ALTERED PROTEASOME EXPRESSION AND NUCLEAR FACTOR (ERYTHROID-DERIVED 2)-LIKE SIGNALING IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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ALTERED PROTEASOME EXPRESSION AND NUCLEAR FACTOR (ERYTHROID DERIVED 2)-LIKE SIGNALING IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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B.S., Neuroscience, Bates College, 2012

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

December 2019
DEDICATION

To my dad, who showed me that listening and going beyond what is required is more important than being the most prestigiously taught person in the room.

And to my mom, who helped me understand that having confidence in yourself and your abilities is what really matters.
ACKNOWLEDGEMENTS

There are many people who have helped get me to this point in my education. I would like to first thank my mentor, Dr. Oscar Bizzozero, for his forbearance as I learned the technical aspects of our work and for reading my verbose drafts of this dissertation. His guidance and determination to find the science in my failed projects and for teaching me how to create a well-rounded story from our findings were essential to my success. I will be forever grateful to him for accepting me into his lab. I am also extremely grateful for having worked with Dr. Jamie Hu, who taught me to always look on the bright side, even in the face of failure, and who always answered my often-silly questions with kindness. I could not have asked for a better coworker/mentor. I cannot thank my committee members – Dr. Kevin Caldwell, Dr. Rebecca Hartley and Dr. Nora Perrone-Bizzozero – enough for their patience and understanding these past five years and for always giving me the most supportive and helpful direction. I would also like to thank the BSGP for taking a chance on me and Dr. Nancy Kanagy for pushing me to finish this degree.

I would like to acknowledge Dr. Jenny Zheng for teaching me western blots and Dr. Rob Oliver for teaching me PCR and for troubleshooting advice – without them, this project would not have been possible. I want to also acknowledge Tamara Howard, who was always willing to lend me her expertise when I needed help. I want to thank my friends in the department of Neurosciences for taking me in as one of their own on many occasions (especially at conferences and on hikes … thank you Don). And thank you to my unofficial second mentor, Michael Haerderle, for instructing me on the basics of journalism, being a wonderful and supportive listener, and teaching me how to be a more grounded version of myself under stress.

Finally, I'd like to thank all of my family and friends for their unending support these last six and a half years. To Chinu and Rashi: thank you getting me into this mess in the first place. To Candace: thanks for being the best friend I could have ever asked for in grad school. To Anna, Amy and Noopur: I’m so thankful for your continuing friendship and support. To my parents: thank you for your love, advice and for never giving up on me. And to my husband, Jacob: I honestly don’t know what I would have done without you.
ALTERED PROTEASOME EXPRESSION AND NUCLEAR FACTOR (ERYTHROID-DERIVED 2)-LIKE SIGNALING IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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ABSTRACT

Multiple sclerosis (MS) is a complex neurological disorder characterized by the interactions between heightened inflammation, oxidative stress and neurodegeneration. We and others have previously demonstrated that proteasome dysfunction and its consequences are also important factors in the pathology of both MS and its rodent model, experimental autoimmune encephalomyelitis (EAE). While proteasome subunit alterations in EAE have been observed, the underlying mechanisms are poorly understood. The first goal of this dissertation was to characterize the mechanisms that regulate proteasome expression and composition in neural cells in EAE and in vitro.

Immunohistochemical analysis of the EAE spinal cord shows changes in proteasome composition in neurons and astrocytes. Molecular and biochemical methods revealed that constitutive proteasome (c-20S) mRNA and protein levels are reduced while immune-proteasome (i-20S) and 11S regulator expression is
augmented in EAE. This altered composition is most likely due to two processes, the first of which is a decline in two transcription factors upstream of c-20S subunits: nuclear factor (erythroid-derived 2) like-1 (Nfe2l1/Nrf1) – which regulates proteasome gene expression – and one of its regulators, pre-B cell leukemia homeobox domain 1 (PBX1). The second is a heightened signal transducer and activator of transcription 1 (STAT-1) / interferon regulatory factor 1 (IRF-1) signaling, which mediates the displacement of constitutive subunits for inducible subunits. Differentiated N2a neurons and C6 astrocytes in vitro display similarly elevated i-20S and 11S levels when exposed to CIII, a pro-inflammatory cytokine cocktail (i.e., interferon-γ, interleukin-1β and tumor necrosis factor-α). Surprisingly, neither exhibit changes in PBX1 / Nrf1 or in c-20S-specific subunit mRNA. Altogether, these data imply that proteasome subunit displacement caused by elevated STAT-1 / IRF-1 is the main mechanism dictating proteasome composition in cultured neurons and astrocytes.

The second goal of this dissertation was to understand how reactive astrocytes participate in neuroinflammation – recent evidence suggests that there are different neurotoxic (A1) and neurotrophic (A2) reactivities. Because A1 astrocytes have been found in the lesions of MS patients, we expanded this idea to our own models. We found A1-reactive astrocytes in the EAE spinal cord, and two pro-inflammatory cytokine-induced reactivities in differentiated C6 astrocytes. The first type (A1-reactive) express heightened NF-κB, complement C3 and Nrf2, while the levels of these constituents in the second (pan-reactive) type are reduced. Furthermore, inhibition of active NF-κB in A1-reactive astrocytes results
in abrogated expression of Nrf2 and complement C3, suggesting that both the astrocytic response to inflammation and Nrf2 expression are connected to NF-κB signaling.

The findings presented in this dissertation shed light on the signaling mechanisms responsible for proteasome regulation in an animal model of MS. They also reveal the differences in these mechanisms and proteasome composition in neurons and astrocytes, as well as diverse reactions by astrocytes during inflammation. We hope that these studies will not only increase our understanding of the pathophysiological processes underlying MS, but also provide the basis for developing new therapeutic approaches to treat this debilitating neurological disorder.
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Chapter 1 – General Introduction

Multiple sclerosis

Multiple sclerosis (MS) is the most common demyelinating disease in humans and the most frequent neurological disorder affecting young adults according to the Centers for Disease Control. In 2010, over 700,000 people in the US were estimated to have MS – women were more likely to be diagnosed than men and there was a higher prevalence in people residing in northern regions in the country compared to southern regions (Wallin et al., 2019). MS is considered to be an autoimmune disease of unknown etiology characterized by severe central nervous system (CNS) inflammation, demyelination, oligodendrocyte cell death and neuronal damage (Lassmann et al., 2007). These neuropathologies lead to various symptoms including decreasing mobility, vision impairment and cognitive deficits among many others.

The time-course of the disease varies according to where a patient falls on the spectrum of MS (Lublin et al., 2014). Considering the onset of disease, clinically isolated syndrome is diagnosed by the appearance of demyelination and lesions in the brain as the result of inflammation. If the disease remains active, the patient then typically develops oscillating phases of remission and relapse – this is considered relapsing-remitting MS. About 80-90% of patients suffer from this form, making it the most common MS type (Lassmann et al., 2012). The disease often continues into secondary progressive MS, in which the periods between remission and relapse shorten while the CNS begins to degenerate, and the patient experiences a continuous neurological decline. In about 10% of cases, the
disease progresses from the onset without any relief in symptoms – this is considered primary progressive MS. There is currently no cure for MS and most therapies are directed at reducing the intensity and number of relapses.

*Experimental autoimmune encephalomyelitis*

The molecular and cellular mechanisms underlying tissue damage in MS are explored using a variety of animal models, but the most common is experimental autoimmune encephalomyelitis (EAE) (Gold et al., 2006). EAE is typically induced in rodents using a myelin protein, such as myelin oligodendrocyte glycoprotein (MOG) or its encephalitogenic peptide (Mendel et al., 1995), along with an immune system enhancer (i.e. Freund’s adjuvant). These injections generate a CNS-specific, demyelinating, autoimmune disorder in the animals similar to MS (Bizzozero and Zheng, 2009). Although EAE is not a perfect model, it shares a number of clinical and pathological features with MS and is routinely used to test therapeutic approaches (Baxter, 2007). The spinal cord is the most affected CNS region in EAE and is pathologically characterized by perivenular infiltration of lymphocytes, macrophage/microglial activation, demyelination and axonal damage (Kuerten et al., 2007). Another prominent pathological characteristic of MS and EAE is astrogliosis and glial scarring (Brosnan and Raine, 2013) – astrocytes in diseased or damaged tissue are often described as reactive since they undergo changes in their phenotype (i.e. become hypertrophic) along with altered molecular signaling in response to the CNS microenvironment (Sofroniew, 2009).
Astrocyte Reactivity

Recently, the study of reactive astrocytes and their function in the CNS has taken a large and complex step forward. Because reactive astrocytes, along with reactive microglia, are often considered to be “first responders” during CNS injury or disease, it is clear that discovering the mechanisms behind their variable functions is essential to understanding their interactions with the rest of the CNS (Anderson et al., 2014; Sofroniew, 2009). The most common marker for reactive astrogliosis is glial fibrillary acidic protein (GFAP) – which was first characterized during a study of MS lesions – although not all astrocyte populations express it to the same extent (Eng et al., 2000; Sofroniew and Vinters, 2010). These cells play critical roles that include participating in CNS development, synapse formation, blood flow regulation and cellular metabolism as well as responding to stressors like inflammation and oxidative stress (Sofroniew and Vinters, 2010).

Historically, astrocyte reactions to stress have been generalized down to reactive or non-reactive based on the amount GFAP they express (Liddelow and Barres, 2017). However, detectable gene expression changes within astrocytes allow for new phenotypic classifications (Zamanian et al., 2012). For example, two distinct types of astrocytes – neuroinflammatory and hypoxic – are generated in vivo using lipopolysaccharide (LPS) injections or middle cerebral artery occlusion (MCAO), respectively. Both share common reactive markers, such as GFAP and vimentin, as well as markers unique to their specific reactivities. The MCAO astrocytes tend to express more neurotrophic genes, as opposed to the LPS astrocytes which express more inflammatory, neurotoxic genes (Zamanian et al.,
These two types were named A1 (i.e. neurotoxic) and A2 (i.e. neurotrophic) astrocytes, similar to the designations of reactive microglia (Fig. 1.1; Liddelow et al., 2017; Martinez and Gordon, 2014). In addition, this same group discovered that the A1 phenotype, characterized by elevated expression of complement C3, could be induced in vitro using either a combination of immune molecules (e.g., complement C1q, interleukin-1α, and tumor necrosis factor-α) or medium from LPS-activated microglia (Liddelow et al., 2017). Culture medium from the A1-reactive astrocytes causes the death of neurons and oligodendrocytes, implying that A1 astrocytes release harmful substances into the CNS microenvironment. In addition, optic nerve crush injury in vivo stimulates A1 reactivity and increases cell death of damaged retinal ganglion cells, and this effect is prevented by genetic ablation of A1 inducing cytokines. The above studies introduced the idea that astrocyte reactivity, particularly in the A1 subtype, is important in the context of CNS injury and disease.

**Figure 1.1** Continuum of astrocyte reactivity (adapted from Liddelow and Barres, 2017) including two major types (A1 and A2), as well as undiscovered types \( (A_n) \), and the corresponding pathways that are upregulated in each type (Zamanian et al., 2012).
Role of reactive astrocytes in neurodegenerative disease

The ground-breaking study by the Barres laboratory not only discovered what triggers A1 reactivity, but also determined that this phenotype could be found in diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and MS (Liddelow et al., 2017). The authors suggested that A1 astrocytes may contribute to these pathologies through the release of neurotoxic factors as well as the loss of normal astrocytic functions, like synapse maintenance and phagocytosis. Likewise, other groups have found that the number of A1 reactive astrocytes rise during aging and that blockage of this cell type – through complement C3 receptor inhibition – can ameliorate pathogenic features in animal models of AD and PD (Clarke et al., 2018; Lian et al., 2016; Yun et al., 2018). Heightened complement C3 was also discovered in optic nerve astrocytes of EAE animals (Tassoni et al., 2019), denoting the presence of A1-reactive astrocytes in this disease.

Many of these studies also implicate the upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in A1-reactive astrocytes as a primary contributor to disease progression, since this factor can bind to the C3 promoter following its activation by tumor necrosis factor-α or TNF-α (Lian et al., 2015). Genetic deletion of astrocytic NF-κB abrogates symptom severity and disease time-course in EAE animals (Brambilla et al., 2009, 2014). A similar protective effect of NF-κB ablation was discovered in animal models of ischemia and spinocerebellar ataxia type 3 (Dvorianchikova et al., 2009; Li et al., 2018). Moreover, a connection between astroglial NF-κB expression and MS has also
been documented. One example is that astrocytes with heightened NF-κB expression have been localized to MS lesions. This particular set of MS patients have a genetic variant in the \textit{NFKB1} locus that has been associated with an elevated risk of developing the disease (Ponath et al., 2018). It is important to note, however, that inflammatory signaling through NF-κB is not the only mechanism by which astrocytes contribute to MS and EAE, as these cells can also cause oxidative damage through NADPH oxidase activation or nitric oxide production (Brambilla, 2019; Mossakowski et al., 2015).

**Oxidative stress in MS and EAE**

Both MS and EAE are multi-dimensional diseases with inflammatory and degenerative features, as well as high levels of reactive oxygen species (ROS) that contribute to cell death in the CNS. Elevated and chronic oxidative stress conditions can cause protein oxidation and aggregation, lipid peroxidation, axonal degeneration and apoptosis. Furthermore, protein oxidation leads to irreversible modifications, like carbonylation, which has been demonstrated in the brains of MS patients (Bizzozero et al., 2005). Under normal conditions, these damaged proteins are degraded by cellular proteases (Bizzozero, 2008). These include the proteasome – the major, ubiquitously expressed proteolytic enzyme complex – various mitochondrial proteases and lysosomal proteinases. The widespread expression of these proteases helps to combat protein dysfunction and aggregation under oxidative stress conditions throughout cellular compartments.
**Structure and function of proteasomes**

The proteasome is a highly conserved proteolytic complex in charge of the degradation of most proteins in the cytoplasmic and nuclear compartments. This proteolytic machinery degrades not only damaged proteins to maintain healthy cellular environments, but also ubiquitinated proteins that are involved in signaling, immunity and apoptosis (Kumar Deshmukh et al., 2019; Rechsteiner, 2005). The 20S core proteasome is comprised of four rings made up of seven subunits each, with the two inner rings formed by β-subunits and the two outer rings formed by the α-subunits (Fig. 1.2; Huber et al., 2012). Of the seven β-subunits, only three contain proteolytic active sites: β1, β2 and β5 have caspase-like, trypsin-like and chymotrypsin-like activity which cleave after acidic, basic and hydrophobic residues, respectively (Coux et al., 1996).

![Diagram of proteasome structure and function](image)

**Figure 1.2 – Constitutive 20S proteasome and immuno 20S proteasome structures, including the α and β rings for each complex as designated by the key at the bottom.**

Thick arrow indicates the trigger of displacement of constitutive β-subunits by inducible β-subunits. The specific catalytic activities of the relevant β-subunits are represented by the following symbols: caspase-like (#), trypsin-like ($) and chymotrypsin-like (*). The 11S regulator or PA28 composition is also shown, with dark pink being PA28α and light pink being PA28β subunits, which can bind to both 20S proteasomes. Adapted from Johnston-Carey et al., (2015).
The α-subunits can recruit other regulatory or co-activator caps, such as the 11S (i.e. PA28) and 19S (i.e. PA700), that bind to one or both ends of the 20S particle (Pickering and Davies, 2012). The 11S particle – a hexamer of alternating PA28α and β subunits – associates with the 20S upon high levels of oxidative stress as well as phosphorylation of the 20S. This activator seems to be necessary for more efficient degradation of oxidized proteins in both the cytoplasm and the nucleus following low doses of hydrogen peroxide (Pickering and Davies, 2012). In contrast, the 19S activator binds to the 20S core to form the 26S proteasome, thus providing ATPase activity as well as substrate selection for degradation of poly-ubiquitinated proteins (Tomko and Hochstrasser, 2013). Another regulator, proteasome inhibitor 31 kilodalton (PI31), has been shown to inhibit peptide generation for antigen presentation (Zaiss et al., 2002). However, with or without regulators, the 20S proteasome can still function in an ATP-independent manner – unlike the 26S proteasome which needs ATP in order to degrade its ubiquitinated substrates (Raynes et al., 2016).

Upon activation of the immune response by inflammation, the three catalytic β-subunits of the 20S proteasome (β1, β2 and β5) are substituted for the respective inducible subunits (β1i, β2i and β5i) (Aki et al., 1994; Hussong et al., 2010; Lundh et al., 2017). This process, called subunit displacement, leads to the replacement of constitutive 20S proteasome (c-20S) for the inducible or immuno-20S proteasome (i-20S) (Früh et al., 1994). The i-20S is thought to be the dominant proteasome form during immune responses, compared to the c-20S and 26S proteasomes (Pickering et al., 2010). In addition, it was recently demonstrated that
the 11S regulator preferentially binds to the i-20S as opposed to the c-20S, whereas the 19S does not prefer one over the other (Fabre et al., 2015). On the other hand, PI31 was shown to prefer binding to the c-20S.

*Transcriptional regulation of proteasome expression*

Because proteasomes respond to changes in the environment, their expression is tightly regulated by a variety of signaling pathways. Of these, inflammation and oxidative stress are two of the more common inducers. Inflammatory cytokines, particularly interferon gamma (IFN-γ) - highly expressed in both MS and EAE - have been shown to increase i-20S proteasomes. IFN-γ is also able to induce the chaperone proteasome maturation protein (POMP) *in vitro*, thereby facilitating the formation of immunoproteasomes (Heink et al., 2005). All three inducible subunits contain interferon-stimulated response elements (ISRE) in their promoters that are responsive to IFN-γ through indirect signaling pathways (Fig. 1.3). When IFN-γ binds to its receptors, the receptors dimerize and activate the associated Janus kinases (Jak) to phosphorylate signal transducer and activator of transcription 1 (STAT-1). In turn, phosphorylated STAT-1 can dimerize and translocate into the nucleus to activate another transcription factor, interferon regulatory factor 1 or IRF-1 (Castro et al., 2018; Wang et al., 2010). IRF-1 has been found to increase expression of all inducible subunits in concert with phosphorylated STAT-1 (Chatterjee-Kishore et al., 1998; Foss and Prydz, 1999; Heink et al., 2005). IFN-γ has also been demonstrated to upregulate the subunits of the 11S regulator (Ahn et al., 1995).
Figure 1.3 – Pathway of IRF-1 activation by IFN-γ/STAT-1 signaling (adapted from Castro et al., 2018). Two IFN-γ receptors, IFN-γR1 (blue) and IFN-γR2 (red), have attached JAK1 (blue rectangles) and JAK2 (red rectangles). Following phosphorylation of each other, STAT-1 docks and is phosphorylated. As a dimer, it then translocates to the nucleus to activate IRF-1 transcription through binding of the interferon-γ activated sequence (GAS) site. IRF-1 is then expressed and localizes to the nucleus where it binds ISRE-containing genes. Note that the IRF-1 promoter can also bind and be activated by NF-κB p65/p50.

Inflammation is not the only trigger of inducible and 11S regulator subunit expression. Extensive research has shown that two nuclear factor (erythroid-derived 2)-like transcription factors, Nrf1 and Nrf2, play a role in activation of these subunits under conditions of oxidative stress. These factors bind to electrophile response elements (EpRE) in the promoters of the genes they regulate – indeed, all proteasome and proteasome regulator subunit genes contain these sequences.
nearby their transcription start sites (Baird et al., 2017). However, the specific localization of the sequence within the genome is more consistent with Nrf1 binding rather than Nrf2. This notion is supported by previous studies showing that Nrf1 is the primary regulator of proteasome subunit gene expression. A form of Nrf1 found in humans, TCF11, induces 26S proteasome gene expression following proteasome inhibition (Steffen et al., 2010). In mouse cells, proteasome inhibition causes Nrf1-mediated synthesis of new subunits, since cells in which Nrf1 is knocked out are not able to produce new subunits (Radhakrishnan et al., 2010). Furthermore, limited proteasome inhibition increases Nrf1 and its various active forms, which then triggers transcription of the 26S proteasome subunits (Sha and Goldberg, 2014). Specific knockout of Nrf1 in the neurons of mouse brains causes direct abrogation of 20S α- and β-subunit expression without oxidative stress playing a major role (Lee et al., 2011), demonstrating the importance of Nrf1 in proteasome expression.

In comparison to Nrf1, Nrf2 seems to be more important during periods of high oxidative stress (Zhang and Xiang, 2016). The 20S proteasome becomes elevated under oxidative stress as a result of increased Nrf2 signaling in both invertebrates and mammals (Pickering et al., 2013). This heightened expression of 20S proteasomes can be due to Nrf2 activation induced by not only electrophilic agents – such as dimethyl fumarate and tert-butyl hydroquinone (tBHQ) – that can react with nucleophilic thiol groups but also various oxidants (Kwak et al., 2003; Lipton et al., 2016). For example, during hydrogen peroxide-induced oxidative
stress, elevated Nrf2 increases both 20S and 11S proteasomal subunit expression (Pickering et al., 2012).

**Proteasome dysfunction in disease**

Disruption of the processes that govern protein homeostasis (i.e., proteostasis) caused by proteasome impairment is a common problem in many pathologies, but particularly in neurodegenerative disease. Because many of these diseases often present with accumulation of mutant or damaged proteins, any change in proteasome function could play a role in the pathology. α-Synuclein, a major player in PD, is known to disrupt proteasome chymotrypsin-like activity in both rat pheochromocytoma PC12 and human neuroblastoma SHSY-5Y cell lines – which have noradrenergic and dopaminergic phenotypes, respectively (Zondler et al., 2017). Reduced proteasome function has also been discovered in the CNS of patients with Huntington’s disease (HD), leading to increases in ubiquitinated proteins (Seo et al., 2004). Furthermore, overexpression of 11S subunits in fibroblasts from HD patients cannot rescue the abrogated proteasome activity. Proteasome dysfunction has also been associated with ALS. *In vitro* studies have shown that acute proteasome inhibition causes superoxide dismutase 1 (SOD-1) to change its conformation to the more toxic, aggregation-prone form commonly found in ALS (Kitamura et al., 2014). Similarly, in the SOD-1G93A mouse model of familial ALS, proteolytic activity of the proteasome is reduced by 50% in the lumbar spinal cord where 20S levels are decreased in motor neurons (Kabashi et al., 2004). Changes in proteasomes, including decreased activity, have also been found in AD (Keller et al., 2000). Isolated 26S proteasomes from mice with
tauopathy have lower proteolytic activity and are directly connected to tau proteins (Myeku et al., 2016). Indeed, tau aggregates reduce the activity of proteasomes isolated from mice without the disease. This coincides with a previous study indicating that the protein substrates in AD are the most likely culprit behind decreased proteasome activity, rather than dysfunction of the proteasome itself (Gillardon et al., 2007). Altogether, changes in either proteasome activity or levels seem to be a common facet of neurodegenerative pathology.

Proteasomes have been theorized to play a role in MS for the past decade, including in our own work. Autoantibodies directed towards multiple proteasome subunits have been found in the serum and cerebral spinal fluid of MS patients (Mayo et al., 2002). Moreover, a mutation in the β1i gene has been connected to a lower risk of MS development as well as higher production of a non-immunogenic myelin peptide (Mishto et al., 2010). Knockout of this same gene in mice causes proteasome activity to substantially decrease in the brain concurrent with higher levels of carbonylated proteins (Ding et al., 2006). Similarly, knockout of the β5i subunit in EAE mice leads to faster onset of more severe symptoms and significant accumulation of poly-ubiquitinated proteins in their brain tissue (Seifert et al., 2010). Our lab has discovered that the activity of the 20S proteasome is significantly diminished in both the gray matter and white matter of MS brains (Zheng and Bizzozero, 2011). In addition, both the cerebella and spinal cords of animals with EAE show similar reductions in activity (Zheng and Bizzozero, 2010a, 2010b) as well as high levels of both oxidized and aggregated protein species (Bizzozero and Zheng, 2009; Dasgupta et al., 2013).
Nrf transcription factors: antioxidant response under normal and disease conditions

The antioxidant response system present in most cells is highly regulated via a family of transcription factors that belong to the cap’n collar basic leucine zipper (CNC-bZIP) family. There are two major designations within this family: the BTB and CNC homology (Bach) proteins and the Nrf proteins. Nrf1 and Nrf2 are known to be ubiquitously expressed throughout tissues and cell types, while NF-E2 p45 and Nrf3 are more tissue specific (Tebay et al., 2015). A common function of all the CNC-bZIP transcription factors is their ability to heterodimerize with small musculoaponeurotic fibrosarcoma (sMaf) proteins and bind to promoter sequences referred to as antioxidant response elements (ARE) or electrophile response elements (EpRE) (Itoh et al., 1997). These sequences can be found in promoters of antioxidant genes as well as genes related to the GSH conjugation phase of drug metabolism (i.e., phase II xenobiotic genes) in which drugs are made more water soluble (Tebay et al., 2015). Nrf1 is known to activate genes similarly to Nrf2 in the presence of stressors like ROS and proteasome inhibition; however, due to multiple Nrf1 isoforms, it can also competitively inhibit Nrf2 from binding or inhibit EpRE activation altogether (Kim et al., 2016). Nrf3 has been shown to respond to stress signals like inflammatory cytokines TNF-α and IFN-γ (Chénais et al., 2005; Kitaya et al., 2007), both of which are prominent in MS and EAE. Due to the large number of genes that are targeted by the Nrf family, loss of function in any of these factors could be catastrophic.
Nrf1 forms and functions

Nrf1 is important for basal control of the antioxidant pathways as well as responses to endoplasmic reticulum stress (Zhang and Xiang, 2016). Knockout studies have revealed that of the three Nrfs, only Nrf1 leads to embryonic lethality in mice when deleted and can cause neurodegeneration in neuronal-specific knockouts (Lee et al., 2011). Nrf1 may also compensate for the loss of both Nrf2 and Nrf3 (Derjuga et al., 2004). However, due to multiple transcripts of its gene – Nfe2l1 – as well as alternative splicing, Nrf1 is an incredibly complex protein with many different forms and accompanying activities (Fig. 1.4). In rodents and humans, the full form of Nrf1 is a 120-kilodalton (kDa) protein called Nrf1α that is glycosylated under basal conditions and translocates to and lodges in the endoplasmic reticulum (ER) membrane (Wang and Chan, 2006). Full length, or p120, Nrf1 is deglycosylated to the p95 form and released into the cytoplasm where it undergoes progressive cleavage to result in many shorter forms, like the activating p85 (Zhang and Hayes, 2013).
Figure 1.4 – Localization and production of the various forms of Nrf1 (adapted from Bugno et al., 2015). Following its transcription, full-length Nrf1/p120 is localized to the ER as a transmembrane protein and glycosylated (red u-shapes). In the absence of stress, p120 is eliminated by proteasomal degradation. Oxidative stress and/or ER stress activate Nrf1, whereby it is deglycosylated and cleaved into the various forms listed. Additionally, some forms (e.g., Nrf1β, Nrf1γ or Nrf1δ) are considered to be alternatively translated. The EpRE activating capability of each isoform is indicated by the color of the name: strong activators (green), weak activators (blue) and dominant negative inhibitors (red). The various Nrf1 isoforms translocate to the nucleus, heterodimerize with a sMaf protein and activate transcription of genes such as proteasome subunits or antioxidant proteins.

Although there is some disagreement as to how some of the shorter forms arise – whether it is due to proteolytic processing or alternative translation initiation – there are a number of shorter forms that still maintain Nrf1’s EpRE binding activity. One of these is a supposedly alternatively translated form has a molecular weight of 55-65 k, depending upon the gel composition. This shorter form, called LCR-F1 or Nrf1β, is considered to be a weak EpRE activator but may have differing activity depending upon the stress signal (Zhang et al., 2014a). For instance, one group found that the p65 form of Nrf1 could act as an EpRE repressor by blocking Nrf2 from binding to the promoters of its target genes (Wang et al., 2007). Finally, two shorter forms that can be generated either by cleavage of Nrf1β or by alternative splicing are Nrf1γ/p36 and Nrf1δ/p25. These smaller proteins are considered to be dominant negative inhibitors of EpRE activators (Zhang et al., 2014a).

*Nrf2 role in cellular function and disease*

Though all of the CNC-bZIP factors can bind to EpRE, Nrf2 is the best studied of these and controls the activation of over 200 stress response genes
(Chorley et al., 2012). These genes are not only related to the antioxidant response, but are also present in autophagy, cytoprotection, metabolism, inflammation, mitochondrial dysfunction and many other processes. Because of the vast regulatory reach of Nrf2, deletion of its gene (Nfe2l2) has significant repercussions including mitochondrial dysfunction, reduced glutathione (GSH) levels, increased susceptibility to chemically induced carcinogenesis and augmented neuronal death in models of neurodegenerative disease (Tebay et al., 2015).

In EAE, the disease is much more severe and has an earlier onset in mice lacking Nrf2 as compared to wild-type mice (Johnson et al., 2010). Moreover, EAE can be treated effectively using dimethyl fumarate (DMF), which increases Nrf2 levels in the CNS and reduces the levels of oxidized proteins and demyelination (Linker et al., 2011). DMF has not only been used to treat EAE, but is also currently being used to treat MS patients (Fox et al., 2012; Linker et al., 2011). When Nrf2 is activated in astrocytes, specifically, it has been shown to promote neuroprotection in various models of neurological disorders, like PD and ischemia (Bell et al., 2011; Vargas and Johnson, 2009). Neurons are known to have very little intrinsic Nrf2 and minimal capacity to produce antioxidants, thus they rely heavily on astrocytic Nrf2 signaling (Baxter and Hardingham, 2016). Furthermore, in the demyelinating cuprizone mouse model of MS, Nrf2 activation via Kelch-like ECH associated protein 1 (Keap1) knockdown in astrocytes led to a decrease in demyelination and increased protection of oligodendrocytes (Draheim et al., 2016).
**Control of Nrf2 levels via translational and post-translational regulation**

Nrf2 levels are tightly regulated both translationally and post-translationally (Fig. 1.5; Bryan et al., 2013). An internal ribosome entry site (IRES) is present in the 5’ untranslated region of Nfe2l2 mRNA, indicating that this protein can undergo cap-independent translation (Li et al., 2010). Under conditions like oxidative stress, the IRES allows the 40S ribosome to bind Nfe2l2 mRNA via phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) (Klann and Dever, 2004). IRES-dependent translation of Nrf2 also seems to be redox sensitive and can balance out with degradation at basal levels (Li et al., 2010). Cap-independent translation is contingent upon the kinases responsible for phosphorylating eIF2α at serine 51, such as general control non-derepressible (GCN), haem-regulated initiation factor 2α kinase (HRI), eIF2α kinase 3 (PERK) and protein kinase RNA-regulated (PKR) (Klann and Dever, 2004).
Following translation, Nrf2 is bound by Keap1, which is responsible for maintaining basal levels of Nrf2 through degradation (Regoli and Giuliani, 2014). Once bound by Keap1, Nrf2 is ubiquitinated by Keap1’s binding partners, Cullin-3 (Cul3) and RING-box 1 (Kobayashi et al., 2004). The ubiquitinated Nrf2 is then transported to the proteasome where it is degraded; due to this tight regulation, Nrf2 protein has a very short half-life (~30 minutes) (Nguyen et al., 2009). Other Keap1 binding partners act oppositely to Cul3 and instead prevent the sequestration of Nrf2 by Keap1. Sequestosome 1 (p62), phosphoglycerate mutase 5 (PGAM5) and parkin 7 (DJ-1) all can attach to the Nrf2 binding site on Keap1 and therefore promote the release and activation of Nrf2 (van Horssen et al., 2010; Komatsu et al., 2010; Lo and Hannink, 2008). Similarly, the cyclin dependent kinase inhibitor p21 can directly block Keap1-mediated degradation of Nrf2 by binding to Nrf2 at the same site that Keap1 would normally attach (Chen et al., 2009). Once a stressor has been detected, such as ROS or electrophilic compounds that react to thiol groups on Keap1, Nrf2 is released from Keap1 by phosphorylation of serine 40 (Bloom and Jaiswal, 2003).

Nrf2 can also be degraded independently of Keap1 via the activity of glycogen synthase kinase 3β (GSK-3β). GSK-3β can phosphorylate and activate Fyn kinase to phosphorylate Nrf2 and signal its shuttling from the nucleus (Jain and Jaiswal, 2007). Levels of GSK-3β and its active phosphorylated form are...
unaltered in EAE (Morales Pantoja et al., 2016), which rules out GSK-3β interference in Nrf2 nuclear translocation and subsequent degradation.

Transcriptional regulation of Nrf signaling

While both Nrf1 and Nrf2 pathways can crosstalk with a number of different transcription factors, this dissertation will focus on those that may be critical to the regulation of proteasome expression and to the behavior of astrocytes. These are the mammalian (or mechanistic) target of rapamycin (mTOR) and the pre-B-cell leukemia homeobox-1 transcription factor (PBX1) for Nrf1, and NF-κB for Nrf2.

As a member of the phosphatidylinositol 3-kinase (PI3K) family, mTOR participates in a wide variety of cellular functions mostly focused on cell growth, survival and proliferation. Specifically, mTOR can be found in two protein complexes (mTORC), mTORC1 and mTORC2, where it acts as the catalytic component. mTORC1 is particularly responsible for regulating metabolic processes that contribute to cellular growth, which also include protein homeostasis (Saxton and Sabatini, 2017). One of the downstream targets of mTORC1 is the sterol responsive element binding protein (SREBP) family of transcription factors, which activate fatty acid and cholesterol synthesis (Porstmann et al., 2008). This is one example of a pathway that has been implicated in Nrf1 activation as well as subsequent proteasome upregulation (Fig. 1.6). Genetic overactivation of mTORC1 or cellular exposure to growth factors is sufficient to upregulate Nrf1 gene and protein expression, thereby increasing gene expression of both 20S and 19S subunits (Zhang et al., 2014b). In addition, knockdown of SREBP1, specifically, results in decreased Nfe2l1 expression. This
transcriptional regulation of Nrf1 and proteasome gene expression could be responsible for maintenance of amino acid levels during periods of cellular growth through enhanced protein degradation.

Figure 1.6 — Pathway of Nrf1 activation by mTORC1/SREBP1 (adapted from Zhang and Manning, 2015). mTORC1 activates transcription of SREBP1, which localizes to the ER membrane and upon activation, it migrates to the Golgi and is cleaved. The cleaved SREBP1 travels to the nucleus and binds to genes with a sterol regulatory element (SRE) – this activates transcription of \textit{Nfe2l1}.

Another protein that is thought to act upstream of Nrf1 is the homeobox protein PBX1. Besides playing a critical role in the development of tissue and organs (Capellini et al., 2011), PBX1 is also important in the differentiation and survival of midbrain dopaminergic neurons (Villaescusa et al., 2016). This study – using chromatin immunoprecipitation (ChIP) in combination with next generation sequencing – discovered that PBX1 can bind to genomic locations proximal to the \textit{Nfe2l1} transcription start site, suggesting \textit{Nfe2l1} as a possible PBX1 target.
Furthermore, patients with PD – in which there is considerable damage to these neurons in the substantia nigra – have lower levels of both PBX1 and Nfe2l1 in this particular neuronal population. Overall, the above literature provides evidence that mTOR and PBX1 are important in the transcriptional regulation of Nrf1 in pathological conditions.

In terms of Nrf2 crosstalk partners that could play a role in disease, one of the most prominent and best studied is NF-κB (Fig. 1.7). NF-κB is a complex of transcription factors that is primarily responsible for pro-inflammatory signaling during immune responses (Oeckinghaus et al., 2011). Activation of NF-κB occurs when immune components, such as TNF-α or interleukin-1β (IL-1β), bind to their respective receptors and signal activation of the IκB kinase (IKK) complex. This complex phosphorylates the IκB protein, which basally binds and sequesters two NF-κB proteins: p50 and p65 or RelA. Once released by phosphorylated IκB, p50 and p65 translocate to the nucleus where they activate genes with κB binding sites. These genes include those of cytokines, chemokines, adhesion molecules and various transcription factors (Pahl, 1999). In addition, oxidative stress has been demonstrated to induce NF-κB activation through its upstream regulatory pathways (Kabe et al., 2005).
Figure 1.7 – Canonical NF-κB activation by IL-1β and TNF-α (adapted from Oeckinghaus et al., 2011) and potential crosstalk with Nrf2 (adapted from Cuadrado et al., 2014). Binding of IL-1β and TNF-α to their receptors, IL-1R (green) and TNFR (orange), respectively, activates a signaling cascade that leads to phosphorylation and activation of the IKK complex. This complex then phosphorylates IκB to release p65/p50 into the cytosol. While IκB is poly-ubiquitinated and degraded by the proteasome, the NF-κB dimer localizes to the nucleus and activates genes with κB sites. This includes Nfe2l2, as it contains these sites – however, other mechanisms have been theorized to activate Nrf2, such as IKK phosphorylation of Keap1 or normal activation of Nrf2 by reactive oxygen species.

It has been suggested that NF-κB regulates Nrf2 at the transcriptional level, since the Nfe2l2 promoter has several κB binding sites in the murine and human sequences (Nair et al., 2008; Rushworth et al., 2012). In support of this idea are the findings (1) that silencing p50 and p65 expression in vitro results in lower Nrf2
expression, and (2) that LPS treatment of both human peripheral monocytic THP-1 cells and normal monocytes leads to p50 and p65 binding of the Nfe2l2 promoter (Rushworth et al., 2012). This study also found that p65 binds the κB site near the transcription start site of the Nfe2l2 locus in lymphoblastoid cells treated with TNF-α. Furthermore, another study observed that macrophages exposed to iron-coated silica nanoparticles – which cause oxidative stress – upregulate the nuclear translocation of both NF-κB and Nrf2, thereby prompting the expression of Nrf2 target genes (Zhang et al., 2017). This group also found that NF-κB inhibition with the compound SN50 reduces the magnitude of both the Nrf2 elevation and that of its targets.

There also appears to be a reciprocal regulation of NF-κB expression by Nrf2, suggesting crosstalk between the two signaling pathways. It has been theorized that Nrf2 can inhibit NF-κB signaling indirectly through activation of heme oxygenase 1 (HO-1), a Nrf2 target (Banning and Brigelius-Flohé, 2005; Soares et al., 2004). Likewise, Nrf2 overexpression produced by treatment with the electrophilic agent sulforaphane decreases both NF-κB activation and the induction of cell death following cytokine exposure, most likely by reducing oxidative stress (Song et al., 2009). Another hypothesis suggests that when Nrf2 is activated, it can trigger transcription of the autophagy adaptor protein p62. In doing so, Nrf2 / p62 signals for the degradation of IκB – indirectly activating NF-κB – as well as enhancing Nrf2 release through degradation of Keap1 (Stępkowski and Kruszewski, 2011). Finally, one study found that knocking out Nrf2 causes a
rise in NF-κB and its signaling (Cuadrado et al., 2014). Overall, it seems that the crosstalk between NF-κB and Nrf2 is complex, but most likely context dependent.

Under basal or stress conditions, both Nrf1 and Nrf2 are vital regulators of their own specific pathways (Ohtsuji et al., 2008; Yang et al., 2005), but they have also been shown to crosstalk with each other. For example, Nrf1 and Nrf2 appear to compete with one another for EpRE-containing genes, depending upon the context. Nrf1 – in particular, Nrf1 p65 – outcompetes Nrf2 for binding sites when both are overexpressed in unstimulated human bronchial epithelial cells (Chepelev et al., 2013). Additionally, knockout of Nrf1α leads to a significant increase in Nrf2 expression, due to the resulting reduction of proteasomes and their degradation of Nrf2 (Qiu et al., 2018). The same study also found that reduction of Nrf2 causes a decrease in Nrf1 mRNA and protein levels. These findings indicate the presence of a complex feedback loop between the two transcription factors as well as intricate relationships with other crosstalk partners.

**Goal of Dissertation**

The primary focus of our laboratory’s work is to identify the pathological mechanisms underlying nervous system damage in MS and the pertinent animal model EAE. Previous studies have revealed that there is a high degree of oxidative stress in both MS and EAE, which leads to the oxidation of many cellular components including proteins. These damaged proteins accumulate due to a failure of the proteolytic systems that normally remove them. Our lab has demonstrated not only that protein oxidation in the form of carbonyls causes protein aggregation and cell death, but also that the proteasome is impaired in both
EAE and MS. The main goals of this dissertation are to 1) determine what upstream signaling could be affecting proteasome expression in EAE, and 2) investigate the specific effects of neuroinflammation on this signaling and its consequences \textit{in vitro}. The general hypothesis of this dissertation is that heightened inflammatory signaling during EAE contributes to dysregulated antioxidant signaling, thereby resulting in altered cellular proteolytic systems and varied astrocytic reactivity.

\textit{Aim 1}

The first aim of this dissertation investigates the expression of proteasome subunits in neural cells and how they are regulated in EAE. In chapter 2, we first show that there is extensive inflammation and oxidative stress in the spinal cords of mice with MOG peptide-induced EAE over the course of the disease. Most importantly, expression of constitutive proteasome and Nrf1 – one of the main transcription factors responsible for c-20S proteasome expression – are diminished in the diseased animals. This could be due to a decrease in one of its own upstream regulators, PBX1, but not to a reduction in mTOR signaling. In contrast, the expression of i-20S and 11S rises in EAE, which is most likely the result of augmented STAT-1 / IRF-1 activation. We conclude that the differential expression of constitutive and immunoproteasomes in EAE spinal cords, particularly in astrocytes and neurons, is probably caused by a combination of subunit displacement and reduced Nrf1 signaling.
**Aim 2**

The second aim explores the regulation of proteasome expression in cultured neurons and astrocytes under inflammatory conditions. Differentiated mouse neuro2a neuroblastoma cells (N2a) – which take on a dopaminergic phenotype – and rat C6 glioma cells were exposed to a mixture of pro-inflammatory cytokines that are elevated in EAE. In both cell types, treatment with the IFN-γ/TNF-α/IL-1β mixture leads to changes in proteasome expression similar to those observed in EAE – heightened i-20S and 11S expression and decreased c-20S expression. However, Nrf1 expression is unchanged upon cytokine exposure, suggesting that, in culture, subunit displacement is the only mechanism responsible for the reduced levels of c-20S particles. While the rise in i-20S expression is due to increased p-STAT-1 signaling, pharmacological inhibition of NF-κB – which also diminishes IRF-1 – in astrocytes has no effect on the cytokine-induced changes in proteasome expression. These findings indicate that STAT-1 can activate i-20S subunit and 11S subunit expression by itself.

**Aim 3**

The final aim of this dissertation studies NF-κB signaling and its effect on Nrf2 expression and cellular reactivity in astrocytes. Results show that A1-reactive astrocytes, characterized by elevated complement C3 and GFAP expression, are indeed present in the EAE spinal cord in the vicinity of inflammatory lesions. Furthermore, we discovered that differentiated C6 cells exposed to pro-inflammatory cytokines generate two different phenotypes that we categorized based on their expression of C3 and NF-κB. The first is an A1-reactive phenotype
that expresses high levels of both C3 and NF-κB. Interestingly, there exists a
subpopulation of astrocytes – named pan-reactive – that upon cytokine treatment
displays unchanged levels of NF-κB compared to control cells. These pan-reactive
cells also have diminished levels of Nrf2 compared to A1-reactive astrocytes,
which have elevated Nrf2 expression. In both cell types, changes in the amount of
Nrf2 protein follow those of the mRNA levels and are most likely due to differential
NF-κB expression. Indeed, pharmacological inhibition of NF-κB signaling in A1
cells leads to a corresponding decrease in the amount of Nrf2 protein. This drug-
induced downregulation of NF-κB in A1-reactive astrocytes also causes a fall in
C3 mRNA expression, causing the cells to behave more like pan-reactive, whose
NF-κB signaling is unresponsive to the cytokine mixture.
Chapter 2 – Decreased levels of constitutive proteasomes in experimental autoimmune encephalomyelitis may be caused by a combination of subunit displacement and reduced \textit{Nfe2l1} expression

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[Accepted for publication by Journal of Neurochemistry]
Abstract

The goal of this study was to determine if subunit displacement and/or alterations in proteasome biosynthesis could explain the changes observed in the levels of constitutive proteasomes (c-20S) and immunoproteasomes (i-20S) in the spinal cords of mice with experimental autoimmune encephalomyelitis (EAE). To this end, EAE was induced in C57BL/6 mice by immunization with MOG\textsubscript{35-55} peptide. Spinal cords were collected at different times during the disease course and used for western blotting, RNA analysis and immunohistochemistry. The results show that, as expression of i-20S and the activator PA28 rise in EAE, there is a concomitant decline in that of c-20S at the mRNA and protein level. These changes are observed in neurons and astrocytes but not oligodendrocytes. The increased amounts of the i-20S-specific subunit β5i and PA28α/β in EAE correlate with the levels of IFN-γ and its downstream effectors p-STAT-1 and IRF-1, but not with those of NF-κB. This suggests the STAT-1/IRF-1 pathway is solely responsible for the induction of these subunits. The decrease in the mRNA and protein levels corresponding to the c-20S-specific subunit β5 may also be due to reduced expression of the nuclear factor (erythroid-derived 2)-like-1 (Nrf1 or Nfe2l1), specifically Nrf1α and Nrf1β. Low Nfe2l1 mRNA expression is unlikely caused by reduced mTOR signaling but could be the result of diminished pre-B-cell leukemia homeobox-1 transcription factor (PBX1) levels. Together, these findings suggest that a combination of subunit displacement and reduced Nrf1 expression may be responsible for c-20S impairment in EAE. The present work provides insights into the dynamics of proteasome expression in the CNS of EAE.
mice and is the first to explore Nrf1 signaling in an inflammatory demyelinating disorder.
Introduction

Proteasomes are large protein complexes that carry out the majority of intracellular proteolysis, and thus play an essential role in regulating cell function and maintaining homeostasis (Rechsteiner and Hill, 2005). The core of these complexes, the 20S proteasome, is made of two outer rings of seven α subunits (α1-α7) and two inner rings of seven β subunits (β1-β7) (Ethen et al. 2007). Each β ring has three proteolytic sites, which display caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities (Coux et al. 1996). Exposure of cells to cytokines, such as interferon-γ (IFN-γ), leads to the replacement of the catalytic β1, β2 and β5 subunits in the constitutive 20S particle (c-20S) by the inducible β1i, β2i and β5i subunits, forming the immunoproteasome 20S particle (i-20S) (Aki et al. 1994). While immunoproteasomes were initially thought to be present exclusively in immune cells - where they participate in the generation of peptides for antigen presentation - they are now known to be expressed in most tissues and to play roles that are unrelated to the immune system (Seifert et al. 2010; Hussong et al. 2010). Under physiological conditions, a fraction of the 20S particle pool is bound to various regulators, which significantly affect its catalytic activity and substrate specificity. One of these activators, PA28 (11S), is also induced by IFN-γ and attaches to the i-20S particle forming a complex (11S/i-20S/11S). This complex produces more peptides of the correct length for the major histocompatibility complex class 1 antigen processing (Rivett and Hearn, 2004). PA700 (19S), the other major activator, binds to the end of 20S core particles to form the 26S
proteasome, which recognizes and hydrolyzes abnormal and misfolded proteins conjugated with polyubiquitin chains (Braun et al., 1999).

While little is known about how proteasome and immunoproteasome expression is controlled under basal conditions, more information is available on how levels of proteasomes are regulated under stress conditions. For example, IFN-γ augments the transcription of the immunoproteasome-specific β subunits (Aki et al., 1994) via binding of STAT-1-dependent interferon regulatory factor-1 (IRF-1) to IFN-γ regulatory elements in the promoter regions of these genes (Foss and Prydz, 1999). In cells containing both c-20S and i-20S particles, a rise in the content of inducible subunits is accompanied by a reduction in the amounts of constitutive subunits by a well characterized process known as subunit displacement (Früh et al., 1994). In addition to post-translational downregulation of constitutive proteasomes in inflammatory conditions, the promoter regions of the genes that encode for the constitutive proteasome subunits and the 11S and 19S activators have electrophile response elements (EpRE) that bind nuclear factor–erythroid-2–related factors (Pickering et al., 2012). These proteins belong to the cap’n’collar subfamily of basic-region leucine zipper transcription factors, and include Nrf1 (Nfe2l1), Nrf2 (Nfe2l2), Nrf3 (Nfe2l3), and the p45 subunit (Motohashi et al., 2002). Both Nrf1 and Nrf2 form heterodimers with small Maf proteins that bind to EpRE and activate transcription (Venugopal and Jaiswal 1998; Biswas and Chan 2010). The amount of Nrf1 augments upon endoplasmic reticulum stress and mild proteasome inhibition by a post-translational mechanism (Bugno et al., 2015; Xiang et al., 2018), whereas Nrf2 levels increase following oxidative stress.
(Kobayashi et al., 2004). While both transcription factors have the potential to increase proteasome subunit expression, Nrf1 alone is required for the upregulation of proteasome genes in response to proteasome inhibition (Radhakrishnan et al., 2010). Additionally, Nrf1 deletion in neuronal cells causes proteasome impairment due to down-regulation of proteasomal genes that encode the catalytic subunits (Lee et al., 2011). Thus, Nrf1 seems to be more important than Nrf2 for promoting constitutive proteasome synthesis.

Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for CNS autoimmune disorder, recapitulating a number of clinical and pathological features of multiple sclerosis (MS) (Gold et al., 2000). Several EAE models exist that mimic the different clinical courses of MS. MOG$^{35-55}$ peptide-induced EAE in the C57BL/6 mouse, the animal model used in this study, is characterized by the presence of inflammatory lesions in the CNS that are present mostly in the spinal cord and the cerebellum (Kuerten et al., 2007). Our laboratory was the first to demonstrate that the peptidase activity of the proteasome is altered in MS brains (Zheng and Bizzozero, 2011) and EAE cerebella (Zheng and Bizzozero, 2010). Furthermore, we discovered that the changes in the levels of the three catalytic subunits of c-20S and i-20S proteasomes account for all the fluctuations of proteasomal peptidase activities during the disease course of EAE (Zheng et al., 2012). The goal of the present study was to determine if subunit displacement and/or alterations in proteasome biosynthesis could explain the changes in the levels of proteasomes and their activators observed in the spinal cords of EAE animals during the inflammatory phase. The results show that, as
expression of i-20S and PA28 rise in EAE, there is a concomitant decline in that of c-20S at the mRNA and protein level. Interestingly, these changes are observed in neurons and astrocytes but not oligodendrocytes. The increase in the amount of the i-20S-specific subunit β5i and PA28α/β in EAE correlate with the levels of IFN-γ and its downstream effectors STAT-1 and IRF-1. Finally, we show that the decrease in the c-20S-specific subunit β5 mRNA levels may also be due to reduced Nrf1 expression. Together, these findings suggest that a combination of subunit displacement and reduced Nrf1 expression may be responsible for c-20S impairment in EAE. The present work provides insights into the dynamics of proteasome expression in the CNS of EAE mice and is the first to explore Nrf1 signaling in an inflammatory demyelinating disorder.

Materials and Methods

Induction of experimental autoimmune encephalomyelitis

Housing and all procedures of the animals were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (protocol #16-200424-HSC). Adult female C57Bl/6 mice (8-week old) were purchased from Envigo (MGI:2161078, previously Harlan Laboratories, Indianapolis, IN). Three to four arriving mice were arbitrarily placed in standard ventilated cages for one week of habituation before EAE induction. No randomization was performed to assign the cages to control or the EAE group. All mice housed in the same cage received the same inoculation. This is necessary because the weaker (EAE) mice could have trouble competing against healthy (control) mice for accessibility to food and water. To induce EAE,
mice (~20 g of body weight) received a subcutaneous injection (200 µl) into the lower back area of custom synthesized MOG\textsubscript{35-55} peptide (200 µg) (21\textsuperscript{st} Century Biochemicals; Marlborough, MA) in saline mixed with complete Freund’s adjuvant (CFA) (1:1) containing 4 mg/ml of heat killed Mycobacterium tuberculosis H37Ra (cat #7001; Chondrex Inc; Redmond, WA). Control mice received saline mixed with CFA without MOG peptide. Two and 48 h after EAE induction, all animals received an intraperitoneal injection of 0.3 µg of pertussis toxin (PTX, cat#180, List Biological Laboratories; Campbell, CA) in 100 µl of saline. Seven days after disease induction, mice received a second immunization on the other flank. This immunization protocol with 2 MOG peptide injections causes a persistent type of EAE, allowing to compare changes that occur in the acute (mostly inflammatory) and the chronic (mostly neurodegenerative) phase of the disease. Food and water were placed on the cage floor to ensure access to nourishment. No measures were taken to minimize possible pain after disease induction because analgesic and anti-inflammatory drugs may affect the disease course. Animals were weighed and examined daily for the presence of neurological signs. Normally, mice that experience a loss of 30% in body weight or have very severe symptoms (moribund stage) are euthanized and removed from the experiment. However, in this study no animal reached that stage and therefore no animal was excluded. A schematic timeline of the study design is depicted in Fig. 1. At various times during the course of the disease, 9 - 60 days post-immunization (dpi), EAE and CFA-injected controls were euthanized by decapitation under inhaled anesthesia of isoflurane (2-3% cat# 430024079, Piramal Critical Care Inc;
La Vergne, TN). The spinal cords were removed using the hydraulic extrusion method. The excised cervical and thoracic sections were flash-frozen for RNA extraction or immediately homogenized in 20 mM sodium phosphate buffer pH 7.5 containing 1 mM EDTA, 0.1 mM neocuproine, 2 mM 4,5 dihydroxy-1,3 benzene disulfonic acid, 1 mM dithiothreitol (DTT), 1x cOmplete™ protease inhibitor cocktail (cat#11873580001; Sigma; St Louis, MO) and 1x PhosSTOP™ phosphatase inhibitor (cat#04906845001; Sigma). Protein homogenates were stored at -80°C until use. Protein concentration was determined with Bradford-based chemistry using Bio-Rad protein assay dye reagent (cat#5000006; Bio-Rad Laboratories; Hercules, CA) with bovine serum albumin as a standard. Lumbar spinal cord sections were used for histological (hematoxylin and eosin staining) and immunofluorescence analysis (see below). This region has more lesions than other spinal cord areas making the pathological changes easy to assess. It is important to note that blinding was not done from the beginning of the study due to the fact that EAE mice exhibit neurological symptoms and thus can be easily identified. However, tissue samples (RNA, protein lysates and histological sections) were assigned with new serial numbers during all experimental procedures. Sample IDs were only revealed to the investigator after data acquisition. This study was not pre-registered.

Subcellular fractionation

Preparation of the crude nuclear fraction was carried out using a modified nuclear extraction protocol adapted from Thermo-Fisher Scientific. Briefly, small spinal cord sections were homogenized at 4°C in 0.5 ml of hypotonic buffer solution
(20 mM Tris-HCl pH 7.4 containing 10 mM NaCl, 3 mM MgCl$_2$, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride and 1x protease inhibitor cocktail) using a glass/teflon Dounce tissue homogenizer (20 up/down strokes). Suspensions were kept on ice for 15 min, mixed with 25 µl of 10% w/v Nonidet P-40 and centrifuged at 3,000 g for 10 min. The supernatant (cytosolic fraction) and the pellet (crude nuclear fraction) were collected, and proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer and analyzed by western blotting.

**Reverse transcription and qPCR**

Snap-frozen spinal cord tissues were homogenized in Trizol® (cat#15596026, Invitrogen; Carlsbad, CA) reagent and total RNA extracted following manufacturer's protocol. Complementary DNA (cDNA) was prepared from DNase I-treated total RNA and synthesized using the SuperScript II First-Strand Synthesis system (cat#18064022, Invitrogen). Quantitative RT-PCR was conducted on the Applied Biosystems 7500 Fast Real-Time PCR System by mixing 20 ng cDNA with 1 µM of gene-specific primers (Table 2.1) and amplified with the Power SYBR Green PCR MasterMix (cat#4367659, Applied Biosystems, Forster City, CA) using a preset program (50˚C for 10 min, 95˚C for 10 min and then 40 cycles of 95˚C/15 sec and 60˚C/1 min). The relative mRNA expression levels for the genes of interest were determined using the comparative $2^{-\Delta\text{Ct}}$ method (Livak and Schmittgen 2001) by normalizing the Ct values of target genes to the geometric mean Ct of 4 house-keeping genes (Gapdh, Hprt1, Rplp0 and Rn18s) (Table 2.1) (Vandesompele et al. 2002). In all cases, the normalized values
from EAE mice were expressed as relative to the average from control animals. The variations of control values are shown in the figures.

**Western blot analysis**

Proteins (5 μg) were separated by SDS–polyacrylamide gel electrophoresis on 4-20% Mini-Protean TGX gels (cat#4561096; Bio-Rad Laboratories; Hercules, CA) and were blotted to polyvinylidene difluoride membranes. Blots were blocked with 3% (w/v) non-fat milk in phosphate-buffered saline solution containing 0.05% (v/v) Tween 20 (PBS-T) and then incubated overnight at 4°C with the primary antibodies listed in Table 2.2. Membranes were rinsed three times in PBS-T and were incubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibodies (Table 2.2). Blots were developed by enhanced chemiluminescence using the Western Lightning ECL™ kit (cat#NEL103; Perkin-Elmer, Boston, MA) and signals captured on blue X-ray films. Films were scanned in a Hewlett Packard Scanjet 4050 and the images were quantified using the NIH Fiji ImageJ 1.52 imaging analysis program (RRID:SCR_003070). Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes or, in the case of the nuclear fractions, by the amount of histone H3. All the antibodies used in this study were validated by the suppliers and detected bands at the predicted molecular weights on western blots.

**Immunofluorescence localization of proteasomes and immunoproteasomes**

To assess cellular distribution of c-20S, i-20S and 11S particles in the lumbar spinal cords of control and EAE mice, we performed double-label immunofluorescence analyses using antibodies against β5, β5i, PA28β and
various cell-specific markers on paraformaldehyde-fixed and paraffin-embedded sections. All the antibodies used for immunohistochemistry are listed in Table 2.3. Briefly, 5 µm-thick sections mounted to slides were deparaffinized and hydrated in down-grade alcohol series. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) with 0.05% Tween-20 using a heat-induced retrieval method. Spinal cord sections were blocked in 4% normal goat serum and then incubated with the primary antibodies at 4°C overnight. The various cell types were detected using mouse monoclonal antibodies against GFAP (glial fibrillary acidic protein) for astrocytes, CA-II (carbonic anhydrase-II) for oligodendrocytes, NeuN (neuronal nuclear antigen) for neurons and CD45 (cluster of differentiation 45) for leukocytes (Table 2.3). Target proteins and cell-specific markers were co-visualized with secondary antibodies conjugated to either a Cy™ 3 fluorophore or an Alexa Fluor® 488 that detect the respective antibody species (Table 2.3). Immunofluorescence images were captured with a Leica TCS SP5 confocal microscope system (Leica Microsystems Inc, Buffalo Grove, IL). Relative fluorescence of β5 and β5i immunoreactivity was quantified with the Leica Application Suite X (RRID:SCR_013673) using the histogram tool. To quantify the relative expression level on the fluorescence-labeled confocal images, individual regions of interest were delineated (hand draw tool) in the green, neural marker-labeled cells and returning the mean grey values in the red (β5 and β5i) channel. For each animal, a total of 8 confocal images from each stained slide (5-50 labeled cells/field) selected at random were analyzed. Values were expressed as the average fluorescence. Data for oligodendrocytes and astrocytes come from both
white matter and gray matter. Data for neurons derive from gray matter and also include motor neurons.

Statistical Analysis

Animal numbers (study size) in each study group (dpi) was determined by power analysis using G*Power 3.1 software (RRID:SCR_013726; Kiel University, Germany) and the following parameters: two-tail analysis, effective size = 2.0, $\alpha = 0.05$ and a power $(1-\beta) = 0.8$. The effective size was calculated assuming a difference between means of 20% and a SD = 0.10, which was taken from similar studies. Results were analyzed for statistical significance with the unpaired Student’s $t$-test utilizing the GraphPad Prism® program 8.1 (RRID:SCR_002798; GraphPad Software Inc.; San Diego, CA) after assessing the normality of the data with the Shapiro-Wilk test. For data that did not follow a normal distribution, statistical significance was determined with the nonparametric Mann-Whitney test. Outliers in the data sets were identified using the GraphPad Prism® ROUT test and assigning a Q value of 1% and were removed before the final statistical analysis.

Results

Pro-inflammatory cytokine expression and oxidative stress are elevated in EAE spinal cords

EAE was induced in female C57BL/6 mice by double immunization with MOG$_{35-55}$ peptide as explained in “Materials and Methods” and shown in Fig. 2.1. Symptoms were graded according to the following scale: 0, no symptoms; 1, tail weakness; 1.5, clumsy gait; 2, hind limb paresis; 2.5, partial hind limb dragging; 3,
hind limb paralysis; 3.5, hind limb paralysis with fore limb paresis; 4, complete paralysis; and 5, moribund. Neurological symptoms and spinal cord pathology start at 14 dpi (7 days after the second injection with MOG peptide), reaching a maximum after 21 dpi (Fig. 2.2a). CFA-injected animals (i.e. controls) do not present any neurological sign or spinal cord damage. In this study, we used a total of 26 control mice and 32 EAE mice. Except for the small sample size at 60 dpi (n = 3), the number of mice in each of the other control and EAE groups ranged between 5 and 8 (Fig. 2.1). This number is very close to the minimum of 6 mice per group predetermined by power analysis using the parameters described in “Materials and Methods”.

As shown in Fig. 2.2b, lymphocyte infiltration in the spinal cord (as detected by staining the sections with hematoxylin and eosin) begins at 14 dpi reaching a maximum at 21 dpi. In contrast, IFN-γ mRNA expression in EAE is already high at 9 dpi (Fig. 2.2c) and remains elevated throughout the course of the illness. TNF-α mRNA levels in EAE are significantly elevated at 21 dpi and thereafter (Fig. 2.2d), while the temporal expression pattern of IL-1β mRNA is similar to that of IFN-γ, except for the values at 9 dpi (Fig. 2.2e). Glutathione levels in the diseased spinal cord decline progressively starting at 21 dpi (Fig. 2.2f). Overall, these data indicate the presence of significant inflammation and oxidative stress in the spinal cords of EAE mice, which are expected to upregulate immunoproteasome and constitutive proteasome expression, respectively.
Reduced c-20S and increased i-20S expression in EAE spinal cords

We next measured the mRNA expression of the immunoproteasome-specific subunit β5i (Psmb8) as well as that of PA28β (Psme2), a component of the 11S activator, in the spinal cord during the course of EAE (9 - 60 dpi). As expected from the temporal mRNA expression pattern of IFN-γ, TNF-α and IL-1β in EAE (Fig. 2.2c-e), Psmb8 and Psme2 mRNA levels in the affected spinal cords become significantly higher than those in controls at 14 dpi, reaching a maximum between 21 dpi and 30 dpi to decrease thereafter (Fig. 2.3a,b). Similar results were obtained for PA28α (Psme1) (data not shown). In contrast, the constitutive proteasome-specific subunit β5 (Psmb5) mRNA expression diminishes in EAE starting already at 9 dpi, with the difference being maximal at 14 dpi - 21 dpi and declining after that period (Fig. 2.3c). Reduced β5 gene expression in EAE was an unexpected finding since constitutive proteasomes are known to be induced by oxidative stress, which is clearly demonstrated by the low glutathione levels in this disorder (Fig. 2.2f).

The amounts of proteasomes and their activators in EAE mice at 30 dpi, measured by western blot analysis of spinal cord proteins, is shown in Fig. 2.4a. The levels of proteasome core particles (c-20S + i-20S), assessed with an antibody that recognizes six of the seven 20S α-subunits (α1-3/5-7), increases in EAE by 75.7 ± 15.0% (p = 0.0043). However, α7-subunit (Psma7) mRNA expression is unchanged in EAE (Fig. 2.4b), suggesting the occurrence of translational and/or post-translational control mechanisms. Proteasome β5i, PA28α and PA28β proteins are greatly augmented in EAE (Fig. 2.4a), which agrees with their
increased gene expression (Fig. 2.4b). The amount of the β5 subunit is reduced by 36.7 ± 7.8% (\( p = 0.0007 \)) in EAE, which follows the 38% decrease in mRNA expression. Levels of the 19S-specific Rpt5 subunit rise by 107 ± 41% (\( p = 0.0260 \)) while there is an 18% reduction in its mRNA levels (\( Psmc3 \)).

Using the intensity values obtained by western blot analysis and purified c-20S and i-20S proteasomes for calibration, we estimated the absolute amounts of each proteasome core particle in control and EAE spinal cord homogenates. The purity of the c-20S particle (positive for β1, β2 and β5 but not for their inducible counterparts) and the i-20S (positive for β1i, β2i and β5i but not for their standard counterparts) was demonstrated by western blotting in our previous publication (Zheng et al., 2012). We found that in control spinal cords, ~86% of core particles correspond to c-20S proteasome (4.67 pmol/mg protein) and the rest (~14%) to the i-20S proteasome (0.73 pmol/mg protein). In contrast, only 33% of the proteasomes in EAE spinal cords at 30 dpi corresponds to the constitutive form (2.98 pmol/mg protein) while 67% (6.10 pmol/mg protein) was in the form of immunoproteasomes. Based on these numbers, the total amount of proteasomes (i.e. c-20S + i-20S) in EAE rises by 68%, which is close to the 75.7% increase observed by western blot analysis using the α1-3/5-7 antibody (Fig. 2.4a).

*Neurons and astrocytes display altered levels of constitutive- and immunoproteasomes*

To ascertain whether the decrease in constitutive proteasome levels in EAE takes place in all spinal cord cells or whether it is limited to a particular cell type (neurons, astrocytes or oligodendrocytes), we conducted a double-label
immunofluorescence confocal microscopy analysis. As shown in Fig. 2.5, images from control and EAE spinal cords at 30 dpi reveal the presence of the c-20S-specific subunit β5 in the cytoplasm of all three major cell types. Staining was more intense in neurons (average intensity = 41) followed by oligodendrocytes (average intensity = 25) and astrocytes (average intensity = 18). Quantification of the images shows that β5 subunit labeling in EAE decreases by 47.0 ± 7.2 % (p = 0.0014) in neurons and by 54.1 ± 15.0% (p = 0.0596) in astrocytes, while levels of this subunit in oligodendrocytes are unchanged (p = 0.3853) (Fig. 2.7a). The reduced amount of β5 in neurons and astrocytes, along with the decreased number of oligodendrocytes in the EAE spinal cord (Hu et al., 2019), is likely the reason for the 36.7% drop in β5 levels observed on western blots of total spinal cord proteins (Fig. 2.4a).

The average β5i intensity for neurons, astrocytes and oligodendrocytes in control spinal cords at 30 dpi are similar (Fig. 2.6). Quantification of the images reveal that β5i subunit labeling in EAE increases by 117 ± 34% (p = 0.0059) in neurons and by 94 ± 47% (p = 0.0749) in astrocytes, while levels of this subunit in oligodendrocytes are unchanged (p = 0.3414) (Fig. 2.7b). It is noteworthy that gray matter astrocytes have the same changes in proteasome composition in EAE as those present in the white matter (data not shown). Similarly, EAE oligodendrocytes do not display any changes in proteasome composition regardless whether they are in gray or white matter (data not shown). It is also interesting to mention that while β5 staining in control and EAE is exclusively cytoplasmic, β5i (particularly in EAE) was also present in the nucleus. This agrees
with a previous observation that IFN-γ promotes the nuclear accumulation of immunoproteasomes in human cervical epithelial HeLa and Hep2 cells (Fabunmi et al., 2000). In addition to neural cells, the immunoproteasome-specific β5i subunit is present in leukocytes located in the more peripheral regions of the spinal cord (Fig. S2.1). In agreement with the western blot data, PA28 was barely expressed in control spinal cords. In EAE, PA28 expression was present in inflammatory cells (not shown) and astrocytes (Fig. S2.2).

*Increased expression of immunoproteasomes is likely due to activation of STAT-1 signaling pathway*

It has been shown that IFN-γ augments the transcription of the immunoproteasome-specific subunits β1i, β2i and β5i (Aki et al., 1994) via binding of STAT-1-dependent IRF-1 to IFN-γ regulatory elements in the promoter regions of these genes (Foss and Prydz, 1999). The expression of PA28 is also increased by IFN-γ (Groepttrup et al., 1995). In addition, immunoproteasome subunits have NFκB response sequences within their promoters (Ferrington and Gregerson, 2012). As shown in Fig. 2.8a,b, the amount of phosphorylated STAT-1 (p-STAT-1) and IRF-1 in the nuclear fraction increases dramatically in the EAE spinal cord measured at 30 dpi, which clearly results from the ~18-fold rise in IFN-γ expression (Fig. 2.2c). In contrast, nuclear levels of NF-κB (p65 subunit) are unaltered in the disease (Fig. 2.8c). In sum, these data strongly suggest that expression of i-20S and PA28 particles in EAE is mainly the result of activation of the IFN-γ / STAT-1 / IRF-1 pathway.
Abnormal Nfe2l1 (Nrf1) signaling may contribute to the reduced c-20S expression in EAE

Nrf1 is a transcription factor that upregulates the expression of all the proteasome subunit genes in a concerted manner, particularly during proteasome impairment (Radhakrishnan et al., 2010; Lee et al., 2013). There are several Nrf1 isoforms that derive from (1) alternative splicing, (2) alternative initiation of translation, and (3) posttranslational processing (e.g., glycosylation, proteolysis) (Bugno et al., 2015; Xiang et al., 2018). We thought of the possibility that Nrf1 expression may be altered in EAE, which could result in decreased synthesis of constitutive proteasomes. To that end, we first measured Nfe2l1 mRNA levels in the spinal cord during the course of EAE using a set of primers that recognizes all the transcripts. As shown in Fig. 2.9a, Nfe2l1 gene expression in EAE declines starting at 9 dpi, with the difference being maximal between 14 and 21 dpi. It is noteworthy that the temporal pattern of mRNA expression of Nfe2l1 is similar to that of Psmb5 (Fig. 2.3). Western blot analysis of cytoplasmic fractions prepared from control and EAE spinal cords at 30 dpi, reveal the presence of six Nrf1-positive bands with molecular masses ranging from 25 kDa to 100 kDa (Fig. 2.9b). Of all these bands, only the 55 kDa band (Nrf1β) is present in the nucleus and its levels are reduced in EAE by 50.0 ± 10.0% (p = 0.0037) (Fig. 2.9c). When the films corresponding to the nuclear fractions are exposed for a longer time, the 100 kDa band (Nrf1α) becomes visible and its levels are also reduced in EAE (Fig. S2.3). Double label-immunohistochemical analysis to identify the cells where Nrf1 is
reduced were inconclusive due to the heterogeneous labeling of the nuclei in control (mostly diffuse) and EAE (mostly punctuate) spinal cords (data not shown).

Aside from stress-related stimuli, Nfe2l1 expression is induced by growth factors through mTORC1-activation of the sterol regulatory element-binding protein 1 transcription factor (Zhang and Manning, 2015). However, we found that the levels of phospho-mTOR are significantly elevated in EAE measured at 30 dpi, implying that other factors may regulate Nrf1 transcription (Fig. 2.10a). One such molecule is the pre-B-cell leukemia homeobox-1 transcription factor (PBX1), which has been recently shown to directly induce Nrf1 gene expression (Villaescusa et al., 2016). Interestingly, at 30 dpi, nuclear PBX1 levels are reduced in EAE spinal cords by 51.7 ± 3.7% (p = 0.0004) (Fig. 2.10b), suggesting that this phenomenon may underlie the Nfe2l1 deficit in the disease.

Proteasome composition is also altered in the cerebella, but not the cerebra, of EAE mice.

Finally, we measured the expression of proteasomes and their activators in the cerebra and cerebella of EAE mice. Lesions in the cerebellum are different, both in location and intensity, than those in the spinal cord, while minimal pathological changes are known to occur in the cerebrum (Kuerten et al., 2007). As shown in Fig. 2.11, the patterns of expression of the proteasome subunits β5, β5i and α, as well as the activator PA28α, in the EAE cerebellum are similar to those found in the spinal cord; specifically, there is an increase in the levels of i-20S and PA28 and a concomitant reduction in the amount of c-20S. However, the magnitude of the changes in the diseased cerebellum is smaller than those in the
spinal cord. For instance, β5i, α1-3/5-7 and PA28α subunit levels in the cerebella of EAE mice rise by 53.6 ± 22%, 17.2 ± 4.4% and 271 ± 33%, respectively (Fig. 2.11), while amounts of these subunits in the EAE spinal cords increase by 1680 ± 585%, 75.7 ± 15.0% and 686 ± 158%, respectively (Fig. 2.4a). Similarly, β5 levels diminish by 25.2 ± 3.8% ($p = 0.0211$) in EAE cerebella versus 36.7 ± 7.8% in EAE spinal cords. In contrast to the findings in the spinal cord, the amount of the 19S subunit Rpt5 in the EAE cerebellum is unaltered. Analysis of EAE and control cerebra revealed no changes in the levels of any of these proteasome subunits except for a modest increase (68.7 ± 22.7%, $p = 0.0174$) in PA28α levels. In sum, the magnitude of the changes in proteasome subunits in EAE are the highest in the spinal cord, followed by the cerebellum and the cerebrum. This correlates with the varying extents of inflammation and damage in these CNS areas. Interestingly, Nrf1β levels are also reduced in the cerebellum but not the cerebrum (Fig. S2.4). Thus, while subunit displacement is likely to account for some of the decrease in the amounts of constitutive proteasomes in EAE, the contribution of Nrf1 deficiency to this phenomenon could also be significant.

**Discussion**

In this study, we describe the changes in the expression of proteasome particles and their activators in the spinal cords of EAE mice. The results clearly show that (1) there is a rise in i-20S and PA28 expression and a decline in c-20S expression in EAE, both at the mRNA and protein level; (2) among the three cell types studied, the changes are observed in neurons and astrocytes but not oligodendrocytes; (3) the increase in the amount of the i-20S-specific subunit β5i
and PA28α/β in EAE correlates with the levels of IFN-γ, p-STAT-1 and IRF-1, but not with those of NFκB, suggesting that STAT-1 and/or IRF-1 dimers are responsible for induction of these subunits; (4) the decrease in the c-20S-specific β5 subunit mRNA and protein levels may also be due to reduced expression of Nrf1α and Nrf1β; and (5) impaired Nfe2l1 mRNA expression is unlikely due to reduced mTOR signaling but could be the result of diminished PBX1 levels.

There is abundant biochemical and immunohistochemical data, including those presented in this study, showing that inflammation is a major feature of the EAE spinal cord. Local inflammation leads to the production of several pro-inflammatory cytokines, particularly IFN-γ, which are known to upregulate immunoproteasome subunits and the proteasome activator PA28 (Aki et al., 1994; Groepttrup et al., 1995). In line with this notion, we found that the amount of i-20S and PA28α/β, both mRNA and protein, is greatly elevated in EAE (Fig. 2.4). This is not surprising as the promoter regions of the inducible proteasome subunits and PA28 subunits contain binding sites for interferon consensus sequence-2 and gamma interferon activated sequence elements that bind STAT-1 and IRF-1 (Chatterjee-Kishore et al., 2000), and the levels of both transcription factors are elevated in the nuclear fractions from EAE spinal cords (Fig. 2.8). The promoter of immunoproteasome and PA28 subunits also contain additional binding sites for other transcription factors, including three regions of consensus sequence for NF-κB binding (Ferrington and Gregerson, 2012). Interestingly, nuclear NF-κB levels were unchanged in EAE, suggesting that IFN-γ is regulating immunoproteasome expression mostly via STAT-1 and IRF-1. The lack of increased NF-κB signaling
in EAE was surprising since the nuclear levels of this transcription factor are known to be up-regulated by pro-inflammatory cytokines like TNF-α (Hayden and Ghosh, 2014) and IL-1β (McDonald et al., 1997) as well as by oxidative stress (Morgan and Liu, 2011), all of which are elevated in EAE (Fig. 2.2d-f). Perhaps NF-κB is elevated in a small subset of cells like microglia or lymphocytes, and therefore such an increase is not detected when one analyzes total spinal cord proteins.

Concomitant with the rise in the concentration of i-20S and 11S particles in EAE, we discovered a reduction in the amount of c-20S, suggesting the displacement of housekeeping proteasome subunits by immuno-subunits. In support of this idea, we observed that neural cells in EAE spinal cords where β5i expression is increased (i.e., neurons and astrocytes) have low β5 expression. Similarly, EAE oligodendrocytes show no changes in either β5i or β5 expression. Moreover, in the EAE cerebellum, where the increase in the amount of β5i is less pronounced than that in the spinal cord, the reduction in β5 levels is smaller. Proteasome subunit displacement is a well-characterized post-translational process achieved by competition of the inducible subunits with the respective constitutive subunits for a common assembly site within the 20S particle (Früh et al., 1994). The preferential incorporation of all three immuno-subunits (β1i, β2i and β5i) is assisted by the proteasome maturation protein (POMP). This protein binds the pro-peptide of β5i with greater affinity than that of β5, resulting in preferred formation of i-20S over c-20S particles (Heink et al., 2005). Interestingly, POMP expression is upregulated by IFN-γ (Burri et al., 2000), whose levels are augmented in EAE (Fig. 2.2c). Furthermore, p-mTOR (Yun et al., 2016), which is
also elevated in EAE (Fig. 2.10a), promotes the formation of immunoproteasomes by making the immuno-subunits available to POMP via PRAS40 activation. Thus, the conditions present in the EAE spinal cord enable the preferential formation of i-20S over c-20S core particles.

It is important to note that immune cells and microglia may contribute to the western blot and qPCR values determined in spinal cord homogenates. Indeed, we found that CD45+ cells contain significant amounts of β5i (Fig. S2.1). Also, we have previously shown that CD3+ cells and microglia in EAE cerebellum express β5i (Zheng et al., 2012). However, even if microglia and immune cells were to express only immunoproteasomes, that would not explain the decrease in the levels of constitutive particles in EAE from 4.67 to 2.98 pmol/mg protein. Thus, the data in the homogenates cannot be explained simply by the contribution of these non-neural cells. Most importantly, the presence of immunoproteasomes in other CNS cells does not change the fact that c-20S is being replaced with i-20S in EAE neurons and astrocytes, as demonstrated by immunohistochemical analysis.

While subunit displacement is likely one important reason for the reduced levels of constitutive proteasomes in EAE, it does not explain why Psmb5 mRNA levels are also diminished in the disease (Figs. 2.3 and 2.4). STAT-3 has been shown to increase Psmb5 mRNA and protein levels, and the amount of constitutive proteasome (Vangala et al., 2014). However, phospho-STAT-3 levels are augmented in EAE spinal cords (Hou et al., 2017), suggesting that reduction of Psmb5 mRNA expression cannot be attributed to impaired STAT-3 signaling. The promoter regions of all the constitutive proteasome subunits and those that
make up the 11S and 19S complexes have EpRE that can bind to and became activated by Nrf1 and Nrf2 (Pickering et al., 2012). However, while both transcription factors can increase proteasome subunit expression, only Nrf1 is required for upregulation of proteasome genes in response to proteasome inhibition (Radhakrishnan et al., 2010). Furthermore, late-stage deletion of Nrf1 in neuronal cells leads to impaired proteasome function due to down-regulation of proteasomal genes that encode the catalytic subunits (Lee et al., 2011). These studies point to the critical role of Nrf1 in the biogenesis of constitutive proteasomes and prompted us to explore the possibility that expression of this transcription factor is diminished in EAE. In the present study, we discovered that Nfe2l1 mRNA levels, utilizing a set of primers that measure all transcripts, are reduced in EAE spinal cords with a temporal pattern similar to that of Psmb5 mRNA. The reason for diminished Nfe2l1 mRNA expression in EAE is unknown but it does not appear to be the result of reduced mTOR signaling (Fig. 2.10a). Interestingly, we found a decline in PBX1 content in EAE spinal cords, which could underlie the reduced Nrf1 expression (Fig. 2.10b). PBX1 has been recently shown to activate Nfe2l1 expression to promote neuronal survival (Villaescusa et al., 2016). Furthermore, those studies also revealed that both PBX1 and Nrf1 are absent in substantia nigra neurons from Parkinson's disease patients, suggesting an impaired PBX1-Nrf1 pathway in neurodegeneration.

The Nfe2l1 gene can yield multiple mRNA transcripts that can undergo alternative initiation of translation generating distinct polypeptide isoforms. These forms can also be modified by glycosylation and proteolytic cleavage, rendering
several bands on SDS-gels with molecular masses ranging from 25 kDa to 140 kDa (Bugno et al., 2015; Xiang et al., 2018). The four major forms are the full-length Nrf1 (Nrf1α, ~100 kDa on SDS-gradient gels), Nrf1β (~55 kDa), generated through in-frame translation, Nrf1γ (~36 kDa), produced by a separate in-frame translation starting codon, and Nrf1δ (~ 25 kDa) that seems to arise from proteolytic cleavage of Nrf1γ (Zhang et al., 2014; Bugno et al., 2015;). Nrf1α exhibits a stronger transactivation activity than Nrf1β (Caterina et al., 1994; Prieschl et al., 1998; Zhang et al., 2014), while Nrf1γ/δ are dominant-negative forms that interfere with the functional association of Nrf1α or Nrf2 to small musculoaponeurotic fibrosarcoma proteins and down-regulate the expression of EpRE-containing genes (Zhang et al., 2014). Our present work reveals that Nrf1β is the major Nrf1 species present in the nuclear fraction along with a small amount of Nrf1α, and that the levels of both of these positive activators of proteasome expression decline in EAE. A number of additional Nrf1-positive bands were detected on western blots of the cytosolic fraction; these include the 85 kDa and 76 kDa species, which are considered proteolytic fragments of Nrf1α, a 46 kDa form (Nrf1β2) that derives from Nrf1β cleavage, and a 25 kDa form that is likely to be Nrf1δ. However, none of these species are present in the nuclear fractions from the spinal cords and thus were not quantified. It is important to note that most studies in the literature assign functionality to Nrf1 isoforms based on their levels in cell homogenates without determining whether or not they are present in the nucleus.
In summary, we have obtained evidence that two important mechanisms, subunit displacement and impaired Nfe2l1 signaling, may be the cause for the reduced amounts of constitutive proteasomes in neurons and astrocytes during inflammatory demyelination. Because constitutive proteasomes and immunoproteasomes are equally effective at removing ubiquitinated (Nathan et al., 2013) and oxidized proteins (Pickering et al., 2010; Zheng et al., 2012), it is fair to conclude that the overall capacity of these cells to eliminate damaged proteins is augmented in EAE and helps prevent the toxic protein accumulation at the peak of the disease. In a previous study on proteasome composition in the EAE cerebellum, we discovered that as the disease progresses from the inflammatory to the neurodegenerative phase, immunoproteasome levels return to normal without being counterbalanced by a rise in the amounts of constitutive proteasomes (Zheng et al., 2012). This suggests that impaired synthesis of constitutive proteasomes in chronic EAE, caused perhaps by deficient Nrf1 signaling, could be an important pathophysiological mechanism.

Acknowledgements

This work was supported by PHHS grants NS082805 from the National Institutes of Health.

Disclosure/conflict of interest

The authors have no conflict of interest.
References


Zhang Y., Qiu L., Li S., Xiang Y., Chen J. and Ren Y. (2014) The C-terminal domain of Nrf1 negatively regulates the full-length CNC-bZIP factor and its shorter isoform LCRF1/Nrf1β; both are also inhibited by the small dominant-negative Nrf1γ/δ isoforms that down-regulate ARE-battery gene expression. *PLoS One* **9**, e109159


Table 2.1 - List of mouse PCR primers used in the study

### Proteasome subunits

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psmb5 / β5</td>
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<td>5'-GATCTGTGGCTGGGATAAGAG-3'</td>
<td>5'-TCCATAACGCGTAAGCATAC-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
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<td>5'-GCTGCTTTCCAACATGATGC-3'</td>
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<td>Psmb8 / β5i</td>
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<td>5'-CCGAGTCCCATTGTGATCCTAC-3'</td>
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<tr>
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<td>Reverse</td>
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<td>5'-CCGAGTCCCATTGTGATCCTAC-3'</td>
</tr>
<tr>
<td>Psme1 / PA28α</td>
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<tr>
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<td>Psma7 / α7</td>
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<td>5'-GCCATACCGTCTTCTCG-3'</td>
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### Antioxidant Defense

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<td>5'-CTCACTTGCTGATGATTACTTCC-3'</td>
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<td>Reverse</td>
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<td>5'-GCCACCTCGCTCTCTG-3'</td>
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<tr>
<td>Il1b / IL-1β</td>
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<td>Reverse</td>
<td>5'-ATCTTTGTGGGCTTGCTCAACT-3'</td>
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### House-keeping genes

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<td>5'-AGGCCCTTCCAAATGCCAAGAGGA-3'</td>
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<td>Reverse</td>
<td>5'-AGGCCCTTCCAAATGCCAAGAGGA-3'</td>
<td>5'-TGTTGATGGGTGTAACCAGCAGAA-3'</td>
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<td>Hprt1 / HPRT1</td>
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<td>5'-GAGGCCTTCCAAATGCCAAGAGGA-3'</td>
</tr>
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<td>5'-GAGGCCTTCCAAATGCCAAGAGGA-3'</td>
<td>5'-TGAGGCTCTACAGCTGCTACCT-3'</td>
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<td>Rplp0 / RPLP0</td>
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<td>5'-CTAATCAGGCGCTGGGACT-3'</td>
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<td>5'-CTCAGCGCTTGACACATCAACTCAGA-3'</td>
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<td>Rn18s / 18S rRNA</td>
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<td>5'-GCCCTACAATACCCATGAACG-3'</td>
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Table 2.2- List of primary and secondary antibodies used for western blot analysis.

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<th>Species/Clonality</th>
<th>Dilutions</th>
<th>Supplier</th>
<th>RRID</th>
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<td>20S α1-3/5-7</td>
<td>Mus / mAb</td>
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<td>Enzo Life Science</td>
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<td>20S β5</td>
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<tr>
<td>20S β5i</td>
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<td>p-mTOR</td>
<td>Rb / mAb</td>
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<td>PBX1</td>
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<td>Lamin B1</td>
<td>Mus / mAb</td>
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<table>
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<th>Secondary Antibodies</th>
<th>Species/Clonality</th>
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<th>Supplier</th>
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<td>Peroxidase-conjugated anti-rabbit IgG</td>
<td>Goat / pAb</td>
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<td>Jackson ImmunoResearch</td>
<td>AB_2307391</td>
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<tr>
<td>Peroxidase-conjugated anti-mouse IgG</td>
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Abbreviations: Mus, mouse; Rb, rabbit; mAb, monoclonal antibody; pAb, polyclonal antibody; RRID, research resource identifier
Table 2.3- List of primary and secondary antibodies used for immunohistochemical analysis.

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<tr>
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<td>PA28β</td>
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<td>Abcam</td>
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<td>GFAP</td>
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<td>CAII</td>
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<td>Santa Cruz Biotechnology</td>
<td>Sc-48351</td>
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<td>NeuN</td>
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<td>CD45</td>
<td>Rat / mAb</td>
<td>1:25</td>
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<table>
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<th>RRID</th>
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<td>711-165-152</td>
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<td>Alexa Fluor® 488-conjugated anti-mouse IgG</td>
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<td>Alexa Fluor® 488-conjugated anti-rat IgG</td>
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<td>715-545-152</td>
<td>AB_2340684</td>
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</table>

Abbreviations: Mus, mouse; Rb, rabbit; mAb, monoclonal antibody; pAb, polyclonal antibody; RRID, research resource identifier.
Figure 2.1 – Schematic diagram showing the timeline of the EAE study design / procedures. MOG, myelin oligodendrocyte glycoprotein; CFA, complete Freund’s adjuvant; qPCR, quantitative polymerase chain reaction; WB, western blot; IHC, immunohistochemistry; dpi, days post-immunization.
Figure 2.2 – Elevated inflammation and oxidative stress during the course of EAE. EAE was induced in C57BL/6 female mice by active immunization with MOG\textsubscript{35-55} peptide in CFA. EAE was induced in C57BL/6 female mice by active immunization with MOG\textsubscript{35-55} peptide in CFA. (a) Animals were monitored daily for signs of clinical disease, and symptoms were scored as indicated in the text. Dots represent the final clinical score of each of the 32 EAE mice used in the study. Control animals did not exhibit neurological symptoms (i.e. score = 0). (b) The number of lymphocytes per spinal cord section were assessed by hematoxylin and eosin staining. (c-e) IFN-\(\gamma\), TNF-\(\alpha\), and IL-1\(\beta\) mRNA levels during the course of the disease were determined by qPCR. (f) Total GSH levels were measured using the enzymatic recycling assay and data shown was taken from one of our previous publications (Morales Pantoja et al., 2016). Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines show the mean ± SEM of 3-8 animals. Asterisks denote values that are statistically different (\(p<0.05\)) from their respective controls.
Figure 2.3 – Altered gene expression of proteasomes, immunoproteasomes and PA28 during the course of EAE (9 – 60 dpi). (a) Proteasome subunit β5i (Psmb8), (b) PA28α (Psme2) and (c) proteasome subunit β5 (Psmb5) mRNA levels in the spinal cords of control and EAE mice were determined by qPCR. Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines show the mean ± SEM of 3-7 animals. Asterisks denote values that are statistically different (p<0.05) from their respective controls as assessed by Student’s t-test.
Figure 2.4 – Reduced expression of constitutive proteasomes and increased expression of immunoproteasomes and the 11S activator in the EAE spinal cord.
(a) Levels of subunits α1-3/5-7, β5, β5i, PA28α, PA28β and Rpt5 in control and EAE spinal cords at 30 dpi were determined by western blot analysis. Left panel shows representative western blots of the analyzed subunits. (b) Psma7 (α7), Psmb5 (β5), Psmb8 (β5i), Psme1 (PA28α), Psme2 (PA28β) and Psmc3 (Rpt5) mRNA levels in the spinal cords of control and EAE mice at 30 dpi were determined by qPCR. In both panels, each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines depict the mean ± SEM of 3-7 animals. Statistical significance was determined by Student’s t-test. NS, not significant.
Figure 2.5 – Representative double-label immunofluorescence images of lumbar spinal cord sections of control and EAE mice depicting the expression of the c-20S-specific subunit β5 in neurons (a), astrocytes (b) and oligodendrocytes (c). Spinal cord sections (5 µm-thick) from control and EAE mice were co-stained with anti-β5 antibody and either anti-NeuN (neurons), anti-GFAP (astrocytes) or anti-CAII (mature oligodendrocytes) antibody, and images were visualized by confocal microscopy. Red channel is for β5 while green channel is for various cell markers. Nuclei were labeled with TO-PRO-3. Bars at lower left of each panel represent 20 µm in length.
Figure 2.6 – Representative double-label immunofluorescence images of lumbar spinal cord sections of control and EAE mice depicting the expression of the i-20S-specific subunit β5i in neurons (a), astrocytes (b) and oligodendrocytes (c). Spinal cord sections (5 µm-thick) from control and EAE mice were co-stained with anti-β5i antibody and either anti-NeuN (neurons), anti-GFAP (astrocytes) or anti-CAII (mature oligodendrocytes) antibody, and images were visualized by confocal microscopy. Red channel is for β5i while green channel is for various cell markers. Nuclei were labeled with TO-PRO-3. Bars at lower left of each panel represent 20 µm in length.
Figure 2.7 – Differential expression of proteasome subunits β5 (a) and β5i (b) in neurons, astrocytes (astros) and oligodendrocytes (oligos) in EAE spinal cords. Double immunofluorescence confocal analysis to estimate the average β5 and β5i fluorescence intensity in neurons, astrocytes and oligodendrocytes was performed as described in “Materials and Methods”. Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines depict the mean ± SEM of 3-7 animals. Statistical significance was determined by Student’s t-test. NS, not significant.
Figure 2.8 – Augmented nuclear levels of p-STAT-1 and IRF-1, but not NF-κB, in the EAE spinal cord. Levels of p-STAT-1 (a), IRF-1 (b) and NFκB (c) in the nuclear fractions from control and EAE spinal cords at 30 dpi were determined by western blot analysis. Band intensities were corrected by the amount of histone H3 in the same gel lanes. Each point represents one animal. Values are expressed relative to controls except for panel b, where IRF-1 was undetectable in the control mice. Bars and horizontal lines depict the mean ± SEM of 3-7 animals. Statistical significance was determined by Student’s t-test. NS, not significant. (d) Representative western blots of the analyzed proteins.
Figure 2.9 – Decreased Nrf1 mRNA and protein in the EAE spinal cord. (a) Nfe2l1 mRNA levels in the spinal cords of control and EAE mice were determined by qPCR. Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines show the mean ± SEM of 3-7 animals. Asterisks denote values that are statistically different (p<0.05) from their respective controls. (b) Cytoplasmic and nuclear proteins from control and EAE spinal cords at 30 dpi were analyzed by western blot using an antibody against Nrf1. The cytoplasmic fraction shows six Nrf1-positive bands with molecular masses ranging from 25 kDa to 100 kDa (arrows) but only the 55 kDa band (Nrf1β) is present in the nuclear fraction. (c) Band intensities corresponding to Nrf1β in the nuclear fraction were corrected by the amount of histone H3 in the same gel lanes. Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines depict the mean ± SEM of 4 animals per experimental group. Statistical significance was determined by Student’s t-test.
Figure 2.10 – High levels of p-mTOR and reduced expression of PBX1 in EAE. (a) The amount of p-mTOR in spinal cord homogenates from control and EAE mice at 30 dpi was determined by western blot analysis. Band intensities were corrected by the amount of Coomassie blue staining in the same gel lanes. (b) PBX1 levels in the nuclear fractions from control and EAE spinal cords at 30 dpi were determined by western blot analysis. Band intensities were corrected by the amount of histone H3 in the same gel lanes. In both panels, each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines depict the mean ± SEM of 4-7 animals. Statistical significance was determined by Student’s t-test. Lam B, lamin B1.
Figure 2.11 – Altered proteasome composition in the cerebella, but not the cerebra, of EAE mice. Levels of subunits β5, β5i, α1-3/5-7, PA28α and Rpt5 in the cerebra and cerebella of control and EAE mice at 30 dpi were determined by western blot analysis. Band intensities were corrected by the amount of Coomassie blue staining in the same gel lanes. Each point represents one animal. Values are expressed relative to controls. Bars and horizontal lines depict the mean ± SEM of 5-6 animals. Statistical significance was determined by Student’s t-test. NS, not significant. Lower right panel shows representative western blots of the analyzed subunits.
Figure S2.1 - Representative double-label immunofluorescence images of lumbar spinal cord sections from EAE mice depicting the high expression of the immunoproteasome subunit β5i in CD45-positive infiltrating leukocytes. Spinal cord sections (5 µm-thick) from EAE mice were co-stained with β5i antibody and CD45 antibody, and images were visualized by confocal microscopy as described in “Materials and Methods”. Nuclei were labeled with TO-PRO-3. Bar at lower left represents 20 µm in length.
Figure S2.2 - Representative double-label immunofluorescence images of lumbar spinal cord sections from EAE mice depicting the expression of the PA28β in neurons, astrocytes and oligodendrocytes. Spinal cord sections (5 µm-thick) from EAE mice were co-stained with anti-PA28β antibody and either anti-NeuN, anti-GFAP or anti-CAII antibody, and images were visualized by confocal microscopy as described in “Materials and Methods”. Red channel is for PA28β while green channel is for various cell markers. Nuclei were labeled with TO-PRO-3. Bar at lower left represents 20 µm in length.
Figure S2.3 – Nrf1α levels are reduced in the EAE spinal cord. (a) Nuclear fractions from control and EAE at 30 dpi were analyzed by western blot. Band intensities were corrected by the amount of histone H3 in the same gel lanes. Values are expressed relative to control and represent the mean ± SEM of 4 mice. Statistical significance was determined by Student’s t-test. (b) Representative western blot.
Figure S2.4 – Nrf1β levels are reduced also in the EAE cerebellum. (a) Nuclear fractions from control and EAE tissues at 30 dpi were analyzed by western blot. Band intensities were corrected by the amount of histone H3 in the same gel lanes. Values are expressed relative to control and represent the mean ± SEM of 6-7 mice. Statistical significance was determined by Student’s t-test. NS, not significant. (b) Representative western blots of the analyzed proteins.
Chapter 3 – Subunit displacement, and not changes in Nfe2l1/2 levels, determines proteasome composition in cytokine-treated neurons and astrocytes

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[Submitted to Neurochemical Research 2019]
Abstract

In this study, we investigated if subunit displacement and/or alterations in proteasome biosynthesis are responsible for the changes in the levels of constitutive proteasomes (c-20S), immunoproteasomes (i-20S) and the activators PA28 and PA700 in neurons and astrocytes cultured with a cytokine mixture (IFN-γ / TNF-α / IL-1β). Exposure of both cell types to cytokines for 24 h increases mRNA and protein expression of the i-20S-specific subunit β5i and PA28α/β and leads to a decline in the amount of the c-20S-specific subunit β5. Since β5 mRNA levels are unchanged by the cytokine treatment, it is fair to conclude that displacement of constitutive β-subunits with inducible β5i subunits is likely the mechanism underlying the decrease in c-20S. As expected, the increase in the amount of the IFN-γ-inducible subunits coincides with elevated expression of phospho-STAT-1 and interferon regulatory factor-1 (IRF-1). However, inhibition of NF-κB signaling in cytokine-treated astrocytes reduces IRF-1 expression without affecting that of i-20S, c-20S and PA28. This suggests that STAT-1 is capable of increasing the transcription of i20S-specific subunits and PA28α/β by itself. The lack of a decrease in proteasome β5 mRNA expression is consistent with the fact that Nrf1 (Nfe2l1) and Nrf2 (Nfe2l2) levels are not reduced by pro-inflammatory cytokines. In contrast, we previously found that there is a significant Nrf1 dysregulation and reduced β5 mRNA expression in the spinal cords of mice with experimental autoimmune encephalomyelitis (EAE). Thus, there are stressors in EAE, other than a pro-inflammatory environment, that are not present in cytokine-treated cells.
Introduction

Proteasomes are multi-enzyme complexes whose sole function is to catalyze the degradation of most intracellular proteins [1]. The core of these complexes, the 20S proteasome, is comprised of two outer α and two inner β rings (i.e. $\alpha\beta\beta\alpha$). Each α and β ring is made of seven α subunits ($\alpha_1$-$\alpha_7$) and seven β subunits ($\beta_1$-$\beta_7$) [2]. While the α rings are the structural components of the proteasome, each β ring has three proteolytic sites, which display caspase-like ($\beta_1$ or $Psmb6$), trypsin-like ($\beta_2$ or $Psmb7$) and chymotrypsin-like ($\beta_5$ or $Psmb5$) activities [3]. The expression, structure and activity of proteasomes are stringently controlled and are constantly adjusted to fulfill cellular requirements. For example, exposure of cells to interferon-$\gamma$ (IFN-$\gamma$), leads to the replacement of the catalytic $\beta_1$, $\beta_2$ and $\beta_5$ subunits in the constitutive 20S particle (c-20S) by the inducible $\beta_1i$, $\beta_2i$ and $\beta_5i$ subunits, forming the immunoproteasome 20S particle (i-20S) [4]. IFN-$\gamma$ promotes this displacement of subunits by two concerted processes. First, it augments the transcription of the $\beta i$ subunits [4] via binding of the signal transducer and activator of transcription 1 (STAT-1)-dependent interferon regulatory factor-1 (IRF-1) to IFN-$\gamma$ regulatory elements in the promoter regions of these genes [5]. Second, it increases the expression of proteasome maturation protein, which binds to the pro-peptides of the catalytic $\beta i$-forms with greater affinity than those of catalytic $\beta$-forms, favoring the formation of i-20S over c-20S particles [6]. Once all of these subunit precursors are incorporated into the proteasome, there is cleavage of the N-terminal pro-peptides from the three pairs of catalytic $\beta$ subunits making the mature proteasome [7].
Proteasome activity is regulated by a number of protein complexes that attach to one or both ends of the $\alpha_\beta_\alpha$ barrel. One of these regulators, PA28 (11S), is also induced by IFN-$\gamma$ and attaches to the i-20S particle forming a complex (11S/i-20S/11S) [8]. PA700 (19S), the other major activator, binds to the end of 20S core particles to form the 26S proteasome, which recognizes and hydrolyzes abnormal and misfolded proteins conjugated with polyubiquitin chains [9]. Other less studied regulators are the 31kDa proteasome inhibitor (PI31 or Psmf1) [10] and the 200kDa proteasome activator (PA200 or Psme4) [11].

Responsibility for up-regulation of genes that encode the subunits of the c-20S particle and the 11S and 19S activators belongs to Nrf1 (Nfe2l1) and Nrf2 (Nfe2l2) [12], which are members of the cap’n’collar subfamily of basic-region leucine zipper transcription factors [13]. In the absence of cellular stress, Nrf1 undergoes endoplasmic reticulum-mediated degradation, while Keap1-bound Nrf2 is degraded by the proteasome. However, upon endoplasmic reticulum stress [14] and oxidative stress [15], the degradation of these transcription factors is reduced, allowing them to translocate to the nucleus where they form heterodimers with small Maf proteins. The Nrf1/Maf or Nrf2/Maf heterodimers then transactivate proteasome genes through electrophile response elements in their promoters [16, 17]. While Nfe2l1 mRNA expression is positively regulated by the mammalian target of rapamycin complex 1 (mTORC1) [18] and the pre-B-cell leukemia homeobox-1 transcription factor (PBX1) [19], that of Nfe2l2 is upregulated by NF-$\kappa$B [20].
We have previously shown that, at the peak of the disease, there is increased expression of i-20S and PA28 and a concomitant decline in c-20S levels in the cerebella and the spinal cords of mice with experimental autoimmune encephalomyelitis (EAE) [21, 22]. In the latter study, we also found that a combination of subunit displacement and reduced Nrf1 expression might be responsible for c-20S impairment in EAE. Furthermore, we speculated that the inflammatory conditions of the EAE spinal cord is likely the cause for Nrf1 impairment and the subsequent decrease in β5 subunit mRNA expression. In the present study, we investigate the effect of a mixture of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-1β), known to be elevated in EAE [22], on the protein and mRNA expression of proteasome core particles and their activators in cultured neurons and astrocytes. We discovered that in neurons and astrocytes exposed to the cytokine mixture, the replacement of c-20S by i-20S occurs by subunit displacement as Psmb5 mRNA levels are unaltered. Moreover, in contrast to EAE, Nrf1 and Nrf2 do not appear to play a role in the cytokine-induced downregulation of c-20S proteasomes in vitro.

Materials and Methods

Cell Culture and Drug Treatments

All incubations were performed in a humidified incubator at 37°C under a 95% air / 5% CO₂ atmosphere. Mouse neuroblastoma cells (Neuro-2A or N2a) (ATCC® CCL-131™) were maintained in minimum essential medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 100 units of penicillin and 0.1 mg/ml streptomycin (Life
Technologies). Differentiation of N2a cells into a neuronal phenotype was achieved by incubation for 24 h with 0.25 mM theophylline (Sigma) and 1 mM N6-2′-O-dibutyryl cyclic-AMP (Sigma) in low serum medium (0.5% FBS). These differentiated cells develop neurites and express neuron-specific markers such as neurofilament heavy chain (NFH) and neuron specific enolase (NSE) (Fig. 3.1a). Cells were then incubated with or without a pro-inflammatory cytokine mixture (CIII) consisting of 5 ng/mL IFN-γ (Sigma), 10 ng/mL IL-1β (Sigma), and 10 ng/mL TNF-α (Sigma) [23]. After 24 h, cells were harvested and used for protein and mRNA analysis.

Rat C6 glioma cells (ATCC® CCL-107™) were sustained in Dulbecco’s modified Eagle’s medium / Ham’s F-12 (Sigma) containing 10% FBS, 2 mM GlutaMAX™ (Life Technologies) and the antibiotic mixture shown above. Cells were differentiated into astrocytes by incubating for 24 h with 0.25 mM theophylline and 1 mM N6-2′-O-dibutyryl cyclic-AMP in low serum medium (1% FBS). These cells display characteristic astrocyte morphology and express glia-specific markers such as glial fibrillary acidic protein (GFAP) and excitatory amino acid transporter-1 (EAAT1) (Fig. 3.1b). Astrocytes were then incubated with or without CIII in the absence or presence of 2 µM IKK-16 (Selleckchem, Houston, TX). As before, cells were harvested after 24 h and used for protein and mRNA analysis.

**Cellular Fractionation**

Preparation of the nuclear fraction was carried out using a modified version of the nuclear extraction protocol developed by Thermo-Fisher Scientific. Briefly, cultured cells were suspended at 4°C in 0.15 ml of hypotonic buffer solution (20
mM Tris-HCl pH 7.4 containing 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 1 mM PMSF and 1x protease and phosphatase inhibitor cocktails) by pipetting up and down several times. Suspensions were kept on ice for 15 min, mixed with 7.5 µl of 10% w/v Nonidet P-40, and centrifuged at 3,000 g for 10 min. The supernatant (cytosolic fraction) and the pellet (nuclear fraction) were collected, and proteins were solubilized in sodium dodecyl sulfate (SDS)-sample buffer and analyzed by western blotting.

Reverse Transcription and qPCR

Cells were lysed using QIAshredder columns (Qiagen, Germantown, MD) and total RNA was isolated from the lysates via the column purification used in the RNeasy® Mini Kit (Qiagen). DNase I-treated RNA was used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 200 ng of RNA were mixed with the 2X reverse transcription master mix, which contains the reverse transcription buffer, a dNTP mixture, random primers, an RNase inhibitor and the MultiScribe Reverse Transcriptase™. The samples were heated as follows: 25°C for 10 min, 37°C for 120 min, and finally 85°C for 5 min to terminate the reaction.

Gene expression levels were quantified using Power SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA) with primers against selected targets made by Integrated DNA Technologies (Coralville, IA) (Tables S3.1 and S3.2). cDNAs (20 ng) were mixed with 1 µM of each primer and amplified with the Power SYBR Green PCR MasterMix according to the manufacturer’s instructions at 50°C for 10 min, 95°C for 10 min and then 40 thermal cycles of 95°C/15 sec and
60°C/1 min. The relative mRNA expression was determined using the comparative 2^{-ΔCt} method [24]. RNA levels in cultured mouse neurons were normalized to the geometric mean of 4 reference genes (Gapdh, Hprt1, Rplp0 and Rn18s) (Table S3.1) [25]. In cultured rat astrocytes, RNA levels were also normalized to the geometric mean of 4 reference genes (Gapdh, Hprt1, Rplp0 and Tbp) (Table S3.2).

**Western Blot Analysis**

Proteins (5 μg) were separated by SDS–polyacrylamide gel electrophoresis on 4-20% Mini-Protean TGX gels (Bio-Rad Laboratories; Hercules, CA) and were blotted to polyvinylidene difluoride membranes. Blots were blocked with 3% (w/v) non-fat milk in phosphate-buffered saline solution containing 0.05% (v/v) Tween 20 (PBS-T) and then incubated overnight at 4°C with the primary antibodies listed in Table S3.3. Membranes were rinsed three times in PBS-T and incubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibodies (Table S3.3). Blots were developed by enhanced chemiluminescence using the Western Lightning ECL™ kit from Perkin-Elmer (Boston, MA). Films were scanned in a Hewlett Packard Scanjet 4050 and the images were quantified using the NIH ImageJ 1.51 imaging analysis program. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. When analyzing proteins from the nuclear fractions, band intensities were normalized by the intensity of histone H3 or HDAC2 in the same lanes. All the antibodies used in this study were validated by the suppliers and detected bands at the predicted molecular weights on western blots.
**Statistical Analysis**

Results were analyzed for statistical significance with the unpaired Student’s *t*-test or two-way ANOVA test utilizing the GraphPad Prism® program (GraphPad Software Inc.; San Diego, CA) after assessing the normality of the data with the Shapiro-Wilk test. No outliers were identified in any of the data sets using the GraphPad Prism® ROUT test and assigning a Q value of 1%.

**Results**

*Reduced c-20S and Increased i-20S/PA28 Expression in CIII-treated Neurons*

The amounts of proteasomes and their activators in control and CIII-treated neurons were measured by western blot analysis (Fig. 3.2a). The mean levels of proteasome core particles (c-20S + i-20S), assessed with an antibody that recognizes six of the seven 20S α-subunits (α1-3/5-7), increases by 38.3 ± 22.7% upon cytokine treatment but the difference is not significant (*p* = 0.1057). Similarly, α7-subunit (Psma7) mRNA expression is unchanged in neurons incubated with the cytokine mixture (Fig. 3.2b). In contrast, the amounts of the i-20S-specific subunit β5i and those of PA28α and PA28β augment in the cytokine-stimulated cells by 589 ± 88% (*p* < 0.0001), 439 ± 101% (*p* < 0.0009) and 414 ± 50% (*p* < 0.0001), respectively (Fig. 3.2a), and this pattern coincides with their increased gene expression (Fig. 3.2b). The amount of c-20S-specific subunit β5 declines by 24.5 ± 5.8% (*p* = 0.0012) in the cytokine-treated neurons (Fig. 3.2a), without changes in its mRNA expression (*Psmb5*) (Fig. 3.2b). Protein and mRNA levels of the 19S-specific subunit Rpt5 (*Psmc3*) are unaltered by the cytokine treatment (Fig. 3.2a, b).
Using the intensity values obtained by western blot analysis and purified c-20S and i-20S proteasomes for calibration, we estimated the absolute amounts of each proteasome core particle in control and CIII-treated neurons. The purity of the c-20S particle (positive for β1, β2 and β5 but not for their inducible counterparts) and the i-20S (positive for β1i, β2i and β5i but not for their standard counterparts) was demonstrated by western blotting in our previous publication [21]. We estimated that, in control neurons, the majority (~90%) of core particles are c-20S proteasomes (12.2 pmol/mg protein) with the rest (~10%) being i-20S proteasomes (1.40 pmol/mg protein). In contrast, only 44% of the proteasomes in CIII-treated neurons correspond to the constitutive form (9.2 pmol/mg protein) while 56% (11.5 pmol/mg protein) is in the form of immunoproteasomes. Based on these numbers, the total amount of proteasomes (i.e. c-20S + i-20S) in neurons rises by 52% upon cytokine treatment, which is close to the 38% increase observed by western blot analysis using the 20S α1-3/5-7 antibody (Fig. 3.2a).

*Increased Amounts of i-20S and PA28 in CIII-treated Neurons Correlates with Augmented STAT-1 / IRF-1, but not with NF-κB Activation*

IFN-γ is known to augment the transcription of the immunoproteasome-specific subunits β1i, β2i and β5i [4] as well as PA28α/β [26]. This occurs via binding of STAT-1-dependent IRF-1 to IFN-γ regulatory elements in the promoter regions of inducible proteasome genes [5]. Immunoproteasome-specific subunits also have NF-κB response sequences within their promoters [7]. As shown in Fig. 3.3a and b, the amounts of phosphorylated STAT-1 (p-STAT-1) and IRF-1 increase dramatically in the nuclear fractions of CIII-stimulated neurons. In
contrast, nuclear levels of NF-κB (p65 subunit) are unaltered by cytokine treatment (Fig. 3.3c). These data strongly suggest that expression of i-20S and PA28 particles in cytokine-treated neurons results from activation of the IFN-γ / STAT-1 / IRF-1 pathway instead of the TNF-α or IL-1β / NF-κB / IRF-1 pathway.

In agreement with the lack of changes in β5 mRNA expression (Fig. 3.2b), neither Nrf1 nor Nrf2 levels are altered in neurons upon cytokine exposure (Fig. 3.3 d, e). This suggests that all the proteasome changes in the stimulated cells are entirely due to subunit displacement, which in turn is the result of increased IFN-γ / STAT-1 / IRF-1 signaling. The absence of changes in Nrf1 and Nrf2 signaling also suggests that the three pro-inflammatory cytokines do not cause significant oxidative and endoplasmic reticulum stress in these cells.

Reduced c-20S and Increased i-20S/PA28 Expression in CIII-treated Astrocytes

The amounts of proteasomes and their activators in control and CIII-treated astrocytes, measured by western blot analysis, is shown in Fig. 3.4a. The levels of proteasome core particles (c-20S + i-20S) rise by 61.8 ± 14.1% (p = 0.0033) upon cytokine treatment. However, α7-subunit (Psma7) mRNA expression is unchanged (Fig. 3.4b), suggesting the occurrence of translational and/or post-translational control mechanisms. Proteasome β5i, PA28α and PA28β protein levels augment in CIII-treated astrocytes by 237 ± 26% (p < 0.0001), 175.2 ± 38.1% (p = 0.0010) and 209.3 ± 30.4% (p < 0.0001), respectively, following their increased gene expression (Fig. 3.4b). The amount of the β5 subunit is reduced by 20.2 ± 7.3% (p = 0.0142) in CIII-treated astrocytes, without changes in its mRNA expression.
mRNA and protein levels corresponding to the 19S subunit Rpt5 are unaffected by the cytokine treatment. Altogether, these data point to subunit displacement as the mechanism underlying the changes in proteasome composition in CIII-stimulated astrocytes.

As we did for neurons (see above), we also calculated the absolute amounts of each proteasome core particle in CIII-treated and untreated astrocytes. In control astrocytes, ~56% of core particles are c-20S proteasomes (5.3 pmol/mg protein) and the rest (~44%) are the i-20S proteasomes (4.2 pmol/mg protein). In contrast, only 27% of the proteasomes in CIII-treated astrocytes correspond to the constitutive form (4.1 pmol/mg protein) while 73% (11.1 pmol/mg protein) are immunoproteasomes. Based on these numbers, the total amount of proteasomes (i.e. c-20S + i-20S) in astrocytes rise by 60% upon cytokine treatment, which is close to the 61.8% increase observed by western blot analysis using the 20S $\alpha$1-3/5-7 antibody (Fig. 3.4a).

**Diminished c-20S Expression in CIII-treated Astrocytes Occurs Despite Increased Nrf2 Signaling**

Consistent with the absence of changes in $\beta$5 mRNA levels (Fig. 3.4b), the amount of the major Nrf1 isoforms (p120 and p65) as well as Nfe2l1 mRNA levels are unaltered in CIII-treated astrocytes (Fig. 3.5a-c). The latter may result from the lack of changes in the expression of p-mTOR and PBX1 (Supplementary Fig. 3.1), which are known to positively upregulate Nfe2l1 mRNA expression [18, 19]. Thus, as in the case of cultured neurons, CIII treatment does not seem to cause endoplasmic reticulum stress in astrocytes. In contrast to Nrf1, levels of Nrf2
protein and mRNA in cytokine-stimulated astrocytes increase by $120.2 \pm 4.7\%$ ($p = 0.0005$) and $83.7 \pm 14.7\%$ ($p < 0.0001$), respectively (Fig. 3.5d, e). This suggests that (1) the pro-inflammatory cytokine mixture causes substantial oxidative stress, which activates Nrf2 signaling, and (2) this transcription factor does not play a significant role in the biogenesis of constitutive proteasomes in this *in vitro* model of inflammation. The presence of NF-κB binding sites in the promoter region of the Nrf2 gene [20] may explain why *Nfe2l2* mRNA expression is also increased in the stimulated astrocytes.

*NF-κB Inhibition and Reduced IRF-1 Signaling have no Effect on c-20S, i-20S or PA28 Expression in CIII-treated Astrocytes*

IKK-16 is a selective IkB kinase β (IKK-β) inhibitor [27] that has been shown to reduce the levels of NF-κB and its downstream effectors [28]. We decided to use this drug to determine the relative contributions of (i) the IFN-γ / STAT-1, (ii) the IFN-γ / STAT-1 / IRF-1 and (iii) the TNF-α or IL-1β / NF-κB / IRF-1 pathways to the biogenesis of immunoproteasomes and the 11S activator in astrocytes. To this end, astrocytes were pre-treated for 1 h with or without 2 µM IKK-16, a concentration that is 50 times the IC$_{50}$ for IKK-β [27]. Cells were then incubated for another 24 h with or without the cytokine mixture. As shown in Fig. 3.6a, CIII treatment increases the amount of nuclear NF-κB (p65) by $335 \pm 76\%$ ($p < 0.0001$) and IKK-16 reduces this activation by $38.8 \pm 13.2\%$ ($p = 0.0276$). IRF-1 levels in cytokine-stimulated astrocytes rise by $986 \pm 195\%$ ($p = 0.0005$), and IKK-16 treatment decreases this expression by $80.4 \pm 8.0\%$ ($p = 0.0203$). As expected, p-STAT-1 levels are greatly elevated in astrocytes incubated with CIII, while IKK-16
has no effect. The above findings suggest that the high IRF-1 levels in astrocytes treated with the cytokine mixture is due to NF-κB rather than STAT-1 activation. As shown in Fig. 3.7, neither the amounts of β5i, β5 nor PA28α in CIII-treated astrocytes change in the presence of IKK-16, suggesting that IRF-1 is not a major contributor to the expression of inducible proteasome genes under these culture conditions.

**Discussion**

IFN-γ is known to up-regulate immunoproteasome-specific subunits and the proteasome activator PA28 [4, 26]. In agreement with this concept, we discovered that the amount of i-20S and PA28α/β, both mRNA and protein, is significantly elevated in CIII-treated neurons (Fig. 3.2) and astrocytes (Fig. 3.4). This was expected since (i) the promoter regions of the inducible proteasome subunits and PA28 subunits contain gamma interferon activated site (GAS) elements that dimers of p-STAT-1 bind to [4, 29, 30] and interferon-stimulated response elements (ISRE) that are activated by IRF-1 [31], and (ii) the levels of both transcription factors are elevated in the nuclear fractions prepared from CIII-stimulated neurons (Fig. 3.3) and astrocytes (Fig. 3.5). The promoter regions of immunoproteasome-specific catalytic subunits and PA28 subunits also contain binding sites for other transcription factors, including three regions of consensus sequence for NF-κB binding [7]. Interestingly, nuclear NF-κB levels are unchanged in CIII-stimulated neurons, suggesting that IFN-γ is regulating immunoproteasome expression mostly via the STAT-1/IRF-1 signaling pathway in these cells. The lack of increased NF-κB signaling in neurons upon CIII stimulation is surprising since
TNF-α [32] and IL-1β [33] are known to up-regulate the amount of NF-κB in the nucleus and both are present in the cytokine mixture. One explanation might be that, in contrast to other types of neurons, the cAMP-differentiated N2a cells used herein express low levels of IL-1β and TNF-α receptors. In contrast to neurons, astrocytes produce high levels of nuclear NF-κB upon exposure to the cytokine mixture (Fig. 3.5). Importantly, inhibition of NF-κB in CIII-treated astrocytes with IKK-16 almost brings back IRF-1 levels to control values (Fig. 3.6) without reducing the amount of i-20S and PA28 and/or increasing the expression of c-20S (Fig. 3.7). These findings, along with the fact that p-STAT-1 levels are unaffected by IKK-16, suggest (i) that IRF-1 expression in stimulated astrocytes is mostly dependent on NF-κB, despite its promoter containing binding sites for both NF-κB [34] and p-STAT-1 [35], and (ii) that in the absence of IRF-1, p-STAT-1 is enough to drive the expression of inducible proteasome and PA28 subunits. The latter conclusion challenges the idea that IFN-γ induction of immunoproteasome-specific subunits is solely mediated by IRF-1 [5] or IRF-1/dephospho-STAT-1 dimers [31].

Concurrent with the rise in concentration of i-20S in CIII-stimulated neurons and astrocytes, there is a reduction in the amount of c-20S, suggesting the displacement of constitutive proteasome subunits by inducible subunits. The observed reduction in β5 levels after just 24 h of incubation with CIII was surprising, since the half-lives of constitutive proteasomes have been reported to range from 40-200 h [6, 36]. In one of these studies, the half-lives of c-20S and i-20S particles in human lymphoblast T2 cells were found to be 133 h and 27 h, respectively, and were independent of IFN-γ [6]. Interestingly, figure 6a in that article shows that the
amount of mature β5 is greatly reduced after just a 24 h-exposure to IFN-γ, but no explanation was provided for this observation. More recently, it was found that suppression of STAT-3 phosphorylation in human prostate epithelial DU-145 cells coordinately decreases the mRNA and protein levels of all the c-20S β subunits in less than 18 h – similar findings were obtained in HeLa, human lung epithelial A-549 and human mammary epithelial MDA-MB-231 cells [37]. These studies suggest that proteasome turnover may be much faster than that determined by pulse-chase experiments. Indeed, incubation of neuron-like PC12 cells with the lysosomal/autophagy inhibitor chloroquine doubles the amount of β5 subunits in just 24 h, indicating high rates of constitutive proteasome synthesis and degradation (Supplementary Fig. 3.2). It is important to note that the possibility of constitutive subunits being exchanged by inducible subunits in the mature proteasome particle has been previously ruled out because no free processed forms of constitutive and inducible subunits has been found, while their free precursors are detectable [38].

Little is known about what regulates the production of the proteasome α-subunits, although it has been postulated that they are always produced in excess and that the rate limiting step in proteasome biosynthesis is the amount of β subunits [39, 40]. In this study, we found that the increase in the amount of proteasome in the CIII-treated neurons and astrocytes, calculated based on the content of β5 and β5i subunits, is similar to that of the total 20S pool, as determined by western blotting using the antibody against the α1-3/5-7 chains. This suggests that α chains are mostly part of mature, functional proteasomes. However, in other
systems this does not seem to be the case. For example, we found in the cerebella of EAE mice, the molar amounts of hemi-proteasomes, determined by quantifying the α-subunits, is almost twice than that calculated using values of mature β subunits [21]. Also, in the lumbar spinal cords of SOD1\(^{G93A}\) transgenic mice, an animal model of amyotrophic lateral sclerosis, the reduction in β5 is not accompanied by an increase in amount of β5i subunits and yet the levels of structural 20S α chains are normal [41]. Similarly, the upregulation of inducible catalytic subunits in the cortices and striata of HD94 mice, an animal model of Huntington's disease, is not followed by an increase in α chain levels despite normal amounts of standard catalytic subunits [42].

PA28 expression augments in response not only to inflammation but also to oxidative stress, inhibition of proteasomal function, generation of misfolded proteins and impairment of mitochondrial function [43, 44]. This suggests that this activator plays roles other than increasing the generation of peptides for antigen presentation. Indeed, PA28 has been found to raise the rate of oxidized protein degradation by core 20S particles, and enhancement of proteasome function by PA28 overexpression has been shown to protect cells against oxidative stress [43, 45, 46]. Therefore, the increase in the levels of proteasomal activator PA28 in CIII-stimulated neurons and astrocytes likely makes these cells more resistant to proteotoxic damage. In cell-free systems, PA28 interacts with and increases the proteolytic activities of both c-20S and i-20S core proteasomes [47]. However, a recent mass spectrometric study on cross-linked proteasome complexes in live cells revealed an unexpected pattern regarding the interactions between
proteasome core particles and the various activators [48]. In that study, the authors show that PA28 associates preferentially with the i-20S particle in both untreated and IFN-γ-treated cells, while PI31 and PA200 are mostly bound to the c-20S proteasome. The 19S cap on the other hand does not have any preference for interacting with c-20S and i-20S proteasome subtypes. Based on this selectivity of activators for core proteasome particles, it is fair to conclude that 11S:i-20S:11S, 11S:i-20S:19S and 19S:i-20S:19S complexes are likely the major proteasome structures in the cytokine-treated cells.

In our previous study, we presented evidence suggesting that both subunit displacement and reduced levels of Nrf1 might be responsible for the reduced c-20S levels in the spinal cords of EAE mice. We also speculated that perhaps inflammatory conditions were behind the impaired Nrf1 signaling in the diseased animals [22]. In this study, we found that neither Nrf1 nor Nrf2 appear to be important in the regulation of constitutive proteasomes in neurons and astrocytes upon a 24 h - exposure to pro-inflammatory cytokines. The lack of changes in proteasome β5 mRNA expression in both cell types reflects the fact that Nrf1 levels are unaltered by pro-inflammatory cytokines. Furthermore, neither mTOR nor PBX1 signaling, the two major pathways that cells use to upregulate Nfe2l1 mRNA expression, is affected by the cytokine mixture in astrocytes. This is in contrast to EAE where we found a significant reduction in PBX1 levels in the spinal cord [22], which may lead to Nrf1 dysregulation and additionally decrease proteasome expression. Thus, there are conditions or stressors in the CNS tissue of EAE mice, other than a pro-inflammatory environment, that cannot be reproduced in cytokine-
treated cells. The obvious next step will be to investigate the abnormal processes that cause PBX1 impairment in EAE.

Acknowledgements

This work was supported by PHHS grants NS082805 from the National Institutes of Health.
References


Figure 3.1 – N2a and C6 cells display specific cellular markers following differentiation. (a) Representative immunofluorescence images of undifferentiated and differentiated N2a cells depicting the expression of neuron-specific NFH (green) and NSE (red) proteins. Nuclei were labeled with DAPI (blue). (b) Representative immunofluorescence images of undifferentiated and differentiated C6 glioma cells showing the expression of astrocyte-specific GFAP (green) and EAAT-1 (red). Nuclei were labeled with DAPI (blue). A description of the primary and secondary antibodies used in these experiments is provided in Table S.3.4.
Figure 3.2 – Reduced expression of constitutive proteasomes and increased expression of immunoproteasomes and the 11S activator in the CIII-treated neurons. (a) Levels of subunits α1-3/5-7, β5, β5i, PA28α, PA28β and Rpt5 in control and CIII-treated neurons were determined by western blot analysis as described in “Materials and Methods”. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. Values are expressed relative to control and represent the mean ± SEM of 7-12 experiments. Left panel shows representative western blots of the analyzed subunits. (b) Psma7 (α7), Psmb5 (β5), Psmb8 (β5i), Psme1 (PA28α), Psme2 (PA28β) and Psmc3 (Rpt5) mRNA levels were determined by qPCR using the primers shown in Table S3.1, and are corrected by the geometric mean of 4 reference genes as described in “Materials and Methods”. Values are expressed relative to control and represent the mean ± SEM of 11 experiments. In all cases, statistical significance was determined by Student’s t-test. NS, not significant.
Figure 3.3 – Augmented nuclear levels of p-STAT-1 and IRF-1, but not NF-κB, Nrf1 and Nrf2, in the CIII-treated neurons. Levels of p-STAT-1 (a), IRF-1 (b) and NF-κB (c), Nrf1 (d) and Nrf2 (e) in the nuclear fractions from control and CIII-treated neurons were determined by western blot analysis. Band intensities were corrected by the amount of histone H3 in the same gel lanes. Values are expressed relative to control and represent the mean ± SEM of 5-7 experiments. Statistical significance was determined by Student’s t-test. NS, not significant. (f) Representative western blots of the analyzed transcription factors.
Figure 3.4 – Reduced expression of constitutive proteasomes and increased expression of immunoproteasomes and the 11S activator in the CIII-treated astrocytes. (a) Levels of subunits α1-3/5-7, β5, β5i, PA28α, PA28β and Rpt5 in control and CIII-treated astrocytes were determined by western blot analysis as described in “Materials and Methods”. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. Values are expressed relative to control and represent the mean ± SEM of 5-9 experiments. Left panel shows representative western blots of the analyzed subunits. (b) Psma7 (α7), Psmb5 (β5), Psmb8 (β5i), Psme1 (PA28α), Psme2 (PA28β) and Psmc3 (Rpt5) mRNA levels were determined by qPCR using the primers shown in Table S3.2, and are corrected by the geometric mean of 4 reference genes as described in “Materials and Methods”. Values are expressed relative to control and represent the mean ± SEM of 7-9 experiments. In both panels, statistical significance was determined by Student’s t-test. NS, not significant.
Figure 3.5 – Nrf2, but not Nrf1, signaling is augmented in CIII-treated astrocytes. Levels of Nrf1 (p120) (a), Nrf1 (p65) (b) and Nrf2 (d) in the nuclear fractions from control and CIII-treated astrocytes were determined by western blot analysis. Band intensities were corrected by the amount of HDAC2 in the same gel lanes. $Nfe2l1$ (Nrf1) (c) and $Nfe2l2$ (Nrf2) mRNA levels (e) were measured by qPCR using the primers shown in Table S3.2 and are corrected by the geometric mean of 4 reference genes as described in "Materials and Methods". In all cases, values are expressed relative to control and represent the mean ± SEM of 3-7 experiments. Statistical significance was determined by Student’s t-test. NS, not significant. (f) Representative western blots of the analyzed transcription factors.
Figure 3.6 – NF-κB inhibition in CIII-treated astrocytes reduces IRF-1 but not p-STAT-1 levels. Astrocytes were incubated with or without CIII in the absence or presence of 2 μM IKK-16 as described in “Materials and Methods”. Levels of NF-κB (a) and IRF-1 (b) in the nuclear fractions were determined by western blot analysis. Band intensities were corrected by the amount of histone H3 in the same gel lanes. The amount of p-STAT-1 (c) was determined by western blotting of the cell homogenates. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. In all cases, values are expressed relative to the untreated control and represent the mean ± SEM of 3-6 experiments. Statistical significance was determined by two-way ANOVA. Asterisks denote values that are significantly different (p < 0.05) from untreated cells. Pound symbols indicate values that are significantly different (p < 0.05) from IKK-16-treated cells. ND, not detected. (d, e) Representative western blots of the analyzed transcription factors.
Figure 3.7 – NF-κB inhibition in CIII-treated astrocytes has no effect on proteasome, immunoproteasome or PA28 levels. Astrocytes were incubated with or without CIII in the absence or presence of 2 μM IKK-16 as described in “Materials and Methods”. Levels of i-20S-specific subunit β5i (a), c-20S-specific subunit β5 (b), and PA28α (c) were determined by western blotting of the cell homogenates. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. In all cases, values are expressed relative to untreated control and represent the mean ± SEM of 6-9 experiments. Statistical significance was determined by two-way ANOVA. Asterisks denote values that are significantly different (p < 0.05) from untreated cells. (e) Representative western blots of the analyzed proteins.
**Figure S3.1- List of mouse PCR primers used in the study**

### Proteasome subunits

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Reverse</th>
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</thead>
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<tr>
<td><strong>Psmb5 / β5</strong></td>
<td>5’-GATCTGTGGCTGGGATAAGAG-3’</td>
<td>5’-TCCATAACGGCCGTAAGCATAC-3’</td>
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<tr>
<td><strong>Psmb8 / β5i</strong></td>
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<td><strong>Psme1 / PA28α</strong></td>
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<tr>
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<td>5’-TGCTTTGTCTGGTCCATCTCG-3’</td>
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<tr>
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<td>5’-ATCATGCAGATCCACTCAC-3’</td>
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<tr>
<td><strong>Psma7 / α7</strong></td>
<td>5’-ACAATGTCTTCTCCTGAACA-3’</td>
<td>5’-GCCATCACGGTCTTCTCG-3’</td>
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### House-keeping genes

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Figure S3.2 - List of rat PCR primers used in the study

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<td><em>Psme2</em> / PA28β</td>
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<table>
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Figure S3.3 - List of primary and secondary antibodies used for western blot analysis.

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<th>RRID</th>
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Abbreviations: Mus, mouse; Rb, rabbit; mAb, monoclonal antibody; pAb, polyclonal antibody; RRID, research resource identifier.
### Figure S3.4 - List of primary and secondary antibodies used for ICC analysis.

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<th><strong>Supplier</strong></th>
<th><strong>Cat#</strong></th>
<th><strong>RRID</strong></th>
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<td>GFAP</td>
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<th><strong>Cat#</strong></th>
<th><strong>RRID</strong></th>
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Abbreviations: Mus, mouse; Rb, rabbit; mAb, monoclonal antibody; pAb, polyclonal antibody; RRID, research resource identifier.
Figure S3.1—Cytokine-treatment does not alter the levels of p-mTOR and PBX1 in astrocytes. Levels of p-mTOR (a) and PBX1 (b) were determined in control and CIII-treated astrocytes by western blot analysis. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. Values are expressed relative to the untreated control and represent the mean ± SEM of 3-6 experiments. Statistical significance was determined by Student’s t-test. NS, not significant. (c) Representative western blots of the analyzed proteins.
Figure S3.2 – The autophagy inhibitor chloroquine increases proteasome β5 levels in neuron-like PC12 cells. PC12 cells were differentiated into the neuronal phenotype with nerve growth factor and were incubated with or without 50 µM chloroquine (CQ) for 24 h. Levels of the c-20S-specific subunit β5 (a) were determined by western blotting of the cell homogenates. Band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. Values are expressed relative to the untreated control and represent the mean ± SEM of 3 experiments. Statistical significance was determined by Student’s t-test. (b) Representative western blot of the analyzed protein.
Chapter 4 – NF-κB signaling determines cellular reactivity and Nrf2 levels in cytokine-treated astrocytes

Kara L. Shanley, Che-Lin Hu, Tamara Howard and Oscar A. Bizzozero

Dept. of Cell Biology and Physiology, University of New Mexico - Health Sciences Center, Albuquerque, New Mexico

[Manuscript in preparation]
Abstract

Dysregulation of the cellular antioxidant response has been implicated in inflammatory disorders like multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). This study investigates the role of pro-inflammatory cytokines, highly expressed in MS and EAE, on nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling in astrocytes. We found that varied populations of reactive astrocytes are present in mouse spinal cords, with neurotoxic A1-reactive astrocytes appearing more in EAE than in controls. Differentiated C6 glioma cells exposed to a combination of interferon-γ, interleukin-1β and tumor necrosis factor-α for 24h produce two populations of astrocytic cells with heterogeneous reactivity. Cells are distinguished by the levels of complement C3 mRNA (C3) and nuclear factor kappa B (NF-κB). Astrocytes with low C3 and NF-κB content, or pan-reactive cells, have reduced Nrf2 protein and mRNA levels. In contrast, cells with elevated C3 and NF-κB expression, or A1-reactive cells, display an increase in the amount of Nrf2 protein and mRNA. Expression of target genes is diminished in pan-reactive cells but elevated in A1-reactive cells, indicating that the changes in Nrf2 have functional consequences. Inhibition of NF-κB signaling in A1-reactive astrocytes with the IκB kinase β inhibitor IKK-16 leads to a reduction of both Nrf2 and C3, mimicking the pan-reactive phenotype. In sum, our results suggest that both astrocytic response to inflammation and Nrf2 expression are connected to NF-κB signaling.
Introduction

The connection between neuroinflammation and oxidative stress is a key pathological characteristic found in many neurodegenerative diseases including Parkinson’s disease, Alzheimer’s disease, and multiple sclerosis (MS). During disease or injury, the release of inflammatory factors promotes the production of oxidants and free radicals, or vice-versa depending on the disorder (Fischer and Maier, 2015). In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, long-lasting neuroinflammation leads to continuous oxidative stress and cellular damage (Mossakowski et al., 2015). Activated microglia in vitro have been shown to induce oxidative stress and cellular injury as well, following the release of pro-inflammatory cytokines (Penta et al., 2013).

The antioxidant response system responsible for combating oxidative stress in most cells is regulated via the nuclear factor (erythroid-derived 2)-like transcription factors (Nrfs) that belong to the cap’n’ collar basic leucine zipper (CNC-bZIP) family. A common function of these factors is their ability to bind to enhancer sequences within the promoters of antioxidant and phase II xenobiotic genes referred to as electrophilic response elements (EpREs) (Itoh et al., 1997). Although all CNC-bZIP transcription factors can bind to EpREs, Nrf2 (Nfe2l2) is the most studied of these and controls the activation of genes related to antioxidant response, autophagy and cytoprotection (Chorley et al., 2012). Genetic knockouts of Nrf2 have severe consequences including reduced levels of essential antioxidants and neuronal death in neurodegenerative disease models (Tebay et al., 2015). Because of the breadth of Nrf2’s transcriptional task, its activation is
Neurodegenerative diseases are known to have different antioxidant responses down to the cellular level (Baxter and Hardingham, 2016; Liddell and Liddell, 2017). Neurons have little endogenous Nrf2 due to rapid degradation of Nrf2 protein, and thus a minimal capacity to produce antioxidants. In contrast, astrocytes have a lower Nrf2 degradation rate (i.e. higher Nrf2 levels), allowing neurons to rely heavily on astrocytes for their antioxidant responses (Baxter and Hardingham, 2016; Jimenez-Blasco et al., 2015). Active neurons produce reactive oxygen species during synaptic transmission that cannot be completely cleared by their own antioxidant defense. Astrocytes are able to sense the enhanced glutamate and calcium secreted by nearby neurons and supplement the neuronal response by activating their own and transferring glutathione precursors to the neurons (Habas et al., 2013; Jimenez-Blasco et al., 2015). Additionally, astrocytes have been shown to promote neuroprotection when Nrf2 is activated or overexpressed in various models of neurological conditions (Bell et al., 2011; Draheim et al., 2016; Sigfridsson et al., 2018; Vargas and Johnson, 2009). However, a previous study has revealed that astrocytes stimulated by conditioned medium from reactive microglia experience a depletion of Nrf2 protein (Correa et al., 2011). Furthermore, C6 glioma cells and primary astrocytes treated with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) have reduced Nrf2 activation that is rescued using the electrophilic drug dimethyl fumarate (Lin et al., 2011).
Recent work has revealed that astrocyte reactivity is more complex than previously thought, and astrocytic responses can be heterogeneous depending upon the stimuli (Liddelow and Barres, 2017). Following certain stressors, like neuroinflammation or hypoxia, distinct subsets of genes become upregulated, allowing the designation of new reactive phenotypes within the astrocyte population (Zamanian *et al*., 2012). Neurotoxic A1 astrocytes react to the inflammatory signaling of active microglia, while neurotrophic A2 astrocytes respond to hypoxic stimuli (Liddelow *et al*., 2017). Additionally, Liddelow *et al.* (2017) found pan-reactive astrocytes that display elevated glial fibrillary acidic protein (GFAP) without concurrent increases in either the A1 or A2 gene subsets. This led to two different theories: 1) astrocyte reactivity may lie within a continuum between A1 and A2, or 2) that there are many more phenotypes than just A1 and A2 (Liddelow and Barres, 2017). Though the reason for these variations in astrocyte reactivity has yet to be determined, A1 astrocytes have been discovered in many neurodegenerative diseases – including MS – and in animal models like EAE (Brambilla *et al*., 2014; Liddelow *et al*., 2017; Ponath *et al*., 2018).

The idea that subpopulations of astrocytes react distinctly during inflammation led us to hypothesize that perhaps Nrf2 expression is variable and connected to the mechanisms regulating these cellular responses. The current study investigates whether there is a link between the role that Nrf2 plays in acute neuroinflammation and astrocytic reactivity in EAE and in cultured astrocytes. We report that there are two distinct subtypes of astrocytic responses *in vivo* and *in vitro*: pan-reactive and A1-reactive. Whereas A1-reactive astrocytes *in vitro*
respond to inflammatory stress with an increase in the expression of Nrf2 and its targets, pan-reactive cells have a reduction in Nrf2 signaling that coincides with a decrease in target gene expression. Nrf2 levels correlate positively with NF-κB expression in the two cell types. Furthermore, inhibition of NF-κB in A1-reactive astrocytes diminishes Nrf2 expression. Finally, we show that down-regulation of NF-κB in A1-reactive astrocytes decreases C3 mRNA expression, causing these cells to behave more like pan-reactive cells. Altogether, our results suggest that both the astrocytic response to inflammation and Nrf2 expression are connected to NF-κB signaling.

Materials and Methods

Materials

Actinomycin D (Act D), cycloheximide (CHX), Dulbecco’s modified Eagle’s/F-12 media (DMEM/F-12), interferon-γ (IFN-γ), interleukin-1β (IL-1β), fetal bovine serum (FBS), N6-2’-O-dibutyryl cyclic-AMP (Bt2AMP), theophylline and tumor necrosis factor-α (TNF-α) were purchased from Sigma Aldrich (St. Louis, MO). GlutaMAX™, penicillin/streptomycin, and trypsin EDTA were obtained from Thermo Fisher Scientific (Waltham, MA). IKK-16 and MG-132 were purchased from Selleckchem (Houston, TX) while epoxomicin was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Induction of experimental autoimmune encephalomyelitis (EAE)

Housing and handling of the animals, as well as the euthanasia procedure, were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.
(protocol 16-200424-HSC). To induce EAE, the following mixture was injected subcutaneously into the lower back area of eight-week-old female C57BL/6 mice (Envigo; Indianapolis, IN): 200 µl of MOG_{35-55} peptide (200 µg) (21st Century Biochemicals; Marlborough, MA) in saline mixed with complete Freund's adjuvant (CFA) supplemented with 4 mg/ml of heat killed Mycobacterium tuberculosis H37Ra (Chondrex Inc; Redmond, WA). Control animals were given CFA without MOG peptide. Two and 48 h after EAE induction, all animals received an intraperitoneal injection of 0.3 µg of pertussis toxin (List Biological Laboratories; Campbell, CA) in 100 µl of saline. Seven days after disease induction, mice received a second immunization with MOG_{35-55} peptide in CFA on the other flank. Animals were weighed and examined daily for the presence of neurological signs. Animals were euthanized 21 days after disease induction, and the spinal cords were removed for immunohistochemistry.

**Immunohistochemistry**

To determine astrocytic reactivity in the spinal cords of control and EAE mice, we performed double-label immunofluorescence analysis using antibodies against GFAP and complement C3 (Supplementary Table 1). Briefly, 5 µm-thick sections mounted to slides were deparaffinized and hydrated in down-grade alcohol series. Antigen retrieval was carried out in 10 mM Tris-HCl buffer pH 7.5, containing 1 mM EDTA and 0.05% Tween 20, using a heat-retrieval method. Spinal cord sections were blocked for 30-40 min in PBS supplemented with 3% BSA and 0.1% Triton X-100, and then incubated overnight at 4°C with primary antibodies in phosphate buffer saline (PBS) containing 2% BSA and 0.1% Tween-
20. GFAP and complement C3 were co-visualized with Dylight 488 donkey anti-mouse and Dylight 549 donkey anti-rabbit secondary antibodies, respectively (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Nuclei were counterstained using DRAQ5 (Thermo Fisher). Images were taken on a Leica TCS SP5 confocal microscope system (Leica Microsystems Inc, Buffalo Grove, IL) and GFAP+/complement C3+ astrocytes were counted using the Leica Application Suite X. Cells were counted using images taken from entire spinal cord sections of control (n = 3) and EAE (n = 3) mice, and the average number of cells per section within each condition was calculated.

**Cell Culture**

All incubations were performed in a humidified incubator at 37°C under a 95% air / 5% CO₂ atmosphere. Rat C6 glioma cells (CCL-107) were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained as a monolayer in DMEM/F-12 supplemented with 10% FBS, 2 mM GlutaMAX, 100 units of penicillin and 0.1 mg/ml streptomycin (Life Technologies, Carlsbad, CA). Cells were differentiated into astrocytes by incubating for 24 h with 0.25 mM theophylline and 1 mM N6-2'-O-dibutyryl cyclic-AMP in low serum medium (1% FBS) as described previously (Zheng and Bizzozero, 2010; Shanley et al., 2019b). This protocol produces morphological characteristics and the expression of cell type specific markers, such as GFAP and excitatory amino acid transporter-1 (EAAT-1), that are reminiscent of astrocytes (Shanley et al., 2019b). Astrocytes were then treated for 24 h with a pro-inflammatory cytokine cocktail (CIII; 5 ng/ml IFN-γ, 10 ng/ml IL-1β, and 10 ng/ml TNF-α) as established by Lin et al. (2011) and
used in our previous study (Shanley et al., 2019b). Several drugs were added to the cells before or after incubation with the cytokine mixture as described below. Following collection, cells were either processed for RNA isolation or for protein quantification according to methods below. Isolated total RNA and protein homogenates were then stored at -80°C until use.

**Subcellular Fractionation**

Preparation of the nuclear fraction was carried out using a modified version of the nuclear extraction protocol developed by Thermo-Fisher. To this end, cultured cells were suspended at 4°C in 0.15 ml of hypotonic buffer solution (20 mM Tris-HCl pH 7.4 containing 10 mM NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1x protease and phosphatase inhibitor cocktails) by pipetting up and down several times. Suspensions were kept on ice for 15 min, mixed with 7.5 µl of 10% w/v Nonidet P-40, and centrifuged at 3,000 g for 10 min. The supernatant (cytosolic fraction) and the pellet (nuclear fraction) were collected, and proteins were solubilized in sodium dodecyl sulfate (SDS)-sample buffer and analyzed by western blotting. Levels of histone deacetylase 2 (HDAC2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured by western blotting to determine the purity of the isolated fractions.

**Reverse-transcription and qPCR**

Total RNA was isolated from lysed cells via the RNeasy Mini Kit (Qiagen; Germantown, MD) using column purification, and RNA concentration was quantified fluorometrically using a Qubit 2.0 Fluorometer (Invitrogen; Carlsbad, CA). cDNA was reverse-transcribed from the purified RNA using the High-Capacity...
cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). Briefly, RNA was incubated with 1U of DNase I at 37°C for 15 min. The enzyme was then inactivated using 5 mM EDTA and heating at 75°C for 10 min. The DNase I-treated RNA (200 ng) was mixed with the 2X reverse transcription master mix, which contains the reverse transcription buffer, a dNTP mixture, random primers, an RNase inhibitor and the MultiScribe Reverse Transcriptase™. The samples were heated as follows: 25°C for 10 min, 37°C for 120 min, and finally 85°C for 5 min to terminate the reaction. A control that was not reverse transcribed was made following the same protocol with pooled RNA from all samples and master mix without reverse transcriptase. Gene expression levels were quantified using Power SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA) with primers against selected targets made by Integrated DNA Technologies (Coralville, IA) (Supplementary Table 2). cDNAs were mixed with 1 µM of each primer and Power SYBR Green PCR Master Mix. Samples were amplified using the following conditions: 50°C for 10 min, 95°C for 10 min and then 40 thermal cycles of 95°C for 15 s and 60°C for 1 min. The relative mRNA expression was determined using the comparative $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001) and values were normalized to the geometric mean of four reference genes (Gapdh, Hprt1, Rplp0, and Tbp).

**Western blot analysis**

Cells were homogenized in 20 mM sodium phosphate, pH 7.5, containing 5 mM EDTA, 0.1 mM neocuproine, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail and 1x phosphatase inhibitor cocktail.
Protein concentration was quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories; Hercules, CA, USA) with bovine serum albumin as a standard. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 4-20% gradient gels (Bio-Rad Laboratories) and blotted to polyvinylidene difluoride membranes. Blots were then incubated overnight at 4°C with the primary antibodies listed in Supplementary Table 1. Membranes were rinsed three times in PBS containing 0.05% Tween-20 and were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse antibody (1:4000; Sigma) or goat anti-rabbit antibody (1:4000; Sigma). Blots were developed by enhanced chemiluminescence using the Western Lightning ECL™ kit from Perkin-Elmer (Boston, MA). Films were scanned in a Hewlett Packard Scanjet 4890 and the images were quantified using the NIH Image 1.63 imaging analysis program. Band intensities were normalized by the amount of Coomassie stain in the corresponding lanes.

**Determination of Nrf2 half-life**

Following the 24 h CIII treatment, cells were incubated with 20 μM CHX to inhibit protein synthesis. Cells were collected at 5 min intervals after the addition of the inhibitor up to 30 min. Proteins were rapidly dissolved in SDS-sample buffer and analyzed by western blotting. Nrf2 intensities at each time point were determined as described above and normalized against those of GAPDH in the corresponding lanes. Values were expressed as percentage of those at 0 min and were log transformed. Nrf2 half-life was calculated using a linear regression analysis of each time-course and the formula: 

\[
t_{1/2} = \left[ y_{interval} - \frac{y_{interval}}{2} \right]/slope.
\]
Measurement of Nrf2 rate synthesis

The rate of Nrf2 synthesis in control and CIII-treated cells were measured by blocking its degradation with either 1 µM epoxomicin or 10 µM MG-132. Briefly, cells were collected at 0, 30 and 60 min after the addition of the proteasome inhibitors and the amount of Nrf2 protein was determined by western blot analysis.

Determination of Nfe2l2 mRNA half-life

To determine the rate of Nfe2l2 mRNA degradation, control and CIII-stimulated cells were treated with 2 µg/mL Act D to block transcription and were collected at 0, 1, 2, 4 and 6 h after the addition of the inhibitor. RNA was isolated from the cells and RT-qPCR was performed as described above. Relative Nfe2l2 mRNA expression was determined using the comparative 2^(-ΔCt) method using the geometric mean of only 3 housekeeping genes (Gapdh, Hprt1, and Rplp0) since Act D was found to affect the expression of Tbp. The half-life of Nfe2l2 mRNA was calculated using the same linear regression analysis as shown above.

NF-κB inhibition

Activation of NF-κB was inhibited through the use of IKK-16, a known IκB kinase inhibitor. Astrocytes were pre-treated with 2 µM IKK-16 (stock prepared in dimethyl sulfoxide or DMSO). After 1 h, the cytokine mixture was added to some of the cultures and incubation continued for an additional 24 h. Cells treated with just DMSO represented the vehicle controls. After incubation, cells were collected for RNA and western blot analysis as described above.
**Statistical Analysis**

Results were analyzed for statistical significance with the unpaired Student’s *t*-test or two-way ANOVA test utilizing the GraphPad Prism® program (GraphPad Software Inc.; San Diego, CA) after assessing the normality of the data with the Shapiro-Wilk test. Data points outside of two standard deviations were considered outliers and removed from the study.

**Results**

*A small but significant number of astrocytes in the spinal cords of EAE mice display A1-like reactivity*

A1-reactive astrocytes have been found in the brains of MS patients, particularly around demyelinating lesions (Liddelow *et al.*, 2017). To determine whether A1-reactive astrocytes are also present in EAE, spinal cord sections were simultaneously immunostained with antibodies against GFAP (a classical astrocyte marker) and complement C3 (a marker of A1-reactive cells). Immunofluorescence confocal microscopy analysis revealed that there is a general increase in both GFAP and complement C3 staining in the EAE spinal cord compared to the CFA-injected controls (Fig. 4.1a). While the majority of astrocytes in the EAE and control spinal cords are GFAP+/C3−, there are double-stained astrocytes noticeably localized around the lesions, which in this model are near the meninges of the ventral areas (Fig. 4.1b). In contrast, control spinal cords have about a quarter of the number of GFAP+/C3+ astrocytes than the EAE spinal cords (*p* = 0.0008) (Fig. 4.1c). These data suggest that astrocyte reactivity in the spinal cords of EAE mice is diverse and that, although they represent a small population
of the cells, there are indeed A1-reactive astrocytes present in EAE. It is interesting to note that C3 is also found in EAE neurons which, along with higher overall C3, is consistent with a previous study showing C3 deposition in the spinal cord of Biozzi ABH mice with chronic/relapsing EAE (Ramaglia et al., 2015).

Pro-inflammatory cytokines induce heterogeneous responses in cultured astrocytes

To tease out the cellular mechanisms underlying astrocyte reactivity under neuroinflammatory conditions, we incubated an astrocyte cell line with a combination of three pro-inflammatory cytokines (IFN-γ, IL-1β, and TNF-α), designated CIII. These are also the three major pro-inflammatory cytokines we found to be greatly elevated in EAE (Shanley et al., 2019a). Using RT-qPCR, we first measured the expression of two genes that have been previously characterized as an A1 reactivity marker – complement 3 (C3) – and as an A2 reactivity marker – pentraxin 3 (Ptx3) (Liddelow et al., 2017). We found that, while C3 mRNA is always upregulated upon exposure of astrocytes to CIII, there were whole experiments where the expression of this A1 marker was not as pronounced (Fig. 4.2a). Therefore, we considered the cells with very high C3 mRNA levels (~100 fold) and high NF-κB levels (see below) as A1-reactive – those with smaller elevation of C3 mRNA levels (~20 fold) and no changes in the expression of NF-κB were considered pan-reactive astrocytes. We also found a significant increase in Ptx3 mRNA expression in the seemingly more reactive population of astrocytes (Fig. 4.2b) but, because the magnitude of increase was much lower than that of C3, we did not consider these cells A2-reactive. The rise in Ptx3 could be due to
the presence of IL-1β and TNF-α in the cytokine mixture, which are known to increase Ptx3 expression (Mantovani et al., 2008).

To further characterize the proinflammatory signaling in these cells, the p65 RelA component of NF-κB was measured by western blot. Previous studies have shown that the A1 astrocyte phenotype can be prevented via the inhibition of NF-κB signaling, which is typically upregulated during inflammation (Li et al., 2018; Lian et al., 2015). As shown in Fig. 4.2c and d, the average levels of NF-κB in the less reactive, pan-astrocyte population is ~ 17% lower than that in control cells, although this change is only borderline significant (p = 0.0715). In contrast, NF-κB p65 levels in A1-reactive astrocytes increase by 34.9 ± 12.1% (p = 0.0073) relative to the controls. These data suggest that even in tightly controlled in vitro models of neuroinflammation, astrocytes can respond heterogeneously both in terms of NF-κB signaling and C3/Ptx3 expression.

*Upon cytokine treatment, Nrf2 expression / activity increase in A1-reactive but not pan-reactive astrocytes*

Because there is substantial crosstalk between NF-κB and Nrf2 signaling (Wardyn et al., 2015), we investigated the expression of the latter in pan- and A1-reactive astrocytes. As shown in Fig. 4.3a and b, A1-reactive astrocytes have significantly elevated Nrf2 levels compared to controls (72.7 ± 30.4%, p = 0.0236), while pan-reactive C6 cells display a reduction in Nrf2 protein expression (26.4 ± 9.2%, p = 0.0078). The variations in Nrf2 expression in A1- and pan-reactive cells seem to allude to a connection between the magnitude of astrocyte reactivity and its antioxidant response.
In both pan- and A1-reactive astrocytes, as well as in control cells, the majority of Nrf2 is present in the nuclear fraction – this represents the active form of the transcription factor (Fig. 4.3c). We also investigated the functional consequences of these Nrf2 by measuring the expression of two Nrf2 target genes: the catalytic subunit of γ-glutamylcysteine ligase (Gclc) and NAD(P)H quinone dehydrogenase 1 (Nqo1). Gclc and Nqo1 were also selected as they are downregulated in EAE (Morales Pantoja et al., 2016). Nqo1 mRNA levels are augmented in the CIII-treated A1-reactive astrocytes by \(212.7 \pm 45.3\%\) (\(p < 0.0001\)) compared to control, whereas Gclc does not change (Fig. 4.4a). Conversely, pan-reactive C6 cells have a decline in both Gclc and Nqo1 mRNA levels by \(51.8 \pm 10.8\%\) (\(p = 0.0031\)) and \(53.6 \pm 12.7\%\) (\(p = 0.0056\)), respectively, compared to controls (Fig. 4.4b). Thus, variations in Nrf2 levels have functional consequences as demonstrated by the altered expression of its target genes.

**Translational and post-translational regulation of Nrf2 are unaffected in CIII-treated pan-reactive astrocytes**

To evaluate whether the diminished Nrf2 protein in pan-reactive astrocytes is due to increased protein degradation, we determined the half-life of Nrf2 in the absence or presence of cytokines. To this end, astrocytes were exposed to the protein synthesis inhibitor cycloheximide (CHX) and Nrf2 levels were then measured at 5-minute intervals for up to 1 h by western blot analysis. Time intervals and length of the experiment were chosen based on a previous study that shows a half-life of \(~ 30\text{ min}\) for this protein (Nguyen et al., 2009). As depicted in Fig. 4.5, the calculated half-life of Nrf2 in the control and pan-reactive astrocytes
are almost identical (20.6 min vs 20.4 min), indicating that increased degradation of Nrf2 is not responsible for the decline in Nrf2 protein expression in these cells. This conclusion is supported by the observations that (i) both Keap-1 and Cul3 levels are not altered by the cytokine treatment and (ii) the amount of active phospho-GSK-3β (Y216) is unchanged in CIII-treated cells (data not shown).

An internal ribosome entry site is present in the 5’ untranslated region of \textit{Nfe2l2} mRNA, indicating that its encoded protein can undergo cap-independent translation (Li \textit{et al}., 2010). To explore whether reduced Nrf2 levels in pan-reactive astrocytes result from decreased translation, cells were incubated separately with two proteasome inhibitors (MG-132 and epoxomicin) to block Nrf2 degradation. We found that, over the course of 1 h of incubation, neither MG-132 (Fig. 4.6a) nor epoxomicin (Fig. 4.6b) changes the rate of Nrf2 appearance in pan-reactive astrocytes compared to unstimulated cells. These data indicate that a reduction in the rate of Nrf2 synthesis is also not the cause for decreased Nrf2 protein levels in the less reactive astrocyte population.

\textit{Changes in Nrf2 protein levels in CIII-treated astrocytes correspond to variations in their gene expression}

We next investigated whether altered gene expression may be underlying the changes in Nrf2 protein levels in cytokine-stimulated astrocytes. \textit{Nfe2l2} mRNA levels were measured in pan-reactive and A1-reactive astrocytes by RT-qPCR. Interestingly, there is a significant decrease (33.7 ± 13.3%; \(p = 0.0442\)) of \textit{Nfe2l2} expression in pan-reactive astrocytes compared to controls. In contrast, \textit{Nfe2l2} mRNA expression augments by 69.4 ± 20.8% (\(p = 0.0033\)) in A1-reactive
astrocytes relative to control cells (Fig. 4.7a). Because Nrf2 protein levels in both cell types (Fig. 4.3) seems to follow the respective mRNA expression (Fig. 4.7a), it is likely that Nrf2 is being regulated at the transcriptional level during neuroinflammatory signaling.

To determine if the Nfe2l2 mRNA stability is increased in A1-reactive astrocytes, cells were exposed to the transcription inhibitor actinomycin D (ActD). Levels of Nfe2l2 mRNA were then measured at different times for up to 6 h by RT-qPCR. Time intervals were selected based on a previous report that determined a half-life of ~3 h for this transcript (Yang et al., 2011). As shown in Fig. 4.7, the Nfe2l2 mRNA half-life in A1-reactive cells is significantly reduced compared to controls (2.63 ± 0.14 h vs 3.54 ± 0.04 h, p < 0.0008). These data indicate that increased Nfe2l2 mRNA levels in A1-reactive cells is caused by increased transcription rather than by reduced mRNA degradation. The reason for the faster removal of Nfe2l2 transcripts in A1-reactive astrocytes is unknown.

Reduction of nuclear NF-κB in A1-reactive astrocytes results in concurrent decrease of Nrf2 and C3 expression

The above experiments suggest a strong link between NF-κB activation and Nrf2 levels. We decided to use IKK-16, a selective IκB kinase β (IKK-β) inhibitor (Waelchli et al., 2006), to determine if NF-κB is indeed responsible for Nrf2 regulation in this model of inflammation. IKK-16 has been previously shown to reduce the levels of NF-κB and its downstream effectors (Ahmmed et al., 2019). To this end, astrocytes were pre-incubated for 1 h with or without 2 μM IKK-16, a concentration that is 50 times the IC₅₀ for IKK-β (Waelchli et al., 2006). Cells were
then incubated for an additional 24 h with or without the cytokine mixture. As shown in Fig. 4.8a and c, CIII treatment increases the amount of nuclear NF-κB (p65) by 335 ± 76% (p < 0.0001) and IKK-16 reduces this activation by 38.8 ± 13.2% (p = 0.0276).

We next measured Nrf2 protein to determine whether decreasing NF-κB levels would also impact the antioxidant response. As presented in Fig. 4.8b and c, Nrf2 levels in A1-reactive cells rise by 406 ± 48% (p < 0.0001) compared to vehicle-treated cells while addition of the IKK-16 decreases this stimulation by 73.7 ± 3.4% (p < 0.0001). These data clearly suggest that NF-κB is an important positive regulator of Nrf2 signaling in these cytokine-treated astrocytes. Furthermore, RT-qPCR revealed that IKK-16 also diminishes the levels of Nfe2l2 mRNA compared to A1-reactive cells by 20.7 ± 10.0% (p = 0.0133), suggesting that NF-κB reduction affects Nrf2 at the transcriptional level in A1-reactive cells.

Finally, we investigated whether NF-κB inhibition has an effect on the expression of C3 and Ptx3 mRNA levels in A1-reactive astrocytes. As show in Fig. 4.9, IKK-16 treatment reduces the transcriptional expression of C3 and Ptx3 by 59.1 ± 7.0% (p < 0.0001) and 83.9 ± 5.9% (p < 0.0001), respectively. This suggests not only that NF-κB signaling is critical for the expression of these reactivity markers but also the NF-κB inhibition makes these A1-type cells behave more like pan-reactive astrocytes.

Discussion

The role of diverse astrocyte populations during disease or after CNS injury has become a topic of great interest. Our study shows that the assortment of
astrocyte responses is not limited to post-mortem tissue or in vivo models of disease, but rather can also be found in an astrocyte-like cell line exposed to inflammatory stress. Following exposure to a combination of pro-inflammatory cytokines, specifically those that are upregulated in EAE (Shanley et al., 2019a), differentiated C6 cells assume either a pan-reactive or an A1-reactive phenotype. The reason(s) for this differential reactivity is unknown, but it does not involve changes in culture conditions (e.g., temperature, oxygen concentration, medium composition), cell passage number or degree of confluency. The two phenotypes were characterized using expression of C3 mRNA and NF-κB (p65), wherein A1-reactive cells have significantly higher levels of both compared to pan-reactive cells (Fig. 4.2). This somewhat matches our findings in the spinal cords of EAE mice, where there are more GFAP+/C3+ astrocytes adjacent to peripheral areas of high immune cell infiltration and pro-inflammatory cytokine production (Fig. 4.1). The term pan-reactive was coined by Liddelow and Barres (2017) to describe astrocytes that are neither A1 nor A2 – clearly the astrocyte population that we designated as pan-reactive does not overexpress the A2-reactive marker Ptx3. We also demonstrated that changes in Nrf2 between both reactivities results from transcriptional level variations – pan-reactive astrocytes have lower mRNA and protein expression while A1-reactive cells display elevated mRNA and protein expression (Fig. 4.3 and 4.8). Changes in Nrf2 transcription are most likely due to differential NF-κB expression in the two cell types. Indeed, the pharmacological reduction of NF-κB protein levels causes a corresponding decrease in the amount of Nrf2 protein (Fig. 4.8). In this aspect, pan-reactive astrocytes resemble neuron-
like N2a cells, whose NF-κB and Nrf2 signaling is unaffected or even reduced in the presence of pro-inflammatory cytokines (Shanley et al., 2019b). Finally, we show that down-regulation of NF-κB in A1-reactive astrocytes also causes a decrease in C3 mRNA expression, causing these cells to behave more like pan-reactive cells whose NF-κB signaling is unresponsive to the cytokine mixture.

Because inflammation is the primary trigger for A1 astrocyte reactivity, the NF-κB signaling pathway has been associated with the toxic function of these cells during disease or injury. One target of this pathway in astrocytes seems to be C3, since enhancing NF-κB levels in cultured astrocytes has been shown to increase C3 levels as well as cause neuronal dysfunction and damage (Lian et al., 2015). Furthermore, in a mouse model of Alzheimer’s disease, amyloid beta production by neurons contributes to augmented NF-κB and C3 in astrocytes (Lian et al., 2016). This response – which results in greater neuroinflammation by interfering with microglial functions – is prevented through inhibition of the C3a receptor (Lian et al., 2015, 2016), thereby implicating A1-reactive astrocytes as a potential origin of neurodegenerative signaling in diseases like Alzheimer’s disease. Moreover, therapeutic use of mesenchymal stem cells and their exosomes following spinal cord injury is able to prevent A1 reactivity in astrocytes by diminishing inflammatory cytokine levels and hindering the nuclear entry of NF-κB (Wang et al., 2018). The above studies all point to the neurotoxic role that NF-κB signaling in A1 astrocytes plays during neuroinflammation and neurodegeneration. However, not all data support that heightened astroglial NF-κB is detrimental. For example, blockage of astrocytic NF-κB signaling in the eyes of a Drosophila model for Machado-Joseph
disease contributes to toxic accumulation of proteins and death of neurons (Li et al., 2018). Furthermore, our study shows that C6 astrocyte-like cells exposed to inflammatory cytokines also upregulate Nrf2 via NF-κB signaling, which could have some beneficial effect during acute neuroinflammation (Chen et al., 2009).

Under physiological conditions, Nrf2 levels are regulated at the post-translational level by Keap1, which directs the transcription factor for proteasomal degradation (Itoh et al. 1999), and by GSK-3β, which increases the removal of Nrf2 by various mechanisms (Jain and Jaiswal 2007, Bryan et al. 2013). Interestingly, the reduction in the amount of Nrf2 in pan-reactive astrocytes is not caused by an increase in its rate of degradation (Fig. 4.5) – a conclusion supported by the lack of changes in the levels of Keap1 and p-GSK-3β. Because there are no changes in the rate of Nrf2 translation upon cytokine treatment and the decrease in Nrf2 protein levels matches that of Nfe2l2 mRNA, it is fair to conclude that Nrf2 in the pan-reactive cells is controlled mostly at the transcriptional level. This also seems to be the case for the Nrf2 upregulation in A1-reactive astrocytes. Supporting this conclusion is the discovery of several conserved regions for NF-κB in the Nfe2l2 promoter and that the amount of Nrf2 in human acute myeloid leukemia cells can be reduced by treatment with NF-κB inhibitors (Nair et al., 2008; Rushworth et al., 2012).

It is well known that cancer cells normally have elevated Nrf2 levels, most likely driven by NF-κB, which protects them from drug-induced death. Although it could be argued that the regulation of Nrf2 by NF-κB is specific to cancer cells, treatment of monocytes with LPS increases Nfe2l2 gene expression, which is
blocked by an IκBα phosphorylation inhibitor (Rushworth et al., 2012). Thus, NF-κB-mediated control of Nrf2 expression does not seem to take place only in tumor cells. Indeed, we have found that while Nfe2l2 mRNA levels are elevated in the spinal cords of EAE mice during the inflammatory phase of the disease (i.e. 14-30 days after MOG immunization), they decline to normal values in the neurodegenerative phase (i.e. 60-90 days after MOG immunization) (Morales Pantoja et al., 2016). Similarly, expression of NF-κB p65 increases dramatically in the spinal cord during the early and peak stages of EAE but diminishes later in the disease (Hwang et al., 2011), suggesting that the relationship between NF-κB and Nrf2 signaling is also present in vivo. While pathological changes in the acute phase of EAE are mainly driven by inflammation from resident microglia and infiltrating immune cells, the damage in the chronic (degenerative) phase appears to result from high levels of oxidative stress (Morales Pantoja et al., 2016; Shanley et al., 2019a). Since Nrf2 signaling in the spinal cords of animals with chronic EAE is unchanged, despite augmented oxidative stress (Morales Pantoja et al., 2016), it is fair to conclude that NF-κB is the major determinant of Nrf2 levels in this model of CNS demyelination.

Astrocyte reactivity is an important contributor to the pathology of MS and its various animal models. It has been previously shown that nervous system demyelination is often preceded by astrocyte-mediated damage – high GFAP levels were recently correlated with MS disease severity and time-course in patients with primary progressive MS (Abdelhak et al., 2019; Sharma et al., 2010). The pathophysiological role of reactive astrocytes and increased NF-κB signaling
in EAE was also demonstrated by the finding that genetic ablation of this transcription factor in astrocytes specifically leads to less pro-inflammatory gene expression during the acute phase of the disease and reduced neurological symptoms (Brambilla et al., 2009). Additionally, neurons and oligodendrocytes within the same microenvironment as the inhibited astrocytes show improved survival, indicating that elevated expression of NF-κB during this disease is harmful (Brambilla et al., 2009, 2014). Although the precise role of A1-reactive and pan-reactive astrocytes in EAE is presently unknown, we have shown that there are at least two distinct populations of these cells in the spinal cord of diseased animals. We have also demonstrated that this mixed reactivity extends to differentiated C6 cells, as determined by varying levels of complement C3 and NF-κB expression. In this *in vitro* system, NF-κB expression drives astrocytic reactivity as well as antioxidant response, similarly to what is seen in EAE. Overall, this study furthers our understanding of the role that this diverse population of glial cells plays in complex diseases like MS.
References


Bloom, D.A., and Jaiswal, A.K. (2003). Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J. Biol. Chem. 278, 44675–44682.


Zhang, Y., Qiu, L., Li, S., Xiang, Y., Chen, J., and Ren, Y. (2014a). The C-terminal domain of Nrf1 negatively regulates the full-length CNC-bZIP factor and its shorter isoform LCR-F1/Nrf1β; both are also inhibited by the small dominant-negative Nrf1γ/δ isoforms that down-regulate ARE-battery gene expression. PLoS ONE 9, e109159.


Figure 4.1—A small but significant number of C3⁺ astrocytes are present in the EAE spinal cord. (a, b) Representative images of double-labeled immunofluorescent lumbar spinal cord sections from control and EAE mice. Sections (5 µm-thick) of lumbar spinal cords were stained with mouse anti-GFAP and rabbit anti-C3 and images were taken using confocal microscopy as described in “Materials and Methods”. Nuclei were detected with DRAQ5 stain. The red channel is representative of C3 staining, the green channel is representative of GFAP and the blue channel is representative of DRAQ5. Bars at lower left represent 50 µm in length. (c) Average numbers of double-labelled astrocytes were counted in EAE and control spinal cords as described in “Materials and Methods”. Values are expressed as the mean ± SEM of 3 animals per condition and statistical significance was calculated using an unpaired Student’s t-test.
Figure 4.2 – Differential expression of C3, Ptx3 and NF-κB (p65) reactivity markers in cultured astrocytes. Levels C3 (a) and Ptx3 mRNA (b) were assessed by qRT-PCR using primers listed in Supplementary Table 2. Values were corrected using the geometric mean of four housekeeping genes as described in “Materials and Methods”. Values are expressed as relative to controls and represent the mean ± SEM of 4-11 experiments per group. NS, not significant. (c) Amounts of NF-κB p65 in pan- and A1-reactive astrocytes were measured as band intensity corrected by the amount of Coomassie blue stain in the respective gel lanes. Values are expressed as relative to controls and represent the mean ± SEM of 10-15 experiments per group. Statistical significance was determined using an unpaired Student’s t-test. (d) Representative western blot of NF-κB (p65).
Figure 4.3 – Altered levels of Nrf2 protein are found in pan- and A1-reactive astrocytes. (a) Representative Coomassie stain and western blots of Nrf2 in each reactive cell type alongside its control. (b) Nrf2 protein levels were determined by western blot with band intensities corrected by Coomassie stain in the corresponding gel lanes. Values are expressed as relative to control and reflect the mean ± SEM of 14-16 experiments per group. Statistical significance was determined using an unpaired Student’s t-test. (c) Representative western blots of Nrf2, HDAC2 and GAPDH in the cytoplasmic (Cyto) and nuclear (Nuc) fractions of pan- and A1-reactive astrocytes showing that in all cases the majority of Nrf2 is in the nucleus.
Figure 4.4 – Differential expression of antioxidant genes in pan- and A1-reactive astrocytes. (a) Gclc and Nqo1 mRNA levels in control and A1-reactive astrocytes. (b) Gclc and Nqo1 mRNA levels in control and pan-reactive astrocytes. mRNA levels were determined by qRT-PCR using primers listed in Supplementary Table 2. Values were corrected using the geometric mean of four housekeeping genes as described in “Materials and Methods” and are expressed as relative to controls. Each bar reflects the mean ± SEM of 4-11 experiments per group. Statistical significance was determined using an unpaired Student’s t-test. NS, not significant.
Figure 4.5 – The degradation rate of Nrf2 protein in pan-reactive and control astrocytes are identical. (a) Representative western blots of Nrf2 and GAPDH expression in control and pan-reactive astrocytes following exposure to 20 µM cycloheximide (CHX). (b) Nrf2 amounts at each time point were calculated using the band intensity corrected by GAPDH in each gel lane and are expressed as percentage of Nrf2 level at time 0 for both controls and pan-reactive cells. Percentages were log transformed for visualization on the graph. (c) The half-life of Nrf2 was calculated for each experiment using the formula shown in “Materials and Methods”. Values are expressed as mean ± SEM of 4 experiments per group. Statistical significance was determined using the unpaired Student’s t-test. NS, not significant.
Figure 4.6 – The rate of Nrf2 synthesis in pan-reactive and control astrocytes are similar. Nrf2 and GAPDH protein levels in control and pan-reactive astrocytes following exposure to 10 µM MG-132 (a) or 1 µM epoxomicin (b) were determined by western blot analysis. In both cases, the intensity of the Nrf2 band was corrected by that of GAPDH in each gel lane. Values are expressed as arbitrary units and represent the mean ± SEM of 5-6 experiments per time-point within each group. Statistical significance was determined using unpaired Student’s t-tests at each time-point.
Figure 4.7 – Altered Nfe2l2 mRNA levels in pan- and A1-reactive astrocytes. (a) mRNA levels of Nfe2l2 were measured by qRT-PCR using primers listed in Supplementary Table 2. Values were corrected using the geometric mean of three housekeeping genes as explained in “Materials and Methods” and are expressed as relative to control. Each value represents the mean ± SEM of 4-11 experiments per group. (b) Nfe2l2 mRNA degradation in A1-reactive cells was determined by qRT-PCR after addition of 2 µg/ml ActD. Values are expressed as percentage of Nfe2l2 mRNA levels at time 0 for both controls and A1-reactive cells. Percentages were log transformed for visualization on the graph. (c) The half-life of Nfe2l2 in control and A1-reactive cells was calculated for each experiment using the formula shown in “Materials and Methods”. Values are expressed as mean ± SEM of 4 experiments per group. Statistical significance was determined using an unpaired Student’s t-test.
Figure 4.8 – Nrf2 mRNA and protein in A1-reactive astrocytes are reduced following inhibition of NF-κB. Astrocytes were incubated with or without CIII in the absence or presence of 2 µM IKK-16 as described in “Materials and Methods”. (a) Levels of NF-κB in the nuclear fractions were determined by western blot analysis. Band intensities were corrected by the amount of histone H3 in the same gel lanes. (b) The amount Nfr2 in cell homogenates was also determined by western blotting. Nrf2 band intensities were normalized by the intensity of the Coomassie blue stain in the respective lanes. NF-κB and Nrf2 levels are expressed relative to the untreated control and represent the mean ± SEM of 6 experiments per group. Statistical significance was determined by two-way ANOVA. Asterisks denote values that are significantly different (p < 0.05) from untreated cells. Pound symbols indicate values that are significantly different (p < 0.05) from cytokine-treated cells. (c) Representative western blots of the analyzed transcription factors. (d) Relative Nfe2l2 mRNA levels were measured by qRT-PCR as described in “Materials and Methods” with values expressed as relative to controls. Values represent the mean ± SEM of 8 experiments per group and statistical significance was determined by two-way ANOVA. Asterisks denote values that are significantly different (p < 0.05) from untreated cells. Pound symbols indicate values that are significantly different (p < 0.05) from cytokine-treated cells.
Figure 4.9 – *C3* and *Ptx3* mRNA in A1-reactive astrocytes is decreased following inhibition of NF-κB. Astrocytes were incubated with or without CIII in the absence or presence of 2 µM IKK-16. Relative *C3* (a) and *Ptx3* mRNA levels (b) were measured by qRT-PCR as described in “Materials and Methods”. Values are expressed as relative to controls and represent the mean ± SEM of 5-6 experiments per group. Statistical significance was determined by two-way ANOVA. Asterisks denote values that are significantly different (p < 0.05) from untreated cells. Pound symbols indicate values that are significantly different (p < 0.05) from cytokine-treated cells.
Table S4.1 – List of primary antibodies used in the study

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<th>Antigen</th>
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<th>Dilution</th>
<th>Supplier (Cat #)</th>
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Table S4.2 – List of PCR primers used in the study

**Astrocyte reactivity markers**

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<td>5’-GAATGAACGACTAGACAAGGCT-3’</td>
<td>5’-CATCAAAATCATCCGACAGCTC-3’</td>
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<tr>
<td>Ptx3 / Pentraxin 3</td>
<td>5’-CGGTGCTGGAGGAACCTG-3’</td>
<td>5’-GCACGCTTCCAAAAATCTTCT-3’</td>
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**Enzymes involved in GSH production – Nrf2 Targets**

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<td>5’-CTTGCTACCCATCCACCA-3’</td>
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<td>5’-GCCAGAGAATGACGTTCATGT-3’</td>
<td>5’-ACGTATGCCACCATGTAGAC-3’</td>
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**Transcription factors regulating the antioxidant response**

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**House-keeping proteins**

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</thead>
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<td>Rplp0 / RPLP0</td>
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Chapter 5 – General Discussion

General Conclusions

Although the cause of MS remains elusive, the mechanisms underlying MS pathology are becoming clearer – for instance, tissue injury in MS tends to result from a combination of DNA/protein damage, inflammation and oxidative stress (Fischer et al., 2013). The same can be said of EAE, as we found heightened inflammation and oxidative stress at the peak of disease in our model – evidenced by the elevated inflammatory cytokine expression and lessened glutathione levels (Fig. 2.1). We have previously shown that this combination of stressors varies at different stages of EAE, with inflammation primarily occurring in the acute phase (Dasgupta et al., 2013). Oxidative stress also tends to start during the acute phase and continues into the chronic phase, as demonstrated by the gradual reduction of glutathione starting at 21 days post-immunization (dpi) and continuing into 60 dpi (Morales Pantoja et al., 2016). In a previous study, our lab discovered that proteasome activity in the EAE cerebellum increases during the acute phase of the disease, but then decreases as the disease progresses (Zheng and Bizzozero, 2010b). This is consistent with the reduced proteasomal activity we found in both white and gray matter from chronic MS patients (Zheng and Bizzozero, 2011). With all of this in mind, the primary objectives of this dissertation were 1) to determine how proteasome expression in EAE is regulated by upstream effectors, and 2) to examine how neuroinflammation influences these effectors in vitro and what would be the cellular and physiological consequences.
The data presented in chapter 2 show that changes in proteasome subunit expression in the spinal cords of EAE mice at the peak of the disease are most likely due to a combination of altered Nrf1 signaling and replacement of constitutive subunits by inducible subunits (Fig. 5.1). At this disease stage, elevated pro-inflammatory cytokines and leukocyte infiltration as well as low glutathione levels are present in the spinal cords. We also observed low c-20S proteasome levels in opposition to high levels of the immunoproteasome and 11S in EAE neurons and astrocytes. The reduction in Nrf1 levels and the resulting abrogation of c-20S subunit synthesis are probably the result of decreased PBX1 expression. On the other hand, elevated p-STAT-1 and IRF-1 levels – likely originating from increased IFN-γ signaling – augment the expression of both i-20S-specific and 11S regulator subunits.
**Figure 5.1 – Chapter 2 Summary:** the number of immunoproteasomes is increased in neurons and astrocytes from EAE spinal cords and is probably due to a rise in p-STAT1 and IRF-1 levels. Immunoproteasome increase is accompanied by a decrease in constitutive proteasomes levels, which correlates with low Nrf1 expression and is likely caused by reduced PBX1 signaling. The displacement of constitutive subunits by their inducible counterparts may also be responsible for alterations in proteasome composition. Chapter 2 provides insights into the dynamics of proteasome expression in EAE and is the first to explore Nrf1 signaling in an inflammatory demyelinating disorder.

Chapter 3 dove deeper into proteasome expression and regulation in cultured astrocytes and neurons exposed to pro-inflammatory cytokines. The experiments therein reveal that subunit displacement is the most probable mechanism underlying the cytokine-induced shift from c-20S to i-20S proteasome in both neurons (Fig. 5.2a) and astrocytes (Fig. 5.2b). The increase in the amount of the IFN-γ-inducible proteasome subunits in neurons and astrocytes coincides with high expression of p-STAT-1 and IRF-1. However, inhibition of NF-κB signaling in cytokine-treated astrocytes – neurons were not studied – reduces IRF-1 expression without affecting that of i-20S, c-20S or 11S. This suggests that STAT-1 is capable of increasing the transcription of i20S-specific subunits and 11S subunits by itself. It is also interesting that the cytokine mixture does not increase Nrf1 mRNA and protein levels, in contrast to EAE, which explains why *Psmb5* mRNA expression is unchanged. This chapter also presents evidence that Nrf2 signaling does not play a role in the expression of constitutive proteasome subunits in this *in vitro* model of inflammation.
Figure 5.2 – Chapter 3 Summary: immunoproteasomes increase while constitutive proteasomes decrease in cultured a) neurons and b) astrocytes exposed to pro-inflammatory cytokines. In both cell types, this switch is due primarily to subunit displacement, since Psmb5 mRNA levels are unaltered. The latter is likely due to the lack of changes in Nrf1 expression. Increased expression of proteasome immuno-subunits and PA28α/β in both cell types is probably caused by IFN-γ-induced activation of the STAT-1 / IRF-1 pathway. However, at least in astrocytes, p-STAT-1 alone is capable of activating the transcription of genes encoding for the i-20S-specific subunit β5i and PA28α/β (11S).
Chapter 4 teased out the relationship between NF-κB and Nrf2 signaling upon inflammation in astrocytes. IHC analysis of EAE spinal cord shows that a small but significant amount of GFAP+ astrocytes also express complement C3 and are therefore considered A1-reactive. We also found that astrocytes of varied reactivity develop in vitro upon cytokine exposure, namely A1-reactive (Fig. 5.3a) and pan-reactive (Fig. 5.3b) phenotypes. A1-reactive astrocytes express high levels of both C3 and NF-κB while pan-reactive astrocytes have smaller increases in C3 and unaffected NF-κB levels. Nrf2 mRNA and protein expression are also high in A1-reactive astrocytes and low in pan-reactive, possibly indicating a direct transcriptional regulation of Nrf2 by NF-κB in response to varying astrocytic reactivity. This is confirmed by a corresponding decline in Nrf2 when NF-κB activation was diminished pharmacologically. Furthermore, NF-κB inhibition in A1-reactive astrocytes results in reduced expression of C3 and Ptx3 mRNA, making the reactivity of these cells more pan-like.
Figure 5.3 – Chapter 4 Summary: A1-reactive astrocytes are part of the astrocyte population in the spinal cords of EAE mice. We also show that a) A1-reactive and b) pan-reactive astrocytes exist in culture following exposure to pro-inflammatory cytokines. The distinct phenotypes seem to depend upon NF-κB signaling and its upregulation of complement C3 and crosstalk with Nrf2. Chapter 4 demonstrates that heterogeneous astrocyte reactivity exists both in vivo and in vitro and connects astrocyte reactivity to NF-κB / Nrf2 crosstalk.

The findings of this dissertation provide additional clues as to why proteasome expression and activity fluctuate in diseases like MS and EAE, particularly those fluctuations pertaining to the inflammatory phases of the disease. These studies also offer a window into cell-specific upstream signaling and proteasome function during neuroinflammation as well as how cellular functions can vary under such conditions. Perhaps understanding how signaling in reactive astrocytes and in neurons affects proteasome function could explain how these cells interact in inflammatory, demyelinating diseases like MS.
Aim 1: Cellular differences in proteasome composition may be contributing to the pathology of acute EAE

As mentioned above, data presented in chapter 2 reveal that the switch from c-20S proteasomes to i-20S proteasomes in acute EAE could be attributed to two mechanisms: 1) subunit displacement, and 2) dysregulated PBX1 / Nrf1 signaling. The i-20S core proteasome, in combination with the 11S regulator, seems to be more efficient than the c-20S particle at producing peptides for major histocompatibility complex (MHC) presentation during immune responses (Johnston-Carey et al., 2015; Tanaka and Kasahara, 1998) and it also participates in the removal of damaged proteins (Zheng et al., 2012). The latter suggests that cells with high content of i-20S and 11S have an increased ability to prevent proteotoxic damage. In our model of EAE, oligodendrocytes are the most sensitive to cell death (Hu et al., 2019) and are also the cells where immunoproteasome expression is unchanged. In contrast, the expression of i-20S in EAE increases in both neurons and astrocytes. Therefore, it is tempting to speculate that augmented immunoproteasome levels in neurons and astrocytes help prevent the demise of these cells at the peak of the disease. In contrast, the lack of changes in proteasome expression in EAE oligodendrocytes may cause these cells to be more sensitive to proteotoxic damage and die at a higher rate.

The other side of proteasome regulation illuminated by the studies in this chapter is the role played by Nrf1 in the control of c-20S proteasome expression. Though we show that the downregulated PBX1 / Nrf1 axis could be one reason for the low c-20S expression, we did not address whether impaired Nrf1 signaling
could lead to other pathological consequences. Nrf1 regulates more than just proteasomal gene expression, as it is heavily involved in the ER stress response and the unfolded protein response (UPR). Genetic knockout of Nrf1 in mouse liver leads to dysregulation of ER stress sensing genes, through binding of an enhancer in the activating transcription factor 6 (Atf6) locus, and the UPR-mediated apoptosis pathway, through an upregulation of DNA damage inducible transcript 3 or CHOP (Baird et al., 2017). Interestingly, augmented CHOP has been found in the hippocampi of EAE mice – this could reflect the downregulation of Nrf1 expression in this disease that we have described here for the first time. In addition, reduced Atf6α is known to cause more severe EAE symptoms (Kamarehei et al., 2019; Stone et al., 2018).

It is important to note that deficient Nrf1 signaling could also increase the expression of i-20S-specific subunits in EAE. It has been shown that microglia in which Nrf1 p120 is knocked down not only has augmented IL-1β and TNF-α expression, but also that of p-STAT-1 (Wang et al., 2018). This suggests that the diminished Nrf1 levels in our MS model could also contribute to further activation of p-STAT-1 and its downstream signaling to increase the synthesis of immuno-subunits.

One caveat of our in vivo studies is that we have focused primarily on the pathological processes underlying acute damage, the reason being that we wanted to discover what consequences the blend of inflammation and oxidative stress have on our targets of interest. However, because the expression of constitutive proteasome worsens over the course of EAE, it will be important to study if Nrf1
also participates in proteasome failure in the chronic animals. Notably, *Nfe2l1* mRNA expression in EAE seems to go back to control values at 60 dpi, suggesting that other factors may be involved in the regulation of proteasomes as disease progresses.

Although the reduction of *Nrf1* in acute EAE seems to be due to its upstream effector, PBX1, the reason for the decrease in the levels of the latter remains unknown. PBX1 is recognized as a downstream target of other transcription factors, such as ecotropic virus integration site-1 (EVI-1) and notch receptor 3 (NOTCH3) (Park et al., 2008; Shimabe et al., 2009). EVI-1 binds to the promoter region of PBX-1, particularly in the context of leukemia (Shimabe et al., 2009), but its expression has not been measured in EAE or MS tissues. NOTCH-3 is probably not involved in PBX1 impairment since it is upregulated in EAE (Jurynczyk et al., 2008). There is one other pathway that could be involved in the downregulation of PBX1 – the RE1 silencing transcription factor/neuron-restrictive silencer factor complex / polypyrimidine tract binding protein 1 (PTBP1) pathway has been implicated in control of PBX1 splice isoform expression (Linares et al., 2015; Tyler et al., 2017). Perhaps there is an overabundance of PTBP1 in EAE, leading to a decrease in both PBX1a (the isoform measured in our experiments) and Nrf1. Additionally, multiple micro RNAs have been identified that modulate the expression of PBX1 (Xu et al., 2013). One of these, miR-486-5p, is upregulated in serum samples from MS patients (Regev et al., 2018).

Finally, while our findings do suggest the participation of the PBX1/Nrf1 signaling pathway in proteasome composition, it will be important to ascertain if
that is indeed the case by utilizing silencing RNA for each transcription factor in both neurons and astrocytes in vitro. Moreover, as with many studies, our in vivo findings would need to be brought back to the disease we are trying to replicate: MS. A few things to establish in this context will be whether the distribution of proteasome subunits in neural cells from MS and EAE tissues are similar and whether PBX1/Nrf1 signaling is also impaired in MS.

**Aim 2: Neuroinflammation alone results in proteasome subunit displacement in neurons and astrocytes in vitro**

The second aim investigated the regulation of proteasome subunit expression in two different neural cell types under inflammatory conditions in vitro. Our studies in chapter 3 demonstrate that both neurons and astrocytes exposed to pro-inflammatory cytokines undergo proteasome subunit displacement, which is different from the combination of dysregulated Nrf1 and subunit displacement that appears to occur in EAE. We also found that astrocytes have a higher percentage of i-20S particles as compared to neurons in vitro. This may indicate that, in the context of acute inflammatory stress, astrocytes have a higher capacity to generate peptides for antigen presentation compared to neurons. However, our study was conducted using differentiated tumor cell lines and the situation may be different in primary cultures of neurons and astrocytes – this will need to be investigated in the future. Additional studies should also be conducted to determine whether the pro-inflammatory cytokine mixture that we used is the best to mimic the cellular environment in EAE. To model oxidative stress, we could employ the glucose / glucose oxidase system, which leads to continuous production of hydrogen
peroxide in the medium (Marinho et al., 2013; Panayiotidis et al., 1999), and determine whether this stressor plays a role in proteasome regulation in these cells. Also, we could combine the mixture of cytokines with an oxidative stress condition in culture to determine whether this combination leads to the same proteasomal dysfunction as observed in EAE.

Just as is the case with EAE, the upregulation of i-20S-specific subunits and 11S subunits in CIII-treated astrocytes and neurons is likely the result of IFN-γ-triggered activation of STAT-1. However, the induction of immuno subunits in astrocytes seems to be independent of IRF-1 – neurons were not studied. We found that the reduction of NF-κB by IKK-16 in CIII-stimulated astrocytes is enough to bring IRF-1 levels down to almost normal values, but p-STAT-1 expression remains elevated along with that of the above-mentioned proteasome subunits. This finding is contrary to previous studies suggesting that the effects of IFN-γ are produced by the partnership of STAT-1 and IRF-1. However, our conclusion is supported by the observation that STAT-1 can itself activate transcription of inducible subunit β1i, while IRF-1 is unable to do so in vitro (Chatterjee-Kishore et al., 2000).

Another question that arises from the findings of this chapter is why Nrf1 mRNA and protein levels are not affected in vitro like they are in vivo. While Nrf1 expression is reduced in the EAE spinal cord, it is unchanged in both neurons and astrocytes exposed to cytokines in culture. One explanation could be that there are stressors in EAE that are not present in the cytokine mixture. Another possibility is that the 24h exposure is not long enough to produce measurable
changes in Nrf1 expression. In this regard, it will be interesting to investigate the effect of a longer period of cytokine exposure (i.e. 72h or more) on PBX1 and Nrf1 levels as well as proteasome composition.

Nrf1 tends to be more involved in controlling homeostatic expression of its target genes, compared to the more inducible response from Nrf2 (Zhang and Xiang, 2016). Perhaps this explains why Nrf1 signaling is not affected by the oxidative stress that likely results from the cytokine exposure. In contrast, the stress caused by CIII-treatment is enough to induce a strong Nrf2 response in cultured astrocytes. The lack of changes in Nrf2 levels in stimulated neurons was an unexpected finding but is consistent with the observation that the Nrf2 pathway in astrocytes is more active compared to that in neurons and oligodendrocytes (Draheim et al., 2016). This also agrees with the idea that astrocytes, via Nrf2 signaling, play a role in providing neurons with protection – such as releasing glutathione for neuronal uptake (Shih et al., 2006).

Cytokines released by activated microglia generate substantial oxidative stress in astrocytes (Chen et al., 2015), through NF-κB-dependent induction of NADPH oxidase (NOX). One study found that a 24-hour treatment with IL-1β and TNF-α is enough to induce high expression of NOX enzymes as well as intracellular ROS production and lipid peroxidation in human astrocytes (Sheng et al., 2013). The above studies indicate that cells that respond to IL-1β and TNF-α and have an active NF-κB signaling pathway (i.e., the differentiated C6 astrocytes) will produce phagocytic NOX activity (Anrather et al., 2006) and increase Nrf2 transcription. Conversely, cells where inflammatory cytokine treatment fails to
activate NF-κB (i.e., the differentiated N2a neurons) will not result in either oxidative stress or upregulation of Nrf2. This places NF-κB at the center of oxidative stress control and the development of an antioxidant response.

**Aim 3: Cellular differences in inflammatory and oxidative stress signaling may play an important role in the pathophysiology of EAE**

The last aim of this dissertation was to explore the link between NF-κB and Nrf2 signaling and its effect on cellular reactivity in cultured astrocytes exposed to pro-inflammatory cytokines. The contribution of reactive astrocytes to the CNS microenvironment in diseases like MS and EAE has become an exciting research topic. In EAE specifically, the astrocytic transcriptome in both the spinal cord and cerebellum shows drastically upregulated immune signaling pathways, with the highest levels being at the peak of disease and declining thereafter (Itoh et al., 2018). The same group also found that the complement pathway is highly augmented in the optic nerve as compared to the spinal cord or cerebellum. This could explain why we only see a smaller subset of A1-reactive astrocytes in the spinal cords of our EAE mice. Both reactive microglia and astrocytes are found in the white matter of the lumbar spinal cord in EAE mice starting at 9 dpi, with high levels of reactivity at 14 and 21 dpi (Valente et al., 2017). This suggests that inflammation activates microglia early on in EAE, which can then activate astrocytes to become A1-reactive. However, a recent study also found elevations in A1-reactive astrocytes present in the EAE spinal cord at 50 dpi, indicating that these cells are still pertinent even in the chronic stage of the disease (Tassoni et al., 2019).
While NF-κB signaling in astrocytes seems to be essential for the development of tissue damage in EAE, genetic inactivation of neuronal NF-κB – through suppression of IκB – does not greatly affect the disease course (Lee et al., 2012). This is consistent with our observations that, in contrast to astrocytes, neuronal NF-κB signaling is unresponsive to cytokine stimulation.

Nrf2 and NF-κB have been demonstrated to interact under a variety of contexts, including inflammation, cancer, proteotoxicity, oxidative stress and others (Sandberg et al., 2014). LPS is known to upregulate gene expression of Nrf2 and prevent cell death in monocytes, which is blocked by inhibiting phosphorylation of IκBα (Rushworth et al., 2012). Though the authors suggest that there could be other signaling partners involved in this regulation, these results provide convincing evidence for NF-κB-mediated regulation of Nrf2 expression. Indeed, our own result of diminished NF-κB and Nrf2 expression following the use of IKK-16 strongly supports this idea. Yet, the crosstalk between Nrf2 and NF-κB is much more complicated than one triggering the transcription of the other. Following LPS stimulation, NF-κB can be upregulated by Rac family small GTPase 1 (RAC1)-IκBα which in turn upregulates Nrf2 – conversely, this amplified Nrf2 response then diminishes the NF-κB response (Cuadrado et al., 2014). The authors speculate that this feedback could be a mechanism to regulate pro-inflammatory signaling, which could explain the increase in Nrf2 in our model as an attempt by A1 astrocytes to reduce their NF-κB response. This also follows with what we described in pan-reactive astrocytes, where NF-κB signaling is already depressed and therefore the compensatory elevation of Nrf2 is unnecessary.
Because Nrf2 downregulates NF-κB signaling and NF-κB activation is critical for astrocyte reactivity, one would expect that abrogation of Nrf2 expression leads to the formation of the more toxic A1-reactive cells. Indeed, Nrf2 knockout mice with EAE have higher levels of demyelination as well as increased reactivity of astrocytes and microglia (Johnson et al., 2010). Furthermore, genetic deletion of Nrf2 in mice causes widespread demyelination as well as astrogliosis across the CNS, resulting in spongiform leukoencephalopathy (Hubbs et al., 2007).

In terms of our A1-reactive versus pan-reactive astrocyte study, there are a few caveats within these experiments that need to be mentioned. The first of these is that we used GFAP to identify astrocytes, which may not be the most comprehensive marker (Liddelow and Barres, 2017). A way to remedy this would be to use an antibody directed against aldehyde dehydrogenase 1 family member L1 (Aldh1l1), which has been shown to stain most astrocytes in vivo as well as C6 cells in vitro (Galland et al., 2019). We should also perform similar studies using primary astrocytes in culture to determine if they would also respond heterogeneously to the cytokine mixture. One reason for this is that C6 cells are technically cancer cells, and most tumor cell lines are known to have higher basal levels of Nrf2 (Tebay et al., 2015), so the Nrf2 response of primary astrocytes could be different.

Although we have established that there are at least two populations of reactive astrocytes in EAE and in vitro, we did not assess the presence of A2-reactive astrocytes. Because these astrocytes are theorized to be beneficial to the greater CNS environment, it will be essential to determine whether they are
present in EAE as well as *in vitro*. We would first need to perform IHC on EAE spinal cord tissue using markers for A2 astrocytes, such as TNF superfamily member 12 (TWEAK) receptor, sphingosine-1-phosphate receptor 3 (S1Pr3) or Ptx3 (Zamanian et al., 2012), and determine whether this ischemia-driven cell subtype is present in EAE. Additionally, it would be of interest to establish whether Nrf2 expression is also elevated in A2-reactive astrocytes generated *in vitro*. In this regard, hyperbaric oxygen preconditioning (HBO-PC) of mice has been reported to increase Nrf2 levels in spinal cord astrocytes and to be protective following ischemic injury. Furthermore, HBO-PC combined with glucose-oxygen deprivation *in vitro* causes a similar Nrf2 boost in primary astrocytes that has been shown to protect co-cultured primary neurons through astroglial release of glutathione (Xu et al., 2014). A separate study discovered that Nrf2 increases in rat brain exposed to hypoxia-ischemia and even more so following HBO-PC (Zhai et al., 2016). Therefore, we could use an *in vitro* model of ischemia, such as an oxygen deprivation or glucose-oxygen deprivation, to assess A2 reactivity markers in our C6 astrocytes as well as to determine if Nrf2 increases similarly to what we found in our A1 phenotype.

**Scientific Impact**

The findings reported in this dissertation help to interpret and extend our previous investigations suggesting that differential changes in composition and activity of the proteasome occur within cellular populations in EAE. These data provide more insight into how the proteasome functions in individual cell types during acute inflammation in the CNS, which will hopefully translate into MS. In
addition, we discovered that astrocytes are more than just part of the first response in the CNS, but that their reactions can be as diverse as the cells themselves. Determining whether pan-reactive or A1-reactive cells are harmful could be essential to understanding how astrocytes behave during complex diseases like MS. By appreciating how individual populations of cells react to inflammation, perhaps we will be able to tailor future treatments for regulation of both proteasome function as well as astrocyte reactivity to help patients suffering from MS.
Appendix A – List of Abbreviations and Acronyms

α7 (Psma7): 20S subunit α7
ActD: actinomycin D
AD: Alzheimer’s disease
ALS: amyotrophic lateral sclerosis
β5 (Psmb5): c-20S subunit β5
β5i (Psmb8): i-20S inducible subunit β5
C3 (C3): complement C3
c-20S: constitutive-20S particle
CFA: complete Freund’s adjuvant
CHX: cycloheximide
CNC-bZIP: cap’n collar basic leucine zipper
CNS: central nervous system
Cul3: cullin-3
Dpi: days post immunization
EAE: experimental autoimmune encephalomyelitis
eIF2α: eukaryotic initiation factor 2 alpha
EpRE: electrophile response element
ER: endoplasmic reticulum
EVI-1: ecotropic virus integration site-1
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GAS: gamma interferon activated site
Gclc, γ-glutamylcysteine ligase
GFAP: glial fibrillary acidic protein
GSH: glutathione
GSK-3β: glycogen synthase kinase 3β
H3: histone H3
HBO-PC: hyperbaric oxygen preconditioning
HD: Huntington’s disease
HDAC2: histone deacetylase 2
Hprt1: hypoxanthine phosphoribosyltransferase 1
i-20S: immuno-20S particle
IL-1β: interleukin-1β
IFN-γ: interferon-γ
IHC: immunohistochemistry
IKK: IκB kinase
IRF-1: interferon regulatory factor-1
ISRE: interferon-stimulated response element
kDa: kilodalton
Keap1: kelch-like ECH associated protein 1
LPS: lipopolysaccharide
MHC: major histocompatibility complex
MOG: myelin oligodendrocyte glycoprotein
mTOR: mammalian target of rapamycin
mTORC: mammalian target of rapamycin complex
MS: multiple sclerosis
NAD(P)H: nicotinamide adenine dinucleotide phosphate
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NOTCH3: notch receptor 3
NOX: NADPH oxidase
Nqo1: NAD(P)H quinone dehydrogenase 1
Nrf1 (Nfe2l1): nuclear factor (erythroid-derived 2)-like 1
Nrf2 (Nfe2l2): nuclear factor (erythroid-derived 2)-like 2
Nrf3 (Nfe2l3): nuclear factor (erythroid-derived 2)-like 3
p62: sequestosome 1
PA28: 11S regulatory particle
PA28α (Psme1): PA28 subunit α
PA28β (Psme2): PA28 subunit β
PA700: 19S regulatory particle
PBX1: pre-B-cell leukemia homeobox-1 transcription factor
PD: Parkinson’s disease
PI31: proteasome inhibitor 31 kDa
POMP: proteasome maturation protein
PTBP1: polypyrimidine tract binding protein 1
Ptx3: pentraxin 3
Rn18s: 18S ribosomal RNA
ROS: reactive oxygen species
*Rplp0*: ribosomal protein lateral stalk subunit P0
*Rpt5 (Psmc3)*: 19S regulatory subunit 6A
RRID: research resource identifier
RT-qPCR: reverse transcription quantitative polymerase chain reaction
sMaf: small musculoaponeurotic fibrosarcoma
SREBP: sterol responsive element binding protein
STAT-1: signal transducer and activator of transcription 1
*Tbp*: TATA-box binding protein
TNF-α: tumor necrosis factor-α
UPR: unfolded protein response
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