Elucidating the effect of aggregated SOD1 on microglia and astrocytes in ALS

Kate Roberts
University of Wollongong

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Elucidating the effect of aggregated SOD1 on microglia and astrocytes in ALS

This thesis is presented as part of the requirement for the award of the degree

Doctor of Philosophy
from the
University of Wollongong
by
Kate Roberts

School of Biological Sciences
Certification

I, Kate Roberts, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the school of biological sciences, University of Wollongong, is wholly my work unless stated otherwise referenced and acknowledged. This document has not been submitted for qualifications at any other academic institute.

Kate Roberts

Date January 2016
Acknowledgements

First of all I would like to thank my supervisor Justin Yerbury for all of his guidance that helped me to be a better scientist. I can’t thank him enough for the opportunity he gave me to continue with my studies after my honours year. His patience and support over these past years when I believed the science gods had it in for me was invaluable.

I would I also like to thank my co-supervisor Iain Campbell and his lab group in Sydney for their support and for teaching me important primary culture techniques. I would also like to thank Brad Turner down in Melbourne for his help with the infusion soluble SOD1. A big thank you also goes out to Martin Engel for his help and guidance when I carried out the injection of aggregated SOD1 experiments.

Thanks to all of the people in lab 210 that always made lab work more interesting and made me laugh many, many times. A special mention goes out to Natalie (Australia’s number 1 RA), Luke, Isabella, Raffa, Lisa, B2, and Jen.

Last but not least I’d like to thank my Mum and brother Luke for all the love and support over the past difficult but fun years of my life studying science. I love you both so much.
Abstract
A large body of literature suggests that amyotrophic lateral sclerosis (ALS) pathology is intimately linked with both aggregated proteins and neuroinflammation; specifically activation, recruitment and dysfunction of microglia and astrocytes. However, there has been little work performed that aimed to understand the impact that aggregates of Cu/Zn superoxide dismutase (SOD1) have on microglia and/or astrocytes.

Mutant SOD1 associated with ALS has recently been shown to activate microglia in a CD14 dependant mechanism when found in the extracellular space, providing one potential explanation of glial activation during disease. Recent work demonstrates a strong link between protein inclusions and cell loss and as a result neuroinflammation in ALS and although they may be made up of different proteins, inclusions are associated with all forms of ALS. With this in mind, the first aim of this project sought to determine if aggregated SOD1 would activate microglia. Recombinant SOD1 was aggregated and this, or soluble non-aggregated forms of SOD1 were then added to EOC.13 microglial cells or primary microglial cells in culture. Although soluble non-aggregated mutant SOD1 has been shown to promote microglial activation in the past, we found that aggregated SOD1 was able to much more efficiently activate microglia in culture when compared with the unaggregated form of mutant SOD1. In addition to CD14 dependant pathways, aggregated SOD1 also bound to the surface of glial cells and was internalized in a lipid raft and scavenger receptor dependent manner. Here, for the first time, we have shown that aggregated mutant SOD1 potently activates microglia.

There is growing evidence to suggest astrocyte dysfunction in ALS. Previous evidence has suggested that accumulation of mutant Cu/Zn superoxide dismutase (SOD1)
initiates activation of ER stress in motor neurons. As protein inclusions have been observed in astrocytes in ALS, the second aim of this project sought to determine if exogenous aggregated mutant SOD1 would affect activation or viability of primary astrocytes. Recombinant mutant SOD1 was aggregated and subsequently added to primary astrocytes in culture. Exogenously added aggregated mutant SOD1 was taken up into the cytosol of astrocytes, remained there for at least 7 days, causing endoplasmic reticulum stress, astrocyte senescence and ultimately cell death. With the use of a panel of endocytosis inhibitors we were able to show that aggregated SOD1 was internalized by primary astrocytes via macropinocytosis. This work, for the first time, links uptake of aggregated mutant SOD1 to dysfunction and toxicity in primary astrocytes.

While the first two chapters showed dramatic effects this was performed in 2D cultures. To demonstrate glial dysfunction in a biological system proteins were infused or injected into the nervous system of mice. Here we report that soluble SOD1<sup>G93A</sup> infused over 5 weeks into the brains ventricles of wild type mice, resulted in no significant impact on anterior horn microglia and astrocytes, when compared to soluble SOD1<sup>WT</sup> infusion. Whereas, 72 hours after injection aggregated SOD1 caused increased microglial activity and caused astrocyte senescence around murine brain ventricles. These results suggest that the aggregated form of SOD1 has a significant impact on the activation state of microglia and astrocyte dysfunction <i>in vivo</i>, and may directly affect disease progression in ALS. As a result, future therapeutics that work simultaneously to prevent SOD1 aggregate induced microglial activation and astrocyte dysfunction may be effective in slowing ALS progression.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Agg</td>
<td>aggregated</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMF-1c-120</td>
<td>amorfix misfolded</td>
</tr>
<tr>
<td>Ast-1</td>
<td>murine astrocyte like cell line</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinichoninic acid</td>
</tr>
<tr>
<td>BiP</td>
<td>binding protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of Differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPZ</td>
<td>chlorpromazine</td>
</tr>
<tr>
<td>Cyt D</td>
<td>cytochalasin D</td>
</tr>
<tr>
<td>DMEM:F-12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-Ethyl-N-isopropyl) amiloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EOC.13</td>
<td>mouse derived microglial cell line</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FALS</td>
<td>familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>Gen</td>
<td>genistein</td>
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GFAP  glial fibrillary acidic protein
HSP  heat shock proteins
IFN-\( \gamma \)  interferon gamma
IGF-1  insulin growth like factor-1
kDA  kilo daltons
IL  Interleukin
IRE1  inositol requiring enzyme 1
LPS  lipopolysaccharides
M\( \beta \)CD  methyl-beta-cyclodextrin
MCP-1  monocyte chemoattractant protein 1
mSOD1  Mutant Superoxide dismutase 1
mRNA  messenger ribonucleic acid
NSC-34  mouse hybrid motor neuron cell line
NTF  neurotrophic factors
ROS  reactive oxygen species
TBST  tris buffered saline Tween
TLR  toll like receptors
TNF-\( \alpha \)  tumor necrosis factor alpha
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PDL  Poly-D-Lysine
Pen-strep  Penicillin-Streptomycin
PFA  paraformaldehyde
PDI  protein disulphide isomerase
PI  Propidium iodide
PMA  Phorbol myristate acetate
MND  Motor Neuron Disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;G37R&lt;/sup&gt;</td>
<td>Superoxide dismutase 1 mutation (glycine 37 changed to arginine)</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;G93A&lt;/sup&gt;</td>
<td>Superoxide dismutase 1 mutation (glycine 93 changed to alanine)</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;A4V&lt;/sup&gt;</td>
<td>Superoxide dismutase 1 mutation (alanine 4 changed to valine)</td>
</tr>
<tr>
<td>SOD1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>Superoxide dismutase 1 wild type</td>
</tr>
<tr>
<td>SALS</td>
<td>sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA binding protein 43</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Thaps</td>
<td>thapsigargin</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>XBP-1</td>
<td>x-box binding protein 1</td>
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Publications and Conference Presentations

Publications:

1. Kate Roberts, Rafaaz Zeineddine, Lisa Corcoran, Wen Li, Iain L. Campbell, and Justin J. Yerbury (2013). Extracellular Aggregated Cu/Zn Superoxide Dismutase Activates Microglia to Give a Cytotoxic Phenotype, GLIA 61(3), 409-419


3. Sharron Hook, Kate Roberts, Janet Kumita and Yerbury, J.J. The interplay of protein aggregates, microglia and neuroinflammation in neurodegenerative disease, Nova science publishes 157-204.

Conferences and workshops

2011  The 22nd international Symposium on ALS/MND- Abstract for a poster accepted and presented

2012 Proteostasis and Disease Symposium- Abstract for a poster accepted and presented.

2012 Proteostasis & Disease Research Centre- Present and contribute to seminars

2013 Rotary Media Training & Presentation Skills Workshop

2014 The 39th Lorne Conference on Protein Structure and Function- Abstract for a poster accepted and presented

2014 Proteostasis and Disease Symposium- Abstract for a poster accepted and presented.

2014 The 25th international Symposium on ALS/MND- Abstract for a poster accepted and presented

2014 Seminar given to the Dobson experimental group meeting at the University of Cambridge
1. Introduction
1.1 Prevalence and types of ALS

Amyotrophic Lateral Sclerosis (ALS) is a lethal, rapidly progressive neurodegenerative disease, which generally develops in patients between 50 and 60 years of age. Estimates suggest that 3-7 people per 100,000 suffer from ALS (Benatar et al. 2006), and in Australia alone approximately 400 people die of ALS each year (Kiernan et al. 2006). ALS is commonly referred to as Motor Neuron Disease (MND) in Australia and the UK but is only one of several motor neuron diseases. ALS involves the degeneration of both the upper and lower motor neurons in the spinal cord, cortex and brain stem, while other motor neuron diseases (such as primary lateral sclerosis, progressive muscular atrophy, progressive bulbar palsy and pseudobulbar palsy) attack only upper or lower motor neurons (Wohlfart et al. 1955, Steele et al. 1964, Pringle et al. 1992, Karam et al. 2010).

The French neurobiologist Jean-Martin Charcot first described the clinical features of ALS almost 140 years ago but the molecular basis of ALS is still not clear. Approximately 90-95% of cases are sporadic (not inherited) and still very little is known about the cause of sporadic disease. However, recently TAR DNA binding protein 43kDa (TDP-43) has been identified as a major component of pathological protein deposits associated with sporadic cases of ALS (Arai et al. 2006). Although familial forms of ALS are less common, with about 5-10% of cases of ALS being inherited from generation to generation (Benatar et al. 2006), they are the most well studied. Since the clinical and pathological profiles are very similar, insights into familial ALS should provide insight into sporadic ALS (Pasinelli and Brown 2006). There are over ten genes with mutations that have been found to be associated with ALS (Table 1.1). Hexanucleotide repeats in the gene C9ORF2 has been found to be the most common
cause of ALS (reviewed in (Ince et al. 2011). Mutations in the Superoxide dismutase 1 (SOD1) gene remains one of the most common causes of ALS and its protein products appearance in protein aggregates is well established. Regardless of cellular function, one hallmark links all forms of sporadic and familial forms of ALS, the appearance of protein aggregates in inclusions in affected tissue.

Table 1.1: Genes and their functions with one or more known mutation which is associated with ALS

<table>
<thead>
<tr>
<th>Genes with mutations known to be associated with ALS</th>
<th>Function of Genes/Proteins</th>
</tr>
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<tbody>
<tr>
<td>Superoxide dismutase 1 (SOD1) (Rosen et al. 1993)</td>
<td>Superoxide-scavenging enzyme</td>
</tr>
<tr>
<td>C9ORF72 (DeJesus-Hernandez et al. 2011)</td>
<td>Yet unknown function</td>
</tr>
<tr>
<td>TAR DNA binding protein (TARDBP) TDP-43 (Arai et al. 2006)</td>
<td>Represses transcription</td>
</tr>
<tr>
<td>Fused in sarcoma (FUS) (Kwiatkowski et al. 2009)</td>
<td>Transcriptional activation</td>
</tr>
<tr>
<td>Angiogenin (ANG) (Greenway et al. 2004)</td>
<td>Stimulates new blood vessel formation</td>
</tr>
<tr>
<td>Optineurin (OPTN) (Maruyama et al. 2010)</td>
<td>May be involved in Transcriptional activation</td>
</tr>
<tr>
<td>Ubiquilin 2 (UBQLN2) (Deng et al. 2011)</td>
<td>Regulates the degradation of ubiquitinated proteins</td>
</tr>
<tr>
<td>Sigma nonopioid intracellular receptor 1 (SIGMAR1) (Al-Saif et al. 2011)</td>
<td>Thought to be involve in lipid transport</td>
</tr>
<tr>
<td>Senataxin (SETX) (Chen et al. 2004)</td>
<td>Thought to be involve in DNA repair</td>
</tr>
<tr>
<td>Alsin (ALS2) (Yang et al. 2001)</td>
<td>May play a role in the development of axons and dendrites</td>
</tr>
<tr>
<td>Vesicle associated membrane protein (VAPB) (Nishimura et al. 2004)</td>
<td>Thought to be involved in vesicle tracking</td>
</tr>
<tr>
<td>Vasolin-containing protein (VCP) (Ishigaki et al. 2004)</td>
<td>Assembly of peroxisomes, 26S proteasome function, vesicle transport and fusion</td>
</tr>
<tr>
<td>D-Amino acid oxidase (DAO) (Mitchell et al. 2010)</td>
<td>May be important in removal of D-amino acids</td>
</tr>
<tr>
<td>Dynactin subunit 1 (DCTN1) (Munch et al. 2004)</td>
<td>Cell division and transport within the cell</td>
</tr>
<tr>
<td>Phosphoinositide 5-phosphatase (FIG4) (Chow et al. 2009)</td>
<td>Thought to be important in vesicle tracking</td>
</tr>
<tr>
<td>Ataxin 2 (ATXN2) (Elden et al. 2010)</td>
<td>May be involved in RNA processing</td>
</tr>
<tr>
<td>Neurofilament, heavy polypeptide (NEFH) (Figlewicz et al. 1994)</td>
<td>Makes a protein component of neurofilaments</td>
</tr>
<tr>
<td>Peripherin (PRPH) (Leung et al. 2004)</td>
<td>Formation of intermediate filaments</td>
</tr>
<tr>
<td>Survival motor neuron, telomeric (SMN1) (Veldink et al. 2001)</td>
<td>Important in supporting motor neurons</td>
</tr>
<tr>
<td>Spastic paraplegia 11 (SPG11) (Daoud et al. 2012)</td>
<td>May play a role in gene expression or protein trafficking</td>
</tr>
</tbody>
</table>
1.2. Protein aggregation

When abnormally folded proteins cannot be degraded or refolded they are prone to aggregation and can form structured, highly organized amyloid fibrils (Serpell et al. 2000) or unstructured, disorganized amorphous aggregates (Dobson 2004). Recent research suggest that the prefibrillar forms of amyloid fibrils may be the most toxic (for an in depth review see (Baglioni et al. 2006)); however, all aggregates are likely to be detrimental, although, the exact mechanisms of toxicity are not fully understood. One proposed mechanism for oligomer toxicity is that exposed hydrophobic residues interact with cell receptors and membranes (Stefani and Dobson 2003, Cecchi et al. 2005, Bolognesi et al. 2010, Campioni et al. 2010), while a possible explanation for inclusion toxicity is the sequestration of vital cellular components such as molecular chaperones (Yerbury et al. 2016).

Amyloid fibrils are a key pathological feature of many diseases and this has led to a need to characterize their formation (Chiti and Dobson 2006). In vitro imaging of amyloid fibrils, using transmission electron microscopy and atomic force microscopy has demonstrated that fibrils consist of 2 to 6 protofilaments, that are 60-120 Å in diameter and of indeterminate length (Serpell et al. 2000). X-ray fiber diffraction has revealed that the ordered structure of fibrils is comprised of β-strands that are perpendicular to the fibrils axis giving it a twisted appearance (Sunde and Blake 1997). Studies involving thioflavin T binding of β-strands have shown that the conversion of proteins into amyloid fibrils involves a lag phase (Naiki et al. 1997, Serio et al. 2000, Pedersen et al. 2004). This is thought to be the time it takes for the nuclei to form; and once the nuclei forms, fibril growth is believed to proceed rapidly during what is termed
the elongation phase until formation reaches a plateau when mature amyloid fibrils are formed (Chiti and Dobson 2006, Wilson et al. 2008). There are also now reported fibrillar aggregates that are not amyloid. SOD1 fibrils might not have a classical amyloid structure and FUS and TDP-43 form fibrils have been called hydrogels or in phase transition (Saini and Chauhan 2014, Patel et al. 2015).

Because the amino acid sequence determines the conformation of a protein, certain changes in the amino acid sequence (through mutation) may result in a change in what is known as the protein folding landscape (Chiti et al. 2002, Gregersen et al. 2006). The protein folding landscape represents how proteins attempt to reach their native state (or functional state) assuming this state is the one with the lowest amount of free energy (Gregersen et al. 2006). The folding funnel can be altered to include two valleys, where mutations in the sequence lead to another pathway that has a free energy minimum lower than that of the native protein (Clark 2004, Gregersen et al. 2006). Clark’s (2004) incorporation of a funnel for the probability of aggregation reflects more accurately the frequent failure of proteins to fold correctly. Changes to the amino acid sequence through the mutation of key residues are thought to increase the chance of protein aggregation that decrease net charge, increase hydrophobic residues and thought to increase the propensity to form β-sheets (Chiti and Dobson 2006). Failure of proteins to reach their native structure is also thought to be detrimental and cause disease due to a loss of function of the misfolded protein and inhibition of normal cell function (Hartl et al. 2011).
1.2.1 Protein quality control

Because correct protein folding is required for proper protein function and thus cell viability, it is not surprising that quality control systems have evolved to inhibit protein misfolding and aggregation (Yerbury et al. 2005). Protein homeostasis (proteostasis), describes the concept of the maintenance of the correct folding, unfolding, trafficking, and turnover of the entire proteome which is important to maintain healthy cellular function (Balch et al. 2008). Protein unfolding occurs when the correct protein conformation is obtained but conditions in the cell are unfavorable and result in folded proteins unraveling (Stefani and Dobson 2003). Unfavorable conditions, such as higher temperature, acidic pH and moderate concentrations of salts, promote unfolding by destabilizing bonds in the native structure (Stefani and Dobson 2003). Protein misfolding on the other hand refers to proteins that may never reach their native conformation and thus cannot perform their normal functions. Chaperones are a major part of the quality control system whose role it is to prevent aggregation and promote efficient protein folding (Hartl et al. 2011). Many heat shock proteins (HSP) are chaperones and are divided into classes based on their approximate molecular mass in kDa. In the cytosol for example, one of the many jobs of HSP70 is its association with polypeptide chains as proteins are being synthesized from the ribosome and thus it assists with folding by energy dependent binding and release (Taylor et al. 2002). Other cytosolic chaperones are sometimes needed for further folding such as HSP90 and HSP60 (with HSP60 more commonly known as a chaperonin) before a protein can reach its native conformation (Hartl and Hayer-Hartl 2002, Hartl et al. 2011). A proportion of all proteins tend not to fold correctly or may unfold as a result of stress. Chaperones, such as the small HSP (sHSP), may bind these misfolded or unfolded proteins in an ATP independent fashion permitting
refolding by other chaperones, or alternatively unfolding for another chance to be correctly folded or eventually degraded (McClellan et al. 2005).

One of the major mechanisms through which cells achieve selective protein degradation is the ubiquitin proteasome system (Schubert et al. 2000). Proteins that are no longer required or proteins that are unable to fold correctly are “tagged” by covalent ligation to ubiquitin (through a multistep pathway involving the formation of a aggresome) and are degraded by the 26S proteasome complex in a process known as aggrephagy (Hershko and Ciechanover 1998). Another pathway that can be employed to degrade aggregated proteins is the lysosome system. Chaperone mediated autophagy, and macroautophagy are both involved in shuttling misfolded proteins and aggregates for degradation via acidic proteases in the lysosome (Cuervo 2010, Filimonenko et al. 2010). While degradation via the ubiquitin proteasome system requires the misfolded protein to be small enough to enter the narrow pore of the proteasome, larger aggregates can be degraded by macroautophagy (Knaevelsrud and Simonsen 2010).

### 1.2.2 Aggregation and its role in ALS

Inclusions containing protein aggregates are a characteristic pathological feature occurring in all forms of human ALS. In humans and transgenic mice with SOD1 related ALS, aggregates of mutant SOD1 have been found in motor neurons and in astrocytes (examples can be seen in Figure 1.1) (Bruijn et al. 1998). It is unclear if SOD1 aggregates are toxic, but their presence is associated with cell death (Bruijn et al. 1998). It is thought that aggregates of SOD1 cause ER stress, protein transport
blockage, inhibition of organelle function, inhibition of proteasome machinery, and decreased chaperone activity which may in turn result in cell death (Bruijn et al. 2004).

Figure 1.1: Neuropathology of ALS. Aggregates found in the spinal cord tissue from patients with a SOD1 mutation A) Inclusions in a spinal motor neuron, B) Inclusions in astrocytes, misSOD1 (green) and the astrocyte marker GFAP (red), C) Aggregates in microglia, misSOD1 (green) and the microglial marker Iba1 (red) (Saberi et al. 2015, Forsberg et al. 2011).

Impaired protein degradation due to the proteasome becoming overwhelmed with excessive amounts of misfolded and aggregated mutant protein in later life is a promising theory of aggregation induced toxicity (Kastle and Grune 2011). It has been shown that mutant SOD1 inhibits the proteasome (Urushitani et al. 2002), with
inhibition thought to be caused by the proteasome becoming “choked” with mutant SOD1 (Cleveland and Rothstein 2001, Urushitani et al. 2002). The impairment of protein degradation would not only affect mutant SOD1, but all other proteins that the proteasome processes in order to achieve normal proteostasis (Pasinelli and Brown 2006). Antibodies to ubiquitin have been found to be immunoreactive to inclusions in all mutant SOD1 mouse models and ALS patients (Bruijn et al. 2004).

Chaperones responsible for correct protein folding, and anti-apoptotic proteins such as B cell lymphoma 2 (Pasinelli et al. 2004), are also found associated with protein aggregates, and this may inhibit correct cell functioning (Pasinelli and Brown 2006). Certain chaperones, such as $\alpha\beta$-crystallin, HSP27, HSP40 and HSP70, have been found to interact with mutant SOD1 (Shinder et al. 2001). HSP70 has been found to interact “transiently” with the mutant SOD1 aggregates and seems not be sequestered in aggregates (Matsumoto et al. 2005). Whereas $\alpha\beta$-crystallin has been shown to be sequestered by the insoluble mutant SOD1 aggregates in mutant SOD1 mice (Wang et al. 2008). With at least a subset of chaperones sequestered into protein aggregates of mutant SOD1, chaperone assisted protein quality control of all proteins is likely to be impaired resulting in impaired proteostasis. The collapse of proteostasis could lead to a multitude of cellular dysfunctions across various pathways reminiscent of the disparate cellular functions reported to be involved in SOD1 mediated cell death (Cleveland and Rothstein 2001). This disruption of normal cellular function may explain why mutant SOD1 is toxic to motor neurons, but it does not explain why motor neuron degeneration is not randomly scattered throughout the CNS but is instead seemingly spread from motor neuron to motor neuron (Shaw 2002).
1.3. SOD1

Copper/zinc superoxide dismutase (SOD1) is a superoxide-scavenging enzyme that is found in the cytosol. SOD1 is a homo-dimer consisting of two polypeptides of 153 amino acids, with each subunit containing one copper and one zinc atom (Cleveland and Rothstein 2001). It was previously known as erythrocuprein (Markowitz et al. 1959) and until 1969 its function was unknown. It was found to have a molecular weight of 34,000, to contain 0.34% copper and it was shown to have an enzymatic function, with experiments showing that it catalyzes the dismutation of superoxide free radicals (McCord and Fridovic 1969). Superoxide free radicals are a by-product of oxidative phosphorylation in the mitochondrion (Pasinelli and Brown 2006). The free radical superoxide needs to be rapidly converted into less reactive molecules because it causes tissue damage by reacting with sulfhydryl bonds, cell membranes and the nucleotides in DNA (Machlin and Bendich 1987). In addition, superoxide can react with nitric oxide (NO\textsuperscript{-}) to produce the dangerous peroxynitrite (Pryor and Squadrito 1995). The copper atom is key to the process of dismutation of superoxide, being reduced and oxidized to produce O\textsubscript{2} and 2H\textsubscript{2}O\textsubscript{2} (Cleveland and Rothstein 2001).

1.3.1 Mutations in SOD1 and its implications

Mutations in the SOD1 gene account for approximately 20% of familial cases of ALS (Benatar et al. 2006). At first it was thought that a mutation in the SOD1 gene caused a decrease in enzyme activity and that this resulted in an increase in reactive oxygen species, which subsequently induced damage to motor neurons (Bowling et al. 1993, Deng et al. 1993). However, in vitro studies showed that different SOD1 mutants had
varying levels of enzymatic activity that did not relate to the rate of progression or to the age of onset of disease (Cleveland et al. 1995). Indeed it was found some mutants that have full enzymatic activity, still cause ALS (Borchelt et al. 1994). Further, increasing the levels of wild type SOD1 and hence the dismutase activity did not slow disease progression (Bruijn et al. 1998). In fact, in one study increasing the wild type SOD1 concentration was shown to accelerate disease in mice (Jaarsma et al. 2008). Transgenic mice over expressing mutant human SOD1 have been shown to develop ALS (Gurney et al. 1994) while mice with a deletion of the SOD1 did not develop symptoms typical of ALS (Reaume et al. 1996). This suggests that mutant SOD1 causes disease through a toxic gain in function. This is consistent with other diseases such as Parkinson’s disease, keratin and collagen diseases which are inherited dominantly and are caused by a toxic gain in function (Gregersen et al. 2006).

Over 100 different mutations of the SOD1 gene associated with ALS have been identified (Pasinelli and Brown 2006). It is thought that all of these mutations are able to destabilize the structure of the SOD1 protein (Borchelt et al. 1994). These various mutations do not occur at one common location (for example, at the active site), instead they have been identified throughout the entire structure (Valentine et al. 2005). It is now commonly acknowledged that mutations in the SOD1 gene are associated with a propensity to misfold and aggregate. However, how misfolded or aggregated SOD1 causes ALS is not fully understood. Nevertheless, it is currently acknowledged that 30% of small interneurons also degenerate in ALS (Cleveland and Rothstein 2001) and that protein inclusions and neuro-inflammation are important features which occur concurrently during the course of disease.
1.4. Neuroinflammation

Neuroinflammation allows the body to rapidly recruit pathogen fighting molecules and cells to the site of insult in the central nervous system (CNS). Acute inflammation in the CNS caused by infections, trauma, stroke and toxins is considered beneficial, as it stops further injury, repairs damage and is short lived. Chronic inflammation in the CNS occurs over an extended period of time, longer than normally would be necessary. Instead of quickly clearing the area of harmful pathogens the prolonged inflammation can be harmful to surrounding healthy tissue and cells; by releasing reactive oxygen species, nitric oxide, tumour necrosis factor alpha (TNF-α), breaking down the blood brain barrier and recruiting immune cells from the periphery when the blood brain barrier is compromised. This is thought to contribute to the progression of many neurodegenerative diseases where neuroinflammation is associated (Hook et al. 2015).

It is necessary for the immune system in the CNS (and for that matter in the periphery) to differentiate between foreign or abnormal cells/molecules and normal healthy cells and their products (Wyss-Coray and Mucke 2002). In the periphery, acquired immunity requires the production of lymphocytes (T cells and B cells) and immunoglobulins to recognize and remember threats. Natural killer cells, macrophages, neutrophils, dendritic cells, basophils and eosinophils are the major cells which contribute to innate immunity in the periphery. However, in the CNS the resident innate immune cells are astrocytes and microglia. Microglia and astrocytes are important in producing many cytokines and many of the components in the complement system of the CNS (McGeer and McGeer 1995).
1.4.1 Evidence of increased expression of neuroinflammatory markers in ALS

There is increasing evidence that inflammation is a major part of ALS pathology. The increased expression of a number of neuroinflammatory markers strongly suggests this is the case. One of these is cyclooxygenase-2 is a pro-inflammatory enzyme which converts arachidonic acid to prostaglandin and is produced in multiple cell types including astrocytes and neurons (McGeer and McGeer 2002). Cyclooxygenase-2 expression can be induced by a number of factors including TNF-α, IL-1β, LPS, elevated calcium levels and multiple growth factors (Consilvio et al. 2004). It has been found to be upregulated in both transgenic mutant SOD1 mouse models and patients with ALS (Almer et al. 2001). It is important in synthesis of Prostaglandin E2 which has been found at levels 2 to 10 times higher in greater than 80% of ALS patients examined compared to the control group (Almer et al. 2002). Prostaglandin E2 induces the release of glutamate from astrocytes and this increase in glutamate has been implicated in contributing to motor neuron loss in ALS (Consilvio et al. 2004, Dave et al. 2010).

Inducible nitric oxide synthase is used by microglia to produce nitric oxide (NO) to protect against various insults to the host. Nitric oxide can be beneficial when there is a bacterial infection as it can inhibit bacterial DNA synthesis, cause double stranded breaks, interact with metal groups in the bacterial enzymes and oxidize bacterial lipids (Lowenstein and Padalko 2004). However, excessive nitric oxide production, as occurs when there is chronic neuroinflammation, is thought to be neurotoxic and has been implicated in a number of neurodegenerative diseases (Liu et al. 2002).
Postmortem spinal cord tissue of 15 patients with ALS, showed increased iNOS immunoreactivity in both astrocytes and neurons when compared to control patients (Sasaki et al. 2000). Indicating increased oxidative damage in the motor system may contribute to neuronal degeneration in ALS (Sasaki et al. 2000).

Interleukin-6 (IL-6), also known as B-cell stimulating factor, is a proinflammatory cytokine in the CNS, predominantly produced by astrocytes and microglia (Sekizawa et al. 1998). Increased levels of IL-6 have been found in the CSF of patients with HIV1, bacterial meningitis and viral encephalitis (Platasalaman 1991). Transgenic mice over expressing IL-6 in the CNS were shown to develop motor impairment and suffer from seizures (Campbell et al. 1993). This transgenic mouse model with increased levels of IL-6 showed increased levels of complement proteins, angiogenesis and breakdown of the blood brain barrier (Campbell et al. 1993). Research also shows that IL-6 may influence inflammation by triggering the differentiation of monocytes to macrophages rather than dendritic cells (Chomarat et al. 2000). This together with evidence of increased levels of IL-6 in neurodegenerative disease suggests that IL-6 may play a role in progression of neurological diseases where increased levels are found. Elevated levels of IL-6 have been found in ALS patients compared to patients with multiple sclerosis or HTLV-1 associated myelopathy (Sekizawa et al. 1998). Recent research shows that proinflammatory cytokines IL-12, IL-15, IL-17 and IL-23 are also elevated in the CSF and serum of patients with ALS (Rentzos et al. 2010a, Rentzos et al. 2010b).
Monocyte chemoattractant protein 1 (MCP-1), also known as chemokine ligand 2 (CCL2), is important in recruiting monocytes, T-cells and dendritic cells to site of inflammation in the CNS. As well as recruitment there is strong evidence to suggest that MCP-1 also plays a role in permeabilisation of the BBB (Stamatovic et al. 2005). An increase of MCP-1 is seen in post mortem tissue and the CSF of patients with ALS (Henkel et al. 2004, Baron et al. 2005, Nagata et al. 2007, Gupta et al. 2011). This data suggests that inflammatory cells are being recruited to damaged areas both from within the CNS and potentially being recruited from the peripheral system.

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine and in the periphery, TNF-α is produced by monocytes and macrophages (Barbara et al. 1996). Originally, it was found to be an important component of the immune response in the periphery, activated against tumor’s (hence the name). However, in the CNS microglia, astrocytes and neurons are all responsible for the production of TNF-α in response to stimuli (Cristina et al. 2012). In the diseased brain an increased level of TNF-α can have a neurotoxic effect. It is found to be elevated in a number of neurodegenerative diseases including Parkinson’s disease, multiple sclerosis and Alzheimer’s disease (Beck et al. 1988, Fillit et al. 1991, Boka et al. 1994). Significantly, higher levels of TNF-α and its receptor sTNFR were found in plasma from patients with ALS compared to controls (Poloni et al. 2000). A subsequent study also found increases in TNF-α and two of its receptors in the plasma of patients with sporadic ALS (Cristina et al. 2012). Interestingly this study found that the higher levels of TNF-α seen in the plasma of patients with ALS is present at disease onset and remains throughout disease progression (Cristina et al. 2012).
Collectively, this data implicates inflammation as an integral part of ALS pathology. The increase in neuroinflammatory markers is consistent with the idea that neuroinflammation plays an important role at disease onset and contributes to progression in ALS patients.

1.4.2. Evidence of increased expression of neuroinflammatory markers in transgenic mutant SOD1 mice

After the initial finding of mutations in the SOD1 gene (Rosen et al. 1993), transgenic mutant SOD1 mice were produced expressing human mutant SOD1 (Gurney et al. 1994). Similar to humans, transgenic mutant SOD1 mice show evidence of protein aggregates, motor neuron loss and neuroinflammation, making them a useful model to utilise when studying ALS (Turner and Talbot 2008).

Studies in transgenic mutant SOD1 mice have shown that increased expression of inducible nitric oxide synthase corresponded with disease progression (Almer et al. 1999). Nitric oxide synthase (iNos) was increased in glial cells in pre-symptomatic and end stage mutant SOD1 mice (Almer et al. 1999). SOD\textsuperscript{G93A} mice have been shown to have higher levels of NOX2 (an isoform of nicotinamide adenine dinucleotide phosphate (NADPH) -oxidase) and superoxide in spinal cord microglia when compared to spinal cord tissue from wild type mice (Wu et al. 2006). NADPH oxidases which include NOX1 and NOX2 generate superoxide by transferring electrons from NADPH to molecular oxygen. Superoxide which can be produced by microglia (see
below) is harmful to neurons (Saez et al. 1987). NOX2 null crossed with mutant SOD1 mice had an extended lifespan compared to mutant SOD1 mice without the NOX2 knockout (Wu et al. 2006, Marden et al. 2007). However, in the Marden (Marden et al. 2007) study NOX2 knockout mice with the SOD1 mutation, were predisposed to lethal eye infections. This potentially suggests that NOX2 is too important to the immune system to be targeted for complete knockout in ALS.

Insulin growth factor 1 (IGF-1) is a neuroprotective factor for motor neurons and it has been shown to extend lifespan in mutant SOD1 mice (Kaspar et al. 2003). IGF-1 delayed astrogliosis, decreased TNF-α release by 59% and extended lifespan (by nine days) in IGF-1 treated mutant SOD1 mice (Kaspar et al. 2003). However, clinical trials involving subcutaneous delivery to patients with ALS unfortunately did not prove beneficial to patients (Sorenson et al. 2008). In fact, IGF-1 gene expression was shown to be up-regulated in leukocytes and microglia in mutant SOD1 mice spinal cord (Chiu et al. 2013). With microglia taken from transgenic mice having significantly higher levels of IGF-1 gene expression in all stages of disease (end stage, symptomatic and pre-symptomatic mice) (Chiu et al. 2008). This, along with the failed clinical studies in humans, suggests that although IGF is upregulated by microglia it cannot adequately protect motor neurons in ALS patients or may contribute to motor neuron loss through an unknown mechanism. Of note reduced IGF-1signaling was shown to protect mice from behavioural impairments associated with the Aβ aggregates in Alzheimer’s disease suggesting the role of IGF-1 in neurodegenerative disease is complex (Cohen et al. 2009).

The early, relatively slow phase of disease progression is eliminated in the absence
of functional CD4+ T cells, accompanied by increased proinflammatory and cytotoxic factors, decreased anti-inflammatory and neurotrophic factors, and decreased survival (Beers et al. 2008). In this same study when mutant SOD1 mice lacking CD4+ T cells had bone transplants from wild type mice, there was a decrease in cytotoxic factors TNF-α, NOX2 and IL-6, and an increase neuroprotection factors including IGF-1, IL-4 and Ym1 (Beers et al. 2008). The immunomodulatory effects of the CD4+ neuroprotective T cells appear to enhance a neuroprotective microglial phenotype with increased anti-inflammatory factors and neurotrophic factors.

Transgenic mutant SOD1 T-cell-deficient (Tcr3/-) mice, have confirmed that T cells play an endogenous neuroprotective role in ALS (Chiu et al. 2008). Reconstitution of mutant SOD1 mice with ex vivo expanded T regulatory or T effector cells from WT donor mice delays the loss of motor function and enhances survival (Banerjee et al. 2008). An increased lymphocyte population was present at 65 days when compared to non-transgenic mice (Chiu et al. 2008). As disease progressed in the transgenic mice further increases in lymphocytes were seen at day 100 and day 135 (Chiu et al. 2008).

Increases in the levels of mRNA expression of IL-1β a proinflammatory cytokine, IL-10 an anti-inflammatory cytokine and IL-6, which can act as both a proinflammatory and anti-inflammatory cytokine have been observed in mutant SOD1 mice (Sekizawa et al. 1998, Nguyen et al. 2001, Hensley et al. 2002). However, data from IL-1β knockout in mutant SOD1 mice demonstrated that the proinflammatory interleukin-1β
was not solely required for ALS disease progression but may modulate the immune response (Nguyen et al. 2001).

A significant increase in TNF-α concentration was observed in the spinal cord of SOD1<sup>G93A</sup> mice compared to wild type mice (Hensley et al. 2003). In addition, when stimulated with LPS, microglia from SOD1<sup>G93A</sup> mice have been shown to secrete more inflammatory mediators, including TNF-α than control mice (Weydt et al. 2004). Increases in TNF-α can be seen prior to symptom onset in SOD1<sup>G93A</sup> mice, indicating that it may play a role at disease onset and not just at the end stages of disease (Hensley et al. 2003).

1.5. The role of microglia in innate immunity and inflammation

Microglia are a component of the innate immune system and are considered to be the macrophages of the CNS. In the healthy CNS, microglia are sometimes called “inactive”, however this label is inaccurate as microglia are constantly using their processes to sample and monitor the extracellular space (Davalos et al. 2005, Nimmerjahn et al. 2005). When the brain is injured or affected by disease, the “less active microglia” in the damaged area undergo rapid activation, which is characterized by cell body enlargement, retracted processes, antigen presentation and phagocytosis, production and secretion of neurotoxic compounds such as; reactive oxygen species (ROS), growth factors and cytokines (Henkel et al. 2009). In addition, they can be neuroprotective through release of neurotrophic factors (NTF), insulin growth like factor-1 (IGF-1) and anti-inflammatory cytokines (Henkel et al. 2009).
Like macrophages found in circulation (Taylor et al. 2002, Benoit et al. 2008, Henkel et al. 2009), microglia have a spectrum of phenotypic states with the extremes being the M1 and M2 phenotypes (Ponomarev et al. 2007, Michelucci et al. 2009). The M1 phenotype, known as classically activated microglia, are activated by proinflammatory cytokines such as interferon gamma (IFN-γ) and by lipopolysaccharides (LPS) on microbes (Michelucci et al. 2009). The classically activated M1 microglia can be neurotoxic because these microglia release reactive oxygen species (ROS), proinflammatory cytokines (TNF-α, IL-1), and reduce trophic factors (Figure 1.2a) (Henkel et al. 2009, Michelucci et al. 2009). The M2 phenotype on the other hand is considered more neuroprotective because it reduces inflammation by blocking the release of proinflammatory cytokines and mopping up endocytic factors after inflammation occurs (Henkel et al. 2009)(Figure 1.2b).
Figure 1.2: **Representations of the M1 and M2 microglial phenotypes neurotoxic and neuroprotective effect on motor neurons.** a) Neurotoxic M1 microglia release reactive oxygen species (NO, O$_2^-$), proinflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6, IL-12, IL-23) directly or indirectly causing motor neuron stress and death. b) Neuroprotective M2 microglia release neurotrophic (NTF) and growth factors (such as IGF-1).

1.6. **Astrocytes role in the CNS**

Astrocytes and oligodendrocytes are the macroglia of the central nervous system. Astrocytes are an important part of the CNS providing metabolic, structural and trophic support to surrounding neurons (Bezzi and Volterra 2001). In healthy human physiology, they maintain homeostasis in the CNS via the uptake and release of ions and neurotransmitters from the extracellular fluid surrounding neurons (Figure 1.3) (Sofroniew and Vinters 2010). Astrocytes also play a critical role in maintenance of the BBB by releasing soluble factors such as basic fibroblast growth factor, glial derived
neurotrophic factor (NTF) and transforming growth factor beta (TGF-β). The release of various neurotrophic factors from astrocytes (such as nerve growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, vascular endothelial growth factor, and insulin growth factor) supports; oligodendrocyte survival, remyelination, neurogenesis and neuronal survival differentiation, function and regeneration (Farina et al. 2007). Astrocytes, also release chemokines and cytokines that are important in myelination (IL-1 and IL-6) and in neural progenitors (CCL2 and CXCL12) (for in depth review (Farina et al. 2007).

Astrocytes can also play a role in immunity in the central nervous system. When astrocytes encounter a foreign stimulus (Figure 1.3; for in depth review (Benveniste 1998) they can be activated, through a process known as astrogliosis, where they undergo significant morphological changes and proliferation. Like microglia (as mentioned previously) astrocytes can be either pro- or anti-inflammatory (Sofroniew and Vinters 2010), and there exists a spectrum of astrogliosis ranging from mild reactive astrogliosis that can be quickly resolved to severe reactive astrogliosis which can result in long lasting changes in the tissue architecture (Sofroniew and Vinters 2010). Reactive astrocytes can release cytokines (IL-6, MCP-1, TNF) and chemokines (CCL2, CCL5, CXCL10 and CXCL12) which contribute to inflammation (Farina et al. 2007).

Astrocytes will also enhance the expression of glial fibrillary acidic protein (GFAP), which is thought to be important in modulating shape, and is commonly used as an astrocyte marker in scientific experiments (Eng et al. 2000, Dong and Benveniste 2001). However, some astrocytes in the normal healthy CNS do not express
detectable levels of GFAP and so an increase in GFAP can used as a marker of increased reactive astrocytes but not an increase in astrocyte population per se (Sofroniew and Vinters 2010). After damage to the CNS they are essential in BBB repair, and in severe cases can form scars to prevent the spread of infection (Sofroniew and Vinters 2010). These glial scars can also prevent damaging inflammatory cells from escaping the site of injury (Sofroniew and Vinters 2010).

Astrocytes can also cause motor neuron injury through non-immune mechanisms. Astrocyte senescence is now thought to be a contributing factor in other neurological diseases such as Alzheimer’s disease (Bhat et al. 2012). Rather than being simply a loss of protective function, senescent cells are functionally altered. One of the most important functions in the CNS is thought to be the maintenance of extracellular glutamate levels. This is because, unlike acetylcholine, which is enzymatically degraded at the neuromuscular junction, uptake and clearance of glutamate occurs via glutamate transporter proteins in astrocytes. The over stimulation by excess glutamate causes excitotoxicity in neurons due to overstimulation and an excess influx of calcium ions (Arundine and Tymianski 2003). Abnormal levels of glutamate have been found in cases of traumatic brain injury and a number neurodegenerative conditions such as Parkinson’s disease, Alzheimer’s disease, multiple sclerosis and ALS (Hyman et al. 1987, Baker et al. 1993, Srinivasan et al. 2005, Griffith et al. 2008). Of interest the only drug widely recommended for use in ALS, Riluzole, is a modulator of glutamate (discussed further in Chapter 7.2) (Miller et al. 2007).
Figure 1.3: **Astrocytes role in the central nervous system.** Different phenotypes of astrocytes and their effect on motor neurons; A) A representation of how astrocytes affect motor neurons in the typical CNS, B) Astrocytes reaction during neuroinflammation possibly resulting in motor neuron stress and death, C) Possible astrocyte dysfunction in senescent astrocytes possibly resulting in motor neuron stress and death.
1.7. The role of non-neuronal cells in ALS

1.7.1 The role of microglia’s in ALS

Numerous studies have shown that microglia are activated and proliferate in ALS patients and ALS mice models (Lampson et al. 1988, Kawamata et al. 1992, Henkel et al. 2004, Turner et al. 2004, Moisse and Strong 2006). As the disease increases in severity, the activation of microglia has also been shown to increase in vivo in ALS patients (Turner et al. 2004). Microglial activation is seen in mutant SOD1 transgenic mice before symptoms appear and throughout disease progression (Hall et al. 1998, Alexianu et al. 2001, Clement et al. 2003). However, as mentioned previously activation of microglia may be either a neuroprotective response or a neurotoxic response. It is unclear whether activation of microglia in ALS is neuroprotective or contributes to rapid disease progression (Henkel et al. 2009). It has recently been suggested that neuroprotective M2 microglia may slow disease progression early during the course of disease, but may be replaced by neurotoxic M1 microglia and may contribute to disease progression at later stages of ALS (Henkel et al. 2009, Appel et al. 2011).

Immunocytochemistry of postmortem tissue from ALS patients demonstrated microglial activation and indicated that an immune response may play a role in motor neuron death (Troost et al. 1993). However, this end stage work does not indicate whether microglial activation occurred during the course of the disease or was evident only at the end stage of disease. Positron emission tomography imaging of ALS patients, using \([^{11}C](R)-PK11195\) and \([^{11}C]\)-PBR28 (with both radiotracers shown to bind specifically to activated microglia), showed an increase in activated microglia in the motor cortex, that correlates with upper motor neuron symptoms seen in patients.
This observed correlation supports previous mouse work which showed that microglial activation is not just an end stage phenomenon and thus may contribute to disease progression in ALS.

Microglia are activated prior to symptom onset but it is still not certain whether they play a neuroprotective or neurotoxic role (Henkel et al. 2006). Ablating microglial expression of mutant SOD1 or transplanting bone marrow from wild-type mice into mutant SOD1 transgenic mice increases the life span of mutant mice (Beers et al. 2006, Boillee et al. 2006). This suggests that microglia play a neurotoxic role during disease progression. In addition, microglia that express mutant SOD1 are more readily activated and induce more motor neuron cell death than WT microglia. Xiao et al (2007) found that upon stimulation with lipopolysaccharide (LPS) mutant SOD1 microglia released more neurotoxic superoxide and nitric oxide when compared to wild type microglia. Microglial cells from mutant SOD1 mice stimulated with LPS secrete more inflammatory mediators, including TNF-α (Weydt et al. 2004), MCP-1, TGF-β (Henkel et al. 2004) and IFN-γ (Ferri et al. 2004), than cells from control mice. When using mixed cultures of motor neurons and microglia this resulted in SOD1G93A expressing microglia causing greater motor neuron death (Xiao et al. 2007).

**1.7.2 Astrocytes role in ALS**

A significant increase in astrocytic activation is seen in mutant SOD1 mice upon disease onset and throughout the early stages of disease progression (Hall et al. 1998). It has also been revealed that astrocytes from neonatal rat spinal cords, become reactive after incubation with CSF from ALS patients (Shahani et al. 1998).
Diminished mutant SOD1 expression in astrocytes did not affect disease onset but delayed microglial activation and slowed disease progression (Yamanaka et al. 2008). This suggests that astrocytes are important for disease progression and may interact with microglia to cause damage to motor neurons in ALS.

There is growing evidence of astrocyte senescence observed in other neurodegenerative diseases including Parkinson’s and Alzheimer’s disease (Bhat et al. 2012, Chinta et al. 2013). In a rat model of ALS an increase in β-galactosidase staining in spinal cord sections was demonstrated suggesting that astrocyte senescence may be deleterious in the mutant SOD1 rat model for ALS (Das and Svendsen 2015). However, the cause of this astrocyte senescence remains unclear.

Although the underlying mechanism of astrocyte senescence and/or dysfunction in ALS remains unknown, evidence that this dysfunction causes, the increased levels of glutamate led to the only approved drug for patients with ALS (Bensimon et al. 1994). Riluzole works by decreasing the levels glutamate, by blocking it’s presynaptic release, which decreases the damage to motor neurons (Miller et al. 2007). However, it only prolongs survival in patients with ALS for 2 to 3 months (Miller et al. 2007). Anti-inflammatory drugs or immune modulating drugs have previously been trialled and some clinical trials are currently underway (Table 1.2) (McGeer and McGeer 2002, Traynor et al. 2006, Habib and Mitsumoto 2011).

<table>
<thead>
<tr>
<th>Drug to treat ALS</th>
<th>Proposed targets of drug</th>
<th>Outcomes and stages of Development and testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>Targets glutamate transport in astrocytes</td>
<td>Phase III trials ongoing</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Targets neuronal apoptosis, and microglia and astrocyte activation</td>
<td>Phase II trials revealed no benefit to patients</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>Targets neuronal apoptosis, and microglia and astrocyte activation</td>
<td>Negative cardiac problems in patients observed</td>
</tr>
<tr>
<td>Edaravone (MCI-186)</td>
<td>Free radical scavenger may have anti-inflammatory and antiapoptotic effects.</td>
<td>Phase III trials (results not yet released)</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Antiangiogenic and immunomodulatory effects</td>
<td>Caused Bradycardia in 50% of patients treated</td>
</tr>
<tr>
<td>Lenalidomide</td>
<td>Antiangiogenic and immunomodulatory effects</td>
<td>Phase II planned</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Inhibitor of protein kinase C</td>
<td>Phase II results promising</td>
</tr>
<tr>
<td>Minocycline</td>
<td>Antimicrobial agent and inhibitor of microglia</td>
<td>Phase III trials revealed harmful effect to patients</td>
</tr>
<tr>
<td>Nimesulide (generic)</td>
<td>COX-2 inhibitor</td>
<td>Phase III trials revealed no benefit to patients</td>
</tr>
<tr>
<td>Celastrol</td>
<td>Suppresses TNF-α, IL-1β and NO− production.</td>
<td>Improved survival of Mutant SOD1&lt;sup&gt;G93A&lt;/sup&gt; mice</td>
</tr>
</tbody>
</table>

Reactive astrogliosis has been found in post mortem spinal cords of ALS patients (Schiffer et al. 1996). This was at least an indication that astrogliosis is present at the end stage of ALS disease. Examination of tissue prior to death was needed to see if astrogliosis occurred prior to the end stage of disease progression. Deuterium-substituted [11C](L)-deprenyl was used to demonstrate astrogliosis in patients with ALS (Johansson et al. 2007). Astrogliosis in patients with ALS is reasonably diffuse occurring in the spinal cord, grey matter and subcortical matter of the brain (Kushner...
et al. 1990, Schiffer et al. 1996). A fairly recent study also indicated that astrocytes that express mutant SOD1 will release factors (undetermined in this study) that are selectively toxic to motor neurons (Nagai et al. 2007) and knock down of SOD1 in astrocytes reduced the toxic effect on motor neurons (Haidet-Phillips et al. 2011).

1.7.3 Other cells that may contribute to dysfunction in ALS

Dendritic cells are antigen presenting cells and play a major role in modulating and initiating the immune response (Banchereau and Steinman 1998). Dendritic cells can exist as “immature” cells which are relatively inactive and unable to activate T cells (Banchereau and Steinman 1998). As well as being activated by IL-1, TNF-α, CD40-L, LPS, Bacteria, viruses and DNA, immature dendritic cells also respond to a large number of chemokines such as chemokine ligand 3, chemokine ligand 4 and MCP-1 (Caux et al. 2000). In tissue and CSF from ALS patients an increased expression of MCP-1 has been observed (Henkel et al. 2004). Elevated immature and activated dendritic cell transcripts were also found in ALS tissue samples this study (Henkel et al. 2004). Interestingly, a correlation was found between an increased level of expression of dendritic cell transcripts and a more rapid progression of ALS (Henkel et al. 2004).

Natural killer (NK) cells are specialized lymphoid cells which can be activated to have cytotoxic effects on abnormal cells (Biron et al. 1999). In particular they are known to recognise some tumour cells and virally infected cells (Moretta and Moretta 2004). When tumour or virally infected cells don’t have enough MHC I on their surface to be recognised by other immune cells, NK cells can rapidly kill these abnormal cells.
NKT cells (which share markers with NK and T cells) have been shown to increase in both the liver and the CNS of mutant SOD1 mice (Finkelstein et al. 2011). This may suggest that peripheral immunity may also play a role in disease progression.

T cells are normally found in the periphery (and not the CNS) where they regulate other immune cells. Examination of the peripheral immune system indicate that during advanced ALS disease, CD4+ T cell activation is significantly decreased compared to control patients (Zhang et al. 2005). In one study, CD4+ T helper cells were found near areas of degeneration in the cortical spinal tract an area responsible for motor neuron signalling (Engelhardt et al. 1993). In this study, CD8+ T suppressor cytotoxic cells were also observed in the ventral horns along with CD4+ T helper cells (Engelhardt et al. 1993). More recent evidence has contradicted this, showing no evidence for CD4+ T-cell response, however they did detect CD8+ in post-mortem tissue suggesting a neurotoxic role in the end stages of disease (Sta et al. 2011).

Monocyte/macrophage cell transcripts levels were found to be increased significantly in spinal cord tissue from sporadic ALS patients compared to controls (Henkel et al. 2004). Previous research has shown that during systemic infection microglia recruit monocytes into the brain through the release of TNF-α (D'Mello et al. 2009). In murine models of Alzheimer’s disease bone marrow derived monocytes were demonstrated to be recruited into the CNS (Simard et al. 2006). There is some evidence in mutant SOD1 mice that the blood brain barrier is comprised at both early and late stages of disease progression which could explain the presence of monocytes, T cells NK cells
during ALS, which are not normally found in the CNS (Garbuzova-Davis et al. 2007, Zhong et al. 2008).

1.8. Disease propagation and extracellular aggregated mutant SOD1 in ALS

While SOD1 is normally an intracellular protein it can also be found outside cells. Evidence of SOD1 secretion is now widespread; SOD1 has been shown to be secreted from various cell types including neuroblastoma (Mondola et al. 1998), thymic derived (Cimini et al. 2002), pituitary derived (Santillo et al. 2007), hepatocyte and fibroblast (Mondola et al. 1996) lines. More importantly it has also been shown to be secreted from a motor neuron cell line (Turner et al. 2005, Gomes et al. 2007), in cerebrospinal fluid of mutant SOD1 rats (Turner et al. 2005), in primary spinal cord cultures of mutant SOD1 mice (Urushitani et al. 2006), in blood plasma (Mondola et al. 2000) and in the cerebrospinal fluid of humans with familial ALS (Jacobsson et al. 2001).

As outlined previously, mutant SOD1 has been shown to inhibit the proteasome (Uversky et al. 2002). When this occurs, cells increase the rate at which they secrete mutant SOD1 (Urushitani et al. 2006). The secretion of mutant SOD1 is mediated by chromogranin interaction (Urushitani et al. 2006). Chromogranins are components of neurosecretory vesicles that are transported in the trans-Golgi network to the cell membrane (Rudolf et al. 2001, Taupenot et al. 2003). This mechanism may explain how mutant SOD1 escapes its intracellular environment (Urushitani et al. 2006).
Mutations to SOD1 have been shown to make it more aggregation prone and mutations in the SOD1 gene are found in a number of familial forms of ALS. In fact aggregation is a common feature in many late onset neurodegenerative diseases including Alzheimer’s disease and Parkinson’s disease. In these neurodegenerative diseases, microglial receptors have also been shown to interact with protein aggregates suggesting that it possible that these two features are be linked (Table 1.3).

Table 1.3: Evidence of receptors shown to interact with aggregated protein and microglia in neurodegenerative diseases

<table>
<thead>
<tr>
<th>Disease and associated aggregate protein</th>
<th>Receptor protein interaction in microglia</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s Aβ</td>
<td>The scavenger receptor for advanced glycation end products (RAGE)</td>
<td>(Deane et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Pyrin domain-containing 3 (NLRP3)</td>
<td>(Halle et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Scavenger receptors (SR-A SR-B)</td>
<td>(Murgas et al. 2012)</td>
</tr>
<tr>
<td></td>
<td>Receptor Complex (CD36,CD47,αβ1 integrin)</td>
<td>(Bamberger et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>FPRL1/FPR2</td>
<td>(Le et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Scavenger receptor CD36</td>
<td>(Coraci et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Toll-like receptors (TLRs) 2-4</td>
<td>(Reed-Geaghan et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>CD14</td>
<td>(Reed-Geaghan et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>CD33</td>
<td>(Malik et al. 2013)</td>
</tr>
<tr>
<td>Parkinson’s α-synuclein</td>
<td>Toll-like receptor 2</td>
<td>(Kim et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>Scavenger receptor CD36</td>
<td>(Su et al. 2008)</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis mutant SOD1</td>
<td>Scavenger receptor</td>
<td>(Roberts et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>CD14</td>
<td></td>
</tr>
</tbody>
</table>
A common hallmark of ALS disease is focal onset followed by a continuous progression of pathology along an anatomical pathway (Ravits and La Spada 2009). Non-ALS related proteins (prion proteins, Tau and α-synuclein) have also been shown to demonstrate anatomical spreading (Clavaguera et al. 2009, Sanders et al. 2014, Peelaerts et al. 2015). In cell culture SOD1 and TDP-43 aggregates have recently been shown to exhibit prion like properties seeding aggregation and spreading from neuron to neuron (Grad et al. 2014, Feiler et al. 2015).

Figure 1.4: **ALS progression and propagation.** ALS disease begins with focal onset (involving upper and lower motor neurons) followed by a continuous spread along an anatomical pathway as the disease progresses (Ravits and La Spada 2009).

Recent studies have shown that SOD1 aggregates in motor neurons can escape the intracellular environment during cell motor neuron death and via exosomes (Grad et al. 2014). This secretion of aggregated mutant SOD1 is thought to be an attempt to
detoxify the cell, by stopping the accumulation of aggregated protein and thus cell death. SOD1 aggregates appear in astrocytes and inhibitory interneurons in close proximity to motor neurons only after inclusion formation in motor neurons in SOD1$^{G93A}$ mice (Rossi et al. 2008, Hossaini et al. 2011). Further, WT astrocytes transplanted into mutant SOD1 mice acquire inclusions (Lepore et al. 2008). This is consistent with the idea that SOD1 aggregates can be released from motor neurons and subsequently taken up by nearby cells. Mounting evidence suggests that microglia and astrocytes contribute to motor neuron death in ALS but what activates these cells remains unknown. One possibility is that protein aggregates formed in motor neurons are subsequently released where they chronically activate microglia and astrocytes. There is precedence for this phenomenon in other diseases such as Alzheimer’s disease where amyloid fibrils activate both microglia and astrocytes.
1.9. Aims

The overarching aim of this work is to examine the possibility that ALS associated protein aggregates (using SOD1 as a model) can interact with and activate microglia and astrocytes specifically:

1. Determine if ALS associated mutant SOD1 aggregates activate primary murine microglia.

2. Determine if ALS associated mutant SOD1 aggregates cause dysfunction in primary murine astrocytes.

3. Establish if extracellular aggregated mutant SOD1 mediates microglial activation and astrocyte dysfunction \textit{in vivo}. 
2. General Methods
2.1 SOD1 purification

SOD1 was produced in *E.coli* using a plasmid containing both human SOD1 and its copper chaperone (SOD1 plasmid was a kind gift from the Oliveberg group, Sweden) as previously described (Lindberg *et al.* 2002). Briefly, cells were cultured at 23°C in the presence of 3 mM CuSO$_4$ and 30 μM ZnSO$_4$ to ensure correct metal loading. The SOD1 protein was further purified by heat denaturation and ammonium sulphate precipitation. Samples were passed over a size exclusion column Superdex 75 (GE Healthcare, UK) equilibrated with 50 mM Tris-HCl 500 mM NaCl buffer pH 7.5. Samples were run at 0.5 mL/minute using 50 mM Tris-HCl 500 mM NaCl buffer pH 7.5. The size exclusion column was operated using AKTA FPLC system (Amersham Biosciences, UK). To analyse purity, a 15% SDS-gel was run under reducing conditions (see chapter 2.2 SDS-PAGE) of relevant fractions, the fractions containing putative SOD1 were pooled and dialysis performed against 10 mM Tris-HCl pH 7.5 at 4°C.

Protein sample pooled from size exclusion purification was direct loaded onto a Q-Sepharose anion exchange column (GE Healthcare, UK) equilibrated with 10 mM Tris-HCl pH 7.5 and eluted with a salt gradient of 0 - 125 mM NaCl pH 7.5. Prior to equilibration, and after use, the column was washed with 10 mM Tris-HCl 1 M NaCl pH 7.5 to remove any unwanted proteins still bound to the column. The column was operated using AKTA explorer (Amersham Pharmacia Biotech, UK). To analyse purity, a 15% SDS-gel was run under reducing conditions of relevant fractions. Fractions containing putative SOD1 (which showed >95% purity) were pooled. Pooled fractions were dialyzed against Phosphate buffered saline (PBS) (160 g/L NaCl, 4 g/L KCl, 22.3 g/L Na$_2$HPO$_4$ (anh), 4 g/L KH$_2$PO$_4$) overnight at 4°C before protein was concentrated
with centricon (5000 Da Cut off) (GE healthcare, UK) to no greater than 10 mg/mL and stored frozen at -20°C.

2.2 SDS-PAGE

SDS-PAGE electrophoresis 15% was performed using a Hoefer Mighty Small II system (Amersham Biosciences, UK), connected to a Bio-Rad power pack 300 power supply (Bio-Rad, USA). Protein samples were combined with 4 x protein sample buffer (0.04 % bromophenol blue, 8 % SDS, 40 % glycerol, 240 mM Tris-HCl, pH 8.8.), 1 μL beta mercaptoethanol (for reducing conditions) and boiled for 5 minutes before loading into wells. Molecular weight markers (Fermentas, Australia) were used to determine the approximate size of bands.

Electrophoresis took place at 120 V until the dye front had reached the bottom of the gel. The gel was then stained with Coomassie blue for 30 minutes. Following staining the gel was destained with destain solution (40% (v/v) Methanol, 10% (v/v) Glacial acetic acid, double distilled H₂O to volume). Gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, USA).

2.3 Protein quantification

To determine the original concentrations of the SOD1 samples a BCA assay was performed. Bovine serum albumin (BSA) (Sigma Aldrich, USA) was used to make protein standards between 0 – 1 mg/mL, which were added to 384 well microplate (Greiner bio-one, USA) in triplicate. A binary dilution of each protein was then carried
out in triplicate on the same plate. BCA working reagent (Sigma Aldrich, USA) was prepared and 80 μL was added to each well. The plate was then incubated at 37°C for 30 minutes before the absorbance was read at 592 nm using a Spectra Max Plus 384 spectrophotometer. A standard curve was prepared using the BSA standards and protein concentrations determined via extrapolation.

2.4 Endotoxin removal

Endotoxin was removed from the recombinantly produced SOD1 using an Endotrap red column (Hyglos, Germany) following the manufacturer’s instructions. Briefly, the column was regenerated using regeneration buffer (commercial buffer based on PBS pH 7.4), equilibrated, using Equilibration buffer (10mM Na2HPO4, 80mM NaCl, pH7.4), before the sample was passed over the column twice to ensure sufficient removal of endotoxin. Equilibration buffer used to elute entire sample.

2.5 Poly D Lysine coating of flasks and chamber slides

To aid in the adhesion of primary astrocytes and microglia to flasks and chamber slides a Poly D Lysine coat (PDL) (Sigma Aldrich, USA) was used. Under sterile conditions 5 μg/mL of PDL in H2O was prepared and added to flasks and chamber slides (enough to cover the surface area of each). PDL was allowed to adhere for 2 hours at 37°C before being washed with sterile H2O. Flasks and chamber slides were then allowed to dry in a biosafety cabinet before use.
2.6 Papain solution

A papain solution was prepared fresh for the enzymatic breakdown of mouse cortex. Papain solution was prepared by dissolving papain (Worthington, USA) (final concentration of 1 mg/mL), L-cysteine (240 μg/mL) and 100 μg/mL DNase I type IV (Sigma) into 1 mL of MEM-HEPES (25mM HEPES). The resulting solution was then incubated at 37°C for 30 minutes before use.

2.7 Western blotting

To confirm the identity of SOD1 after purification and to determine internalisation of biotinylated aggregated SOD1G93A, western blots were performed. SDS-PAGE analysis was performed under reducing conditions (as described above) with pre-stained markers (Fermentas, Australia). Western transfer was then performed, onto nitrocellulose membrane (Biotrace, Mexico), utilizing fibre pads and blotting paper pre-soaked in western transfer buffer (3.1 g Tris base/L, 14.4 g glycine/L, 20 % (v/v) methanol). The trans-blot apparatus was placed in the Mini-Protean II electrophoresis cell (Bio-Rad, USA) which was filled with western transfer buffer. The transfer was performed at 100 V for 1 hour at 4°C, while stirring, using the Bio-Rad power pack 300 power supply (Bio-Rad, USA). After transfer, the nitrocellulose membrane was placed in heat denatured casein (1% (w/v) heat denatured casein and 0.01% (w/v) thimerosal in PBS) overnight to block unreacted sites, before western blotting was undertaken. The membrane was washed with PBS triton X-100 0.1% before each antibody treatment (anti-SOD1 1:1000, secondary HRP 1:1000) and the chemiluminescent was detected using the enhanced chemiluminescence kit (Pierce, USA) and Amersham Hyperfilm (GE Healthcare, UK).
To determine levels of BiP from cell lysates western blots were also performed under slightly different conditions to those described above. The transfer was performed at 24 V overnight at 4°C, while stirring, using the Bio-Rad power pack 300 power supply (Bio-Rad, USA). After transfer, the nitrocellulose membrane was rinsed in TBST (Tris Buffer Solution Tween; 14.61g NaCl/L, 1.18g Tris/L, 6.35 g Tris HCl/L, 2mL Tween per liter of milli Q at pH 7.5) and placed in TBST containing 5% milk powder for blocking (Diploma, New Zealand). The membrane was washed with TBST before each antibody treatment (anti BiP 1:1000, secondary 1:500) and the chemiluminescent was detected using the enhanced chemiluminescence kit (Pierce, USA) and Amersham Hyperfilm (GE Healthcare, UK).

2.8 Cytokine release
To test for cytokine release of three major cytokines observed to be upregulated in ALS TNF-α and MCP-1 and IL-6 were measured (Sekizawa et al. 1998, Poloni et al. 2000, Nagata et al. 2007). Supernatant was obtained from microglia and primary astrocyte cultures that had been treated with aggregated SOD1G93A (and supernatant from positive and negative controls). Supernatants were then snap frozen using liquid nitrogen and stored at –80°C until assayed via ELISA. Mouse TNF-α, IL-6 and MCP-1 ELISA Ready-Set-Go kits (ebiosciences USA) were used to measure the amount of cytokine released from primary microglia and astrocytes.

Briefly, a Corning Costar ELISA plate was coated with 100 μL/well of capture antibody (1:250 dilution of purified anti-mouse IL-6 or MCP-1 in coating buffer). The plate was sealed and incubated overnight at 4°C. Wells were aspirated and washed and the
plate blotted on absorbent paper. To block wells 200 μL/well of 1 × Assay Diluent was added to the plate and incubated at room temperature for 1 hour. Wells were aspirated and washed 5 times. Recombinant mouse IL-6 or MCP-1 standard (supplied with the kit), was diluted to 1000 pg/mL and serially diluted (performed in triplicate final volume of 100 μL/well) to make a standard curve. 100 μL of supernatant from the cells was added to wells in triplicate the plate sealed and incubated at 4°C overnight. Wells were aspirated and washed, 100 μL/well of detection antibody (biotin conjugate anti-mouse TNF-α, MCP-1 or IL-6 1:250 dilution in 1 × assay diluent) was added, and the plate was sealed and incubated at room temperature for 1 hour. Wells were washed as previously described, before 100 μL/well of Avidin-HRP (1:250 dilution in 1 × assay diluent). Plate was sealed and incubated at room temperature for 30 minutes. Wells were aspirated and washed and 100 μL/well of substrate solution was added and the plate incubated at room temperature for 15 minutes. 50 μL of stop solution was added to each well. The absorbance of the wells at 450 nm was measured using a Spectra Max Plus 384 spectrophotometer. A standard curve was prepared from the TNF-α, IL-6 or MCP-1 standards and the levels of TNF-α IL-6 and MCP-1 determined.

2.9 Biotinylation of aggregated SOD1

For use in separate experiments (non-ELISA based experiments), Biotin reagent was made up to a concentration of 40 mg/mL in DMSO. The biotin solution was then combined with aggregated SOD1G93A using a 0.25 mg per mg of aggregated SOD1G93A. This solution was then incubated with gentle agitation at room temperature for 2 hours. Excess unbound biotin was removed via dialysis (3x) in PBS pH 7.4.
2.10 Mean fluorescent intensity determination of confocal images
Mean fluorescent intensity of confocal images was determined via image j software analysis measuring mean fluorescence intensity per cell following a previously published method (https://sciencetechblog.files.wordpress.com/2011/05/measuring-cell-fluorescence-using-imagej.pdf).
3. Extracellular aggregated Cu/Zn superoxide dismutase activates microglia to give a cytotoxic phenotype
This chapter is based on a peer reviewed journal (with slight adaptations) published in the journal *Glia* in 2013.

**Extracellular Aggregated Cu/Zn Superoxide Dismutase Activates Microglia to Give a Cytotoxic Phenotype**

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**Contributions**

Kate Roberts—Designed and performed experiments, analysed data and wrote paper.

Rafaa Zeineddine—Performed and analysed experiments presented in Figure 3.8 and 3.9

Lisa Corcoran—Assisted with primary culture generation

Wen Li—Provided assistance and training in primary culture experiments

Iain L Campbell—Provided supervision and assistance at laboratories in University of Sydney and contributed to editing the paper.

Justin Yerbury—Designed experiments, analysed data and wrote paper.
3.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating motor neuron disease characterised by the selective death of motor neurons in the motor cortex and spinal cord. Motor neuron death is associated with the formation of protein inclusions and activation and proliferation of astrocytes and microglia (Pasinelli and Brown 2006). The actual cause(s) of most cases of ALS are undefined, however approximately 10% of cases are inherited (familial ALS; fALS). The best-studied fALS cases are from families possessing mutations in the gene encoding copper/zinc superoxide dismutase (Cu/Zn SOD, SOD1). It is thought that mutant SOD1 causes pathology through a toxic gain of function and there is increasing evidence that the misfolding of mutant SOD1 underlies disease pathology (Wang et al. 2002). The precise mechanism by which SOD1 mutants are toxic is unknown. Recent research demonstrates the importance of non-neuronal cells, such as microglia, in disease progression (Pramatarova et al. 2001). In fact, activation of microglia can be observed before the onset of weakness and significant motor neuron loss in transgenic mice expressing human mutant SOD1 (Alexianu et al. 2001). Data from experiments conducted in wild type/mutant SOD chimeric mice demonstrate that mice with wild type glial cells live longer, suggesting that glial cells containing mutant SOD1 contribute to disease (Clement et al. 2003). Microglial activation in mutant SOD1 mice increases throughout the duration of the disease and remains evident in the late stages of the disease (Hall et al. 1998, Alexianu et al. 2001, Henkel et al. 2006).

Recent work demonstrates that mutant SOD1 can be secreted into the extracellular environment, where it is toxic to motor neurons via activation of microglia (Urushitani et al. 2006). This indirect toxicity is likely to be due to the secretion of toxic mediators
such as reactive oxygen species (ROS), proinflammatory cytokines and glutamate (Bal-Price and Brown 2001, Henkel et al. 2006). Evidence for mutant SOD1 secretion is now widespread; mutant SOD1 has been shown to be secreted from a range of cell types (COS-&/NIH3T3 cells (Urushitani et al. 2006), hepatocyte and fibroblast (Mondola et al. 1996), neuroblastoma (Mondola et al. 1998), thymic derived (Cimini et al. 2002), and pituitary derived (Santillo et al. 2007). In addition, and more importantly, it has also been shown to be secreted from a motor neuron cell line (Turner et al. 2005, Gomes et al. 2007), primary spinal cord cultures from mutant SOD1 expressing mice (Urushitani et al. 2006), and has been found in cerebrospinal fluid (CSF) of mutant SOD1 rats (Turner et al. 2005), in blood plasma (Mondola et al. 2000) and in CSF (Jacobsson et al. 2001) of fALS patients.

Although SOD1 forms intracellular inclusions, there are lines of evidence that suggest that SOD1 aggregates can escape the intracellular environment. SOD1 aggregates appear in astrocytes and inhibitory interneurons in close proximity to motor neurons only after inclusion formation in motor neurons in G93A mice (Rossi et al. 2008, Hossaini et al. 2011) consistent with their release and subsequent uptake. In addition, one report has established that approximately 6-7% of transplanted wt astrocytes were found to contain detectable ubiquitin positive inclusions after transplantation in mutant SOD1 mice (Lepore et al. 2008). Moreover, a recent report has demonstrated that in N2a cells in culture SOD1 aggregates can transfer from cell to cell, requiring a release of aggregates (Munch et al. 2011). Regardless, during phagocytic removal of dead or dying motor neurons during ALS microglia will inevitably encounter inclusions of aggregated protein. Since protein aggregates associated with other neurodegenerative diseases such as Alzheimer’s can activate microglia (Casal et al.
2002), we wondered whether SOD1 protein aggregates would also effectively trigger microglia activation. In the current study we investigated the response of microglia to SOD1 aggregates compared to that of soluble wild type and mutant SOD1. We show that aggregated SOD1 is a potent activator of microglia, for the first time linking protein aggregation and gliosis in ALS.
3.2 Methods

3.2.1 EOC.13 cell cultures

LADMAC (American type culture collection, USA) and EOC.13 (American type culture collection, USA) cells originally frozen in liquid nitrogen were thawed quickly in a container with ethanol which was placed in 37°C water bath. Cells were then transferred to 15 mL falcons which were topped up to 10 mL with Dulbecco’s Modified Eagle Medium F12 (DMEM:F12) (Invitrogen, Australia) before being pelleted in a bench top centrifuge for 5 minutes at 301 g at 22°C. Supernatant was then removed and 5 mL of growth media (see below) was used to resuspend cell pellets.

LADMAC cells growth media contained 10 % Foetal Bovine Serum (FBS) (Bovogen Biologicals Australia) and 90 % DMEM: F12. In the case of EOC.13 cells growth media contained 10 % FBS, 20 % LADMAC conditioned media (containing the necessary Colony Stimulating Factor 1 (CSF-1)) and 70% DMEM F12. Cells were then transferred to a T-25 tissue culture flasks (Greiner bio-one, Germany) and grown in a CO₂ incubator at 37°C until 80 % confluent.

LADMAC conditioned media was generated by culturing LADMAC cells until they were 80 % confluent and subsequently incubating for 3-5 days and collecting the supernatant. The collected supernatant was then removed and filtered for use in EOC.13 growth media or frozen at -20°C until needed.

3.2.2 Primary microglial cell cultures

Primary microglial cultures were prepared as described previously (Carter et al. 2007). Briefly, postnatal mouse forebrain (days 1–2) was stripped of meninges, finely
chopped, and dissociated in papain before being cultured for 7-10 days on poly-d-lysine-coated culture flasks in DMEM (Invitrogen, Australia) supplemented with 10% FBS. After establishment of the astrocyte monolayer, flasks were shaken for 1 h to obtain enriched microglia cultures (Figure 3.1).

3.2.3 Purification of recombinant SOD1

See Chapter 2.

3.2.4 SOD1 aggregation

Purified SOD1G93A protein was incubated at 0.5 mg/mL in 10 mM KH$_2$PO$_4$ in the presence of 20 mM Dithiothreitol (DTT), 5 mM Ethylenediaminetetraacetic acid (EDTA) at 37°C and a pH of 7.4 whilst shaking for 62 hours. For thioflavin T measurements the same conditions were used with the addition of 50 μM Thioflavin T. Solutions were added to a 384 well microplate and fluorescence was examined using a Polarstar omega (BMG Labtech, Australia) fluorescent plate reader using an emission filter of 490 +/- 10 nm and excitation filter of 440 +/- 10 nm.
Figure 3.1: **Experimental outline of primary microglia culture preparation.** Mating’s were set up between wildtype C57BL/6 mice and after 3-4 weeks pups were obtained. Brain tissue was then harvested from 1-2 day pups, meninges removed and cortex digested using papain. Cells were then cultured for one week before cells were split and cultured for a future 2 weeks before microglia were obtained by shaking at 225 rpm for 3 hours.
3.2.5 Circular Dichroism

All samples were dialysed against 10 mM Na₂HPO₄, pH 7.4 at 4°C. Samples were analysed using the Jasco Model J-810 spectropolarimeter linked to a CDF-4265/L Peltier system (Jasco, Canada), with 6 reads used to obtain an average spectrum. Spectra of phosphate buffer alone was subtracted from that of protein samples.

3.2.6 SOD1 enzymatic activity In-gel-zymography

SOD1 variants were loaded onto a 10% native PAGE gel which was run at 100 V for 2 hours at 4°C. The gel was then incubated with 0.6 mg/mL Nitroblue tetrazolium (NBT) in the dark while rocking for 30 minutes and subsequently washed before being incubated with 0.45% tetramethyl-ethlenediamine (TEMED) and 10 μg/mL riboflavin and rocked for 30 minutes before being exposed to light.

3.2.7 Exogenous SOD1 treatment

In order to produce resting EOC.13 microglia, LADMAC supernatant was withdrawn and the cells incubated in 10% FBS in DMEM: F12 for 72 hours before treatment. After 72 hours 5 μg/mL of recombinant SOD1 (WT, G93A soluble and aggregated) protein or the same volume of PBS as a negative control were added to individual wells in 10% FBS in DMEM: F12. Primary microglia were treated with 5 μg/mL of recombinant SOD1 for 24 hours. Supernatants were collected at various timepoints over a 24 hour period and snap frozen in liquid nitrogen and stored at -80°C. In some experiments cells were pretreated with either 5 mg/ml polyinosinic acid (poly(i)) or a neutralising anti-CD14 antibody (Abcam; 1 mg/ml). Mitosox Red was used as per the manufacturer’s instructions to measure superoxide production in living cells (Invitrogen, Australia), data was collected using FACS diva (BD Bioscience, USA) and
analysed using Flow Jo (Tree star, USA). Cytokine concentration in supernatants were measured using Ready-Set-Go kits (eBioscience, USA) as per the manufacturer’s instructions (eBioscience, USA).

3.2.8 Cell surface binding

SOD1 protein was added to EOC.13 cells that had been removed from the flask with 5 mM EDTA in PBS. Cells were resuspended in Hank’s buffered salt solution (HBSS, 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1 mM MgSO₄, 4.2 mM NaHCO₃) containing either 20 μg/mL of SOD1 protein sample (native SOD1G37R, SOD1G93A, SOD1A4V, SOD1WT and aggregated SOD1G93A, SOD1A4V) or the unrelated intracellular enzyme GST or no protein as controls. Cells were then incubated on ice for 30 minutes before being washed with HBSS. A 1:200 dilution of anti-Cu/Zn SOD1 rabbit antiserum in PBS containing 0.1 % BSA (Millipore, USA) and subsequent sheep anti-rabbit-FITC (Chemicon, Australia) was used to detect bound SOD1. In some instances cells were pretreated with with either 25 μg/ml cytochalasin D, 100 μM EIPA or 5 mM MβCD or pretreated with either 500 μg/ml Mannan, 300 μg/ml Fucoidan, 500 ng/ml LPS in PBS azide for 30 min at 4°C, followed by 20 μg/mL of aggregated SOD1G93A for 30 min at 4°C. In the case of biotinylated SOD1G93A and SOD1A4V aggregates, EOC.13 cells were incubated with 10 μg/ml SA-Alexa 488 (Chemicon, Australia). Propidium iodide (Sigma Aldrich, USA) was added to each sample just before analysis by flow cytometer for exclusion of dead cells from the analysis. Data was collected using FACS diva (BD Bioscience, USA) and analysed using Flow Jo (Tree star, USA). Alternatively cells were grown on cover slips and treated as above before being fixed using ice cold paraformaldehyde in PBS for 15 min at room temperature, washed in PBS and subsequently mounted in Citifluor AF1.
(Citifluor, UK) and imaged using a Leica TCS confocal scanning microscope (Leica, Germany).

### 3.2.9 Cell viability assays

Conditioned media from aggregated SOD1<sup>G93A</sup> treated primary microglia were added to NSC-34 cells to make a final concentration of 50% (v/v). NSC-34 cells were incubated in 50% conditioned media for 48 hours before using calcein-AM as a probe for cell viability. Briefly, cells were exposed to 1 μM calcein-AM and incubated at 37°C for 30 min. After 30 min the fluorescence of the wells (485+/−10 nm excitation and 520+/− 10 nm emission) was measured using a fluostar optima microplate reader. In some experiments cells were co-treated with a neutralizing anti-TNF-α antibody (Abcam), hemoglobin or SOD1<sup>WT</sup>.

### 3.2.10 Statistics

Data were expressed as mean +/- SEM. Statistical significance was evaluated with one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test using Prism 5.0 GraphPad Software (San Diego, CA). A value of P < 0.05 was considered significant.
3.3 Results

3.3.1 SOD1 aggregates at physiological pH and temperature to form fibrils

In order to examine the effect of exogenous SOD1 on microglia we purified recombinant human SOD1 from *E. coli*. To confirm that the SOD1 was folded correctly we used circular dichroism and in-gel zymography. Purified SOD1 had a CD spectra (Figure 3.2A) similar to that reported previously (Stevens *et al.* 2010) consistent with the purified product having a correctly folded secondary structure. In addition, recombinant SOD1 was a functional superoxide scavenger (Figure 3.2B) demonstrating a functional tertiary fold. Consistent with work of others (Chattopadhyay *et al.* 2008) we found that incubation of SOD1 with 20 mM DTT and 5 mM EDTA produced SOD1 aggregates that were thioflavin T positive (Figure 3.3C) and contained fibrillar structures (Figure 3.2D). The kinetics of aggregation were consistent with that of other fibril forming systems exhibiting a lag phase followed by growth phase and subsequent plateau (Figure 3.2C). We observed that the more pathogenic variant A4V had a higher propensity to aggregate than did the G93A variant. Under the conditions tested we did not observe any significant aggregation of the wild type SOD1 (Figure 3.2C).
Figure 3.2: **Recombinant mutant SOD1 is more aggregation prone than wt SOD1.**

**A:** Circular dichroism spectrum of 5 μM purified SOD1 proteins (SOD1AAV, SOD1G93A, SOD1G37R, and SOD1WT) in 10 mM Na₂HPO₄, pH 7.4. **B:** In-gel-zymography assay measuring enzymatic activity of SOD1AAV, SOD1G93A, SOD1G37R, and SOD1WT. A total of 2 μM SOD1 samples were run at 100 V for 2 h at 4°C using native gel electrophoresis. Gel was stained with NBT/Riboflavin/TEMED to determine dismutase activity with achromatic bands indicating activity. **C:** Aggregation of SOD1 was measured using a thioflavin T assay. Purified SOD1 was incubated at 0.5 mg/mL in 10 mM KH₂PO₄ in the presence of 20 mM DTT, 5 mM EDTA, and 50 μM thioflavin T. **D:** TEM micrograph of SOD1G93A at 0.5 mg/mL incubated in 10 mM KH₂PO₄, 20 mM DTT, 5 mM EDTA was loaded onto a carbon-coated nickel grid and negatively stained using 2% (w/v) uranyl acetate.

### 3.3.2 Aggregated SOD1 activates EOC.13 cells

EOC.13 cells were used in this study as a model for microglia to exclude any artefacts caused by LPS contamination introduced from bacterially produced recombinant SOD1. EOC.13 cells are derived from the C3H/HeJ mouse (TLR4 defective) and thus are unresponsive to LPS (Walker et al. 1995). Previous studies have found that soluble mutant SOD1 incubated with primary microglia can increase TNF-α secretion.
compared to wild type SOD1 in a CD14/TLR4 dependant mechanism (Urushitani et al. 2006, Zhao et al. 2010) however, the lack of CD14 in mice did not decrease microglia activation suggesting that there may be other receptors involved in vivo. The fact that the EOC.13 model is TLR4 defective makes it a useful model for identifying other activation pathways. In the current study recombinant human SOD1, either aggregated or soluble and correctly folded, was added to cells and incubated for 24 hours. The cell supernatants were then examined for TNF-α content. We found that in the presence of SOD1, EOC.13 cells increased TNF-α secretion (Figure 3.3) in a fashion that was consistent with the SOD1 mutants aggregation propensity (WT<G37R<G93A<A4V SOD1; 16.91 +/- 0.62, 17.72 +/- 0.9897, 21.08 +/- 0.9179, 22 +/- 2.479 respectively). However, these differences were not statistically significant. When EOC.13 cells were incubated with aggregated G93A or A4V SOD1 there was a > 5 fold increase in TNF-α levels found in supernatant (Figure 3.3). Indicating that aggregated SOD1 is a much more efficient activator of EOC.13 cells when compared to soluble SOD1. In addition, incubation of EOC.13 cells with aggregated SOD1 increased ROS production as measured by mitosox red (Figure 3.4). The specific ability of aggregated SOD1 to trigger TNF-α secretion was time (Figure 3.5) and concentration dependant (Figure 3.6). In this model the appearance of TNF-α in the supernatant was independent of both MCP-1 and IL-6 (Figure 3.7).
Figure 3.3: **Aggregated SOD1 induces TNF-α release from EOC.13 microglia.** Microglial cell line EOC.13, were grown for 72 h in the absence of CSF-1 followed by incubation with 5 μg/mL SOD1 (SOD1<sup>WT</sup>, SOD1<sup>G37R</sup>, SOD1<sup>G93A</sup>, SOD1<sup>A4V</sup> and preaggregated SOD1<sup>G93A</sup> and SOD1A4V). The supernatants were harvested at 24 h, and at intervals, and TNF-α levels measured with Mouse TNF-α ELISA Ready-Set-Go kit. *Denotes statistically significant increase when compared with WT SOD1-treated cells (P < 0.05). (Work performed during honours included in (Roberts et al. 2013).

Figure 3.4: **Incubation of EOC.13 cells with aggregated SOD1 increases ROS production.** Microglial cell line EOC.13, were grown for 72 h in the absence of CSF-1 followed by incubation with 5 μg/mL SOD1 (SOD1<sup>WT</sup>, SOD1<sup>G93A</sup>, and preaggregated SOD1<sup>G93A</sup>). Mitosox red fluorescence was measured using flow cytometry. *Denotes statistically significant increase when compared with wild type SOD1-treated cells (P < 0.05).
Figure 3.5: **TNF-α secretion triggered by aggregated SOD1 is time dependant.** Microglial cell line EOC.13, were grown for 72 h in the absence of CSF-1 followed by incubation with 5 μg/mL SOD1 (SOD1\textsuperscript{WT}, SOD1\textsuperscript{G93A} and preaggregated SOD1\textsuperscript{G93A}). The supernatants were harvested at 24 h, and at intervals, and TNF-α levels measured with Mouse TNF-α ELISA Ready-Set-Go kit.

Figure 3.6: **TNF-α secretion triggered by aggregated SOD1 is concentration dependant.** EOC.13 cells were incubated with 5–100 μg/mL aggregated G93A SOD1 for 24 h and supernatants collected and analyzed for TNF-α levels. *Denotes statistically significant increase when compared with wild type SOD1-treated cells (\(P < 0.05\)).
Figure 3.7: **TNF-α in the supernatant was independent of both MCP-1 and IL-6.** A-B: EOC.13 cells were incubated with 5 μg/mL SOD1 for 24 hours and supernatants collected and analysed for MCP-1 and IL-6 levels.

### 3.3.3 Aggregated SOD1 interacts with cell surface and is internalised by EOC.13 cells

Given that aggregated mutant SOD1 promoted a TNF-α response from the EOC.13 cells we sought to characterise their interaction with aggregated SOD1. To determine if aggregated SOD1 would bind to the cell surface it was incubated with cells on ice for 30 minutes before subsequent immunodetection. Using both flow cytometry and confocal microscopy we observed substantial binding of SOD1 to the cell surface of
EOC.13 cells (Figure 3.8 A & B). We observed an increase in fluorescence for aggregated SOD1 treatments (geomean of 25.4) (Figure 3.8 B). However, no binding of the control protein GST was detected (geomean of 4.97 compared to 4.71 for no treatment controls). Confocal microscopy revealed that the fluorescence detected was initially present at the surface of the cell (Figure 3.8 A; 30 minutes). However, after 60 minutes of incubation the recombinant human SOD1 was no longer detected on the cell surface (Figure 3.8 A, Non-permeabilised), but was detected inside cells after permeabilisation. Cell lysates of EOC.13 cells treated with aggregated SOD1 confirm uptake of aggregates (Figure 3.8 C). In addition, the aggregates remain in cells even after 24 hours with minimal degradation (Figure 3.8 C).

3.3.4 Inhibition of lipid raft formation and scavenger receptors reduces the aggregated SOD1 binding to EOC.13 cells

In an attempt to further characterise the binding and internalisation of SOD1 aggregates to EOC.13 cells we first tested a small panel of endocytosis inhibitors. We pre-incubated EOC.13 cells with either cytochalasin D, EIPA or MβCD which are inhibitors of actin cytoskeleton rearrangement, macropinocytosis and lipid raft formation respectively. Only pre-treatment with MβCD was able to decrease the amount of SOD1 aggregate binding. Moreover, using confocal microscopy of permeabilised cells as a measure of internalisation, only pre-treatment with MβCD was able to inhibit internalisation of aggregated SOD1 (Figure 3.9). In addition, to narrow down the receptor(s) that may be important to aggregated SOD1 binding to the cell surface we pre-incubated cells with either LPS, fucoidan or mannan to compete for binding to the LPS receptor, scavenger receptors and mannose receptors respectively. Pre-incubation of cells with fucoidan was able to significantly (p < 0.05)
supress the amount of cell surface binding of aggregated SOD1. Although the amount of aggregated SOD1 bound to cells was decreased by pre-incubation with LPS this was not significant.

Figure 3.8: **Aggregated SOD1 binds to the cell surface and is internalized into EOC.13 microglia.** **A:** Scanning confocal micrographs of aggregated SOD1 proteins interacting with EOC.13 cells. Cells were incubated with 20 lg/mL of aggregated G93A SOD1 for either 30 or 60 min on ice. At 30 min, cells were fixed and stained for human SOD1. At 60 min, cells were either fixed or fixed and permeabilised before probing for human SOD1. **B:** Quantitative analysis of the binding of SOD1 proteins to EOC13 cells using flow cytometry after 30 min incubation on ice. In addition, control protein GST (20 lg/mL) was also added to the EOC.13 cells for 30 min on ice and cells subsequently probed for GST binding. Results are geometric means (standard error of triplicates and are representative of at least three independent experiments. * Statistically significant increase when compared with cells not treated with SOD1 (P < 0.05). **C:** Western blot of cell lysates from EOC.13 cells incubated in the presence of SOD1 aggregates. Biotinylated SOD1 aggregates were added on to cells and incubated for 24 h. At various times, cells were lysed and lysates analysed by Western blot. SOD1 aggregates were reduced with b-mercaptoethanol and full length SOD1 and any degradation fragments were detected using SA-HRP.

*(Work performed by Rafaa Zeineddine included in (Roberts et al. 2013)).*
3.3.5 Aggregated SOD1 interacts with and is internalised by primary microglia

Given that aggregated SOD1 interacted with EOC.13 cells we sought to characterise the interaction of SOD1 aggregates with primary microglia. Due to the avidity of the microglia to the coated culture dishes, we were unable to obtain meaningful flow cytometry data. To determine if aggregated SOD1 would bind to the cell surface of primary microglia, aggregates were incubated with cells on ice for 30 minutes before subsequent immunodetection. Using confocal microscopy we observed substantial cell surface fluorescence suggesting binding of SOD1 to the cell surface (Figure 3.10a; quantified in g). After 60 minutes of incubation the aggregated SOD1 could be

Figure 3.9: Aggregate binding is dependent on lipid raft formation and scavenger receptors. A: EOC.13 cells were pretreated with either 25 μg/mL cytochalasin D, 100 μM EIPA or 5 mM MβCD for 30 min at 4°C, followed by 20 μg/mL of aggregated G93A SOD1 for 30 min at 4°C. Cells were then probed for human SOD1 and analyzed by flow cytometry. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference (P < 0.05). B: EOC.13 cells were pretreated with either 500 μg/mL Mannan, 300 μg/mL Fucoidan, 500 ng/mL LPS in PBS azide for 30 min at 4°C, followed by 20 μg/mL of aggregated G93A SOD1 for 30 min at 4°C. Cells were then probed for human SOD1 and analyzed by flow cytometry. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference (P < 0.05). (Work performed by Rafaa Zeineddine included in Roberts et al. 2013).
detected inside cells after permeabilisation (Figure 3.10b). To determine if the mechanism of binding was similar to that of the EOC.13 cells we pretreated the cells with either fucoidan, LPS or MβCD. In contrast to the EOC.13 cells we observe a significant decrease of aggregated SOD1 binding after pre-treatment with LPS (Figure 3.10e & g) (p < 0.05). Consistent with our EOC.13 data we also observed a significant decrease in internalised SOD1 after pre-treatment with fucoidan and a smaller but still significant decrease after pre-treatment with MβCD (Figure 3.10c,d & g; p < 0.05).

Figure 3.10: Inhibition of mutant SOD1 binding in murine primary microglia. Aggregate binding is dependent on lipid raft formation and scavenger receptors in primary microglia. A-G: primary microglia cells were pretreated with either 25 μg/mL cytochalasin D, 100 μM EIPA or 5 mM MβCD for 30 min at 4°C, followed by 20 μg/mL of aggregated G93A SOD1 for 30 min at 4°C. Cells were then probed for human SOD1 and analyzed by confocal microscopy. G: Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference (P < 0.05). * Denotes statistically significant difference (P < 0.05).

3.3.6 Aggregated mutant SOD1 activates primary microglia

To confirm that the aggregated SOD1 dependant release of TNF-α is not restricted to the EOC.13 cell line we examined the ability of aggregated SOD1 to stimulate TNF-α
release from primary microglia. To minimise LPS contamination we used LPS affinity chromatography to separate any remaining LPS from the preparations. In addition, recombinant wild type SOD1 purified in the same manner was used as a negative control. Consistent with previous work (Urushitani et al. 2006, Zhao et al. 2010) we found that G93A SOD1 elicited a significantly increased TNF-α response from microglia compared to wild type SOD1 (p<0.05). Importantly, when compared to the mutant SOD1 dependant response we observed that pre-aggregated G93A SOD1 promoted a >3 times greater increase in secreted TNF-α over the same time period. This was significantly greater than TNF-α released after both wild type and mutant SOD1\textsuperscript{G93A} treatment of primary microglia (p<0.05) (Figure 3.13). In contrast to the EOC.13 model the aggregated SOD1 also induced an increase in MCP-1 release from primary microglia (Figure 3.11). In addition, aggregated SOD1 induced significant increases in superoxide production as measured by mitosox red (Figure 3.12). In order to verify that TNF-α release was dependant on lipid raft, scavenger receptor and CD14 dependant mechanisms we pre-incubated cells with either the scavenger receptor ligand polyinosinic acid, or a CD14 neutralising antibody. Polyinosinic acid was used in this assay as fucoidan and MβCD were both toxic to cells over an extended period (data not shown). TNF-α release increased after treatment with aggregated SOD1\textsuperscript{G93A}, however it also was increased after incubation with polyinosinic acid when added alone, as a result pre-treatment with this ligand did not inhibit TNF-α release. In contrast, TNF-α was decreased when cells were pretreated with anti-CD14 (Figure 3.14). While our data suggests that the uptake of aggregates, and thus proinflammatory response could be blocked by both scavenger receptors and CD14, we confirm this occurs only in the case of CD14.
Figure 3.11: Aggregated SOD1 induces MCP-1 release from primary microglia. 

**A-B**: Primary microglia cells were incubated with 100 μg/mL SOD1 (SOD1\textsuperscript{WT}, SOD1\textsuperscript{G37R}, SOD1\textsuperscript{G93A}, SOD1\textsuperscript{A4V} and preaggregated SOD1\textsuperscript{G93A}). The supernatants were harvested at 24 h, and cytokine levels measured with Mouse IL-6 and MCP-1 ELISA Ready-Set-Go kits. *Denotes statistically significant increase when compared with wild type SOD1-treated cells \((P < 0.05)\).
Figure 3.12: *Aggregated SOD1 induces increased superoxide production in primary microglia.* A-D: Primary microglia cells were incubated with 100 μg/mL preaggregated SOD1$^{G93A}$. The superoxide production was measured using mitox red. *Denotes statistically significant increase when compared with wild type SOD1-treated cells ($P < 0.05$).

### 3.3.7 Aggregated SOD1 induces release of soluble neurotoxic factor

Since microglial activation has been implicated in toxicity of extracellular mutant SOD1 on mixed primary brain cultures we took the supernatants of SOD1 activated microglia and added them to mouse motor neuron like NSC-34 cells. After 48 hours of incubation the viability of the cells treated with supernatant from aggregated SOD1$^{G93A}$ treated
microglia had significantly dropped (~25%, p<0.05) (Figure 3.15). Although the viability of cells incubated in soluble mutant G93A SOD1 induced microglia supernatant dropped around 10% this was not significant. In an attempt to determine the toxic factor in conditioned media we co-incubated either a neutralizing anti-TNF-α antibody (5 μg/ml), 50 μg/ml hemoglobin or μg/ml wild type SOD1 with conditioned media to block toxicity from TNF-α, nitric oxide and superoxide respectively. However, we observed no change in cell viability after any of the pre-treatments used (Figure 3.16).

Figure 3.13: Aggregated SOD1 induces TNF-α release from primary microglia. Primary microglia were incubated with 5 μg/mL SOD1 (SOD1WT, SOD1G93A, and pre-aggregated SOD1G93A) for 24 h. The supernatants were harvested at 24 h hours and TNF-α levels measured with Mouse TNF-α ELISA Ready-Set-Go kit. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference (P < 0.05).
Figure 3.14: **Blocking of CD14 receptor reduces the level of aggregated SOD1 induced TNF-α release from primary microglia.** Primary microglia were pre-treated with either polyinosinic acid or anti-CD14 antibody before incubation with 5 μg/mL pre-aggregated SOD1G93A for 24 h. The supernatants were harvested at 24 h and TNF-α levels measured with Mouse TNF-α ELISA Ready-Set-Go kit. Results are means and standard error of triplicate experiments. * Denotes statistically significant difference ($P < 0.05$).

Figure 3.15: **Soluble factors from primary cells are toxic to NSC-34 neuronal cells.** Supernatants collected from SOD1-treated primary microglia were added to cultures of motor neuron like NSC-34 cells to give 50% v/v with fresh media. Cultures were incubated for 48 h before analysing cell viability using Calcein-AM. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference from PBS CM ($P < 0.05$).
Figure 3.16: Aggregated SOD1 induces TNF-α release from primary microglia which is toxic to motor neuron like NSC-34 cells. NSC-34 cells were treated with either anti-TNF-α antibody, hemoglobin or wild type SOD1 before added in combination with conditioned media from aggregated SOD1 (Agg. G93A CM)-treated microglia or PBS-treated microglia (PBS CM). Results are means ± standard error of triplicate experiments. * Denotes statistically significant difference ($P < 0.05$).
3.4 Discussion

Two of the most prominent hallmarks of ALS pathology are: (i) inclusions consisting of aggregated proteins and (ii) gliosis. There is accumulating evidence to suggest that both protein aggregates (Watanabe et al. 2001, Wang et al. 2002, Wang et al. 2008, Johnson et al. 2009, Fushimi et al. 2011, Guo et al. 2011) and glia play an active role in ALS disease pathogenesis (Pramatarova et al. 2001, Clement et al. 2003, Turner et al. 2004, Beers et al. 2006). However, there has been almost no attempt to link these two aspects of disease. Although SOD1 inclusions are exclusively intracellular, there is evidence that these inclusions can escape from the cytoplasm and come into contact with nearby cells (Münch et al. 2011). Although extracellular soluble mutant SOD1 has been shown to activate microglia (Urushitani et al. 2006, Zhao et al. 2010), the effects of microglia coming into contact with SOD1 aggregates is unknown. Here, we investigated the effect of aggregated SOD1 on microglial activation and subsequent toxicity to motor neuron like cells. We show, for the first time, that microglia become activated when they come in to contact with aggregated SOD1. Binding and uptake of aggregated SOD1 can be inhibited by fucoidin and LPS, suggesting that signalling is dependent on scavenger receptor and CD14 binding. We also show that soluble factors secreted by aggregate activated microglia are toxic to motor neuron like cells. These findings are consistent with aggregates released from dead or dying cells playing a role in propagation of pathology in ALS.

All cases of ALS are associated with protein aggregates in the form of inclusions that are found in surviving motor neurons or in nearby astrocytes (Miller et al. 2004). There is a range of proteins that can be found associated with inclusions including gene
products of some fALS genes such as FUS, TDP-43, Optineurin, Ubiquilin2, and SOD1 (Neumann et al. 2006, Deng et al. 2010, Maruyama et al. 2010, Forsberg et al. 2011). SOD1 inclusions can only be found in SOD1 FALS cases, but recent work suggests misfolded SOD1 can be found in SALS motor neurons (Bosco et al. 2010, Forsberg et al. 2011). In this study, we used recombinant human SOD1 produced in E. coli to examine the response of microglia to ALS associated aggregates. Circular dichroism and enzymatic zymography demonstrated that human SOD1 used for this study was correctly folded. The secondary structure measured in this study was similar to that previously published (Stevens et al. 2010) and the purified protein showed enzyme activity. Reduction of the disulphide bond and chelation of the metal ions promoted aggregation of recombinant SOD1 as previously shown (Chattopadhyay et al. 2008). A comparison of the rate of aggregation of three recombinant SOD1 mutants showed that their aggregation propensity correlated with the rate of disease progression with A4V being the most rapid and G37R the slowest (Wang et al. 2008). Under the conditions tested, there was little aggregation from the wild type SOD1, suggesting that the mutants are more aggregation prone. The aggregates produced by these conditions included fibrillar structures as shown previously (Chattopadhyay et al. 2008).

It has been established that SOD1 can be secreted from a range of cells and can be found outside cells in vivo (Jacobsson et al. 2001, Turner et al. 2005, Urushitani et al. 2006). However, aggregates of SOD1 accumulate exclusively intracellularly, and there is no evidence to suggest that the secreted SOD1 actively aggregates outside of cells. Recent work demonstrates that SOD1 aggregates can escape the intracellular
compartment, by an unknown mechanism, into the extracellular space (Münch et al. 2011). The consequences of this will be dependent on the cell type that the released aggregates come in to contact with. In the example of neurons, it has been shown that aggregates of mutant SOD1 can seed further aggregation of mutant SOD1 in native neurons it has entered (Münch et al. 2011). In this study, although soluble SOD1, both wild type and mutant, did not induce significant increases in EOC.13 cell activation, aggregated G93A SOD1 promoted a fivefold increase in TNF-α release in these cells. The response is dose dependant and can be detected as early as 6 h after addition of aggregates. In addition, this response seemed to be independent of IL-6 and MCP-1 release in EOC.13 cells. EOC.13 cells treated with aggregated SOD1 also showed an increase in ROS production suggesting that the response was not restricted to TNF-α release.

To confirm that the observed effects of aggregated SOD1 were not restricted to a single cell line, further studies were carried out using primary microglial cultures. Previous studies had shown that microglia could become activated in the presence of unaggregated mutant SOD1 (Urushitani et al. 2006, Zhao et al. 2010). Consistent with this, we found that upon incubation with unaggregated mutant SOD1 primary microglia significantly increased their secretion of TNF-α compared with those incubated with SOD1WT. Consistent with our results from EOC.13 cells, primary microglia also released significantly more TNF-α upon treatment with aggregated mutant SOD1 compared with SOD1WT or dimeric unaggregated SOD1G93A. In contrast, primary cells also responded by releasing significant amounts of MCP-1 and increasing production of superoxide. To eliminate any artefacts due to LPS, all recombinant protein was
prepared using LPS affinity chromatography reducing the amount of any LPS present in the sample. In addition, the fact that wild type and mutant SOD1 were purified under identical conditions suggests LPS plays no role in the release of TNF-α in these experiments. Further evidence that LPS is not involved is the fact that EOC.13 cells do not respond to LPS but do respond to aggregated mutant SOD1. The data presented here is the first example of ALS associated aggregates activating microglia. However, it has been previously shown that microglia can be activated by aggregates of Aβ (Casal et al. 2002, Maezawa et al. 2011) and α-synuclein (Zhang et al. 2005) associated with Alzheimer’s disease and Parkinson’s disease, respectively. Collectively, these data suggest that there may be a common feature of these aggregates that is responsible for microglial activation. Although we only tested SOD1 aggregates it is likely that all ALS associated aggregates, including TDP-43 positive aggregates, activate microglia.

A range of receptors have been shown to play a role in the binding of protein aggregates, such as those made from the amyloid-β (Aβ) peptide, to the cell surface of microglia (Bamberger et al. 2003, Fu et al. 2012). Scavenger receptors CD36 (Coraci et al. 2002) and SR-A (ElKhoury et al. 1996), the receptor for advanced glycation end products (RAGE) (Yan et al. 1996), integrin α6β1- integrin and integrin associated CD47 (Bamberger et al. 2003), and CD14 and Toll-like receptors (TLR) 2/4 (Reed-Geaghan et al. 2009) have all been shown to be involved in microglial interaction with aggregated Aβ. The interaction between microglia and protein aggregate is generally not a simple one to one receptor to ligand interaction with many receptors coming together to form large complexes that include signalling receptors.
such as TLR2/4. This process of receptor complex formation, in some cases, is dependent on membrane organization such as lipid raft formation (Zeng et al. 2003). We show that aggregated SOD1 binding is inhibited by pretreatment with MβCD, fucoidan, and LPS, suggesting that binding is dependent on lipid raft formation, scavenger receptors and CD14. Scavenger receptors have been shown to preferentially bind to proteins that have been modified by ROS or advanced glycation, or chemical denaturants (Gowen et al. 2001, Horiuchi et al. 2003). In addition, fucoidan has been shown to inhibit the binding of aggregated proteins such as Aβ (Fu et al. 2012) and misfolded proteins in complex with extracellular chaperones (Wyatt et al. 2011). Suggesting that scavenger receptors play an important role in binding to misfolded or aggregated proteins in vivo. Although CD14 is best known for its ability to bind and promote phagocytosis of pathogens, its pattern recognition capability is likely to be responsible for binding to protein aggregates such as Aβ. We present data here to suggest that CD14 is involved in the binding of aggregated SOD1 and thus activation of and release of TNF-α by primary microglia. The data are consistent with previous data suggesting that CD14 can form a large multireceptor complex that include scavenger receptors, such as CD36 and SR-A, in the recognition of protein aggregates such as those made from Aβ. These large complexes have been shown to include signalling co-receptors such as the TLRs that trigger intracellular cascades resulting in robust immune reactivity including TNF-α release (Reed-Geaghan et al. 2009). Thus, it is likely that the receptor complex involved with aggregated SOD1-induced activation includes TLR co-receptors. Interestingly, in our EOC.13 model activation was able to proceed in the absence of a functional TLR4 suggesting that other TLR are also likely to be involved. Indeed, scavenger receptors such as CD36 can combine with TLR2/6 to produce responses to pathogen (Hoebe et al. 2005).
It had previously been shown that extracellular mutant SOD1 was toxic to motor neurons in culture. This was found to be dependent on microglia activation by extracellular mutant SOD1. In this work, supernatants from microglia that have been activated by contact with aggregated SOD1 were toxic to motor neuron-like cells in culture consistent with a role of activated microglia in the cell death of motor neurons in ALS. We used a neutralizing anti-TNF-α antibody, hemoglobin, and wild type SOD1 to suppress toxicity from TNF-α, nitric oxide, and superoxide, respectively. However, we were unable to identify a specific toxic soluble factor. Although the toxic factor in the supernatant is unknown, it remains possible that a combination of stressors could be responsible for the loss of cell viability. Regardless, the release of toxic factors after aggregated SOD1 dependant activation of microglia causes stress on motor neuron-like cells and may induce further release of protein aggregates causing a positive feedback loop.

Research demonstrates the importance of non-neuronal cells in the pathogenesis of ALS (Hall et al. 1998, Alexianu et al. 2001, Henkel et al. 2006). Current thought is that there are fundamental differences underlying disease initiation and disease progression in ALS. This line of thought is based on results showing that mutant SOD1 was important in neurons for disease initiation and in glia for disease progression in a mouse model of ALS (Boillee et al. 2006). Although the role of protein aggregation in disease pathology has not been clarified, SOD1 aggregates are certainly toxic to the neurons they accumulate in culture (Matsumoto et al. 2005, Soo et al. 2009). It is possible then that protein aggregation and inclusion formation are linked with the
initiation event in ALS. The work presented here provides a potential link between SOD1 aggregation and microglial activation, and thus provides evidence to suggest protein inclusions may also be linked to disease progression. As a result, silencing SOD1 aggregate induced microglial activation may be effective in slowing ALS progression.
4. Extracellular aggregated Cu/Zn superoxide dismutase causes astrocyte senescence
4.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating motor neuron disease characterised by the selective death of motor neurons in the motor cortex and spinal cord. The actual cause(s) of most cases of ALS are undefined, however approximately 10% of cases are inherited (familial ALS; fALS). The best-studied fALS cases are from families possessing mutations in the gene encoding copper/zinc superoxide dismutase (Cu/Zn SOD, SOD1). It is thought that mutant SOD1 causes pathology through a toxic gain of function, and there is increasing evidence that the misfolding of mutant SOD1 underlies disease pathology (Wang et al. 2002). The precise mechanism by which SOD1 mutants are toxic remains unclear.

It has previously been shown using co-culturing techniques that astrocytes play an important role in the mutant SOD1 mouse model for ALS. In these studies, wild type astrocytes were found to be beneficial to mutant SOD1 neurons when cultured together (Yamanaka et al. 2008). Conversely, mutant SOD1 astrocytes were found to cause wild type motor neuron degeneration in both mice and rat cultures (Yamanaka et al. 2008, Diaz-Amarilla et al. 2011, Rojas et al. 2014). Astrocytes derived from ALS patient post-mortem tissue, and induced pluripotent stems generated from ALS patients’ fibroblasts, were also found to be toxic to motor neurons in cell culture (Haidet-Phillips et al. 2011, Meyer et al. 2014). Transplantation of SOD1G93A glial precursor cells, which differentiated into astrocytes, was shown to induce motor neuron degeneration in mice (Papadeas et al. 2011). Interestingly, this study was able to demonstrate in mice, that astrocyte dysfunction with reduced glutamate transport expression accompanied the motor neuron loss (Papadeas et al. 2011). This
dysfunction in astrocytes has also been previously demonstrated in motor cortex and spinal cord tissue from ALS patients (Rothstein et al. 1995).

Recent studies have shown that SOD1 aggregates in motor neurons can escape the intracellular environment during cell motor neuron death and via exosomes (Münch et al. 2011, Grad et al. 2014). SOD1 aggregates appear in astrocytes and inhibitory interneurons in close proximity to motor neurons only after inclusion formation in motor neurons in SOD1G93A mice, (Rossi et al. 2008, Hossaini et al. 2011). Further, WT astrocytes transplanted into mutant SOD1 mice acquire inclusions (Lepore et al. 2008). This is consistent with the idea that SOD1 aggregates can be released from motor neurons and subsequently taken up by nearby cells. However, the effect of uptake of inclusions on astrocytes remains unclear.

There is substantial evidence of endoplasmic reticulum stress occurring in neurons in ALS patients and mutant SOD1 mice (Gonatas et al. 1992, Atkin et al. 2006, Atkin et al. 2008, Saxena et al. 2009, Sun et al. 2015). In neuronal cells aggregated SOD1 has recently been shown be taken up via macropinocytosis, inhibit protein transport between the ER-Golgi apparatus which leads to ER stress and cell death (Sundaramoorthy et al. 2013, Zeineddine et al. 2015). When the endoplasmic reticulum encounters misfolded proteins it mounts the unfolded protein response (UPR) in an attempt to relieve stress (Korennykh et al. 2009). The UPR is comprised of three main stress sensors, the transmembrane proteins PERK, IRE1 and ATF6 (Manie et al. 2014). Each of which activates downstream signalling pathways that
reduce ER load and increase ER capacity to deal with misfolded proteins (Hetz 2012). Inositol requiring enzyme 1 (Ire1), is a transmembrane protein which possesses both kinase and endoribonuclease activity. The activation of Ire1 endoribonuclease activity results in the excision of an intron from X-box binding protein (XBP-1). This causes a frameshift in XBP-1 creating an alternate isoform which regulates the expression of genes for protein quality control (Jaronen et al. 2014). This makes splicing of XBP-1 a good target when identifying ER stress and subsequent UPR within cells. Researchers have previously shown increased amount of spliced XBP-1 in neuron like cells expressing mutant SOD1, indicating ER stress (Oh et al. 2008).

Since other types of protein aggregates associated with neurodegenerative diseases such as Alzheimer’s have been shown to trigger both cell death (Brera et al. 2000) and senescence in astrocytes (Bhat et al. 2012), we wondered whether mutant SOD1 protein aggregates effected astrocyte function and survival. In the current study we investigated the effect of uptake and persistent presence of SOD1 aggregates had on primary astrocytes. Our results demonstrate, for the first time, that aggregated SOD1 enters astrocytes via macropinocytosis, causing endoplasmic reticulum stress and senescence and/or cell death. Based on these results it is proposed that protein aggregates may directly contribute to astrocyte dysfunction and thus disease progression in ALS.
4.2 Methods

4.2.1 Primary astrocyte isolation

Primary astrocytes were prepared using the same steps during weeks 1 to 4 as described previously for primary microglia. However at week 5 the mixed glial culture was incubated with magnetic CD11b beads (to remove microglia) before being passed over a macs sort column (Miltenyi Biotech, Australia) to isolate primary astrocytes (Figure 4.1). To confirm pure cultures of primary murine astrocytes (as opposed to a mixed glial culture) GFAP and CD11b antibodies (Millipore, Australia) were used to analyse astrocyte and microglia populations within samples.

Week 1      Set up mating pair

Week 2-3    Remove male (monitor female for signs of pregnancy)

Week 4      Obtain tissue from 1-2 day pups (or set up new mating pair if no pregnancy results)

Week 5      Pass mixed glia culture (incubated with cd11b magnetic beads) over macs sorting column

Figure 4.1: Experimental overview of extraction of primary astrocytes and purification from mixed murine glial cultures. Cortex tissue from 1-2 day mice pups was chopped up and digested with papain solution before established mixed glial population was passed over a macs sorting column to obtain purified astrocyte cultures.
4.2.2 Freeze thaw lysis

Cells were harvested, washed in PBS and the cell suspension was centrifuged at top speed (13,000xg) for 45 seconds before being resuspended in FT Lysis buffer (600mM KCl, 20mM Tris-Cl, 20% Glycerol and protease inhibitor cocktail). Cells were then frozen using liquid nitrogen and then thawed, this was repeated three times. After the cell lysates was thawed, 250 units of DNase was added and incubated at room temperature for 10 minutes. Protein concentration was then measured using a BCA assay. Samples were then stored at -20°C until ready to use.

4.2.3 Astrocyte uptake of aggregated SOD1

Aggregated SOD1\textsuperscript{G93A} with a biotin label was added to astrocyte cultures for 2 hours at 37°C. Cells were washed 3 times with PBS (between each subsequent step) and then fixed with 4% PFA for 15 minutes at room temperature. To permeabilise cell membranes, tween 20 (0.5%) was added to the cells for 10 minutes. A 5% BSA solution (in PBS) was added for 20 minutes to block non-specific binding. SA-Alexa488 diluted in PBS (1:500) (Chemicon, Australia) was added for 1 hour to detect biotinylated aggregated mutant SOD1. To confirm the presence and purity of astrocyte culture, cells were incubated with anti-mouse GFAP antibody raised in rabbit (1:300), or rat anti-mouse CD11b (1:400) for 1 hour. Fluorophores anti-Rabbit Alexa 488 and anti-Rat Alexa 488 (1:500) were added to cells and incubated for 1 hour. Cover slips were added to slides using Mounting media (Prosci Tech, Australia) and CoverGrip coverslip sealant (Biotium, USA) was used before slides were stored at -20°C. Slides were imaged on the Leica TCS SP5 confocal microscope (Leica, Germany) using imaging software LAS AF.
Primary astrocytes were also allowed to settle and grow in 6 well plates (for 48 hours before treatment). Cells were washed with PBS between each step of the experiment. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (and PBS as a negative control) was incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Cells were then lysed and the protein concentration of each sample was measured using a BCA assay. 40 μg of total protein/well was loaded onto an SDS page gel. Equal loading was confirmed by examining total protein per lane and measuring actin levels across all samples using an anti-actin antibody. Western blots were carried out incubating blots for 1 hour with SA-Alexa488 (1:500) (Chemicon, Australia) to detect biotinylated aggregated SOD1\textsuperscript{G93A}.

4.2.4 Inhibition and detection of macropinocytosis

Primary astrocytes were grown on chamber slides (Lab Tek, Australia) for 48 hours in DMEM with 10% FCS and pen-strep (Invitrogen, Australia). Cells were washed with PBS between each step of the experiment. Inhibitors genistein (gen) (185 μM), methyl-beta-cyclodextrin (MβCD) (5mM), chlorpromazine (CPZ) (5μM) or 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (100μM) were added to the astrocytes for 30 minutes at 37°C. Biotinylated aggregated SOD1\textsuperscript{G93A} (100μg/mL) was added for 30 minutes at 37°C. Cells were then fixed with 4% PFA for 15 minutes at room temperature. To permeabilise the cell membrane 0.5% triton X-100 in PBS was incubated with the cells for 20 minutes at 4°C. A solution of 5% BSA in PBS was used to block non-specific binding of antibodies. To probe for the biotinylated aggregated SOD1, SA-Alexa 488
(1:500) was incubated with the cells for 60 minutes at 4°C. Treated primary astrocytes were then imaged on the Leica TCS SP5 confocal microscope (Leica, Germany) using imaging software LAS AF.

4.2.5 Detecting the cellular location of aggregated SOD1

Primary astrocytes were grown on coverslips for 48 hours in phenol free DMEM with 10% FCS and pen-strep (Invitrogen, Australia). Cells were washed with PBS between each step of the experiment. The astrocytes were incubated with biotinylated aggregated SOD1<sub>G93A</sub> for 2 hours at 37°C. Cells were then fixed using PFA 4% for 20 minutes at room temperature. To permeabilise all cell membranes, cells were incubated with 0.5% triton X-100 in PBS for 30 minutes at 4°C. Alternatively, to selectively permeabilise only the outer cell membrane, the cells were treated with 10 μM Digitonin (Holmuhamedov and Lemasters 2009) in PBS for 10 minutes at 4°C (Figure 4.2). A solution of 5% BSA in PBS was used to block non-specific binding of antibodies. To probe for the biotinylated aggregated SOD1, SA-Alexa 488 (1:500) was incubated with the cells for 60 minutes at 4°C. Treated primary astrocytes were then imaged on the Leica TCS SP5 confocal microscope (Leica, Germany) using imaging software LAS AF.
Figure 4.2: **Triton X-100 and digitonin permeabilisation of the cell membrane.** Triton X-100 permeabilises all cellular membranes allowing for detection of molecules in the cytosol and in membrane bound compartments, whereas digitonin selectively permeabilises only the plasma membrane allowing specific detection of cytosolic molecules (green spots vs grey spots). This can be used to determine the cellular location of molecules detected with fluorescently labelled antibodies (as marked on the schematic as green).

### 4.2.6 ER stress

Primary astrocytes were grown on chamber slides for 48 hours in DMEM with 10% FCS and pen strep (Invitrogen, Australia). Cells were washed with PBS between each step of the experiment. The astrocytes were transfected with a XBP-1 Venus plasmid, a reporter of ER stress, (2.5 μg/μL) using lipofectamine (Iwawaki *et al.* 2004) (Figure 4.3). The ER stress reporter contains Ire1 endoribonuclease splicing sites, which upon activation of IRE1, the recombinant XBP-1 mRNA is spliced and results in an in-frame translation of a venus fluorescent protein. Aggregated SOD1<sup>G93A</sup>, soluble SOD1<sup>G93A</sup>, soluble SOD1<sup>WT</sup> (and PBS as a negative control) were incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO<sub>2</sub> incubator at 37°C for 72 hours (protected from light). Cells were fixed with 4% PFA for 15 minutes at room and stored at 4°C for 24 hours before imaging on the Leica TCS SP5 confocal microscope (Leica, Germany) using imaging software LAS AF.
Figure 4.3: Measuring endoplasmic reticulum (ER) stress using the XBP-1 reporter plasmid. After ER stress occurs, inositol requiring-1 (IRE-1) becomes activated and splices the mRNA of XBP-1 producing a Venus fluorescent protein (Iwawaki et al. 2004).

To further test for ER stress, levels of BiP were examined after treatment of primary astrocytes with SOD1. An increase in levels BiP, from basal levels, is an established indicator of ER stress (Oslowski and Urano 2011). Primary astrocytes were allowed to settle and grow (using DMEM with 10% FCS and pen-strep) in 6 well plates for 48 hours before treatment. Cells were washed with PBS between each step of the experiment. Aggregated SOD1G93A, soluble SOD1G93A, soluble SOD1WT (and PBS as a negative control) were incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO2 incubator at 37°C for 72 hours. Cells were then lysed and the protein concentration of each sample was measured using a BCA assay. 40 μg of total protein/well was loaded onto an SDS page gel. Equal loading was confirmed by examining total protein per lane and measuring actin levels across all samples using anti β-actin (1:1000). Western blots were carried out incubating blots overnight at 4°C with rabbit anti-BiP and for 1 hour with goat anti rabbit Alexa HRP (1:500) (Abcam, Australia).
4.2.7 Astrocyte Senescence

Initially, to test for astrocyte senescence, cells were probed using a novel antibody recognising a cell surface antigen increased on senescent cells (N. Cashman, Patent pending). Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (50 μM H\textsubscript{2}O\textsubscript{2} as a positive control and PBS as a negative control) were incubated with primary astrocytes for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Cells were fixed before probing for senescent cells using AMF-1c-120 a rabbit mAb (1:1000) (Courtesty of Neil Cashman) and an anti-rabbit Alexa 488 Ab (1:500) for 60 minutes at 4°C. Confocal microscopy and analysis using image J was used to obtain the average mean fluorescence intensity across triplicates.

To confirm astrocyte senescence a commercial β-Galactosidase staining kit was used (Genesearch, Australia). Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (50 μM H\textsubscript{2}O\textsubscript{2} as a positive control and PBS as a negative control) were incubated with primary astrocytes for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Cells were fixed and stained using the commercial β-Galactosidase staining kit. Light microscope images of senescent astrocytes were taken for each treatment. The absorbance of the 96 well plate was then was read at 490 nm using a Spectra Max Plus 384 spectrophotometer to obtain averages for 6 replicates (per treatment) of at least two independent experiments.
4.2.8 Astrocyte cell death

Primary astrocytes were grown on chamber slides (company) for 48 hours in DMEM with 10% FCS and pen strep (Invitrogen, Australia). Cells were washed with PBS between each step of the experiment. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (and PBS as a negative control) were incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Cells were then treated as per the Image-iT Live Red Caspase Detection Kit protocol (Invitrogen, Australia). Sytox green was used to stain nucleus and Flica reagent was used to detect caspase activation.

In addition, primary astrocytes were allowed to settle and grow (using DMEM with 10% FCS and pen strep) in 6 well plates for 48 hours before treatment. Cells were washed with PBS between each step of the experiment. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, (and PBS as a negative control) were incubated with the cells for 2 hours at 37°C. Media (phenol red free) was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Ethanol was added to cells and allowed to dry before media was replaced (positive control for cell death with the highest reading designated as 100% cell death). Propidium iodide (3 μM) was added to the cells before determining the level of fluorescence (485 nm excitation, 620 nm emission) on the FLUOstar OPTIMA plate reader (BMG Labtech).
4.2.9 Statistics

Data is expressed as mean +/- SEM. Statistical significance was evaluated with either an unpaired t-test, or a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test using Prism 5.0 GraphPad Software (San Diego, CA). A value of P < 0.05 was considered significant.
4.3 Results

4.3.1 Astrocytes take up aggregated SOD1

Given previous evidence that SOD1 inclusions can be found in astrocytes near to motor neurons (Hovden et al. 2013) and that wild type transplanted astrocytes take up aggregates in SOD1 mice (Lepore et al. 2008) the internalisation of aggregated SOD1\textsuperscript{G93A} in primary astrocytes was examined. An astrocyte culture of high purity was confirmed (Figure 4.4 and 4.5) with a consistent lack of, or very little CD11b reactivity. Using confocal microscopy it was observed, that incubation of aggregated SOD1\textsuperscript{G93A} (40 μg/mL) resulted in its association with GFAP positive cells in foci not present in PBS treated controls (Figure 4.6). Additional experiments also demonstrated that SOD1\textsuperscript{G93A} protein aggregates stimulate a small but significant increase in GFAP when added to astrocyte cultures (Figure 4.7). To confirm that the aggregates had entered the cell, rather than bound to the cell surface, a cross sectional image of primary astrocytes containing SOD1 aggregates was obtained. Aggregated SOD1\textsuperscript{G93A} can be seen within the boundary of the cell membrane, as determined by transmission images, both in the xz and yz cross section images (Figure 4.8).

In order to better quantify internalisation of aggregated mutant SOD1 the mean fluorescence intensity of individual cells was calculated. Astrocyte cultures treated with aggregated SOD1\textsuperscript{G93A} showed significantly greater mean fluorescence intensity compared to astrocytes treated with PBS (Figure 4.9). While the amount of fluorescence varied substantially in the aggregate treated cultures, the background fluorescence in PBS treated samples showed little variation. This observation is consistent with i) astrocytes taking up variable amounts of aggregated SOD1 and ii)
with the fact that not all astrocytes took up the aggregated material. To confirm internalisation western blot analysis on primary astrocyte cell lysates, previously treated with aggregated biotinylated SOD1\textsuperscript{G93A}, was carried out. Western blots showed a smear greater than 150 kDa and some large aggregate trapped in the stacking gel (Figure 4.10) in treated but not control lysates. When quantified, the density of the band in the aggregate samples was found to be significantly greater than the control sample (Figure 4.10). Some endogenous biotin signal can be observed in the control sample and the aggregated biotinylated SOD1\textsuperscript{G93A} sample at 120 kDa and was considered background biotinylated protein (Figure 4.10) (Tytgat et al. 2015).

Figure 4.4: **Statistics of scanning confocal micrographs determining purity of cultures by staining for microglia.** Primary cultures were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Less than 5% of cells have Iba1 staining in purified astrocyte cultures compared to 50-85% staining in mixed primary cultures.
Figure 4.5: **Scanning confocal micrographs determining purity of cultures by staining for microglia.** Primary astrocytes and EOC.13 microglia were fixed then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250).

Figure 4.6: **Scanning confocal micrographs of primary astrocyte internalisation of aggregated SOD1\(^{G93A}\).** Cells were incubated with 40 \(\mu\)g/mL of aggregated SOD1\(^{G93A}\) for 2 hours at 37°C. After 2 hours, cells were fixed and permeabilised before probing for Aggregated SOD1\(^{G93A}\) (SA-Alexa 488) as indicated in green and glial fibrillary acidic protein (Anti- rabbit Alexa 633) staining indicated by the red colour. White dotted lines represent cell boundary as determined by transmission images.
Figure 4.7: **Statistics of scanning confocal micrographs to determine the level of GFAP fluorescence between treatments.** Aggregated SOD1<sup>G93A</sup>, H<sub>2</sub>O<sub>2</sub> or PBS, was added to primary astrocytes for 72 hours at 37°C in a CO2 incubator. Astrocytes cells were then permeabilised before probing for the astrocyte marker GFAP using a rabbit GFAP antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are mean fluorescence (AU) ± standard error. * denotes significant difference.

![Graph showing statistics of scanning confocal micrographs](image)

Figure 4.8: **Cross section of primary astrocytes showing the internalisation of aggregated SOD1.** The astrocytes were incubated with aggregated SOD1<sup>G93A</sup> with a biotin label for 2 hours at 37°C. They were then fixed using PFA 4%. A solution of 5% BSA in PBS was used to block non-specific binding of antibodies. To probe for the biotinylated aggregated SA-Alexa 488 (1:500) was incubated with the cells. Confocal z stack was used to obtain a cross section of primary astrocytes. Red dotted lines represent cell boundary as determined by transmission images.

![Cross section of primary astrocytes](image)
Figure 4.9: **Internalisation of biotinylated aggregated SOD1$^{G93A}$ by murine primary astrocytes.** The astrocytes were incubated with aggregated SOD1$^{G93A}$ with a biotin label for 2 hours at 37°C. They were then fixed using PFA 4%. To permeabilise all cell membranes 0.5% triton X-100 in PBS was incubated with the cells for 20 minutes. A solution of 5% BSA in PBS was used to block non-specific binding of antibodies. To probe for the biotinylated aggregated SOD1$^{G93A}$, SA-Alexa 488 (1:500) was incubated with the cells. Confocal microscopy and analysis using Image J was used to obtain the average mean fluorescence intensity *** denotes significant difference between aggregated SOD1$^{G93A}$ treatment and the negative control.

Figure 4.10: **Western blot of cell lysates from primary astrocytes incubated in the presence of SOD1$^{G93A}$ aggregates.** Primary astrocytes were incubated with 40 μg/mL of biotinylated SOD1$^{G93A}$ for 30 minutes at 37°C. Cells were then removed using trypsin and washed before being lysed on ice for 30 min in 0.1% SDS Tris NaCl lysis buffer. Lysates were then analysed by western blot and biotinylated aggregated SOD1$^{G93A}$ was detected using a streptavidin HRP. Actin was used as a loading control and density was determined through image j analysis. Results are means ± standard error of triplicates and representative of at least two independent experiments. *Denotes statistically significant increase when compared with the negative control (PBS) ($P < 0.01$). Arrow indicates background endogenous biotin signal in cultures.
4.3.2 Clatherin independent macropinocytosis of aggregated SOD1\textsuperscript{G93A} in primary astrocytes

To further characterise the internalisation of aggregated SOD1\textsuperscript{G93A} into primary astrocytes a small panel of inhibitors of endocytosis were used. Primary astrocytes were incubated with either Gen, M\textbeta\textsuperscript{CD}, CPZ or EIPA which are inhibitors of caveolae mediated endocytosis, lipid raft formation, clatherin mediated endocytosis and macropinocytosis respectively (Zeineddine \textit{et al.} 2015). There was no significant difference between aggregate internalisation when incubated alone or in the presence of M\textbeta\textsuperscript{CD}, Gen or CPZ (Figure 4.11). However, internalisation of aggregated SOD1\textsuperscript{G93A} into primary astrocytes was significantly inhibited by incubation with EIPA \(P<0.001\) (Figure 4.11). However, inhibition of uptake with EIPA did not totally block SOD1 interaction with the cells surface. In primary astrocytes treated with EIPA aggregated SOD1\textsuperscript{G93A} can be seen on the cell surface but it is not internalised (Figure 4.12).
Figure 4.11: Inhibition of aggregated SOD1\textsuperscript{G93A} clatherin independent macropinocytosis in primary astrocytes. Primary astrocytes were isolated from murine mixed glial cultures, before being treated with inhibitors (CPZ, EIPA, Gen and MβCD) and then incubated with 40 μg/mL biotinylated aggregated SOD1\textsuperscript{G93A} to investigate internalisation. Cells were fixed and permeabilised before probing for Aggregated SOD1\textsuperscript{G93A} (SA-Alexa 488). Results are means ± standard error of triplicates and representative of at least two independent experiments. *Denotes statistically significant increase when compared with wild type SOD1-treated cells (P < 0.001).
Figure 4.12: **Cross section of primary astrocytes treated with inhibitors of endocytosis and incubated with aggregated SOD1G93A.** Astrocyte cultures were incubated with inhibitors of endocytosis (EIPA, MβCD, CPZ, Gen or PBS for Agg positive control) for 30 minutes before the addition of aggregated SOD1G93A with a biotin label and a further 1 hour incubation at 37°C. They were then fixed using PFA 4%. A solution of 5% BSA in PBS was used to block unspecific binding of antibodies. To probe for the biotinylated aggregated SA-Alexa 488 (1:500) was used. Confocal z stack was used to obtain a cross section of primary astrocytes.

### 4.3.3 Cytokine release

Given the association of astrogliosis with ALS (Sekizawa *et al.* 1998, Rentzos *et al.* 2010a, Rentzos *et al.* 2010b) and our observation that astrocytes can take up SOD1 aggregates resulting in small increases in GFAP, we next looked to see if SOD1 aggregates would induce cytokine release associated with ALS. IL-6 and MCP-1 are two immunologically important indicators of astrocyte activation and also important ALS inflammatory mediators (Sekizawa *et al.* 1998, Nagata *et al.* 2007). To examine
the effect of exogenous aggregated SOD1 on astrocyte activation, IL-6 and MCP-1 levels were examined using commercial ELISA kits. In the presence of aggregated SOD1, primary astrocyte secretion of IL-6 and MCP-1 did not significantly increase above basal levels after 72 hours (Figure 4.13).

Figure 4.13: Aggregated SOD1 does not cause IL-6 or MCP-1 cytokine release when incubated with primary murine astrocytes. Aggregated SOD1\textsuperscript{G93A} and PBS (as a negative control) were incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\(_2\) incubator at 37°C for 72 hours. MCP-1 and IL-6 release was measured using a company ELISA kit with commercial standards (ebiosciences, USA). No significant difference (NS).

4.3.4 Accumulation over time and cellular localization of aggregated SOD1\textsuperscript{G93A}

To determine the fate of aggregated SOD1\textsuperscript{G93A} internalised by astrocytes a time course assay was used and SOD1 aggregated associated fluorescence was analysed via confocal microscopy. Aggregated SOD1\textsuperscript{G93A} was shown to remain within primary astrocytes for up to 168 hours after aggregates were first added, without apparent significant reduction in fluorescence or average size of the particle (Figure 4.14). To examine where aggregated SOD1\textsuperscript{G93A} was located within primary astrocytes a selective permeabilisation experiment was performed. While triton X-100
permeabilises all cellular membranes digitonin only permeabilises the outer cell membrane (Nizard et al. 2007). While triton X-100 would allow the detection of SOD1 aggregates in the cytosol and within membrane bound compartments such as endosomes or lysosomes, digitonin will only permeabilise the plasma membrane allowing detection of SOD1 aggregates, using SA-Alexa 488 in the cytosol. Given that fluorescent particles were observed after digitonin permeabilisation in addition to those cells treated with triton X-100, we conclude that aggregated SOD1\textsuperscript{G93A} can be found present in the cytosol of primary astrocytes after uptake (Figure 4.15).
Figure 4.14: **Uptake of aggregated SOD1$^{G93A}$ overtime.** The astrocytes were incubated with aggregated SOD1$^{G93A}$ with a biotin label for 2 hours at 37°C. Media was then replaced and cells incubated in a CO$_2$ incubator at 37°C for a total of 24, 48, 72, 96, 120, 144 and 168 hours. They were then fixed using PFA 4% for 20 minutes at room temperature. To permeabilise all cell membranes 0.5% triton X-100 in PBS was incubated with the cells for 30 minutes at 4°C. A solution of 5% BSA in PBS was used to block non-specific binding. To probe for the biotinylated aggregated SOD1$^{G93A}$, SA-Alexa 488 (1:500) was incubated with the cells for 60 minutes at 4°C.
4.3.5 Aggregated mutant SOD1 induces ER stress in primary astrocytes

Previous work has suggested that internalisation of SOD1 aggregates in to neuroblastoma cells causes ER stress leading to cellular dysfunction and death (Sundaramoorthy et al. 2013). To examine if internalisation of aggregated SOD1 leads to ER stress in primary astrocytes, cells were transfected with the ER stress reporter XBP1-venus. Treatment of primary astrocyte cultures with aggregated SOD1$^{G93A}$ lead to a statistically significant increase in venus fluorescence, as did cells treated with a known ER stress inducing agent thapsigargin, in comparison to the PBS only control.
Addition of soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} did not induce venus expression above that of the PBS control (Figure 4.16), indicating soluble forms of SOD1 do not induce ER stress. Another marker of UPR induced ER stress is the increase in abundance of the ER Chaperone Bip (Olsowski and Urano 2011). Addition of aggregated SOD1, but not soluble mutant or wild type SOD1, significantly increased the levels of Bip when compared to the controls (Figure 4.17).

Figure 4.16: Aggregated SOD1\textsuperscript{G93A} results in endoplasmic reticulum stress in primary astrocytes. XBP-1 splicing (indicated here by green fluorescence) was used to measure endoplasmic stress induced UPR. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (and PBS as a negative control) was incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours (protected from light). Cells were fixed with 4% PFA for 15 minutes at room and stored at 4°C for 24 hours before imaging on the confocal microscope. *** Denotes statistically significant difference P<0.001.
Figure 4.17: **UPR induced Endoplasmic reticulum stress after treatment with aggregated SOD1\textsuperscript{G93A}.** Western blot of cell lysates from primary astrocytes incubated in the presence of 40 ug/mL SOD1\textsuperscript{G93A} aggregates (Agg), soluble SOD1\textsuperscript{G93A} (G93A) and soluble wild type SOD1 (WT) for 72 hours (thapsigargin (Thaps) used as a positive control and PBS as a negative control). Blots were probed for the ER stress marker binding immunoglobulin protein (BiP) and β-actin was used as a loading control. A) Representative western blot for BiP (n= 6 replicates) and β-actin as a loading control. B) Representative SDS PAGE gel (n= 6 replicates) showing whole cell lysates from primary astrocyte treatments C) Results are means ± standard error of 6 replicates and representative of at least two independent experiments with 6 separate western blots performed with PBS band density across each western blot given the density of 1 which all other results were compared to in that western. Then all 6 were compared.
Band density was normalised against PBS across the different western blots. * Denotes statistically significant difference $P<0.05$.

4.3.6 Aggregated mutant SOD1 induces senescence in primary astrocytes

Astrocyte senescence has been shown to be accelerated in rat models for ALS and other neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease (Bhat et al. 2012, Chinta et al. 2013, Das and Svendsen 2015). To determine if aggregates of mutant SOD1 could induce senescence in primary astrocytes taken from neonatal mice two methods were used. Initially, a novel antibody recognising a cell surface antigen associated with senescent cells was used (N. Cashman, Patent pending) (Figure 4.18). Only when primary astrocyte cultures were treated with aggregated SOD1$^{G93A}$, not soluble mutant or wildtype SOD1, was there an increase in staining, consistent with aggregates inducing senescence. To confirm that the antibody was detecting senescent cells, astrocyte cultures were treated with H$_2$O$_2$ to induce senescence (Bitto et al. 2010). To corroborate these findings a commercially available β-Galactosidase kit that detects senescence was used. Primary astrocytes were also shown to exhibit increased levels of β-Galactosidase when treated with aggregated SOD1$^{G93A}$ and H$_2$O$_2$ when compare to controls (Figure 4.19).
Figure 4.18: Scanning confocal micrographs of AMF-1c-120 staining in primary astrocytes treated with SOD1. **A** Aggregated SOD1<sup>G93A</sup>, soluble SOD1<sup>G93A</sup>, soluble SOD1<sup>WT</sup> (50μM H<sub>2</sub>O<sub>2</sub> as a positive control and PBS as a negative control) was incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO<sub>2</sub> incubator at 37°C for 72 hours. Cells were fixed before probing for senescent cells using AMF-1c-120 a rabbit mAb (1:1000) and an anti-rabbit Alexa 488 ab (1:500) for 60 minutes at 4°C. **B** Confocal microscopy and analysis using Image J was used to obtain the average mean fluorescence intensity per cell. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes significant difference p<0.05.
Figure 4.19: Activation of senescence in primary astrocytes treated with aggregated SOD1. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{WT} (50\,\mu M H\textsubscript{2}O\textsubscript{2} as a positive control and PBS as a negative control) was incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Cells were fixed and stained using a commercial β-Galactosidase staining kit. A) Light microscope images of senescent astrocytes in each treatment. B) The 96 well plate was incubated at 37°C for 24 hours before the absorbance was read at 490 nm using a Spectra Max Plus 384 spectrophotometer. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference p<0.05.
4.3.7 Aggregated mutant SOD1 results in an increase in Cell death

Given that exogenously applied aggregates were toxic to motor neuron like NSC-34 cells (Sundaramoorthy et al. 2013), we next tested whether uptake of aggregates affected astrocyte viability. Initially to determine if aggregated SOD1\textsuperscript{G93A} and/or soluble SOD1\textsuperscript{G93A} was toxic to astrocytes a propidium iodide (Pi) cell death assay was performed. After 72 hours the percentage cell death of astrocytes treated with aggregated SOD1\textsuperscript{G93A} was significantly greater than the negative control (Figure 4.20). In contrast soluble mutant SOD1 did not increase Pi staining from controls (Figure 4.20).

![Bar chart showing percentage cell death](image)

Figure 4.20: An increase in the percentage of cell death after treatment with aggregated SOD1\textsuperscript{G93A}. Aggregated SOD1\textsuperscript{G93A}, soluble SOD1\textsuperscript{G93A} (ethanol used as 100% cell death and PBS as a negative control) was incubated with the primary astrocytes for 2 hours at 37°C. Media was then replaced and cells incubated in a CO\textsubscript{2} incubator at 37°C for 72 hours. Results are means ± standard error of triplicates and representative of at least two independent experiments. * Denotes statistically significant difference p<0.05.

Due to the increase in cell death after treatment with aggregated SOD1\textsuperscript{G93A} we next examined caspase activation to determine if cell death occurred via apoptosis. To do this a fluorogenic caspase substrate was added to cells. Using confocal microscopy,
we observed substantial Flica fluorescence for both the aggregated SOD1$^{G93A}$ and soluble SOD1$^{G93A}$ treated astrocytes but no increase was detected for wildtype SOD1 (Figure 4.21).

Figure 4.21: Scanning confocal micrographs of activation of caspases 3 and 7 in primary astrocytes treated with SOD1. Aggregated SOD1$^{G93A}$, soluble SOD1$^{G93A}$, soluble SOD1$^{WT}$ (and PBS as a negative control) was incubated with the cells for 2 hours at 37°C. Media was then replaced and cells incubated in a CO$_2$ incubator at 37°C for 72 hours. Staining perform as per Image-iT Live Red Caspase Detection Kit protocol (Invitrogen). Sytox green was used to stain nucleus (green) and Flica reagent was used to detect caspase (seen in red). N=6 * Denotes statistically significant difference.
4.4 Discussion

There is mounting evidence from ALS patient studies (both most mortem tissue and live in vivo imaging) that both protein aggregates and glia play an active role in ALS disease pathogenesis (Pramatarova et al. 2001, Watanabe et al. 2001, Wang et al. 2002, Clement et al. 2003, Turner et al. 2004, Beers et al. 2006, Wang et al. 2008, Johnson et al. 2009, Fushimi et al. 2011, Guo et al. 2011). However, there has been very few studies that have tried to examine and link these two aspects of disease. Recent studies have shown that SOD1 aggregates in motor neurons can escape the intracellular environment during motor neuron death (Grad et al. 2014). Interaction between aggregates and microglia has been established not only in ALS (Roberts et al. 2013) but also in other neurodegenerative diseases, indicating that activation of non-neuronal cells by protein aggregates may play a role in disease progression (Le et al. 2001, Bamberger et al. 2003, Deane et al. 2003, Halle et al. 2008, Su et al. 2008, Reed-Geaghan et al. 2009, Murgas et al. 2012, Kim et al. 2013).

Here we investigated the effect of aggregated mutant SOD1 on astrocyte dysfunction and subsequent toxicity. We show for the first time, uptake of aggregated SOD1 causes astrocyte senescence and death. Uptake of aggregated mutant SOD1 can be inhibited by EIPA suggesting that endocytosis of aggregated mutant SOD1 by astrocytes occurs via clatherin independent macropinocytosis. We also showed for the first time, that internalisation of pre-aggregated SOD1G93A caused ER stress resulting in UPR induction in primary astrocytes. Aggregates were observed to be maintained in the cytosol of astrocytes for prolonged periods of time. Given prolonged ER stress is known to cause the activation of caspases and subsequent apoptotic cell death, the
findings presented here are consistent with the idea that aggregates released from
dead or dying neurons, could play a role in astrocyte dysfunction in ALS.

While previous studies have shown that SOD1 aggregates can escape the intracellular
environment (Münch et al. 2011, Grad et al. 2014), in the current work, SOD1
aggregates were made from recombinant protein to allow us to study the effects of
protein aggregates in isolation from the extracellular milieu. In order to visualise
internalisation of the aggregated protein a biotin label was added to the aggregates to
allow detection with streptavidin conjugates. Aggregated SOD1\textsuperscript{G93A} was found to
rapidly enter astrocytes within 2 hours. In contrast, after internalisation the aggregated
protein was shown to remain within cells for a least a week in astrocyte cultures. This
suggests that internalised aggregates of SOD1\textsuperscript{G93A} persist for an extended period of
time and astrocytes are not able to readily clear them.

In a previous study aggregated mutant SOD1 was shown to interact with scavenger
receptors and be internalised in a lipid raft dependent manner in microglia (Roberts
et al. 2013). In contrast to microglia, aggregated SOD1 internalisation in primary
astrocytes was found occur via clatherin independent macropinocytosis. Entry via
macropinocytosis is exploited by a range of pathogens including protozoa, bacteria
and viruses (Lim and Gleeson 2011). Interestingly, consistent with our results,
infectious prion proteins (and prion like proteins) have also been shown to utilise
macropinocytosis to gain entry into CHO cells (a cell line derived from a Chinese
hamster’s ovary), N2a cells (neuroblastoma cell line), HeLa cells (human
adenocarcinoma cell line) and NIH3T3 cells (mouse fibroblast cells) (Magzoub et al.
2006, Wadia et al. 2008, Forget et al. 2013). Aggregates of mutant SOD1 have also
been shown to activate micropinocytosis in neurons (Zeineddine et al. 2015).

The effect of internalised aggregated mutant SOD1 on astrocytes would at least
partly depend on its final location within the cell. The present study determined that
after internalisation aggregated SOD1$^{G93A}$ could be found in the cytosol. This is
consistent with the Alzheimer’s associated protein tau, which has been shown to
gain entry into cytoplasm of a neuronal precursor cell line and displace tubulin (Frost
et al. 2009). In a similar manner, the current study showed uptake of SOD1
aggregates and little overlap with GFAP suggesting displacement of cytoskeleton
elements. Previous work involving prion proteins showed that aggregated proteins in
the cytoplasm were able to perturb the cytosolic protein quality control pathway
(Chakrabarti et al. 2011). Further work is required to fully determine what the effect
of translocation of SOD1 aggregates into the cytosol has on cellular homeostasis.
However, due to previous evidence that showed aggregate uptake stimulated ER
stress in neuron-like NSC-34 cells (Sundaramoorthy et al. 2013), the present study
examined if SOD1 aggregates induced ER stress and subsequent UPR in primary
astrocytes.

Increased levels of ER stress markers (downstream products of the IRE1 ER stress
pathways) can be found in ALS patient and transgenic mutant SOD1 mice tissue (Atkin
et al. 2006, Atkin et al. 2008). Up regulation of the PERK pathway has recently been
reported to occur selectively in motor neurons (Sun et al. 2015). Research has also been able to show up regulation of PDI in microglia and in anterior horn astrocytes from mutant SOD1 transgenic mice, indicating that ER stress may also affect glial cells (Jaronen et al. 2013, Jaronen et al. 2014). These studies importantly suggest a link between ALS and ER stress but the cause of ER stress remains unclear. In the current study, addition of aggregated ALS linked mutant SOD1 caused increases in UPR markers consistent with ER stress in isolated primary astrocytes. We were able to utilise a XBP-1 plasmid that when spliced by IRE1 produces an in frame venus fluorescent protein, to monitor activation of UPR. Only aggregated SOD1G93A and not soluble forms of SOD1, induced UPR in treated primary astrocytes. In addition, the increased levels of BiP presented here, also indicated ER stress in astrocytes treated with aggregates. Consistent with our work, previously published experiments, instead looking at neuronal cells, showed ER stress after addition of aggregated SOD1 (Sundaramoorthy et al. 2013). Sudaramoorthy’s (et al. 2013) study reveals how aggregated SOD1 inhibits protein transport between ER-Golgi, causing Golgi fragmentation and ER stress, leading to the eventual cell death observed. Although the current study did not examine ER-Gogli transport, this will likely be an important avenue to follow up in future studies.

The initial response of the cell when the endoplasmic reticulum encounters misfolded proteins is to mount a protective response in an attempt to restore proteostasis (Korennykh et al. 2009). However, if homeostasis is not restored, the cell can activate a cell death signalling cascade due to prolonged activation of IRE1 and CHOP (Szegedi et al. 2006). ER stress induced apoptosis also activates calpain mediated
caspase 12 activation (Nakagawa and Yuan 2000). Our study showed that aggregated SOD1G93A, introduced to astrocytes in culture, lead to both the prolonged accumulation of biotinylated aggregates, ER stress, and an increase in the activation of caspases leading to an increase in cell death. While astrocytes are known to be dysfunctional and dying the mode of cell death is unknown.

In addition to cell death pathways, astrocytes have also been shown to initiate cellular senescence in response to various stresses (Bitto et al. 2010). The astrocyte senescence induced after the addition of aggregated SOD1 is consistent with β-galactosidase staining of astrocytes seen in spinal cord sections from mutant SOD1 rats (Das and Svendsen 2015). It is also consistent with growing evidence of astrocyte senescence observed in other neurodegenerative diseases including Parkinson’s and Alzheimer’s disease (Bhat et al. 2012, Chinta et al. 2013).

The increase in GFAP (reactive astrocyte phenotype) is also consistent with other studies looking at ALS and aging astrocytes in vitro (Díaz-Amarilla et al. 2011, Kohama et al. 1995, Morgan et al. 1997). The results presented here suggest that the senescent phenotype represents subpopulations of astrocytes in the cultures treated with aggregates. This increase in both senescence and GFAP has also been noted in end stage mutant SOD1G93A mice (Das and Svendsen 2015).
This is not the first time ER stress and senescence has been linked. This link has been found in an Ataxia telangiectasia disease model (another disease of the central nervous system that affects movement) (Liu et al. 2005). Our results overall are consistent with this previous research, with astrocyte cells showing markers of ER stress, with increased senescence and cell death. However, most experiments presented in this chapter were carried out after 72 hours incubation. It will therefore be important to determine whether or not senescence occurs prior to cell death in this experimental paradigm.

Previous work demonstrates the importance of non-neuronal cells in the pathogenesis of ALS (Alexianu et al., 2001; Hall et al., 1998; Henkel et al., 2006). Current thought is that there are fundamental differences underlying disease initiation and disease progression in ALS. This line of thought is based on results showing that mutant SOD1 was important in neurons for disease initiation and in glia for disease progression in a mouse model of ALS (Boilee et al., 2006). Although the role of protein aggregation in disease pathology has not been clarified, cells with SOD1 aggregates are more likely to undergo cell death in culture models (Matsumoto et al., 2005; Soo et al., 2009). The work presented here links these two observations and suggests that astrocytes that have taken up SOD1 aggregates experience ER stress, become senescent, and may undergo cell death, which would result in reduced trophic support for motor neurons (Figure 4.22). As a result, blocking SOD1 aggregates from causing astrocyte dysfunction may be effective in slowing ALS progression.
Figure 4.22: Proposed pathway of aggregated SOD1 induced astrocyte dysfunction and cell death. Neuronal cell death is associated with protein aggregation. Upon cell death or dysfunction, aggregates can be released and taken up by nearby astrocytes. SOD1 aggregates are taken up by astrocytes results in ER stress, senescence and cell death, potentially leading to reduced trophic support of motor neurons. A reduction in trophic support may leave motor neurons vulnerable to further protein aggregation and cell death and subsequent release of aggregates in a toxic feedback loop.
5. Aggregated mutant SOD1 activates microglia and induces astrocyte senescence when injected into the brains of mice
5.1 Introduction

Increasingly, the importance of aggregates and non-neuronal cells in neurodegenerative is being recognised and studied. There is growing evidence that in the presence of extracellular deposits or plaques there is a direct interaction between microglia and aggregated proteins resulting in activation (Mattiace et al. 1990). Aβ_{1-42} oligomers obtained from post-mortem tissue at low concentrations (5-50 nM) were demonstrated to activate microglia in a similar fashion to their in vitro-formed counterparts (Maezawa et al. 2011). Nitric Oxide was released from the microglial cells but an observed absence of many of the pro-inflammatory factors that are often associated with microglial activation, such as PGE₂, TNF-α, IL-6 and IL-1β was noted.

In AD post mortem brain tissue and in AD mouse models, activated microglia also accumulate in the areas of the brain harbouring impaired neurons and astrocytes (Busche et al. 2008, Kuchibhotla et al. 2009) and are found surrounding plaques (McGeer et al. 1987, Mattiace et al. 1990, Meyer-Luehmann et al. 2008).

Post-mortem brains of patients previously diagnosed with Parkinson’s disease, show the highest loss of dopaminergic neurons in the substantia nigra pars compacta (Agid 1991, Henry et al. 2009) an area which has previously been reported to have the highest density of microglial cells in the brain (Lawson et al. 1990). In Parkinson’s disease, activated microglia have been reported in brain tissue, surrounding dopaminergic neurons (McGeer and McGeer 2008, Nakadate and Tanaka-Nakadate 2015). When mice had α-synuclein aggregates injected into their brains, microglia were observed to take up the aggregates and become activated (Boza-Serrano et al. 2014).
A number of studies have now shown that microglia are activated and proliferate in ALS patients and ALS mice models (Lampson et al. 1988, Kawamata et al. 1992, Henkel et al. 2004, Turner et al. 2004, Moisse and Strong 2006). As the disease increases in severity, the activation of microglia has also been shown to increase (Turner et al. 2004, Henkel et al. 2009). Microglial activation is seen in mutant SOD1 transgenic mice before symptoms appear and throughout disease progression (Hall et al. 1998, Alexianu et al. 2001, Clement et al. 2003). Immunocytochemistry of postmortem tissue from ALS patients demonstrated microglial activation and indicated that an immune response may play a role in motor neuron death (Troost et al. 1993). Positron emission tomography imaging of living ALS patients, using \([^{11}C](R)\)-PK11195 and \([^{11}C]\)-PBR28 (with both radiotracers shown to bind specifically to activated microglia), showed an increase in activated microglia in the motor cortex, that correlates with upper motor neuron symptoms seen in patients (Banati 2002, Turner et al. 2004, Zürcher et al. 2015). This data is consistent with microglial activation playing an important role in disease progression.

A significant increase in astrocytic activation is seen in mutant SOD1 mice upon disease onset and throughout the early stages of disease progression (Hall et al. 1998). It has also been revealed that astrocytes from neonatal rat spinal cords, become reactive after incubation with CSF from ALS patients (Shahani et al. 1998). While culture studies show lowering SOD1 expression in astrocytes lowers their toxicity to motor neurons, diminished mutant SOD1 expression in astrocytes did not affect disease onset but delayed microglial activation and slowed disease progression.
(Yamanaka et al. 2008). This evidence suggests that a complex relationship could be occurring between SOD1, astrocytes and microglia in ALS to affect motor neurons.

There is increasing evidence that the misfolding of mutant SOD1 underlies disease pathology (Wang et al. 2002). However, the precise mechanism by which SOD1 mutants are toxic remains unclear. There are a number of mutations in SOD1 that have been shown to cause destabilisation of the structure of the protein, resulting in the mutant forms of SOD1 having a greater propensity to aggregate (Borchelt et al. 1994). Although there is substantial evidence of both SOD1 aggregates and activation of glial cells in patient pathology, very little research has focused on how SOD1 aggregates affect astrocytes and microglia when introduced directly \textit{in vivo}.

As astrocytes and microglia have been shown to play a role in ALS we aimed to determine if extracellular mutant SOD1 directly affected these cells when injected into the brain of wild type mice (wild type mice were used to reduce confounding effects of glial activation already existing in SOD1 transgenics). We found that while soluble WT or mutant infused in to the lateral ventricle of mice had no effect on glial staining even after 5 weeks of infusion, single injections of aggregated SOD1 resulted in robust changes in glia in the brain. Importantly, we find that this \textit{in vivo} data supported the \textit{in vitro} results of increased microglial activity and astrocyte senescence after the addition of aggregated SOD1$^{G93A}$.
5.2 Methods

5.2.1 Animal housing and monitoring

Mice were ordered from Animal Resource Centre and housed in the University of Wollongong animal facility for 1 week for acclimatization (AESOP001; AESOP002). After surgery mice were housed individually to avoid over grooming or chewing of implants from other mice.

Mice were monitored daily to check for any signs of distress from the surgery. Monitoring examined posture, activity, eating/drinking, gait, respiration, hydration, coat condition, grooming and surgical wound appearance.

5.2.2 Brain surgery for soluble infusions using an Alzet pump

Recombinant SOD1 was purified as previously outlined and LPS depleted using an LPS removal column (Chapter 2). It was determined that aggregated forms of SOD1 would clog the tubing in the Alzet pumps and was therefore injected in a subsequent experiment (see below). The protein was then infused over 5 weeks into the ventricle of the mice, while weight and motor function was monitored (kindly performed by Dr Brad Turner).

A scalpel was used to open the skin at the top of the skull, to allow ease of access and to minimise gluing of the skin to the skull. The location of the catheter and pump was calculated to bregma using the mouse/rat brain atlas (Paxinos G 2001). A drill
(Foredom, USA) designed for mouse surgery was used to drill a hole into the skull. The pump catheter was lowered into the brain before being raised 2 mm to allow the application of glue around the base of the Alzet pump (Dureck, US). The incision was sutured and a subcutaneous injection of an anti-inflammatory Meloxicam (APEX laboratories, Australia) was injected. A stock solution of the analgesic Metacam was made up at 100 μg/mL and the amount of Metacam used was determined for each mouse. In this treatment 8 wild type and 8 transgenic 60 day old mice were used. PBS, SOD1^{WT} and SOD1^{G93A} were added to the alzet pumps at 3.2 mg/mL and 250 μL was infused over 42 days. Therefore, the pumps infused at 0.15 μL/hour, with the endpoint being when mice were ~100 days old. An overview/timeline of this procedure can be seen in Table 5.2. Note however, that a number of PBS treated mice had to be euthanized for ethical reasons after 90 days and tissue was not collected. This meant that not enough replicates of the PBS control were available to do any meaningful statistical analysis. Given that infusion of the soluble wild type SOD1 was shown in previous experiments to have neuroprotective effects the experiment was continued and any effect of SOD1^{G93A} was judged on differences from SOD1^{WT} treatment.

5.2.3 Intracranial guide cannula surgery

For a more detailed methodology see previously published method (Engel et al. 2015). Briefly, the correct location for the guide cannula was calculated to bregma using the mouse/rat brain atlas (Paxinos G 2001). A drill (Foredom, USA) designed for mouse surgery was used to drill a hole into the skull. Three more shallow holes, which were used to secure the cannula, were drilled part way into the skull but not all the way.
through. A fine needle was then used to pierce through the delicate meningeal membranes before the guide cannula was inserted. It was then secured using the screws and the first layer of super glue. It was then further secured using dental cement and another layer of super glue. A dummy cannula to prevent entry of foreign debris was inserted and the mouse was removed from the stereotaxic device. The surgical area was treated with betadine (an antiseptic solution) and a subcutaneous injection of an anti-inflammatory Meloxicam (APEX laboratories, Australia) was given. A stock solution of the analgesic Metacam was made up at 100 μg/mL and 100 μL was introduced into mice using an intraperitoneal injection.

The animals were then monitored for any adverse signs to the surgery before being left to recover. For 1 week post-surgery mice were monitored daily as previously described.

Mice were allowed to recover from day 2 to 6. On day 7, 2 μL the recombinant protein aggregates (100 μg/mL) or PBS (vehicle control) were injected into the brains of mice using a small volume μL syringe. The dummy cannula was replaced after injections and mice monitored as previously described for 72 hours. An overview/timeline of this procedure can be seen in Table 5.1.
5.2.4 Tissue collection

Mice were humanely sacrificed using a slow-fill CO$_2$ inhalation technique to avoid unnecessary suffering (see AESOP008) and they were rapidly perfused transcardially with 0.9% saline and in some cases followed by ice-cold PBS- buffered 4% PFA, pH 7.4. Brains and spinal cords were rapidly removed from the animals for immunohistochemistry or biochemical analysis. For experiments where aggregates of SOD1 were injected into the ventricle, only brains were collected and analysed as aggregates are not diffusible structures. Spinal cords were analysed in the soluble SOD1 infusion experiments as it had previously been shown using SOD1$^{WT}$ that protein infused in to the ventricle reached the spinal cord in this time frame (Turner et al. 2005).
Table 5.1: **Summary of injection of SOD1\textsuperscript{G93A} aggregates into wildtype mice over a 5 week period.**

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Acclimatization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>Intracranial guide cannula surgery and monitoring of mice</td>
</tr>
</tbody>
</table>
| Week 3       | **Study**  
|              | Injection of protein and monitoring of mice |
| Week 3-5     | Tissue collected  
|              | Tissue sectioning  
|              | Tissue Staining  
|              | Confocal imaging |

Table 5.2: **Summary of infusion of soluble SOD1\textsuperscript{WT} and soluble SOD1\textsuperscript{G93A} protein into wild type mice over a 13 week period.**

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Acclimatization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>Alzet pump surgery and monitoring of mice</td>
</tr>
<tr>
<td>Week 3+9</td>
<td>Infusion of protein and monitoring of mice</td>
</tr>
</tbody>
</table>
| Week 10-13   | Tissue collected  
|              | Tissue sectioning  
|              | Tissue Staining  
|              | Confocal imaging |
5.2.4 Immunological analysis of brain sections

Due to injection of SOD1 aggregates into the ventricle of the mouse brain lateral sections examined were limited to the area of the brain surrounding the ventricle (Figure 5.2). Brains from mice were sectioned via a Leica cryostat at 20 μm per section and placed onto slides. Tissue sections were then thawed and a PAP pen was used to draw a hydrophobic barrier around the brain sections to contain solutions added to brain slices. A solution of 4% PFA in PBS was added to brain sections at room temperature for 15 minutes. The samples were then wash 3 times with PBS between each of the following steps. A permeabilisation solution (0.001% DMSO, 0.01% triton X-100, 0.02% BSA made up in PBS), followed by a 5% BSA was used in a blocking step. The primary antibodies; rabbit anti-Iba1 (1:250) (Wako, USA) used to identify microglia, rabbit anti GFAP (1:250) used to stain for astrocytes; AMF-1c-120 a rabbit mAb (Courtesy of Neil Cashman) (1:250) was used to probe for senescent cells, were incubated with the brain sections overnight in a humidified chamber at 4°C. Secondary detection antibodies; goat anti rabbit IgG Alexa 647 (1:250), was incubated for 1 hour after washing with PBS. Cover slips were added to slides using Mounting media (Prosci Tech, Australia) and CoverGrip coverslip sealant (Biotium, USA) was used before slides were stored at -20°C. Slides were imaged on the Leica TCS SP5 confocal microscope (Leica, Germany) using imaging software LAS AF.

To minimise tissue injury that may conceal aggregated dependant gliosis (due to gliosis caused by tissue injury from needle insertion) aggregates were injected into the ventricles. Due to the location of PBS and aggregated mutant SOD1 injections (and
the shorter 72 hour time frame before collection not allowing for anticipated infusion throughout the CNS as was the case with the Alzet pump experiments), the area around the ventricles were targeted for imaging using a tile scan and Z stack process.

Figure 5.2: Examples of the ventricle area targeted in the brains of mice injected with aggregated SOD1 (Paxinos G, 2001).
5.3 Results

5.3.1 Infusion of SOD1\textsuperscript{G93A} does not increase microglial activity when compared to SOD1\textsuperscript{WT} infusion

Previous studies showed that Alzet pump infusion of recombinant wild type SOD1 efficiently reached spinal cord neurons and provided some benefit to the G93A rat model of ALS (Turner \textit{et al.} 2005). In vitro studies have shown that soluble recombinant mutant SOD1 can activate microglia (Roberts \textit{et al.} 2013). Here we extended this work by infusing wild type and mutant SOD1 in to the lateral ventricle of mice to determine if soluble mutant SOD1 would activate microglia in spinal motor neurons (Roberts \textit{et al.} 2013). Microglial activity in the anterior horn was examined after a 5 week infusion with SOD1\textsuperscript{WT} and SOD1\textsuperscript{G93A} by staining for Iba-1 as used previously (Ahmed \textit{et al.} 2007). Given Iba-1 is present on resting microglia it can be observed in both SOD1\textsuperscript{WT} infused mice spinal cord tissue and in SOD1\textsuperscript{G93A} infused mice spinal cord tissue (Figure 5.3). However, when the level of Iba-1 staining was quantified there was no significant difference between samples taken from mice infused with SOD1\textsuperscript{G93A} and mice infused with SOD1\textsuperscript{WT} (Figure 5.4).

Microglia morphology in the anterior horn after infusion with SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} was also examined. Microglia (indicated by blue arrows) in the anterior horn of the spinal cord sections previously treated with either SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A}, were shown to have similar morphology (Figure 5.5).
Figure 5.3: Scanning confocal micrographs of microglial activity in SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} treated murine spinal cord tissue. ALZET pumps containing SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} were used to deliver treatments to wild type mice over a 5 week period and the spinal cord tissue harvested. Tissue sections were cut to 30 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Images shown are representative of 18 images from 6 individual mice. The anterior horn is outlined and indicated by (AH). Scale bars in blue are 50 μm.
Figure 5.4: Quantification of scanning confocal micrographs of Iba-1 stained microglia in SOD1\textsuperscript{G93A} or SOD1\textsuperscript{WT} treated murine spinal cord tissue. ALZET pumps containing SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} were used to deliver treatments to wild type mice over a 5 week period. Tissue sections were cut to 30\(\mu\)m sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error are representative of 6 individual mice (3 mice infused with SOD1\textsuperscript{WT} and 3 mice injected with SOD1\textsuperscript{G93A}). Results were not statistically significant different (NS).
Figure 5.5: Black and white confocal micrographs of microglial morphology in SOD1<sup>G93A</sup> and SOD1<sup>WT</sup> treated murine spinal cords. ALZET pumps containing SOD1<sup>WT</sup> or SOD1<sup>G93A</sup> were used to deliver treatments to wild type mice over a 5 week period and the spinal cord tissue harvested. Tissue sections were cut to 30 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Images shown are representative of 18 images from 6 individual mice. The anterior horn is outlined in red and indicated by (AH). Blue arrows point to microglia of interest.

5.3.2 Infusion of SOD1<sup>G93A</sup> does not result in increased Glial fibrillary Acidic Protein when compared to SOD1<sup>WT</sup> infusion

An increase in GFAP, an astrocyte marker (Eng 1985), has previously been reported in post-mortem spinal cord tissue from patients diagnosed with ALS (Fujita <i>et al.</i> 1998). In addition, results from the previous chapter indicate that when added to astrocyte cultures SOD1 protein aggregates stimulate a small but significant increase in GFAP (Chapter 4). Therefore, the abundance of GFAP in the anterior horn (where the motor neuron population is most prominent) was examined after a 5 week infusion with SOD1<sup>WT</sup> and SOD1<sup>G93A</sup>. GFAP staining can be observed in both the SOD1<sup>G93A</sup> infused mice spinal cord tissue and in SOD1<sup>WT</sup> infused mice spinal cord tissue (Figure 5.6).
However, upon quantification there was no significant difference in the level of GFAP staining between those samples taken from mice infused with SOD1\textsuperscript{G93A} and those from mice infused with SOD1\textsuperscript{WT} (Figure 5.7).

Figure 5.6: Scanning confocal micrographs of GFAP stained astrocytes in SOD1\textsuperscript{G93A} or SOD1\textsuperscript{WT} treated murine spinal cord tissue. ALZET pumps containing SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} were used to deliver treatments to wild type mice over a 5 week period and the spinal cord tissue harvested. Tissue sections were cut to 30 \( \mu \text{m} \) sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the astrocyte marker GFAP using a rabbit GFAP antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Images shown are representative of 18 images from 6 individual mice. The anterior horn is outlined and indicated by (AH). Scale bars in blue are 50 \( \mu \text{m} \).
Figure 5.7: **Quantification of scanning confocal micrographs of GFAP stained astrocytes in SOD1\textsuperscript{G93A} or SOD1\textsuperscript{WT} treated murine spinal cord tissue.** ALZET pumps containing SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} were used to deliver treatments to wild type mice over a 5 week period. Tissue sections were cut to 30 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error are representative of 6 individual mice (3 mice infused with SOD1\textsuperscript{WT} and 3 mice injected with SOD1\textsuperscript{G93A}). Results were not statistically significant different (NS).

5.3.3 Infusion of SOD1\textsuperscript{G93A} does not result in increased AMF-1c-120 staining when compared to SOD1\textsuperscript{WT} infusion

The previous chapter demonstrated that the level AMF-1c-120 staining was consistent with β-Galactosidase staining for senescence. AMF-1c-120 staining was therefore examined in the anterior horn after a 5 week infusion with SOD1\textsuperscript{WT} and SOD1\textsuperscript{G93A}. AMF-1c-120 is thought to recognise a cell surface antigen specific to senescent cells (N. Cashman, Patent pending). AMF-1c-120 staining can be observed in both SOD1\textsuperscript{G93A} infused mice spinal cord tissue and in SOD1\textsuperscript{WT} infused mice spinal cord tissue (Figure 5.8). Quantification of AMF-1c-120 staining indicated that there was no
significant difference in putative senescence staining between samples taken from mice infused with SOD1\textsuperscript{G93A} and mice infused with SOD1\textsuperscript{WT} (Figure 5.9).

Figure 5.8: **Scanning confocal micrographs of astrocyte senescence in SOD1\textsuperscript{G93A} or SOD1\textsuperscript{WT} treated murine spinal cord tissue.** ALZET pumps containing SOD1\textsuperscript{WT} or SOD1\textsuperscript{G93A} were used to deliver treatments to wild type mice over a 5 week period and the spinal cord tissue harvested. Tissue sections were cut to 30μm sections using a Leica cryostat. Tissue sections were then probed for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and an anti-rabbit Alexa 647 ab (1:250). Images shown are representative of 18 images from 6 individual mice. The anterior horn is outlined and indicated by (AH). Scale bars in blue are 50 μm.
Figure 5.9: Quantification of scanning confocal micrographs of astrocyte senescence activity in SOD1^{G93A} or SOD1^{WT} treated murine spinal cord tissue. ALZET pumps containing SOD1^{WT} or SOD1^{G93A} were used to deliver treatments to wild type mice over a 5 week period. Tissue sections were cut to 30μm sections using a Leica cryostat. Tissue sections were then probed for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error are representative of 6 individual mice (3 mice infused with SOD1^{WT} and 3 mice injected with SOD1^{G93A}). Results were not statistically significant different (NS).

5.3.4 Infusion of SOD1^{G93A} does not result in physical symptoms in mice

When determining deterioration of motor function and disease progression in mice, body weight and latency to fall are commonly measured (Carter et al. 1999, Hayworth and Gonzalez-Lima 2009). While these animals were not transgenic we nonetheless tested for any signs of changes in wellbeing during the infusion protocol. There was no significant difference over time within the group or between infusion of SOD1^{G93A} and SOD1^{WT} treatments when measuring latency to fall (Figure 5.10). All mice demonstrated a normal increase in weight as they grew older however there was no significant difference in body weight between treatments (Figure 5.11) (kindly...
performed by Dr Bradley Turner). This data suggests that the infusion of both WT and mutant forms of SOD1 are well tolerated in non-transgenic mice.

Figure 5.10: **Motor function of mice after infusion of PBS, soluble SOD1$^{WT}$ or soluble SOD1$^{G93A}$.** ALZET pumps containing PBS, SOD1$^{WT}$ or SOD1$^{G93A}$ were used to deliver treatments to both wild type and SOD1$^{G93A}$ expressing mice over a 5 week period. Latency to fall was used as an indicator of symptomatic mice (kindly performed by Dr Bradley Turner).

Figure 5.11: **Body weight of mice after infusion of PBS, soluble SOD1$^{WT}$ or soluble SOD1$^{G93A}$.** ALZET pumps containing either PBS, SOD1$^{WT}$ or SOD1$^{G93A}$ were used to deliver treatments to both wild type and SOD1$^{G93A}$ expressing mice over a 5 week period. Body weight was used as an indicator of symptomatic mice (kindly performed by Dr Bradley Turner).
5.3.5 Injecting aggregated SOD1$^{G93A}$ results in increased microglial activity

Given that SOD1 aggregates are several fold more potent activators of microglia we wanted to determine if these aggregates would activate microglia in vivo. Given that the aggregates were not in solution and are were likely to block the tubing in Alzet pumps a short-term single injection experiment was performed. Iba-1 staining around the ventricles was examined after injection with aggregated SOD1$^{G93A}$ compared with control animals injected with PBS. Quantification revealed that there was a significant increase (Figure 5.12) in iba-1 staining in mouse brains injected with aggregated SOD1$^{G93A}$ (Figure 5.13) compared to those injected with PBS (Figure 5.14). Confocal images show that the increase in the density of iba-1 staining (when compared to PBS controls) is concentrated around the ventricles of aggregated SOD1$^{G93A}$ treated mice, where there is an apparent increase in the density of Iba-1 staining when compared to PBS control microglia (Figure 5.13 and 5.14). The significant increase in iba-1 could also suggest an increase in the number of microglia although this could not be accurately quantified (i.e. too difficult to distinguish individual cells amongst all the cell processes).

Microglia morphology around the ventricles after injection with aggregated SOD1$^{G93A}$ was also examined. In tissue surrounding the ventricles of PBS treatment samples, microglia (indicated by blue arrows) have much smaller bodies with fine, hard to detect processes. In the aggregated SOD1$^{G93A}$ treatment samples, microglia (indicated by red arrows) have larger bodies with thick processes (Figure 5.15). The strong Iba-1 staining in the ependymal of aggregated SOD1$^{G93A}$ treatment samples is also striking (Figure 5.15).
Figure 5.12: Quantification of scanning confocal micrographs of microglial in aggregated SOD1\textsuperscript{G93A} or PBS treated murine brains. Mice were injected with aggregated SOD1\textsuperscript{G93A} or PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error of 72 individual images (see merged scanned images of ventricles Figure 5.13 and 5.14) and representative of 6 individual mice (3 mice injected with PBS and 3 mice injected with Aggregated SOD1\textsuperscript{G93A}). * Denotes statistically significant difference p<0.05.
Figure 5.13: Scanning confocal micrographs of Iba-1 stained microglia in aggregated SOD1G93A treated murine brains. Mice were injected with aggregated SOD1G93A, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V).
Figure 5.14: **Scanning confocal micrographs of Iba-1 stained microglia in PBS treated murine brains.** Mice were injected with PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V).
Figure 5.15: **Black and white confocal micrographs of microglial morphology in aggregated SOD1\(^{G93A}\) and PBS treated murine brains.** Mice were injected with aggregated SOD1\(^{G93A}\) or PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20\(\mu\)m sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the microglial marker Iba-1 using a rabbit Iba-1 antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). The border between brain and ventricle is outlined and the ventricle indicated by green dotted line. Some microglia of interest are indicated by blue and red arrows.
5.3.6 Injecting aggregated SOD1\textsuperscript{G93A} does not result in increased Glial fibrillary Acidic Protein Staining

Given the previously observed small increase in GFAP associated with adding SOD1 aggregates to astrocyte cultures, we sought to determine if introducing aggregated SOD1 into the brain of mice caused an increase in GFAP immunofluorescence. GFAP staining was imaged and quantified after injection with aggregated SOD1. Both aggregated SOD1\textsuperscript{G93A} (Figure 5.16) and PBS (figure 5.17) treated sections showed GFAP stained astrocytes surrounding the ventricles. However, there was no significant difference in GFAP staining surrounding the ventricles of mice injected with aggregated SOD1\textsuperscript{G93A} and mice injected with PBS alone (Figure 5.18).

![Figure 5.16: Quantification of scanning confocal micrographs of GFAP stained astrocytes in aggregated SOD1\textsuperscript{G93A} or PBS treated murine brains. Mice were injected with aggregated SOD1\textsuperscript{G93A} or PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20\textmu m sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the astrocyte marker GFAP using a rabbit GFAP antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error of 72 individual images (see merged scanned images of ventricles Figure 5.17 and 5.18) and representative of 6 individual mice (3 mice injected with PBS and 3 mice injected with Aggregated SOD1\textsuperscript{G93A}). NS denotes no significant difference.](image-url)
Figure 5.17: Scanning confocal micrographs of GFAP stained astrocytes in aggregated SOD1\textsuperscript{G93A} treated murine brains. Mice were injected with aggregated SOD1\textsuperscript{G93A}, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the astrocyte marker GFAP using a rabbit GFAP antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V). Scale bars in white are 200 μm and scale bars in blue are 50 μm.
Figure 5.18: **Scanning confocal micrographs of GFAP stained astrocytes in PBS treated murine brains.** Mice were injected with PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20μm sections using a Leica cryostat. Tissue sections were then permeabilised before probing for the astrocyte marker GFAP using a rabbit GFAP antibody (1:250) and a secondary anti rabbit Alexa 647 (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V). Scale bars in white are 200 μm and scale bars in blue are 50 μm.
5.3.7 Injecting aggregated SOD1\textsuperscript{G93A} increases AMF-1c-120 staining

Transgenic rats with the human mutant SOD1 gene have previously been shown to have an increased rate of astrocyte senescence (Das and Svendsen 2015). In addition, an increase in markers of astrocyte senescence has also been found in post-mortem tissue from patients diagnosed with Alzheimer’s disease (Bhat et al. 2012). Therefore, we hoped to determine if aggregated protein, in this case SOD1, found in patients with ALS, would cause astrocyte senescence in mice when introduced \textit{in vivo} using a novel antibody recognising a cell surface antigen specific to senescent cells (N. Cashman, Patent pending).

A significant increase (Figure 5.19) in immunodetection using AMF-1c-120 was observed in the brains of mice injected with aggregated SOD1\textsuperscript{G93A} (Figure 5.20) compared to those injected with PBS (Figure 5.21). The results in Chapter 4 are consistent with the idea that this antibody detects changes that correspond to astrocyte senescence, and \textit{in vivo} after aggregate injection it strongly stains the ependyma (consistent with GFAP staining) and the underlying white matter. This suggests that aggregate treatment increases detection of astrocytes using AMF-1c-120 suggesting astrocyte senescence \textit{in vivo}. We also noted that there is apparent intracellular staining of normal neurons using AMF-1c-120 outside of this region.
Figure 5.19: Statistics of scanning confocal micrographs of astrocytes stained for senescence marker (AMF-1c-120) in aggregated SOD1\textsuperscript{G93A} or PBS treated murine brains. Mice were injected with aggregated SOD1\textsuperscript{G93A} or PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then probed for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and a secondary anti rabbit Alexa 647 (1:250). Results are means ± standard error of 72 individual images (see merged scanned images of ventricles Figure 5.20 and 5.21) and representative of 6 individual mice (3 mice injected with PBS and 3 mice injected with Aggregated SOD1\textsuperscript{G93A}). * Denotes statistically significant difference P<0.05.
Figure 5.20: Scanning confocal micrographs of astrocytes stained for senescence marker (AMF-1c-120) in aggregated SOD1<sup>G93A</sup> treated murine brains. Mice were injected with aggregated SOD1<sup>G93A</sup>, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then probed for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and an anti-rabbit Alexa 647 ab (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V). Scale bars in white are 200 μm and scale bars in blue are 50 μm.
Figure 5.21: Scanning confocal micrographs of astrocytes stained for senescence marker (AMF-1c-120) in PBS treated murine brains. Mice were injected with PBS, monitored for 72 hours and the brain tissue harvested. Tissue sections were cut to 20 μm sections using a Leica cryostat. Tissue sections were then probed for astrocyte senescence using AMF-1c-120 a rabbit mAb (1:250) and an anti-rabbit Alexa 647 ab (1:250). The border between brain and ventricle is outlined and the ventricle indicated by (V). Scale bars in white are 200 μm and scale bars in blue are 50 μm.
5.4 Discussion

There is growing evidence from patient studies that both protein aggregates and glia have a role to play in ALS disease pathogenesis (Pramatarova et al. 2001, Watanabe et al. 2001, Wang et al. 2002, Clement et al. 2003, Turner et al. 2004, Beers et al. 2006, Wang et al. 2008, Johnson et al. 2009, Fushimi et al. 2011, Guo et al. 2011). However, there is a limited amount of in vivo evidence that examines and links these two aspects of disease. Recent studies have shown that SOD1 aggregates in motor neurons can escape the intracellular environment during motor neuron death providing a rationale for glial-aggregate interaction (Grad et al. 2014). Interaction between aggregates and glia has been established previously, not only in ALS (Roberts et al. 2013 and see Chapter 4), but also in other neurodegenerative diseases including Alzheimer’s and Parkinson’s disease (Le et al. 2001, Bamberger et al. 2003, Deane et al. 2003, Halle et al. 2008, Su et al. 2008, Reed-Geaghan et al. 2009, Murgas et al. 2012, Kim et al. 2013). In previous studies investigating other neurodegenerative diseases, the respective protein aggregates (e.g. Aβ_{1-42} and α-synuclein aggregates) have been injected into mice to discover the impact these aggregates had on glia (Maezawa et al. 2011, Boza-Serrano et al. 2014). However, similar studies have not previously been carried out to determine the effect introducing aggregated SOD1 has on astrocytes and microglia. Here we investigated the effect of aggregated mutant SOD1 on microglial activation and astrocyte function in vivo. We show for the first time, while soluble forms of SOD1 have no effect of glial markers, microglia activation and astrocyte dysfunction are significantly increased after exposure to aggregated mutant SOD1.
Aggregated SOD1\textsuperscript{G93A} was previously shown to potently activate both EOC.13 cells and primary microglia in vitro (Roberts \textit{et al.} 2013). In the current study, we injected aggregated SOD1\textsuperscript{G93A} into the central nervous system of mice to examine the response of microglia \textit{in vivo}. Consistent with our previous \textit{in vitro} work introducing aggregated SOD1\textsuperscript{G93A} increased microglia activity after just 72 hours exposure to the aggregates. Interestingly, the microglial morphology in tissue from the aggregated SOD1 treated brains was notably altered compared to the control brains. What has previously been classified as ramified non-reactive microglia (Kettenmann \textit{et al.} 2011, Hook \textit{et al.} 2015) (small bodies can be seen but the thin processes are difficult to see here) were observed in the PBS control treated brains. However, microglia from the brains of mice injected with aggregated SOD1 could be classified as hypertrophic microglia (large bodies think stumpy processes) (Kettenmann \textit{et al.} 2011, Hook \textit{et al.} 2015). These results are consistent with another aggregation associated animal model, in this case modelling Alzheimer’s disease (Stalder \textit{et al.} 1999). In this study they also found hypertrophic microglia in areas with dense amyloid plaques (Stalder \textit{et al.} 1999). Together, these results suggest that if aggregated SOD1 inclusions are released from neurons in ALS patients, the interaction of aggregates and microglia will result in their activation. Given the presence of microgliosis around degenerating neurons in ALS it is likely that at least some of the activation previously observed \textit{in vivo} (Turner \textit{et al.} 2004) is a result of glia-aggregate interactions.

While aggregated SOD1 added to primary astrocytes in culture resulted in small but significant increases in GFAP, there was no significant increase in GFAP
immunoreactivity in mice injected with aggregated SOD1. It is possible that this was due to differences in the experimental models. In the cultures of primary astrocytes, they are isolated from other cells, and the small effects seen in vitro that don’t translate to the in vivo work may be due to factors (such as NO or other signals) produced by astrocytes that will further activate other astrocytes in culture but that may be taken up by microglia or other cells in the three dimensional setting of the mouse brain (Brahmachari et al. 2006). In the complex environment of the brain there is a possible interplay between astrocytes and other cells, indeed the affinity of different cell types for aggregated SOD1 may mean that astrocytes receive a much lower dose compared to the controlled dose applied to enriched astrocyte cultures in in vitro experiments.

There was also no significant difference in GFAP staining in spinal cords of mice undergoing infusion of SOD1 over 5 weeks between mutant and wild type SOD1. This suggests that an increase in GFAP is not induced by the soluble mutant form or the aggregated form of the SOD1 protein. Previous work has reported an increase in GFAP in the anterior horn of post mortem spinal cord tissue from patients diagnosed with ALS (Fujita et al. 1998) the current work suggests that aggregates may not contribute to this signal. However, more recent evidence suggests that loss of GFAP could accelerate disease progression in a mutant SOD1 transgenic mouse model of ALS (Yoshii et al. 2011), the work presented here is consistent with the idea that uptake of aggregates from dead or dying neurons may contribute to astrocyte loss and thus disease progression in ALS.

There is growing evidence of astrocyte senescence observed in other neurodegenerative diseases including Parkinson’s and Alzheimer’s disease (Bhat et
al. 2012, Chinta et al. 2013). Here we show that aggregated SOD1 resulted in increased staining with AMF-1c-120, which in Chapter 4 correlated to astrocyte senescence. This is consistent with recently published results that showed β-galactosidase staining in spinal cord sections from mutant SOD1 rats (Das and Svendsen 2015). It is also consistent with our previous results in primary culture using the novel senescence antibody (N. Cashman, Patent pending) and β-galactosidase to detect senescence after the addition of aggregated SOD1.

While aggregated SOD1 affected glia in short term experiments, infusion of soluble forms of SOD1 did not. A statistical increase in microglial activity or astrocyte senescence could not be measured in spinal cord samples from mice infusion with SOD1G93A compared SOD1WT infused mice. This is inconsistent with work previously performed in primary microglia which showed a small but significant increase in microglial activity after the addition of soluble SOD1G93A (Zhao et al. 2010, Roberts et al. 2013). Microglia may be over activate in vitro and the small effects seen in vitro that don’t translate to the in vivo work may be due to inhibitory factors produced by astrocytes and neurons in mice brains (Neumann et al. 1998, Hoek et al. 2000, Chang et al. 2001, Haider et al. 2001). However, this lack of difference in both microglial activity or astrocyte senescence between in vivo samples is consistent with the lack of significant difference between treatments when measuring latency to fall and body weight.
Previous research has shown that mutant SOD1 was important in neurons for disease initiation and in glia for disease progression in a mouse model of ALS (Boillee et al., 2006). Although the role of protein aggregation in disease pathology has not been clarified, SOD1 aggregates have been shown to be toxic to the neurons when they accumulate in culture (Matsumoto et al., 2005; Soo et al., 2009). The results presented here, in both the in vitro and in vivo studies (Chapters 3, 4 and 5), show for the first time that introduction of aggregates of mutant SOD1 causes astrocyte senescence and microglial activation. This work provides a potential link between SOD1 aggregates and microglial activation and astrocyte dysfunction, and thus provides evidence that protein inclusions may also be linked to disease progression in a non-cell autonomous manner apart from neuron to neuron transmission observed in other studies (Grad et al. 2014, Ayers et al. 2016). As a result, in the future stopping SOD1 aggregates from causing microglial activation and astrocyte dysfunction may be effective in slowing ALS progression.
6. Conclusions and future directions
It is becoming increasingly acknowledged that protein aggregates and neuroinflammation have an important role to play in many neurodegenerative diseases (Hook et al. 2015). And although the presence of aggregates and neuroinflammation has been well documented in ALS the direct effect of these aggregates on microglia and astrocytes has not been closely examined in vitro or in vivo.

The specific aims of the study presented in this thesis were 1) to determine if ALS associated mutant SOD1 aggregates activate primary murine microglia, 2) to determine if ALS associated mutant SOD1 aggregates cause dysfunction in primary murine astrocytes, and 3) to establish if extracellular aggregated mutant SOD1 mediates microglial activation and astrocyte dysfunction in vivo. These aims were addressed in chapters 3, 4 and 5 respectively.

Microgliosis is now well documented in ALS, both in vitro and in transgenic mice models and more importantly in vivo in ALS patients (Lampson et al. 1988, Kawamata et al. 1992, Henkel et al. 2004, Turner et al. 2004, Moisse and Strong 2006). In ALS models, microglia have previously been shown to exhibit neurotoxic phenotype markers such as increased levels of TNF-α and IL-23 (Poloni et al. 2000, Rentzos et al. 2010a). This neurotoxic microgliosis would put already stressed motor neurons under increased strain and potentially contribute to ALS progression through the release of neurotoxic factors and potentially toxic aggregates from microglia (Figure 6.1) (Appel et al. 2011). Protein inclusions made of SOD1 (and other proteins) in ALS post mortem tissue has also been well-established and a propagation of protein
aggregates and neuropathology has been proposed (Shibata et al. 1996, Forsberg et al. 2011).

Figure 6.1: Representation of the possible role that aggregates play in ALS. SOD1 aggregates are taken up by microglia causing microglia to switch to a neurotoxic phenotype. SOD1 aggregates taken up by astrocytes causing astrocyte; ER stress, senescence and cell death, potentially leading to reduced trophic support of motor neurons, motor neuron cell death and release of aggregates. Aggregates represented in black, neurotoxic microglia in green, senescent astrocytes in yellow and motor neurons in blue.
Therefore, as both protein inclusions and microgliosis are associated with ALS, the first aim of this project sought to determine if aggregated SOD1 would activate microglia. Recombinant SOD1 was aggregated and this, or soluble non-aggregated forms of SOD1 were then added to EOC.13 microglial cells or primary microglial cells in culture. Although soluble non-aggregated mutant SOD1 had been shown to promote microglial activation in past studies (Zhao et al. 2010), we for the first time found that aggregated SOD1 was able to much more efficiently activate microglia in culture when compared with the unaggregated form of mutant SOD1. In addition to CD14 dependant pathways, we were able to show that aggregated SOD1 also bound to the surface of glial cells and was internalized in a lipid raft and scavenger receptor dependent manner. Identifying the precise mechanism by which aggregated SOD1 activates microglia to produce a neurotoxic phenotype is an important step in understanding how microgliosis in ALS, results in increased stress to already stressed and dying neurons (Figure 6.1).

Mutant Cu/Zn superoxide dismutase (SOD1) has previously been shown to reduce the expression of glutamate transporter 1 in astrocytes, likely resulting ultimately, in excitotoxicity for surrounding neurons (Ferraiuolo et al. 2011). Dysfunctional astrocytes could also affect the uptake and release of lactate, ATP, glycine adenosine and various growth factors (Figure 6.1) (Sofroniew and Vinters 2010). Like the aforementioned microglia from post mortem tissue, the presence of aggregates is similarly seen in astrocytes from ALS patients (Shibata et al. 1996, Kato et al. 2000, Forsberg et al. 2011). As protein inclusions and astrocyte dysfunction are thought to be an important part of ALS, the second aim of this project sought to determine if
exogenous aggregated mutant SOD1 would induce astrocyte dysfunction in vitro. Recombinant mutant SOD1\textsuperscript{G93A} was aggregated and subsequently added to primary astrocytes in culture. The results showed that exogenously added aggregated mutant SOD1\textsuperscript{G93A} is taken up into the cytosol of astrocytes, sustained for an extended period of time, causing endoplasmic reticulum stress, astrocyte senescence and ultimately cell death. This work, for the first time has shown that exogenously aggregated mutant SOD1 causes dysfunction and toxicity in primary astrocytes. These results suggest that there may be a potential link between protein aggregation and astrocytic dysfunction in ALS.

The work presented here also showed, with the use of a panel of endocytosis inhibitors, that aggregated SOD1 was internalized by primary astrocytes via macropinocytosis. As mentioned previously with the mode of entry into microglia, the mode of entry of aggregated SOD1 into astrocytes could also represent a target for future drugs in the treatment of ALS. As microglia, astrocytes, motor neurons and possibly other cell types are able to take up aggregates, the search for a treatment based on cellular uptake might prove difficult. Although, one might imagine that modulating aggregate uptake may be possible given that microglia use phagocytosis and neurons/astrocytes use micropinocytosis. Given it is detrimental to both neurons (Matsumoto et al. 2005) and astrocytes to take up SOD1 aggregates it might be possible to divert aggregates to microglia by using micropinocytosis inhibitors. However, this would potentially increase microgliosis so it would have to be delivered in combination with a therapeutic strategy to alter the microglia phenotype from M1 towards M2.
As microglia and astrocytes have been shown to play a key role in the healthy functioning of the central nervous system, the third aim of this thesis was to gain a better understanding of the impact that aggregated SOD1 and soluble SOD1 has on microglia and astrocytes in vivo. Here we report that soluble SOD1^{G93A} infused over 5 weeks into wild type mice, resulted in no significant impact on anterior horn microglia and astrocytes, when compared to soluble SOD1^{WT} infusion. Whereas, 72 hours after injection aggregated SOD1 caused increased microglial activity and astrocyte senescence surrounding ventricles. One might predict that infusion of aggregated SOD1 over five weeks would more accurately simulate the prolonged progression what occurs in transgenic mutant mice (and hence more closely simulate disease progression in ALS patients). We would have liked to do a similar infusion of the aggregated in this manner, however, this would have clogged the Alzet pumps and therefore an alternative short-term experiment was performed with direct injections via the intracranial guide cannula. This method was used to limit the local inflammation due to injection as the implantation of the cannula allowed inflammatory markers time to return to basal levels. In future experiments, a longer experiment examining the effects of injected aggregated SOD1 through time would be vital to determine if microglial activation persists and astrocyte function remains impaired.

Taken together with current literature, the data presented here suggest a model (Figure 6.1) in which 1) neurons trigger ALS by accumulating SOD1 aggregates (Matsumoto et al. 2005, Soo et al. 2009), 2) the dead or dying neurons release aggregates which can be taken up by neurons, astrocytes or microglia ((Zeineddine
et al. 2015), Chapter 3 and Chapter 4)), 3) these aggregates induce a neurotoxic phenotype in microglia, and 4) cause senescence and death in astrocytes which may result in a decrease in trophic support for neurons. The work presented here provides evidence of the detrimental effect that the addition of SOD1 aggregates can have, causing microglial activation and astrocyte dysfunction. As a result, future therapeutics that prevents both SOD1 aggregate induced microglial activation and astrocyte dysfunction may be effective in slowing ALS progression.
7. Bibliography


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8. Appendix
Figure 8.1: A 15% SDS-PAGE gel of fractions obtained from size exclusion column. Gel was run at 120 V. Fractions of 2 mL were collected and every second fraction eluted between 50-80 mL was analysed. Samples of SOD1 that had a purity greater than 95% were then pooled and western blotting was performed to confirm the presence of SOD1.
Figure 8.2: Native mass spectrum of SOD1 wild-type. Briefly, purified SOD1 at a concentration of 5 uM in 200 mM NH4OAc (pH 6.8) was nano-electrosprayed into a SYNAPT G1 HDMS mass spectrometer. This figure is an expansion of the dissociated 6+ charge state monomer under mild activation energy (40 V Trap). The major species in this spectra is a peak with a mass of 15930 Da which corresponds to a fully metalated SOD1 monomer. Other unlabeled peaks in this spectra correspond to various oxidative modifications made to Cys111 during purification (Mass spectrometry performed by Luke McAlary).