature)- and 3 month (mature) rats, indicating that there are intrinsic differences in the immune system of rats of these two ages. Such age-related differences need to be taken into consideration when testing therapeutics on immature experimental models for clinical conditions that manifest and affect neurones in predominantly mature or ageing individuals such as Alzheimer's Disease, Parkinson's Disease and MND.

From our data on non-operated 3m and 24m rats (Chapter 4), we propose that the greater and more rapid loss of injured motoneurones seen in 7d rats, is associated with pre-existing lower levels of CNS cytokines compared to 3m and 24m rats. Significant age-effects were noted with 11 out of the 12 assayed cytokines by univariate ANOVAs. The view that ageing only affects pro-inflammatory cytokines (Michaud, Balardy et al. 2013) however, may be too simplistic, because of the 11 cytokines with significant age effects, of which IL-1α, IL-1β, IL-2, IL-6, IL-12p70, TNF-α, IFNγ and GM-CSF are known to be pro-inflammatory, whereas IL-4, IL-5, IL-6 and IL-13 are anti-inflammatory (Berger 2000, Shimada and Hasegawa-Ishii 2011). The discriminant function analysis and plot showed that the first function discriminated the pups and aged rats from the adult group and the only cytokine to affect this function was GM-CSF. GM-CSF is produced by neurones, astrocytes and ependymal cells as a cytokine that stimulates microglial cell growth (Ridwan, Bauer et al. 2012). In both pups and aged rats, polar extremes of GM-CSF levels were noted and this could be taken to mean that the lower GM-CSF levels in pups, correlates with lower microglial activity and reduced production of pro-inflammatory mediators that are potentially

neuroprotective (Parajuli, Sonobe et al. 2012). This could leave injured motoneurones more likely to die. This view could be tested in 7d rats by artificially elevating cytokines before nerve avulsion to see if this confers neuroprotection, as we have already done in 3m rats (Chapter 5). However, pilot experiments would be needed to determine (i) how much the cytokines need to be elevated and (ii) the duration of inflammatory priming prior to nerve injury; to create an optimal inflammatory milieu to confer neuroprotection. Recent studies have suggested that priming/conditioning lesions are the mechanisms by which chronic, low grade inflammation may be capable of promoting neuroprotection and repair (Qiu, Cafferty et al. 2005, Salegio, Pollard et al. 2011, Blesch, Lu et al. 2012). The intrinsic growth capacity of the CNS is thought to be repressed for proper synaptic development in the transition period of an immature to the mature system and this intrinsic growth capacity is thought to be reactivated by conditioning/priming injuries in the PNS but not the CNS (Abe and Cavalli 2008, Salegio, Pollard et al. 2010). As a result, the conditioning lesion theory has been limited to pseudounipolar neurones of the sensory system where a priming injury to the peripheral process, prior to injury of the central process, promotes central axonal regeneration. Drawing on this model, CNS neuroprotection in the present study may be effected by age-related glial priming/conditioning in the CNS (Reynolds, Hendry et al. 2001). Given that motoneurone survival mechanisms are known to change with age, perhaps glial priming/conditioning provides a better environment for mature motoneurones to survive injury (Schweizer, Gunnersen et al. 2002).

We report significant differences in the cytokine responses between inflamed adults and pups. This indicates that intrinsic age-related differences exist in the way the innate immune system responds to immune challenge. In plasma, priming the mature rats for

8 hours yielded high mean concentrations in the most number of cytokines (5 out of 12), compared to their immature counterparts. While in the brainstem, priming the mature rats for 2-, 4- and 24 hours yielded high mean concentrations in the most number of cytokines (3 out of 12), compared to their immature counterparts.

A notable exception to this was IL-10 where inflamed pups had consistently higher levels of this anti-inflammatory cytokine in their CNS compared to inflamed adults. This could be taken to indicate that the immature innate anti-inflammatory response to an immune challenge is more robust than in mature animals. This would be in keeping with our suggestion that pro-inflammatory cytokines are neuroprotective.

The present study shows age-related changes in pro-as well as anti-inflammatory cytokines. This highlights the need to consider age-related changes in the general inflammatory signature, rather than changes in one or two selected cytokines, as generally occurs in discussions centred around current inflammaging theories (Franceschi 2007, Franceschi and Campisi 2014). In addition to the possibility of age-related differences in the levels of a myriad of cytokines, there are likely to be age-related differences in the way the immune system responds to immune challenges. This has major implications for our current understanding of “neuroinflammaging” and neurodegenerative diseases such as MND that are predominantly studied using immature experimental models. Overall, our results indicate that immature experimental models of neuronal degeneration that are either based on neuroinflammaging or used to support it, are unlikely to transpose to neuronal degeneration in the ageing animal.

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7 General Discussion

Extensive literature into pathogenetic mechanisms of many neurodegenerative diseases, such as Motor Neuron Disease, focuses on the deprivation of neurotrophic support, neuroglial involvement and inflammaging as culprit causes of pathological manifestations in motoneurones (Henriques, Pitzer et al. 2010, Philips and Robberecht 2011, Staszewski and Prinz 2014, Hooten, Beers et al. 2015). As a consequence, motoneurones are thought to eventually undergo neuronal death leading to the myriad of phenotypical presentations of neurodegenerative conditions. Such neurodegenerative conditions could be multifactorial and delving deeper into these hypotheses may provide a converging point as a novel area of investigation for the development of therapeutic modalities. One such common denominator/converging axis seems to be the concept of ageing. Despite focussed investigations into this field, published work has suffered from inconsistent findings, given the widely accepted theory that ageing is mostly detrimental to neuronal cell populations (Dauer, Huang et al. 2011, Michaud, Balardy et al. 2013). These opinions have encouraged a simplistic presentation of either increasing or decreasing levels of individual inflammatory cytokines to be extrapolated to the whole animal in healthy ageing and disease. Within this thesis, I aimed to investigate 3 potential mechanisms of neuroprotection: (i) a potent neurotrophic factor, MGF24, capable of rescuing mature or adult motoneurones (Chapter 2); (ii) early perineuronal changes following two peripheral injuries (transection vs. avulsion) that respectively resulted in motoneuronal survival or death (Chapter 3); and (iii) the complex interplay of ageing and inflammatory cytokines, in both CNS parenchyma surrounding facial motoneurones and peripheral blood plasma (Chapters 4 to 6).

7.1 Neuroprotection mechanisms of the MGF neurotrophic factor

Using the facial nerve avulsion model, in Chapter 2, I demonstrated that the IGF-1 receptor and Protein Kinase C may not be the main mechanisms employed by MGF, a potent neurotrophic factor, in rescuing facial motoneurones that are destined to die after injury. Instead, my preliminary immunohistochemical study, in accordance with an extensive body of literature, suggests that the PI3k-Akt-mTOR pathway may mediate the neuroprotection of facial motoneurones in our injury model (Li, Xu et al. 2003, Ohta, Arai et al. 2011, Saxena, Roselli et al. 2013). We could follow this up with the same experiments employed with the IGF-1receptor- and PKC- inhibitors by using a PI3k inhibitor such as wortmannin to determine if it blocks MGF-mediated neuroprotection of motoneurones (Zhuang, Xu et al. 2004, McNamara and Degterev 2011, Ueda, Nakahara et al. 2013). Unfortunately, as our study came across underpowered, further investigation is required before IGF-1r and PKC can be concretely ruled out. It also cannot be discounted that Chapter 2 is specific to MGF's neuroprotection of facial motoneurones and other signals downstream of PKC or other IGF-1 receptors have been exempted from the study. Furthermore, motoneurone subtypes can respond differently to various trophic factors (Terrado, Monnier et al. 2000) thus caution must be exercised if extrapolating MGF's neuroprotective mechanisms to motoneurone populations other than facial motoneurones. Future studies could look at the IGF-1 receptor antibody binding affinity *in-vitro* and *in-vivo* while kinase activity could be examined using multiplex assays to look at levels of phosphorylated proteins, at multiple time-points, for optimal detection. However, such experimental approaches would require generation of parallel cohorts of animals, given these methods require cell culture- and fresh-frozen tissue while stereological counts of motoneurones (that we studied as an outcome) required perfusion-fixed tissue. Further

assessment should investigate mechanisms of potent neurotrophic factors, to generate data that could potentially be utilised to modulate neurotrophic factor efficacy in protecting different subsets of motoneurones.

7.2 Perineuronal glial activity and synaptic response to injured motoneurone

Along with neurotrophic factors and their ability to prevent motoneuronal death, perineuronal activity is another factor thought to indicate the fate of a motoneurone post-injury (Torvik and Skjörten 1971, Dauer, Huang et al. 2011, Yamada, Nakanishi et al. 2011). Chapter 3 investigates perineuronal glial activity and synaptic responses at an early stage (7 days) post-motoneurone injury. Motoneuronal survival was studied in the context of nerve transection while motoneuronal death was rendered by nerve avulsion (Torvik and Søreide 1975). We found increased immunostaining for astrocytes and microglia surrounding injured motoneurones compared to uninjured motoneurones. However, there was no significant difference in gliosis around transected- and avulsed-motoneurones. This suggests glial immuno-peroxidase staining, at least when semi-quantified, does not predict the fate of motoneurones 7 days post-injury. We also found no difference in immunostaining for synapses across normal uninjured and injured groups, which is in contrast to previous immunofluorescence synaptophysin studies in the confocal microscope (Yamada, Nakanishi et al. 2011). While this discrepancy could be due to strain differences (Sprague-Dawley vs. Wistar rats), or the redistribution of synapses within the facial nucleus, it is likely due to methodological issues. These include attempting to immunoperoxidase-stain and semi-quantify microscopic synaptic structures in a large volume and using large depth of tissue section (100µm sections generated for stereological counts). In addition to using other methods to assess expression of protein/RNA (e.g. Western Blots, ELISAs, PCR or DNA sequencing),

tissue sampling at alternative time-points (before or after 7 days) may have enhanced detection of perineuronal glial and synaptic responses that could further indicate the fate of injured motoneurones. Understanding the perineuronal responses in correlation with the fate of injured motoneurones, could provide us with better insight into early clinical manifestations of MND and according intervention strategies to halt disease progression (Tyzack, Sitnikov et al. 2014).

7.3 Inflammatory cytokine expression and immune-priming

Some clinical and experimental manifestations of MND and experimental motoneuronal degeneration have been related to changes in inflammatory mediators and cells that produce them (Beers, Henkel et al. 2011, Beers, Zhao et al. 2011, Kano, Beers et al. 2012, Henkel, Beers et al. 2013, Zhao, Beers et al. 2013). Findings reported in Chapter 4 demonstrate an age-related polarity of inflammatory cytokines in peripheral blood and CNS tissue surrounding the facial motoneurones in aged rats, such that low levels of peripheral inflammation were seen with high levels of CNS (brainstem) inflammation. While the peripheral result contradicts previous studies (Michaud, Balardy et al. 2013), most of the current literature is based on studies of a selected few cytokines (usually 1 to 3 cytokines) rather than a broad assessment of an inflammatory signature. Furthermore, CNS tissue was typically analysed with the intrinsic blood still within it. This would influence true CNS cytokine concentrations since the brain has an extensive vascular network. Thus, we obtained CNS parenchyma tissue surrounding the motoneurones of interest after perfusing the brain with saline. This resulted in brain tissue largely devoid of any blood for cytokine analysis. Moreover, multiplexing allowed us to simultaneously assay 12 cytokines in the CNS and peripheral blood from the same animals, under the same conditions, which allows multiple comparisons to be

made. However, it must be noted that our findings on the inflammatory environment of the CNS, are limited to the brainstem parenchyma surrounding facial motoneurones. It is possible that different conditions exist around other cranial- or spinal- motoneurones and that strain and species variations exist. This could also be investigated using the present methodology to generate comparison data.

CNS region-specific variations in cytokine expression levels outside lower motoneurone regions are also known to exist (Beers, Zhao et al. 2011). With respect to MND, future experiments could compare cytokines in frontal cortex and temporal lobe. This might provide an insight into recent associations of MND with fronto-temporal dementia in the context of inflammation (Piguet 2013). Notwithstanding the above, our focus on the brainstem parenchyma around facial motoneurones was intentional. The injury model used in our study targeted Sprague-Dawley facial motoneurones and aimed to mimic in adult rats, the intrinsic CNS inflammatory environment around motoneurones seen in aged rats. This was done by intraperitoneal injection of Lipopolysaccharide as an immune priming agent (Chapter 5). Immune mechanisms have been implicated in experimental models of motoneurone degeneration. Thus, in MND mouse models, motor weakness presents initially in the hindlimbs and is delayed in forelimbs. Beers, Zhao et al. (2011) showed that MND mice with hindlimb motor weakness had elevated levels of Th1 immune response markers in the lumbar spinal cord (that supplies hindlimbs). However, even prior to forelimb motor weakness, Th2 responses had already begun mounting in the cervical spinal cord. Beers et al (2011) attributes this response to an attempted protective response in the cervical cord to anticipated “distress” or injury to cervical cord motoneurones. While this concept of CNS immune-priming is fairly novel and still highly debated (Salegio, Pollard et al. 2011), peripheral nervous system priming has been shown to benefit central axonal

regeneration as evidenced by multiple studies (Qiu, Cafferty et al. 2005, Abe and Cavalli 2008). Our findings from Chapter 5 identified that immune-priming the adult environment with Lipopolysaccharide did not affect the post-injury survival of adult motoneurones. However, this calls into question whether our “glial priming” was sufficient enough to mount a protective response. As challenging the immune system with the Lipopolysaccharide endotoxin alone could be either unnecessary (i.e. another agent is needed) or insufficient (i.e. a greater dose-, additional immune challenges-, or longer period of challenge is required) to recreate these presumptive age-related priming/conditioning in the CNS. Chapter 6 highlighted the finding that immature rats presented with lower levels of peripheral and CNS-brainstem cytokines compared to mature rats. We correlated this with the well-documented rapid and abundant loss of axotomised motoneurones in immature rats (Hamburger and Levi-Montalcini 1949, Lieberman 1974, Hamburger 1977, Greensmith and Vrbova 1996, Johnson, Goldspink et al. 2011) and proposed that lower levels of CNS inflammatory cytokines may contribute to increased motoneuronal degeneration in immature systems, given that the opposite is true for aged/mature rats, where high levels of CNS cytokines correlated with the least and slowest motoneuronal degeneration. When we compared inflamed immature rats with inflamed mature rats, we also observed intrinsic differences in the innate immune system and its response to the immune challenge. Mature rats presented with higher cytokine levels in both peripheral blood and CNS with the notable exception of IL-10. IL-10 as an anti-inflammatory cytokine was consistently the only elevated cytokine in inflamed immature rats compared to inflamed mature rats. Future studies require comprehensive investigation into functional implications of intrinsic differences between immune-primed immature rats and mature rats, given that a commonly used experimental model of MND is an immature mouse model with genetic

mutations in the superoxide dismutase 1 gene (Philips and Rothstein 2015). The SOD1 mouse model is the most studied cause of familial MND (fMND) even when only 5-10% of MND patients have fMND, of which only 1-2% have the SOD1 mutation (Van Den Bosch 2011, Philips and Rothstein 2015).

The work presented as part of this thesis has contributed novel and significant findings to research in the area of motoneuronal degeneration and survival. However, the full interplay of functional implications of age-related changes in motoneuronal degeneration and neurodegenerative diseases such as MND still remains unclear. Investigations of age-related factors in neuronal degeneration should take priority in the field of neurodegenerative research given the increasing impact of age-associated diseases on populations worldwide (Franceschi and Campisi 2014). Since advancing age is the single biggest risk factor in age-related neurodegenerative disease, understanding of processes fundamental to ageing effects of the nervous system is likely to facilitate the development of experimental models that better reflect human disease. This would hopefully lead to the establishment of novel targeted-therapy strategies and potentially a multitude of clinical applications with the ultimate outcome of effectively translating research from “bench” to “bedside”.

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8 Appendices

8.1 Presentations and abstracts arising from thesis

1. **V Katharesan,** Q-Z Ong, MD Lewis, R Vink and IP Johnson, "Age-related changes in motor systems of rats and the effect of inflammatory cytokines on adult motoneurones", International Symposium on ALS/MND – 26th Annual Meeting 2015, Orlando, USA.

2. **V Katharesan**, Q-Z Ong, M D Lewis, R Vink and I Johnson, "A different perspective on Motor Neuron Disease: Age-related differences in the motor systems of rats", Florey International Postgraduate Research Conference 2015, Adelaide Australia.

3. **V Katharesan**, Q-Z Ong, R Vink and I Johnson, "Extent of motoneuronal survival across the rat lifespan", Motor Neuron Disease Australia Research Meeting 2014. Melbourne, Australia.

4. **V Katharesan**, Q-Z Ong, R Vink and I Johnson, "Changes in peripheral musculature and extent of motoneuronal survival across the rat lifespan", Florey International Postgraduate Research Conference 2014. Adelaide, Australia.

5. **V Katharesan** and I Johnson, "Potency of a muscle-derived isoform of IGF-1 for the rescue of facial motoneurones and insight into its mechanism of action", Australasian Neuroscience Society – 34th Annual Meeting 2014. Adelaide, Australia.

6. I Johnson, **V Katharesan**, K Mitris, Q-Z Ong, "Research on Motor Neuron Disease at the University of Adelaide", Neurodegenerative Disease Expo 2014. Adelaide, Australia.

7. **V Katharesan**, I Johnson, R Li, A Evans, "Novel features of adult rat facial motoneurone rescue by a muscle-derived isoform of IGF-1", International Symposium ALS/MND – 24th Annual Meeting 2013. Milan, Italy.

8. **V Katharesan,** I Johnson, R Li, A Evans, "Non-IGF-1 receptor dependant rescue by an isoform of IGF-1 isolated from active muscle", Australasian Neuroscience Society – 33rd Annual Meeting 2013. Melbourne, Australia.

9. I Johnson, **V Katharesan**, S Khan, G Goldspink, "Rescue of motoneurones: an ageing problem", International Motoneuron Meeting – 2012. Sydney, Australia.

10. I Johnson and **V Katharesan**, "Neurotrophic rescue differs with age", Adelaide Centre for Neuroscience Expo - 2012. Adelaide, Australia.

8.2 Invited Talks arising from thesis

1. **V Katharesan**, A Tenakoon, S Deery and I Johnson, “Current Research on Motor Neuron Disease at the University of Adelaide”, Motor Neuron Disease Association of South Australia, MND Community Christmas Lunch, Adelaide Sailing Club, upcoming 15th December 2016.

2. **V Katharesan**, “Motor Neuron Disease”, RadioAdelaide Interview with Katrina Hall, https://radio.adelaide.edu.au/viythia-katharesan-motor-neurone-disease/

3. **V Katharesan** and I Johnson, “Research on Motor Neuron Disease at the University of Adelaide”, Motor Neuron Disease Association of South Australia Annual General Meeting, Hilton, Adelaide, 27th October 2015.

4. **V Katharesan**, Q-Z Ong, R Vink and I Johnson, “Age-related changes in the peripheral target and extent of motoneuronal survival after neurotrauma”, 5th Australian Neurotrauma Symposium, University of South Australia, 8th October 2015.

5. **V Katharesan**, “The young and bold are not the same as the golden old”, Three Minute Thesis Finals, The University of Adelaide, 8th September 2015.

6. **V Katharesan**, “A different perspective on Motor Neuron Disease”, Neurosurgical Research Foundation Annual General Meeting 2015, The University of Adelaide, 12th August 2015.

7. **V Katharesan**, “My experience as a new user of Bio-plex technology: analysis of inflammatory cytokines”, BioPlex Pacific User Group Meeting, The University of Adelaide, 27th May 2015.