Janus kinase 1 drives endoplasmic reticulum stress-induced transcriptional reprogramming in astrocytes

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Janus kinase 1 drives endoplasmic reticulum stress-induced transcriptional reprogramming in astrocytes.

Savannah Graham Sims
Dissertation submitted to the School of Medicine
At West Virginia University
In partial fulfillment of the requirements for the degree of
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In
Immunology and Microbial Pathogenesis

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Keywords:
Astrocytes, endoplasmic reticulum, JAK-STAT, neuroinflammation, cytokines, signaling, central nervous system, unfolded protein response

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Abstract
Janus kinase 1 drives endoplasmic reticulum stress-induced transcriptional reprogramming in astrocytes.

Savannah G. Sims

Neurological and neurodegenerative diseases are heterogenous and devastating diseases with limited therapeutic options and no cures. The broad, long-term goal of this project was to elucidate therapeutic targets for neurodegenerative conditions that attenuate damaging inflammation while leaving the beneficial immune response intact and avoiding broad immunosuppression. Inflammation and the accumulation of misfolded proteins are associated with a wide variety of neurological diseases. Here, we have examined how the accumulation of misfolded proteins shapes inflammatory signaling in the glial cell population astrocytes. Astrocytes are the most populous cell in the central nervous system (CNS) and provide physical and trophic support to the CNS. Proper astrocyte function is paramount for a healthy brain. Recent evidence indicates endoplasmic reticulum (ER) stress and inflammation are linked. ER stress occurs when the protein folding capacity of the cell is overwhelmed, resulting in the initiation of the unfolded protein response (UPR) to regain homeostasis. However, unresolved UPR activation leads to cell death and aberrant inflammation. Further, astrocytes are relatively resistant to ER stress-induced cell death. We have found that UPR activation in astrocytes activates JAK1-dependent inflammatory gene expression. Canonical JAK1 signaling is initiated by ligand binding of a cytokine receptor that results in Signal Transducers and Activators of Transcription (STAT)-dependent inflammatory gene expression. Using RNA sequencing of primary murine astrocytes, we have demonstrated that JAK1 regulates approximately 10% of ER stress-induced gene expression. However,
we found JAK1 initiates different gene expression based on the activating stimulus. In response to ER stress, JAK1 regulates a distinct subset of gene expression that we hypothesize does not rely on JAK1-dependent phosphorylation of STATs. Instead, we have described a noncanonical role for JAK1 in response to ER stress that utilizes the transcription factor activating transcription factor (ATF) 4. ATF4 is expressed in response to ER stress and other types of cell stress. We demonstrate here that JAK1 and ATF4 coimmunoprecipitate, suggesting a physical interaction between these two proteins. Further, we showed via ChIP-seq that JAK1 is required for ATF4 to bind transcription start sites in promoter regions. Here, we have demonstrated a mechanism by which JAK1 regulates ER stress-induced gene expression in astrocytes in a noncanonical mechanism. Future directions of this project will focus on understanding the physiological consequences of this pathway in vivo in models of neuroinflammation.
Dedication

To my Mom and Dad - all of this is dedicated to you. Because of your guiding force every day of my life, I never truly questioned my ability to achieve my goals. Thank you for believing in me, for instilling in me the value in hard work and dedication. Thank you for listening. Thank you for always prioritizing education. Thank you for ensuring that I had access to every opportunity. I am so grateful for each of you. All I am or ever will become is a direct product of your inspiring, caring, and loving parenting. I am so lucky to be your daughter.

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“Science and everyday life cannot and should not be separated.”

Dr. Rosalind Franklin, PhD
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"You lose your curiosity when you stop learning." – Katherine Johnson

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Table of Contents
Janus kinase 1 drives endoplasmic reticulum stress-induced transcriptional reprogramming in astrocytes .......................................................... 1
Abstract ........................................................................................................... ii
Dedication .......................................................................................................... iv
Acknowledgements ............................................................................................ v
Table of Contents ............................................................................................... xii
List of Figures .................................................................................................... xiv
List of Tables ...................................................................................................... xv
List of Nomenclature .......................................................................................... xvi
Chapter 1 Introduction and Background ............................................................ 1
Introduction ........................................................................................................ 1
Endoplasmic Reticulum Stress and the Unfolded Protein Response .................... 9
Unfolded Protein Response and Astrocytes ..................................................... 14
IRE1 in astrocytes ........................................................................................... 15
PERK in astrocytes .......................................................................................... 17
ATF6 and OASIS in astrocytes ......................................................................... 22
Non-cell autonomous effects of ER stressed astrocytes .................................... 23
Conclusions ....................................................................................................... 27
Bibliography ...................................................................................................... 28
Chapter 2 Janus Kinase 1 Is Required for Transcriptional Reprograming of Murine
Astrocytes in Response to Endoplasmic Reticulum Stress ................................ 37
Abstract .......................................................................................................... 37
Introduction ....................................................................................................... 38
Materials and Methods ................................................................................... 40
Results ............................................................................................................... 46
Discussion ......................................................................................................... 58
Supplementary Material .................................................................................. 64
Bibliography ...................................................................................................... 66
Chapter 3 JAK1 directs ATF4-dependent gene expression ................................. 71
Abstract .......................................................................................................... 71
Introduction ....................................................................................................... 72
Methods ........................................................................................................... 75
Results ............................................................................................................. 81
Discussion ........................................................................................................ 90
<table>
<thead>
<tr>
<th>Chapter 4 Conclusions and Perspectives</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conclusions and Summary</td>
<td>96</td>
</tr>
<tr>
<td>Discussion and Future Directions</td>
<td>101</td>
</tr>
<tr>
<td>Canonical and Noncanonical JAK1 signaling</td>
<td>101</td>
</tr>
<tr>
<td>The in vivo role of JAK1 in astrocytes</td>
<td>110</td>
</tr>
<tr>
<td>Bibliography</td>
<td>118</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5 Appendix I: Review Article Full Text</th>
<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title: The role of endoplasmic reticulum stress in astrocytes</td>
<td>127</td>
</tr>
<tr>
<td>Abstract</td>
<td>127</td>
</tr>
<tr>
<td>Table of Contents Image:</td>
<td>129</td>
</tr>
<tr>
<td>Main Points:</td>
<td>129</td>
</tr>
<tr>
<td>Introduction</td>
<td>129</td>
</tr>
<tr>
<td>IRE1 in astrocytes</td>
<td>136</td>
</tr>
<tr>
<td>PERK in astrocytes</td>
<td>138</td>
</tr>
<tr>
<td>ATF6 and OASIS in astrocytes</td>
<td>144</td>
</tr>
<tr>
<td>Non-cell autonomous effects of ER stressed astrocytes</td>
<td>146</td>
</tr>
<tr>
<td>Discussion and Perspectives</td>
<td>150</td>
</tr>
<tr>
<td>Bibliography</td>
<td>154</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1 Astrocytes produce a variety of JAK-STAT dependent cytokines. ..........................2
Figure 1.2 JAK1 is well-established to signal through a variety of cytokine receptors. ...............4
Figure 1.3 The canonical Unfolded Protein Response (UPR) is activated by 3 trans-ER membrane sensors: IRE1, PERK, and ATF6. Figure was created in Biorender. .........................10
Figure 2.1 JAK1 is required to drive ER stress-induced IL-6 expression and does not affect canonical PERK signaling .................................................................47
Figure 2.2 JAK1 regulates approximately 10% of ER stress-induced gene expression. ..............48
Figure 2.3 JAK1 is required for full engagement of PERK-dependent gene expression. ..........51
Figure 2.4 ER stress induces a unique JAK1-dependent gene expression profile that is distinct from OSM-induced JAK1-dependent gene expression ........................................54
Figure 2.5 JAK1 and ATF4 cooperatively regulate a subset of ER stress-induced genes ..........55
Figure 2.6 A subset of ER stress-induced JAK1-dependent signaling is insensitive to kinase inhibition of JAK1. ........................................................................................................57
Figure 2.7 Summary of ER stress-induced JAK1-dependent signaling .................................60
Figure 3.1 JAK1 kinase inhibition does not ablate most JAK1-dependent gene expression in response to ER stress .........................................................................................................81
Figure 3.2 ER stress-induced gene expression does not require STAT3. ..............................83
Figure 3.3 JAK1 and ATF4 interact between amino acids 248 and 275 of ATF4, and this interaction does not require ER stress .................................................................85
Figure 3.4 JAK1 is expressed in the nucleus in response to ER stress .......................................88
Figure 3.5 JAK1 is required for ATF4-promoter interactions ................................................89
Figure 4.1 JAK1 regulates basal and inflammation-induced C3 .............................................102
Figure 4.2 Development of BioID - a potential future direction to identify novel JAK1-interacting molecules ..............................................................................................................107
Figure 4.3 JAK1 global inducible in vivo knockouts .................................................................113
Figure 4.4 Summary of JAK1-dependent signaling in response to ER stress ........................115
Figure 5.1 The canonical Unfolded Protein Response (UPR) is activated by 3 trans-ER membrane sensors: IRE1, PERK, and ATF6 .................................................................133
Figure 5.2 Proposed mechanisms by which endoplasmic reticulum stress signaling in astrocytes impacts the overall CNS environment .........................................................149
List of Tables
Table 2-1 Top ER stress induced JAK1 dependent genes. ..................................................64
Table 5-1 Endoplasmic reticulum (ER) stress-induced changes in astrocytes ................153
List of Nomenclature

- ACM: astrocyte conditioned media
- AD: Alzheimer’s disease
- ALS: amyotrophic lateral sclerosis
- AP-1: activator protein 1
- Apo: apolipoprotein
- ATF: Activating transcription factor
- ATP: adenosine triphosphate
- BBB: blood brain barrier
- BCA: bicinchoninic acid assay
- BiP: binding immunoglobulin protein
- BSA: bovine serum albumin
- C/EBP: CCAAT/enhancer binding protein
- CCL: chemokine ligand
- cDNA: complementary deoxyribonucleic acid
- cGMP: cyclic guanosine monophosphate
- ChIP: chromatin immunoprecipitation
- CHOP: C/EBP homologous protein
- CM: conditioned media
- CNS: central nervous system
- CNTF: ciliary neurotrophic factor
- DNA: deoxyribonucleic acid
- EAE: experimental autoimmune encephalomyelitis
- eIF2α: eukaryotic initiation factor 2 α
- ELISA: enzyme-linked immunosorbent assay
- ER: endoplasmic reticulum
- ERAD: endoplasmic reticulum associated degradation
- GADD45: growth arrest and DNA-damage-inducible protein
- GCN2: general control nonderepressible 2
- GDNF: glial cell line-derived neurotrophic factor
- GFAP: glial fibrillary acidic protein
- GRP: glucose regulated protein
- HRI: heme regulated inhibitor
- IB: immunoblot
- IFN: interferon
- IL: interleukin
- iNOS: inducible nitric oxide synthase
- IP: immunoprecipitation
- IRE1: inositol requiring enzyme 1
- ISR: integrated stress response
- ISRIB: integrated stress response inhibitor
• JAK: Janus kinase
• JNK: c-jun N-terminal kinase
• LIF: leukemia inhibitor factor
• LPS: lipopolysaccharide
• LTD: Long term depression
• LTP: long term potentiation
• MAPK: mitogen-activated protein kinase
• MCAO: middle cerebral artery occlusion
• MS: Multiple Sclerosis
• NF-κB: nuclear factor-κB
• NGF: nerve growth factor
• NO: nitric oxide
• OASIS: old astrocyte specifically induced substance
• ORF: open reading frame
• OSM: Oncostatin M
• p: phosphorylated
• PCR: polymerase chain reaction
• PD: Parkinson’s disease
• PERK: protein kinase R like endoplasmic reticulum kinase
• PI3K: phosphatidylinositol 3-kinase
• PKR: protein kinase R
• RNA: ribonucleic acid
• RNAseq: RNA sequencing
• RT-qPCR: quantitative reverse transcription polymerase chain reaction
• scRNAseq: single cell RNA sequencing
• SERCA: sarcoendoplasmic reticulum calcium transport ATPase
• shRNA: short hairpin RNA
• siRNA: small interfering RNA
• SOD: superoxide dismutase
• STAT: signal transducer and activator of transcription
• TBST: Tris buffered saline Tween 20
• Thaps: thapsigargin, inhibitor of sarcoendoplasmic reticulum (SR) calcium transport ATPase
• TNF: tumor necrosis factor
• TRIB3: tribbles homolog 3
• TTC: triphenyl tetrazolium chloride
• Tunic: tunicamycin, inhibitor of N-glycosilation
• Tyk: tyrosine kinase
• UPR: unfolded protein response
• VWM: Vanishing White Matter Disease
Introduction and Background

Introduction

Neurodegenerative diseases are characterized by the progressive loss of neuronal function, cognitive decline, and eventual death. As reported in 2017, the estimated financial burden of neurological diseases is approximately $800 billion a year [1, 2]. The largest risk factor of suffering from a neurological disease is age. Twenty five percent of the United States population will reach the age of 65 by 2050, meaning a large portion of the population will be at great risk for developing a neurodegenerative disease [3]. No curative treatments are available, and the prevalence of these diseases is continuing to rise with a globally aging population. While neurological and neurodegenerative diseases are widely heterogeneous, we have focused our interests in evaluating molecular signaling pathways in astrocytes, cells that comprise over half of the cellular composition of the Central Nervous System (CNS), that serve as common links between multiple neurodegenerative diseases to help identify mechanisms that could expand the knowledge base to develop pragmatic post-diagnosis treatment.

Astrocytes are glial cells that support proper neurological function in the Central Nervous System (CNS), in part, by providing structural support for neuronal synapses and blood vessels, participating in electrical and chemical transmission, and providing trophic support via soluble factors. Dysregulation of astrocyte function contributes to neurological decline in CNS diseases. Astrocytes comprise a large portion of the CNS [4, 5]. Historically, astrocytes were viewed as a homogenous population that primarily played a role in structural support to the CNS, however, technical advances and meticulous
experimentation have shown that astrocytes are a heterogeneous and dynamic population of CNS-resident cells, playing important roles in both homeostasis and disease. For example, astrocytes support synapse formation and function through both physical interactions and secreted molecules [6]. Astrocytes play a role in synaptic pruning during development, a process which is essential for proper neural development [7]. Astrocytes also support synaptic function by regulating homeostasis of ions (Ca^{2+}, Cl^-, K^+), water transport, and neurotransmitter reuptake and recycling [8, 9].

In addition to their supportive role, astrocytes respond to insult and injury, can promote neurotoxicity, and direct CNS inflammation by promoting microglial activation and leukocyte trafficking. Inflammation, particularly proinflammatory cytokines such as interleukin (IL) - 6, IL-1, tumor necrosis factor (TNF) -α, and the complement system, play an important role in neurological diseases and are associated with worsened neurological outcomes [10-14]. Astrocytes are key directors of inflammation within the CNS. It is well established that astrocytes undergo transcriptional and phenotypical changes in response to injury, called astrogliosis [9]. During astrogliosis, astrocytes are more

Figure 1.1 Astrocytes produce a variety of JAK-STAT dependent cytokines.

In addition to their supportive role, astrocytes respond to insult and injury, can promote neurotoxicity, and direct CNS inflammation by promoting microglial activation and leukocyte trafficking. Inflammation, particularly proinflammatory cytokines such as interleukin (IL) - 6, IL-1, tumor necrosis factor (TNF) -α, and the complement system, play an important role in neurological diseases and are associated with worsened neurological outcomes [10-14]. Astrocytes are key directors of inflammation within the CNS. It is well established that astrocytes undergo transcriptional and phenotypical changes in response to injury, called astrogliosis [9]. During astrogliosis, astrocytes are more
proliferative, glial fibrillary acidic protein (GFAP) expression increases, signaling molecules and cytokines are upregulated, the extracellular matrix remolds, and changes in ability of astrocytes to properly regulate synapses and the blood brain barrier (BBB) occur. Reactive astrocytes have opposing roles on disease states. For example, reactive astrocytes worsen spinal cord injury, but during ischemia, reactive astrocytes promote overall neural recovery [15-22].

Reactive astrocytes are known to contribute to the pathology of neurological disease, in part, by producing soluble inflammatory mediators such as IL-1β, TNF-α, CXCL1, CXCL10, CCL2, CCL7 CCL20, IL-6, IL-10, and IL-15 to modulate the inflammatory milieu of the CNS. Many of these cytokines and chemokines rely on Janus Kinase (JAK) – Signal Transducer and Activators of Transcription (STAT) signaling (Figure 1.1). The JAK-STAT family is an important signaling pathway that is comprised of four JAK proteins (Tyk1, JAK1, JAK2, JAK3) and seven STATs (STAT1, STAT3, STAT4, STAT5a, STAT5b, STAT6) proteins. The JAK-STAT family is responsible for coordinating immunological responses to a multitude (reportedly over 70) of cytokines, chemokines, and growth factors. Because each JAK and STAT protein can homo- or heterodimerize to drive transcription, the various combinations of these JAKs and STATs provides great complexity in this signaling pathway. Historically, the JAK-STAT signal transduction cascade was discovered by examining the antiviral responses of interferons (IFN) and gene expression. IFNs were discovered as a secreted product that interfered with viral replication [23]. IFN receptors do not have intrinsic kinase activity, so these receptors rely on JAKs to mediate phosphorylation and activation of STATs.
To mediate cytokine induced signaling, JAK proteins are constitutively associated with the cytoplasmic portions of cytokine receptors that span the plasma membrane. Cytokine receptors are activated upon ligand (cytokine) binding. Cytokine receptors bound to their cytokines undergo a conformational change that brings JAK proteins into proximity. JAKs auto phosphorylate and this phosphorylation occurs on an activation loop to increase the intrinsic kinase activity of JAKs [24]. JAKs then phosphorylate the cytokine receptor on tyrosine residues. These tyrosine residues are then recognized by STAT proteins because STATs contain SH2 domains. STATs are recruited to the cytokine receptor via these SH2 domains where JAKs can phosphorylate STATs [25, 26]. Phosphorylated STATs can hetero- or homodimerize. Upon dimerization, a nuclear localization sequence is exposed. STAT dimers translocate to the nucleus and bind promoter regions to initiate cytokine-induced gene expression [27].

Figure 1.2 JAK1 is well-established to signal through a variety of cytokine receptors.
The JAK-STAT signaling cascade has been widely studied and associated with many diseases, including neurodegenerative diseases [28]. There are a number of pharmaceutical inhibitors in use and in clinical trials designed to inhibit JAKs and STATs primarily applied to treatment of cancer and immune disorders [29]. These JAK inhibitors are also undergoing clinical trials for many autoimmune diseases such as rheumatoid arthritis and irritable bowel disease, as well as neoplastic diseases (58). Five JAK inhibitors have received FDA approval (28). Baricitinib (Olumiant), tofacitinib (Xeljanz), and upadacitinib (Rinvoq) are currently approved to treat rheumatoid arthritis. Ruxolitinib (Jakafi/Jakavi) is approved by the FDA to treat myelofibrosis and polycythemia vera. Fedratinib (Inrebic) is also FDA approved to treat myelofibrosis [30, 31]. Inhibitors of STAT3 and STAT5 also are utilized as research tools and are undergoing investigation as potential cancer treatment (59,60). In addition to clinical applications, the availability of these inhibitors is useful for basic research to determine the significance of JAK-STAT signaling in the context of neurological disease. However, it is important to note that all JAK inhibitors will have broad impacts in all cells that express the targeted JAK proteins. For example, JAK inhibitors will likely make patients taking these medications more susceptible to certain viral, bacterial, or fungal infections. Therefore, patients will require monitoring and counseling concerning these treatments [32, 33].

Mouse knockout experiments have helped us gain critical knowledge on the role of JAKs and STATs in vivo. JAK1 knockouts in mice are perinatal lethal and newborn pups exhibit loss of hematopoietic function [34, 35]. JAK2 knockouts are embryonic lethal [36]. JAK3 knockout mice exhibit severe combined immunodeficiency with a loss of all mature B and T cells [37, 38]. Tyk2 knockouts are viable but experience major anti-viral
immune deficits and lack responses to type I interferons, IL-12, and IL-23 family cytokines [39]. Much of this animal work has been strengthened in human studies. Loss of function mutations in JAK1 result in primary immunodeficiency accompanied by frequent infections. Some cancers are associated with JAK1 loss of function. Further, mutations in JAK1 that cause a gain of function result in hyper eosinophilic syndrome [40] and other immune problems, including reports in cancers [41-43], complex autoinflammatory syndrome [44], and, atopic dermatitis [40], psoriasis [45]. Loss of function mutations in JAK2 have not been characterized, which is consistent with JAK2 knockouts being embryonic lethal in mice. However, JAK2 gain of function mutations are associated with many cancers including myeloproliferative neoplasms, leukemia, and lymphoma. Consistent with JAK3 knockouts in mice, JAK3 loss of function mutations in humans also causes SCID. Gain of function mutations are associated with development of leukemia. Tyk2 loss of function mutations are associated with primary immunodeficiency, however, Tyk2 loss of function is associated with protection against autoimmunity. Gain of function mutations in Tyk2 are not established.

JAK1 and JAK2 are the primary JAK signaling proteins in the CNS; expression of Tyk2 and JAK3 is primarily restricted to cells of hematopoietic lineage. JAK-STAT signaling is required for astrocyte development. In one of the first studies to demonstrate this, it was shown that ciliary neurotrophic factor (CNTF) receptor ligation activates JAK1-dependent phosphorylation of STAT1 and STAT3 to commit neuronal precursor cells to the astrocyte lineage [46, 47]. CNTF is a member of the IL-6 family signaling pathway, and other similar cytokines, IL-6 and LIF, can trigger similar effects [48]. Further, mice lacking the LIF receptor have reduced numbers of astrocytes [49]. Taken together, these
studies suggest that JAK-STAT signaling is relevant in astrocytes and early expression is vital for development.

Because JAK-STAT signaling is necessary for astrocyte development, this provides a clear physiological role for these proteins in the CNS. However, aberrant JAK-STAT signaling is reported in neoplastic disease. Alterations in JAK-STAT signaling is observed in grade III/IV gliomas and medulloblastoma. In primary high-grade gliomas, STAT3 is constitutively activated in 60% of these malignant tumors and is driven by EGFR-mediated phosphorylation of JAK2. Further, STAT3 expression positively correlates with tumor grade, suggesting that STAT3 is oncogenic in the brain. These data suggest that there is potential to target JAK-STAT signaling components in gliomas to sensitize these tumors to effective treatment with anti-EGFR therapies. Constitutive activation of STAT3 is thought to drive proliferation and survival of glioblastoma and glioma cells. [50-52].

In addition to brain cancers, JAK-STAT signaling is activated in traumatic and ischemic CNS injury. Importantly, STAT3 is required for GFAP expression. GFAP is a primary marker of astrogliaosis, an astrocytic phenotype associated with disease and injury and required for glial scar formation (astrogliosis) [17, 53, 54]. Astrocytes in models of AD, HD, and MS also express higher levels of phosphorylated STAT3 compared to controls [55]. Additionally, JAK inhibition ameliorates disease progression in rodent models of PD and MS [56, 57]. In the APP/PS1 AD mouse model, deletion of STAT3 in astrocytes was associated with decreased expression of amyloid-β in the brain and reduced disease burden. Further, a STAT3 inhibitor reduced cognitive problems in the AD-prone mice.
This suggests that STAT3-driven astrogliosis may drive disease progression in AD. [58].

Despite the clear involvement of JAK-STAT signaling in a variety of neurological diseases, the mechanisms, and cell-specific contributions of JAK proteins has not been greatly explored. JAK1 is known to associate with the common γ receptor of type I interferon receptors, the IL-2 family receptors, IL-4 family receptors, and the gp130 receptors of the IL-6 cytokine family, the cardiotrophin receptor, and the IL-10 family receptors (Figure 1.2). The cell-specific contributions of JAK1-driven neuroinflammation are unknown. Therefore, we are interested in understanding JAK-dependent mechanisms in astrocytes in response to cellular stress that is associated with a variety of neurological diseases.

We have identified JAK1 as a specific regulator of endoplasmic reticulum stress-induced inflammation in astrocytes. JAK1 in neurons is associated with proprioception. Interestingly, the JAK1 inhibitor oclacitinib is used in veterinary applications to treat dogs with pruritus associated with allergic dermatitis [59]. Understanding the cell-specific contributions of JAK1 signaling has been difficult due to limitations of in vivo models.

Neurodegenerative diseases are accompanied by inflammation and cell stress, including compromised protein structure and function. Well-established links between misfolded proteins and several neurodegenerative diseases exist including but are not limited to Alzheimer’s disease (amyloid-β, tau), Parkinson’s disease (α-synuclein), Huntington’s disease (Huntingtin with expanded glutamine repeats), spongiform encephalopathies (prions). However, therapeutically targeting misfolded proteins has been unsuccessful in clinical trials [60]. When there is an excessive accumulation of
misfolded or unfolded proteins in the ER lumen, endoplasmic reticulum stress occurs. We have identified endoplasmic reticulum stress as a stimulus that promotes inflammatory astrocytes.

Astrocytes have been demonstrated to express many inflammatory signaling molecules, and activation contributes to the inflammatory environment of the CNS [61]. Further, ER stress has been explicitly linked to initiating inflammation. For example, ER stress augments inflammatory responses stimulated by the bacterial cell wall component lipopolysaccharide (LPS) and can directly drive activation of the acute phase response [62, 63]. Taken together, accumulating evidence suggests that chronic ER stress and subsequent signaling may drive inflammation in astrocytes and contributes to neurological disease.

**Endoplasmic Reticulum Stress and the Unfolded Protein Response**

Secreted and membrane bound proteins are translated and processed in the endoplasmic reticulum (ER). Within the ER, proteins mature by folding into the proper tertiary and quaternary structure and acquire necessary post-translational modifications. The ER is also critical for membrane lipid production and for the regulation of intracellular Ca$^{2+}$ [64]. Often, proteins within the ER fail to fold into the correct form. Fortunately, the cell has intrinsic quality control mechanisms that eliminate misfolded proteins, such as chaperone-mediated folding [65] and ER associated degradation (ERAD) [66, 67]. However, when these control mechanisms are overwhelmed, misfolded proteins accumulate in the ER lumen. The aberrant accumulation of misfolded proteins and concomitant induction of ER stress has been observed in many diseases and cell types [68, 69]. ER stress occurs when a cell can no longer keep up with the demand to fold
proteins due to the number of misfolded proteins in the ER lumen. ER stress initiates a highly conserved adaptive mechanism called the unfolded protein response (UPR). The intracellular signaling stimulated by ER stress is aimed at restoring homeostasis; however, if the stress is not alleviated, prolonged ER stress can drive cell death and inflammation which may contribute to pathology [69].

ER stress can occur transiently in physiological conditions when there is an increased demand for protein secretion, or in pathogenic states where ER stress occurs due to genetic mutations, oxidative stress, ischemia, or other maladaptive cellular states. Although UPR activation is necessary to maintain homeostasis and clearly plays a role in

Figure 1.3 The canonical Unfolded Protein Response (UPR) is activated by 3 trans-ER membrane sensors: IRE1, PERK, and ATF6. Figure was created in Biorender.
homeostatic processes, tight regulation of the UPR is paramount for maintaining cellular health. Persistent activation of the UPR is reported in multiple diseases, including diabetes, cancer, and neurodegeneration [70-72].

There are three known proteins which sense the accumulation of misfolded proteins and transmit distinct signals to the cytosol and nucleus to modify transcriptional and translational programs to cope with ER stress, summarized in Figure 1.3. These trans-ER membrane proteins are inositol requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor (ATF) 6. These enzymes are maintained in their inactive state through interaction with the ER-resident protein chaperone glucose regulated protein (GRP) 78 (also known as binding immunoglobulin protein (BiP)) [73]. GRP78 binds broadly to hydrophobic residues that are exposed by misfolded proteins [74]. Excess misfolded proteins recruit GRP78 away from the luminal domains of PERK, IRE1, and ATF6 allowing activation [75, 76]. PERK and IRE1 can also directly interact with misfolded proteins which contributes to its activation via a ligand-receptor type interaction [77-81]. Figure 1.3 provides an overview of the UPR signal transducing molecules.

IRE1 is the most evolutionarily conserved UPR initiator and contains both kinase and endoribonuclease domains. Following release of GRP78, IRE1 oligomerizes in the ER membrane facilitating trans-autophosphorylation of IRE1 which increases RNase activity [82-84]. IRE1 then splices the mRNA of x-box-binding protein (XBP1) to remove a small stop codon-containing intron which allows translation of the functional transcription factor [85]. Additionally, the RNase activity of IRE1 mediates regulated IRE1-
dependent decay (RIDD), in which a subset of ER-targeted mRNAs are degraded [86, 87]. Collectively, IRE1 drives XBP-1-dependent gene expression that includes ER chaperones and, through RNA degradation, reduces nascent polypeptide entry into the ER to reduce the folding demand [87, 88].

PERK is a trans-ER membrane serine/threonine kinase which is activated by misfolded proteins in the ER lumen. Following release of GRP78, PERK dimerizes and trans- and auto-phosphorylates to increase its kinase activity [73, 89]. PERK phosphorylates the eukaryotic initiation factor (eIF) 2α which leads to binding and inhibition of the guanine nucleotide exchange factor (GEF) eIF2B. This prevents formation of the complex needed to load the 43S ribosome with methionine, thus preventing translation initiation [90, 91]. Under these conditions, some proteins are selectively translated. For example, activating transcription factor (ATF) 4 is translated when eIF2α is phosphorylated. ATF4 translation can lead to expression of CHOP (encoded by the gene ddit3). In many cases, CHOP acts as a proapoptotic factor. Overall, PERK activation in response to ER stress reduces the protein load on the ER, and if mechanisms fail to restore homeostasis, initiate cell death.

ATF6 is a transmembrane glycoprotein that is a member of the basic leucine-zipper proteins (bZIP) transcription factor family. Upon the accumulation of misfolded proteins and disassociation of GRP78, ATF6 localizes to the golgi apparatus where is cleaved by site-1 and site-2 proteases [92], revealing a nuclear localization sequence. Subsequently, ATF6 translocates to the nucleus and binds promoter sequences to initiate gene expression of ER protein chaperones and UPR regulators to increase folding
capacity of the ER [93]. Overall, IRE1, PERK, and ATF6 are activated in response to the accumulation of misfolded proteins within the ER lumen to promote efficient protein folding through the upregulation of protein chaperones and by reducing the folding burden on the ER by eliminating influx of mRNA and polypeptides. If these mechanisms are insufficient, persistent UPR activation will promote apoptosis to eliminate the irreparably damaged cell.

ER stress can occur transiently in physiological conditions when there is an increased demand for protein secretion, or in pathogenic states, where ER stress occurs due to genetic mutations, oxidative stress, ischemia, or other maladaptive cellular states. ER stress has been best characterized in cells harboring high secretory capacities such as pancreatic β cells and antibody-producing B cells. For example, cell specific knockout of PERK in β-cells results in hyperglycemia and pancreatic atrophy, suggesting that PERK is required for correct β-cell function. This indicates that pancreatic β-cells experience physiological levels of ER stress to efficiently fold insulin precursors to meet the demand to maintain proper blood glucose concentrations. IRE1 also down regulates insulin at the mRNA expression level in vitro. Thapsigargin (an inhibitor of the sarco/endoplasmic reticulum Ca$^{2+}$ ATPase that induces ER stress by depleting the ER stores of calcium) and glucose induces the degradation of insulin mRNA. Expressing a kinase dead mutant of IRE1 in these cells abrogated this effect. This demonstrates that IRE1 can degrade insulin mRNA under ER stress conditions when the demand to produce insulin is high [94]. However, dysregulated or chronic UPR activation in these cells has been shown to contribute to pancreatic β-cell loss in type I and type II diabetes.
Additionally, antibody-producing B cells also require UPR signaling molecules. IRE1 is required for B cell maturation and for development of immunoglobulin. This was initially observed utilizing mice lacking XBP1. These mice do not make mature immunoglobulin responses, indicating that B cell maturation and plasma cell differentiation requires physiological activation of the IRE1-driven UPR pathway. IRE1α is required for B cell immunoglobulin gene arrangement, B cell receptor formation, and splicing XBP-1 for final differentiation, but IRE1α is not required for pro-B cells to arise. When stimulated with lipopolysaccharide (LPS), B cells upregulated UPR-related genes, likely to recruit aid to efficiently meet the demand to process and fold immunoglobulin proteins for large-scale antibody secretion [95]. Although UPR activation is necessary to maintain homeostasis and clearly plays a role in homeostatic processes, tight regulation of the UPR is paramount for maintaining cellular health. Persistent activation of the UPR is present in multiple diseases, including diabetes, cancer, and neurodegeneration [70-72].

Unfolded Protein Response and Astrocytes

Typically, the UPR is activated transiently to restore homeostasis, however, chronic UPR activation has been implicated in a wide variety of diseases affecting the CNS (CNS) including, but not limited to, AD, MS, and prion disease, PD, and HD [96-108]. Perturbations in astrocyte function are now implicated in nearly all neurological diseases [9]. This highlights the importance of astrocytes in maintaining and directing neurological integrity. However, the mechanisms by which cellular stressors initiate astrocyte dysfunction that contributes to disease are not well understood. Recently, single cell RNA sequencing (scRNAseq) revealed that subpopulations of astrocytes that are
expanded during Experimental Autoimmune Encephalomyelitis (EAE) have increased UPR signaling, suggesting that the UPR is associated with neuroinflammatory disease [109]. Additionally, overexpression of spliced XBP1 in astrocyte-like glial cells in C. elegans extends life span of the species [110]. Astrocytes express all the initiating sensors of the UPR (IRE1, PERK, and ATF6) and express a unique ER stress sensitive molecule, old astrocyte specifically induced substance (OASIS). Further, astrocytes are largely resistant to aberrant ER stress-induced cell death, suggesting a unique role for UPR signaling in astrocytes.

IRE1 in astrocytes
IRE1 signaling has been linked to cell death and inflammation in the CNS. Evidence of active IRE1 signaling has been reported in post-mortem human tissue in clinically confirmed cases of AD, HD, and glioma in addition to many mouse in vivo and in vitro disease models. In an immortalized astrocytic cell line, SVGA, cells infected with HIV-1 require IRE1 signaling to activate JNK and activator protein (AP) -1 to induce cell death [111]. Further, nitric oxide (NO) has also been demonstrated to activate IRE1-dependent signaling in human glioma cell lines. Treating human astrocytoma (CRT-MG) cells with an NO donor and the ER stress inducer, thapsigargin, increased apoptosis that coincided with IRE1 nuclease activity, IRE1/TRA F2 complex formation, and p-JNK1/2 levels, implying that treatment of NO subsequently activates the IRE1-α/TRA F2/JNK pathway. IRE1 knockdown confirmed that intracellular NO affects IRE1-dependent phosphorylation of CREB in human glioma cells [112]. Together, this suggests pathogenic stimuli (viral infection and reactive nitrogen species) can activate the IRE1 arm of the UPR and contribute to cell death in in vitro astrocytoma cell lines.
*In vivo* evidence suggests that IRE1 signaling in astrocytes is associated with the neurodegenerative diseases AD and MS. In brain tissue from AD patients, phosphorylated IRE1 is increased and correlates with disease severity based on Braak Staging, a pathology-based characterization of AD [100, 113, 114]. To investigate the role of IRE1 in a mouse model of AD, IRE1 was deleted in the nervous system using Nestin-cre and crossed with the 5XFAD genetic model of AD. Genetic deletion of the RNase domain of IRE1 significantly reduced amyloid deposition and astrocyte activation. Further, deficiency of IRE1 signaling improved synaptic function and long-term potentiation, suggesting restored memory and learning capacity of the mice. This led to the amelioration of disease hallmarks including Aβ1-42 production, amyloid plaque deposition, and cognitive deficits. Additionally, deletion of IRE1 reduced astrogliosis, based on GFAP staining, in the 5XFAD hippocampus. In this case, attenuation of gliosis may be through a direct effect on astrocytes or due to reduced overall disease burden [100]. Further AD studies are needed to delineate the astrocyte-specific contributions of IRE1 signaling.

In a large pharmacogenetic screen to identify signaling pathways involved in pathogenic neuroinflammation in MS, astrocytes were stimulated *in vitro* with TNF-α and IL-1β, two cytokines known to be associated with the pathogenesis of EAE and MS. Here, Wheeler et. al determined that IRE1 is phosphorylated and XBP1 was spliced, suggesting activation of IRE1 signaling during astrocyte-mediated neuroinflammation. To confirm this *in vivo*, this study used cell-specific lentiviral delivery of short hairpin (sh) – RNA targeting the gene that encodes for IRE1 (ern1) to knockdown expression in astrocytes during active EAE, which reduced disease severity. These studies demonstrated that abrogating
expression of IRE1 under control of the astrocyte selective GFAP promoter ameliorated EAE disease course and reduced inflammatory mediators produced by astrocytes [115]. This suggests that IRE1 signaling in astrocytes is pathogenic in the murine EAE model of MS.

These studies, collectively, imply that IRE1 signaling in astrocytes can be activated by various stimuli and that activated IRE1 can integrate with many signaling pathways that promote inflammation or cell death.

PERK in astrocytes
Activated PERK signaling has been reported in a variety of neurological diseases including AD, MS, prion disease, neurotropic viral infection, and ALS [98, 105, 116, 117]. Using immunocytochemistry to analyze brain tissue of human MS samples, the UPR proteins GRP78, XBP-1, and CHOP were increased in acute MS lesions [118]. In models of prion disease, neuronal cell lines were infected with PrP, the misfolded protein associated with prion disease. Prion infected neurons were more susceptible to cell death, and targeting PERK signaling in in vivo models of prion disease is protective [104, 105, 108]. Some reports show that prolonged expression of CHOP is pro-apoptotic, but this has not directly been demonstrated in primary astrocytes or in vivo models [119, 120]. However, the Venezuelan Equine Encephalomyelitis Virus (VEEV) induces apoptosis of the astrocyte-like glioblastoma cell line (U87Mg) through CHOP expression that is activated by PERK [121]. PERK knockdown in primary astrocytes reduces viral load of VEEV, but there is no difference in viral load between U87Mg cells with or without PERK expression [122].
Further, evidence of activated PERK signaling in astrocytes has been reported in neuropathological studies of human AD and PD brains [98, 123]. Additional studies have established that PERK is phosphorylated in glial cells in brains from tauopathy-associated dementias [117]. In a 2014 study, Devi and Ohno examined the role of a hemizygous PERK knockout crossed to the genetic AD model, 5XFAD. Genetic PERK ablation reduces phosphorylated eIF2α and ATF4. PERK haploinsufficiency in 5XFAD mice partially rescued memory loss in a behavioral fear conditioning model. These cognitive improvements coincided with a reduction in amyloid-β plaque burden in hippocampal and cortical regions of 5XFAD mice. Importantly, the maladaptive effects of PERK signaling were specific to onset of AD; there were no measured cognitive changes in unaffected PERK+/− mice compared to control animals [124].

In sporadic ALS and in the transgenic ALS mouse model that expresses mutant superoxide dismutase (SOD)G93A, immunohistochemistry staining of spinal cords demonstrated that many astrocytes, along with other cell types, expressed CHOP, suggesting that PERK signaling is activated in astrocytes in ALS [116]. Another study modeling ALS in mice demonstrated that astrocytes are activated, as quantified by GFAP immunofluorescence staining. Here, mice expressing wild type human SOD, which has been reported to spontaneously aggregate and model spontaneous ALS, were exposed to the pharmacological N-linked glycosylation inhibitor (tunicamycin) to induce UPR activation, which was shown to increase SOD1 aggregation. Importantly, wild type littermates did not have a significant increase in GFAP staining upon tunicamycin treatment [125]. This suggests that SOD aggregation and UPR activation enhance GFAP
expression, which is associated with a reactive astrocyte phenotype, in a murine ALS model.

Additionally, Vanishing White Matter Disease (VWM) demonstrates the importance of downstream PERK signaling in astrocytes. VWM is a leukencephalopathy in which dysfunctional astrocytes are thought to drive pathogenesis [126]. VWM is caused by an autosomal recessive mutations in eIF2B, which reduce function and cause prolonged suppression of protein translation in response to stimuli that promote eIF2α phosphorylation [127, 128]. This highlights a role for phosphorylated eIF2α-driven translational repression in preserving astrocyte homeostasis and directly links signaling components downstream of PERK to neurological disease.

To date, multiple reports link UPR-dependent PERK signaling in astrocytes to inflammatory gene expression and/or neurotoxicity [103, 129-131]. ER stress-inducing pharmacological agents thapsigargin and tunicamycin promote phosphorylation of eIF2α in primary murine astrocytes. [103, 129, 132, 133]. A 2014 study demonstrated that gene expression of inflammatory markers (IL-6, CCL2), astrocyte markers (GFAP, OASIS), and ER stress-related genes (GRP78, CHOP, PERK, ATF4) are upregulated throughout the course of EAE in brain and spinal cord tissue. Downstream markers of PERK activation such as phosphorylation of eIF2α and CHOP expression are exhibited in thapsigargin-treated astrocytes concomitantly with upregulation of IL-6, CCL2, and CCL20. Additionally, ER stress augmented IL-6 expression induced by IL-6 or oncostatin M (OSM) in a PERK-dependent fashion. This suggests that astrocytes may contribute to the UPR and inflammatory response seen in CNS tissue during EAE. It is important to note
that these inflammatory proteins are induced at the protein level even under conditions of phosphorylated eIF2α, which functions to attenuate translation, demonstrating that these proteins are translated during UPR activation [131].

PERK is an important driver of inflammatory gene expression in astrocytes in response to ER stress. A partial (heterozygous) or complete (homozygous) genetic loss of PERK in primary astrocytes was associated with a lower astrocyte-driven expression of IL-6, CCL2, and CCL20 analyzed by qPCR or ELISA. Further, primary astrocytes treated with thapsigargin and a PERK inhibitor, GSK2606414, reduced production of cytokines and chemokines measured by ELISA. This demonstrates that PERK activation contributes to both transcriptional and translational activation of inflammatory mediators in astrocytes [129]. Therefore, unresolved UPR activation may contribute to prolonged, aberrant inflammatory activation via PERK signaling that may contribute to the non-resolving nature of neurological diseases.

Cytokines such as IL-6, which is driven by PERK activation in astrocytes rely on JAK-STAT signaling to exert their effects. JAK-STAT signaling has been directly linked to astrocyte-driven pathology in neurodegenerative and neurological diseases.

PERK signaling activates downstream signaling in a JAK1 dependent mechanism, and inhibiting JAK1 kinase activity reduced ER stress-induced inflammatory gene expression. Importantly, JAK1 inhibition does not impact all ER stress-induced gene expression. Further, it has been shown that PERK activates JAK1 to drive a subset of gene expression that is distinct from those induced by the JAK/STAT activating cytokine OSM [133]. This demonstrates that UPR signaling modulates inflammatory responses in
a manner distinct from traditional inflammatory signaling. Taken together, this evidence suggests that PERK and JAK-STAT signaling in neurodegenerative disease models may promote aberrant inflammation. Targeting PERK signaling in astrocytes may be a mechanism to selectively attenuate immune responses in neurological diseases.

Targeting the UPR to selectively attenuate inflammation is supported by work in other cell types. For example, UPR signaling in macrophages activates proinflammatory cytokine signaling via the IRE1 pathway. Here, ER stress activates the nucleotide-binding oligomerization domain-containing protein (NOD) 1/2 and sXBP1 in an IRE1 dependent manner. Contrary to macrophages, PERK drives IL-6 expression in astrocytes. This highlights that the UPR regulates inflammation using distinct mechanisms in different cell types [134, 135]. ER stress-induced IL-6 production in astrocytes differs from macrophages in that it requires PERK and JAK1 but is independent of IRE1 and nuclear factor-κB (NF-κB) [129, 131]. Additionally, endothelial cells produce IL-6 in response to ER stress, but here, this IL-6 expression is dependent on both ATF4 and sXBP1 [136]. ER stress induced IL-6 expression in astrocytes does not rely on ATF4 signaling, as demonstrated using siRNA-mediated knockdown of ATF4 in primary astrocyte cultures [133]. This illustrates the need for more careful investigations regarding the nuances of UPR signaling in various cell types. For example, the UPR in the CNS literature focuses heavily on neurons and oligodendrocytes, however, these findings may not apply to astrocytes. Although astrocytes induce IL-6 and other inflammatory molecules in a PERK-dependent fashion, this is not the case for other IL-6 family members. Importantly, ciliary neurotrophic factor (CNTF) is downregulated upon ER stress induction in cultured astrocytes [137]. This suggests trophic support from astrocytes can be restricted by the
UPR. Indeed, it has been shown that ER stressed astrocytes lose trophic support for neuronal synapse formation [103].

Collectively, multiple studies have demonstrated that PERK signaling promotes an astrocyte-driven inflammatory response. Although inflammation provides a beneficial and restorative role, chronic inflammation is thought to contribute to neurological disease. PERK signaling in astrocytes may be a target to selectively attenuate damaging inflammation while retaining beneficial inflammatory signaling in the CNS. Further studies and conditional deletion of PERK and downstream signaling components in astrocytes are needed to solidify the role in disease models.

ATF6 and OASIS in astrocytes

ATF6 and old astrocyte specifically induced substance (OASIS (CREB3L1)) are bZIP transcription factors similarly activated in response to ER stress. OASIS is a molecule primarily expressed in astrocytes in the CNS. Upon activation, it is transported to the golgi apparatus, is cleaved, and the N-terminal domain promotes expression of ERAD-associated genes [138]. ATF6 is activated (cleaved) in embryonal astrocytes during differentiation suggesting a role for ATF6 in astrocyte development [139]. OASIS is also important for astrocyte differentiation. In mice lacking OASIS, astrocyte development was impaired. OASIS was shown to bind the promoter of glial cells missing transcription factor 1 (Gcm1) and promote Gcm1 expression. Gcm1 may regulate GFAP promoter methylation allowing transcriptional activation. The reduced expression of Gcm1 in OASIS-/- mice may, in part, underlie the reduced astrocyte differentiation [139].

To date, few studies have been performed examining the role of ATF6 in astrocytes during disease states. In a murine model of ischemic stroke, middle cerebral artery occlusion
(MCAO), ATF6 knockout mice exhibited reduced infarct area as analyzed by the metabolic stain triphenyl tetrazolium chloride (TTC). Concomitantly, ATF6α knockout mice had reduced STAT3 activation and expression of GFAP in the ischemic area of the brain 3 days post MCAO as measured by immunoblotting [140]. This study suggests that ATF6α is protective during ischemia and ATF6α knockout is associated with reduced GFAP expression.

OASIS activation has also been linked, in astrocytes, to AD disease mechanisms. Apolipoprotein E (ApoE) is a protein involved in catabolizing triglycerides and the ApoE4 allele is strongly associated with the development of AD, although causal associations between ApoE4 and AD are not fully known, reviewed in [141]. Primary astrocytes expressing mutant APOE, to model human ApoE4, exhibit reduced ApoE expression and increased UPR activation, including cleavage of OASIS and genes downstream of the IRE1 and PERK pathways. This suggests that ApoE can induce cleavage of OASIS and activate the UPR in astrocytes and promote neuronal toxicity [142]. Collectively, there are limited studies on the role of ATF6 and OASIS, however, these studies demonstrate that activation must be well-regulated for proper astrocyte function.

Non-cell autonomous effects of ER stressed astrocytes
In a 2017 study, Sprenkle et. al was the first to describe that astrocytes can transmit ER stress to other cell types, a phenomenon that was previously described in cancer cells and termed transmissible ER stress (TERS) [143, 144]. This suggested that UPR activation in astrocytes can induce UPR signaling in neighboring cells. In this study, astrocyte conditioned media (ACM) collected from astrocytes treated with the ER stress-inducing agent thapsigargin or tunicamycin was transferred to HT-22 hippocampal
neuronal cells. The cells that were exposed to thapsigargin treated ACM exhibited higher gene expression and protein levels of GRP78, spliced XBP1, and CHOP, indicating that astrocytes secrete a soluble factor that stimulates an ER stress response. Further, this study showed that neurons experiencing ER stress also secrete a molecule that induces ER stress in cultures of neurons, astrocytes, and microglia [132, 145]. This study identified that UPR activation can be transmitted between cells of the nervous system. These studies are consistent with previous work that demonstrated that ER stress is also transmissible between cancer or myocardial cells and macrophages, which also respond to ER stress by producing inflammatory molecules, albeit these mechanisms are distinct from those identified in astrocytes [132, 144, 146]. ER stressed astrocytes, through a PERK-dependent process, also increase microglial expression of IL-1β and IL-6 [131]. Independently of PERK, ER stressed astrocytes reduce microglial expression of arginase, CD206 and insulin like growth factor 1 [129]. Together, these data indicate that in response to ER stress, astrocytes can shift microglia to an inflammatory phenotype. Additionally, Wheeler and colleagues demonstrated that XBP1 knockdown in astrocytes decreases the number of monocytes that traffic to the CNS during EAE. Macrophages that trafficked to the CNS during EAE in the GFAP-driven XBP1 knockdown had reduced expression of inflammatory genes involved in IL-6 signaling, NF-κB signaling, and chemokine signaling. Similarly, microglia in the astrocyte specific XBP1 knockdown had reduced proinflammatory gene expression in comparison to EAE animals with XBP1 expression astrocytes [115].

Astrocyte conditioned media from healthy astrocytes is known to support synaptogenesis [147-149]. To determine if UPR activation impacts the ability of astrocytes
to support synapses, Mallucci and colleagues collected astrocyte conditioned media (ACM) from thapsigargin-treated astrocytes. By immunostaining pre and post synaptic terminals, ACM from UPR activated astrocytes was shown to reduce synaptogenesis. Further, inhibiting PERK pharmacologically restored the ability of ACM to promote synapse formation, suggesting that UPR activation via PERK inhibits astrocyte-mediated neurotrophic functions. Further, this study tested if targeting PERK-eIF2α signaling \textit{in vivo} could be neuroprotective. Using mice that over express prion protein (PrP) and succumb to prion infection. Astrocyte specific lentiviral overexpression of GADD34, an eIF2α-specific phosphatase, was markedly protective in prion-infected mice. GADD34 overexpression (to reduce PERK signaling) in astrocytes prevented neurodegeneration in the hippocampus, had an increased number of pyramidal neurons, reduced astrocyte reactivity based on morphology and GFAP staining, and extended the life span of these mice in comparison to control PrP animals. This study shows both \textit{in vitro} and \textit{in vivo} that UPR activation via the PERK pathway alters the transcriptome and secreted molecules of astrocytes and this is linked to a reduction in neuronal synapse formation. (Smith et al., 2020). These studies expand upon and corroborate the previous findings that PERK inhibition is protective in prion infection [97, 99, 104, 105]. Further, this suggests that UPR-activated astrocytes have pathogenic roles in prion infection and identifies PERK signaling as a central driver in this process.

Consistent with the notion that astrocytes have a significant role in directing the milieu of the inflammatory environment in the CNS, viral infections have also shown to induce the UPR in astrocytes, leading to pathogenic non cell-autonomous astrocyte dependent pathology. The HIV protein Tat has been shown to induce ER stress in
astrocytes leading to GFAP-dependent neurotoxicity (Fan & He, 2016). Inflammation and expression of the human endogenous retrovirus protein, syncytin-1, promote ER stress in astrocytes in MS [150]. This study demonstrated that ER stress proteins were upregulated in MS patient brains, along with the human endogenous retrovirus protein (HERV) syncytin-1. Syncytin -1 induces splicing of XBP1 and leads to downstream inflammation. These mechanisms were confirmed by transfecting primary human fetal astrocytes with syncinctin-1. This induced splicing of XBP1, indicating that the IRE1 pathway is activated. Further, Nos2 was concomitantly upregulated and contributed to oligodendrocyte toxicity in the EAE model. Together, this suggests that IRE1 signaling is stimulated by the HERV protein syncytin-1 to initiate a sXBP1-dependent nitric oxide and neuroinflammatory response.

Additionally, Zika virus has been shown to activate the UPR in astrocytes. ZIKV infection of astrocytes caused an over expression of UPR-related genes BiP, XBP1, CHOP, and growth arrest and DNA damage-inducible protein (GADD) 34. Under these conditions, cell viability was decreased, RNA metabolism genes and micro-RNAs were downregulated, however, astrocyte-derived soluble factors glial cell line-derived neurotrophic factor (GDNF) and neuronal growth factor (NGF) were upregulated, highlighting that some molecules were still being translated under ER stress conditions [151]. However, these results are associated with UPR activation, and direct evidence for the non-cell autonomous action of UPR signaling in astrocytes still requires investigation. These results lay the groundwork for further studies examining the role of ZIKV and other neurotrophic viral infections in astrocytes.
In summary, astrocytes play a critical role at directing the overall CNS environment due to their close physical and trophic connection to other CNS cells as well as blood vessels. UPR activation is emerging as an important process by which astrocytes influence the survival, activation and function of other CNS resident and infiltrating cells.

Conclusions

Astrocytes, the most populous glial cell, mount potent inflammatory responses and have been shown to aberrantly contribute to neurological pathology. Critically, limited therapies and cures exist for most neurological diseases. Therefore, it is logical to assume that identifying novel therapeutic targets to regulate disease-associated signaling cascades is vital for the design of effective treatments. Overall, due to the well-established association of protein aggregation and accumulation in neurological disorders and the recent advances in astrocyte biology, understanding how astrocytes experiencing ER stress influence the CNS environment may be a critical link in understanding signaling pathways that contribute to neurological dysfunction. Our overarching hypothesis is that fine-tuning astrocyte mediated responses to cell stress may be beneficial in ameliorating aberrant inflammation that worsens neurodegeneration. Here, we have studied the mechanisms that link endoplasmic reticulum (ER) stress and inflammation to identify novel therapeutic targets for neurodegenerative diseases.

The UPR has been studied in many disease states and cell types, however, the cell-specific roles of the UPR in the CNS is a relatively emerging field. ER stress has been primarily characterized in the CNS focusing on neurons and oligodendrocytes [130, 152]. As more studies are performed, it is evident that UPR activation has diverse roles in each CNS cell type. For example, EAE is ameliorated by PERK activation in oligodendrocytes,
but PERK knockdown in astrocytes had no effect on the development of EAE [115, 153]. Although the UPR is known to activate pathways that have been associated with apoptosis, there is little evidence that UPR signaling in astrocytes induces cell death. Instead, UPR-activated astrocytes are posited in a unique position to contribute to the inflammatory environment of the CNS because astrocytes are the most populous glial cell, can be neurotoxic, and direct CNS inflammation by promoting microglial activation and leukocyte trafficking [154-156]. Inflammatory and reactive astrocytes are attributed to neurotoxicity in many disease models. Understanding how astrocytes are fine-tuned to produce these neurotoxic responses is of vital importance; neuronal loss cannot be overcome and leads to motor and cognitive decline.

In this work, we identify that ER stress and astrocyte-driven gene expression changes are linked by the protein JAK1. In response to ER stress, the PERK signaling pathway of the UPR can drive JAK-STAT dependent inflammatory signaling. Further, we have identified that JAK1 can utilize alternative transcription factors to initiate a distinct set of gene expression. ATF4 is selectively expressed in response to cellular stressors, including ER stress. We have identified that JAK1 and ATF4 interact, and that JAK1 knockdown inhibits ATF4 binding to promoter sequences, suggesting that JAK1 and ATF4 cooperatively drive gene expression.

Bibliography


Janus Kinase 1 Is Required for Transcriptional Reprograming of Murine Astrocytes in Response to Endoplasmic Reticulum Stress
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Abstract

Neurodegenerative diseases are associated with the accumulation of misfolded proteins in the endoplasmic reticulum (ER), leading to ER stress. To adapt, cells initiate the unfolded protein response (UPR). However, severe or unresolved UPR activation leads to cell death and inflammation. The UPR is initiated, in part, by the trans-ER membrane kinase PKR-like ER kinase (PERK). Recent evidence indicates ER stress and inflammation are linked, and we have shown that this involves PERK-dependent signaling via Janus Kinase (JAK) 1. This signaling provokes the production of soluble inflammatory mediators such as interleukin-6 (IL-6) and chemokine C-C motif ligand 2 (CCL2). We, therefore, hypothesized that JAK1 may control widespread transcriptional changes in response to ER stress. Here, using RNA sequencing of primary murine astrocytes, we demonstrate that JAK1 regulates approximately 10% of ER stress-induced gene expression and is required for a subset of PERK-dependent genes. Additionally, ER stress synergizes with tumor necrosis factor-α (TNF-α) to drive inflammatory gene expression in a JAK1-dependent fashion. We identified that JAK1 contributes to activating transcription factor (ATF) 4-dependent gene expression, including expression of the genes growth arrest and DNA damage (GADD) 45α and tribbles (TRIB) 3 that have not previously been associated with JAK signaling. While these genes are JAK1 dependent in response to ER stress, expression of GADD45α and TRIB3 are not induced by the JAK1-activating cytokine, oncostatin M (OSM). Transcriptomic analysis revealed that JAK1 drives distinct transcriptional programs in response to OSM stimulation versus ER stress. Interestingly, JAK1-dependent genes induced by ER stress in an
ATF4-dependent mechanism were unaffected by small molecule inhibition of JAK1, suggesting that, in response to UPR activation, JAK1 initiates gene expression using noncanonical mechanisms. Overall, we have identified that JAK1 is a major regulator of ER stress-induced gene expression.

Introduction
Prevalent diseases including neurodegenerative disorders, cancer, obesity and diabetes are associated with the accumulation of misfolded proteins in the endoplasmic reticulum (ER) [1]. Under normal conditions, molecular chaperones within the ER fold proteins, an essential step in the maturation of proteins destined for membranes or secretion. Misfolded proteins can result from a multitude of origins including inflammation, reactive oxygen species (ROS), or genetic mutations [2, 3]. Misfolding can ultimately result in loss of protein function and deleterious effects to the cell. In eukaryotes, the ER has an intricate monitoring system to ensure each protein is properly folded before being exported to its ultimate destination. If a protein is misfolded, mechanisms are in place to re-fold or degrade the aberrant polypeptide. However, when misfolded proteins overwhelm these mechanisms, this results in a disruption of homeostasis, referred to as ER-stress and activation of the unfolded protein response (UPR). The UPR is a highly conserved stress response tasked with restoring homeostasis or initiating apoptosis [4].

The UPR is mediated by three ER transmembrane sensor proteins: inositol requiring enzyme-1 (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor (ATF) 6. Under unstressed conditions, the molecular chaperone glucose regulated protein (GRP78) interacts with and maintains each of these proteins in an inactive conformation. When unfolded proteins accumulate, GRP78 is recruited away from these ER transmembrane proteins, promoting oligomerization and conformational changes in PERK and IRE1, which then likely interact with misfolded polypeptides initiating enzymatic activity [2, 4-7]. Once active, PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) to reduce protein translation and alleviate
the influx of nascent polypeptides into the ER [8]. Concomitantly, the UPR promotes the activation and/or expression of transcription factors such as ATF6, X-box binding protein 1 (XBP1) and ATF4 to drive the expression of ER chaperones to restore function [2]. ER stress has been widely studied in neurons because it is often associated with neuronal death in models of neurological diseases. Increasing evidence indicates ER stress also affects astrocytes. Astrocytes are the most populous glial cell and respond to external stimuli by promoting production of inflammatory cytokines [9]. In previous studies, humanized ApoE4 and amyloid-β drive ER stress and astrocyte dysfunction. α-synuclein and mutant LRRK2, associated with PD, work together to drive ER stress and Ca\(^{2+}\) disruption in astrocytes [10]. Inflammation and expression of the human endogenous retrovirus protein, syncytin-1, promote ER stress in astrocytes in MS [11]. Consistent with this, our previous work has indicated that neuroinflammation and STAT3 phosphorylation concomitant with ER stress in the MS mouse model of experimental autoimmune encephalomyelitis (EAE) [12]. Additionally, we have recently shown that ER stress is transmissible between cells of the CNS. We showed that neurons experiencing ER stress can alert neighboring cells, including astrocytes, by inducing an ER stress response in those cells [13]. Together, these studies suggest that astrocytes are impacted by ER stress in neurological diseases and may contribute to the associated pathologies.

In addition, the UPR stimulates an inflammatory response to possibly alert neighboring cells to an impending danger and to recruit immune cells [3, 13, 14]. However, this inflammation may contribute to the pathology of diseases involving ER stress [3, 15, 16]. The UPR has been linked to primary signaling molecules contributing to inflammation such as nuclear factor κB (NF-κB), the mitogen activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK), and simulates an acute phase response [15]. The UPR has also been shown to promote the production of cytokines and chemokines, including the pleiotropic cytokine, IL-6 [12, 17, 18]. Typically, IL-6 exerts its action by binding to its cell membrane receptor and activating a Janus kinase (JAK) and
signal transducer and activator of transcription (STAT) cascade to modulate gene expression [19]. We have previously shown a PERK-dependent mechanism of JAK1 activation leading to IL-6 production, uncovering another connection between ER-stress and inflammation [12].

The JAK-STAT pathway is an integral signal transduction pathway in modulating inflammatory gene expression and immunological function [20]. Loss of function studies have shown that the 4 JAKs (JAK1, JAK2, JAK3 and Tyk2) and 7 STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) are essential for lymphoid development, T and B cell development, erythropoiesis, defense against viral and bacterial infections, as well as neural function [21, 22]. While the majority of effects elicited by JAK activation are attributed to the activation of STAT proteins, JAKs also integrate with other signaling pathways including phosphatidylinositol 3-kinase (PI3K) signaling and the MAPK pathway [23]. Furthermore, cell stressors such as hypoxia, reactive oxygen/nitrogen species, and ER stress activate JAK signaling through receptor-dependent and independent mechanisms [12, 24-26].

It is well established that JAK1 is required for responsiveness to interferons, the IL-6 family of cytokines and IL-2, among others, as well as various forms of cell damage. Considering the integral relationship between ER stress and inflammation, we hypothesized that JAK1 may also be a critical signaling node controlling transcriptional changes in response to ER stress. Consistent with this hypothesis, we have identified that JAK1 regulates approximately 10% of the genes induced by ER stress. In addition to its traditional role downstream of cytokine receptors, JAK1 modulates expression of a distinct subset of genes in response to ER stress.

Materials and Methods
Mice and Primary Cell Preparations

C57Bl/6, PERK floxed and CAGG-CreERTM mice were purchased from The Jackson Laboratory and bred and housed in the animal facility at West Virginia University under the care
of the animal resources program. Primary murine astrocytes were prepared as previously described [27]. Astrocytes were cultured in Dulbecco’s modified eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 16 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES; Gibco), 1X non-essential amino acids (Corning), 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco), and 50 µg/ml gentamicin (Lonza). Astrocytes were separated from microglia by shaking at 200 RPM for 1.5 h. Cells were then trypsinized (0.05%, Gibco) for 5 min at 37°C, collected in media and centrifuged for 5 min at 300g. Cells were then seeded into multi-well plates and stimulated after 48 – 72 h.

**Antibodies and Reagents**

Primary antibodies used were: Anti JAK1 (3344), JAK2 (3230), P-εIF2α (3398), εIF2α (5324), P-STAT3 (9145), STAT3 (12640), Lysine-specific histone demethylase 1 (LSD1) (2184), ATF4 (11815) from Cell Signaling; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (MAB374) from Millipore; JAK1 (610231) from BD Transduction Laboratories; transferrin receptor (TfR) H68.4 (13-6800) from Thermo Fisher; and CCAAT-enhancer-binding protein homologous protein (CHOP) (sc-7351) from Santa Cruz. Cytokines used were: oncostatin (OSM) M and tumor necrosis factor (TNF) – α from R&D systems. Thapsigargin and tunicamycin used were from EMD Millipore and AZD1480 was supplied from Santa Cruz Biotechnology. Puromycin was supplied from Fisher Scientific.

**Immunoblotting**

Cells were washed twice with phosphate buffered saline (PBS) and lysed with lysis buffer (20 mM 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 7.5; 150 mM NaCl; 2 mM Ethylenediaminetetraacetic acid (EDTA); 2 mM Ethylene-bis(oxyethylenenitrilo)tetaacetic acid (EGTA); 0.5% Nonidet P-40 (NP-40)) containing 1X phosphatase and protease inhibitor cocktail.
(Pierce) as previously described [28]. Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Equal amounts of protein from each sample were solubilized in Laemmli sample buffer and heated for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the membranes were blocked in 5% milk/tris buffered saline with tween-20 (TBST), followed by an overnight incubation at 4°C with primary Ab diluted in 5% bovine serum albumin (BSA) or milk in TBST, according to the manufacturer’s recommendation. Horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse (1:4000 dilution) secondary Ab (Jackson Immuno Research) were incubated for 1 h at room temperature, followed by detection with enhanced chemiluminescence. Membranes were imaged digitally using a ChemiDoc Touch (Biorad). Immunoblot images were analyzed using ImageLab software (BioRad). When applicable, quantification of immunoblot images were quantified by obtaining volumetric measurements in ImageLab.

qRT-PCR
RNA was isolated using 1 ml of TRizol (Sigma-Aldrich) according to the manufacturer’s instructions. RNA was quantified using a NanoDrop (NanoDrop Technologies), and 1 µg of RNA was used for cDNA synthesis using Moloney Murine Leukemia Virus reverse transcriptase (Promega). The cDNA was analyzed by quantitative PCR performed using probe-based gene expression assays (IDT or Themo Fisher) in a Stratagene MX3005P or Applied Biosystems Quant Studio 3. Reactions were carried out in 20 µL and analyzed using the ΔΔCt method.

Protein translation
Protein synthesis was estimated by measuring puromycin incorporation using a modified method based on [29]. Briefly, cells were incubated with puromycin (5 µg/ml) for 5 min followed by washing in cold PBS and lysed with lysis buffer. One microgram of protein was spotted in duplicate or triplicate on nitrocellulose and allowed to dry. The membrane was then
immunoblotted (dot blot) using an anti-puromycin antibody (Millipore) at 1:5000 dilution in 5% milk/TBST. Dots were quantified using ImageLab software (Biorad).

**RNA sequencing and Bioinformatics**

RNA was quantified by Qubit fluorometer. RNA quality was assessed by Bioanalyzer Nano chip. All RIN values were greater than 8. Libraries were built using 750 ng RNA and KAPA stranded mRNA kit as per manufacturers protocol. The libraries were then quantified with the Qubit and run on the Bioanalyzer using a High Sensitivity DNA chip to determine average size. They were then pooled at an equimolar ratio and sequenced (paired end (PE) 100bp) on the HiSeq 2500 at Marshall University. RNA seq was also performed externally by Genewiz. Analysis was performed using CLC Biomedical Genomics Workbench and Ingenuity Pathway Analysis (Qiagen). Non-coding or non-annotated genes were not included in analysis. Gene ontology was analyzed using ShinyGO v0.60 [30]. Full data sets are available at NCBI Sequence Read Archive (SRA) # SRP129889.

**Immunoprecipitation**

Protein lysates were collected in lysis buffer. Anti-rabbit Dynabeads (15 µl per sample, Invitrogen) were coated with 1 µg of α-ATF4 antibody overnight. Beads with the α-ATF4 antibody were washed with PBS with 0.1% BSA 3 times. Protein (750 µg) was then incubated with the Dynabeads for 3 h and washed 2 times with 0.5% NP-40 lysis buffer and 2 times with PBS with 0.1% BSA. Protein was eluted by incubating the Dynabeads in 1X Laemelli Buffer at 95°C for 5 minutes.

**Cellular Fractionation**

Nuclear and cytoplasmic fractions were obtained by collecting cells in 0.05% NP-40 buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, 0.05% NP-40 with 1X protease and phosphatase inhibitors) and centrifuged at 2700 x g for 10 min at 4 °C. Supernatants were
collected and centrifuged at 17,000 x g for 15 min at 4 °C to obtain cytoplasmic fractions. The pellet containing nuclei was washed twice in 200 µl of wash buffer (5 mM HEPES, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 250 mM sucrose, 0.1% BSA, with 1X protease and phosphatase inhibitors). The pellet was then resuspended in wash buffer and layered on top of 1 ml of 1 M sucrose (with protease and phosphatase inhibitors), and centrifuged at 2700 x g for 10 min at 4 °C. The nuclear pellet was washed in the 0.05% NP-40 lysis buffer. The nuclear proteins were extracted by resuspending the pellet in nuclear extraction buffer (20 mM HEPES pH 7.4, 1.5 mM MgCl₂, 0.2 mM EDTA, 10 mM β-glycerophosphate, 300 mM NaCl with 1X protease and phosphatase inhibitor) and incubating on ice for 30 min. The nuclear fractions were subsequently centrifuged at 17,000 g for 15 min at 4°C. The supernatant was saved as nuclear extract.

**siRNA Transfections**

Primary astrocytes were transfected with the indicated small interfering (si) RNA (50 pmols per 35 mm well) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol. Cells were used for experiments 48–72 h after transfection. The siRNAs used in this study include Control (non-targeting) siRNA, JAK1 siRNA #1 (sequence: GCUCGAAACCGAAUCAUCA), JAK1 siRNA #2 (sequence: CACUGAUUGUCCACAAUATT), JAK2 siRNA (sequence GGACUAUAUGUGCUACGAUTT), ATF4 siRNA #1 (sequence: GCUGCUUACUAUACUCUAATT), ATF4 siRNA #2 (sequence: GCCUAGGGUCUUAGUGATT).

**ELISA**

Culture supernatants (100 µL, undiluted) were collected and assayed by ELISA for murine IL-6 (Biolegend) according to the manufacturer’s protocol.

**Statistics**
Data are the means of at least three independent experiments. Significance, indicated by * where $p < 0.05$, was determined by one-way analysis of variance (ANOVA) with post hoc analysis or by Student’s T test. RNA-seq significance was determined using Empirical Analysis of Differential Gene Expression (EDGE) test [31, 32].
Results

In this study, we have used primary murine astrocytes as a model to study the role of JAK1 in the ER stress response. Astrocytes are resistant to the cytotoxic effects of prototypical ER stress inducing agents but respond with a robust UPR and inflammatory response [12], making them ideal to study signaling and gene expression without overt cell death. We have previously shown that ER stress-induced IL-6 expression requires PERK and JAK1 in astrocytes [12]. To extend these findings, we tested if JAK2 could also regulate IL-6. We focused on testing JAK1 and JAK2 because other JAKs (JAK3 and Tyk2) are lowly expressed in astrocytes [33]. As shown in Figure 2.1A, ER stress induced by thapsigargin (thaps) drives production of IL-6 and siRNA-mediated knockdown of JAK1 abrogated ER stress-induced IL-6 production, while JAK2 knockdown had no effect in comparison to the control (non-targeting) siRNA. Other JAK proteins, Tyk2 and JAK3, are lowly expressed in astrocytes, suggesting they do not play an appreciable role in ER stress-induced signaling [33]. Next, to understand how JAK1 affects ER stress-induced signaling, we tested if JAK1 or JAK2 could modulate canonical PERK signaling. JAK1 or JAK2 was knocked down in astrocytes, and the cells were exposed to thaps for 4 h to induce ER stress. Knockdown of JAK1 and JAK2 was highly effective and selective, but this had no significant impact on PERK-dependent eIF2α phosphorylation or CHOP expression (Figure 2.1B). Because JAK2 had no effect on driving thaps-induced IL-6 production and did not affect the UPR signaling pathway, we chose to focus our studies solely on JAK1. Moreover, eIF2α phosphorylation leads to translational repression, and this is also unaffected by JAK1 knockdown (Figure 2.1C). JAK/STAT signaling drives transcriptional changes, therefore, we tested if JAK1 could regulate expression of UPR
signal transducers and ER chaperones. As shown in Figure 2.1D, ER stress increased the expression of PERK, ATF6, and the oxidoreductase ER oxidoreductin-like beta (Ero1lb) dependent on JAK1. These data indicate that JAK1 activation is dispensable for

**Figure 2.1 JAK1 is required to drive ER stress-induced IL-6 expression and does not affect canonical PERK signaling.**

A. Primary astrocytes were transfected with control (CTL), JAK1, or JAK2 siRNA for 48 h and then treated with thapsigargin (thaps) (1 µM) for 24 h and analyzed by immunoblot and ELISA. B. Astrocytes were transfected as in (A) followed by thaps (1 µM) treatment for 4 h and analyzed by immunoblot. C. Astrocytes were transfected with CTL or JAK1 siRNA and treated with thaps (1 µM) for 90 min. In the last 5 minutes of treatment, puromycin (5 µg/ml) was added to cultures. Cell lysates were analyzed by dot blot using anti-puromycin antibody and quantified. UT = Untreated. D. Astrocytes were transfected as in (A), treated with thaps (1 µM) for 4 h then analyzed by RT-qPCR. UT = Untreated. E. Primary astrocytes were transfected with one of two distinct JAK1 siRNAs and treated with thaps (1 µM), TNF-α (5 ng/ml), or both thaps and TNF-α for 4 h and analyzed by RT-qPCR. N = 3. *p ≤ 0.05. Data are represented as means ± standard deviation.
PERK dependent signaling that leads to translational repression, and imply a unique role for JAK1 in the regulation of the ER stress response. UPR signaling has also been shown...
to augment already ongoing inflammatory responses, including NF-κB signaling [34-36]. To determine if JAK1 plays a role in mediating synergy between inflammatory and UPR signaling, we treated astrocytes with thaps and the proinflammatory cytokine, tumor necrosis factor (TNF) – α, which engages NF-κB signaling [37]. Here, we also chose a second JAK1-targeting siRNA to corroborate our findings that this response is specific to JAK1. We show that inflammatory gene expression (IL-6, CCL2, and CCL20) responds in a synergistic manner to ER stress and TNF-α. Further, this synergy is JAK1 dependent, highlighting the role of ER stress in influencing astrocyte-dependent inflammatory responses. (Figure 2.1E). We have defined synergy here, as ER stress having a more than additive effect on the TNF-α stimulated inflammatory response. Overall, these data suggest that JAK1 drives transcriptional regulation during UPR activation in astrocytes.

These findings, with our previous work showing that JAK1 regulates IL-6, CCL2 and CCL20 expression, led us to hypothesize that JAK1 has an important role in regulating the transcriptional response to ER stress [12]. To test this globally, we used RNA sequencing (RNA-seq). Astrocytes were transfected with control or JAK1 siRNA followed by treatment with thaps for 4 h. Global changes in the transcriptome were then analyzed by RNA-seq. As shown in the volcano plot in Figure 2.2A, ER stress induces transcriptional reprogramming including upregulation of the prototypical UPR genes CHOP (ddit3), ATF4 and XBP1 (Figure 2.S1). When JAK1 was knocked down in ER stressed cells, this appeared to change the expression of many genes when compared to thaps alone based on t-test p-values (Figure 2.2B). These data suggested that both ER stress and JAK1 had a significant impact on the overall gene expression profile. To test this, we used principal component analysis (PCA) that revealed that each of the
treatment groups had a unique expression profile, indicating that JAK1 regulates the overall response to ER stress (Figure 2.2C). We next investigated the global impact of JAK1 on ER stress-induced gene expression using stringent statistical analysis. We identified all of the genes significantly (EDGE test p < 0.05) upregulated by 1.5 fold or greater in response to ER stress. We then identified all the ER stress-induced genes that are JAK1 dependent. These were genes significantly upregulated by ER stress and significantly reduced by 1.5 fold or greater by JAK1 knockdown. Overall, more than 450 genes were increased by ER stress and approximately 10% of these genes were regulated by JAK1 (Figure 2.2D). These data indicate that JAK1 has a significant (p = 2.01 x 10^{-14} by hypergeometric probability \[38\]) and unexpectedly large role in the regulation of ER stress-induced gene expression. To examine the most strongly induced genes, we identified the top 50 ER stress-induced genes (Figure 2.S1). This list included well-established genes known to be robustly induced by ER stress including tribbles 3 (TRIB3), CHOP, and ATF3 [39]. We then compared this gene set to the ER stress induced genes that are JAK1-dependent (Figure 2.S1). This identified CCL20, which we previously identified as JAK1-dependent as well as many genes not previously associated with JAK1 signaling. By comparing these two analyses, we identified that 15 (30%) of the top 50 ER stress-induced genes are JAK1 dependent (Figure 2.2D). This is a highly significant overlap (p = 6.14 x 10^{-21} by hypergeometric probability). These included adrenomedullin 2 (Adm2), CCL20, Prostaglandin-endoperoxide synthase 2 (Ptgs2), Nuclear Protein 1 (Nupr1) and Regulator of G Protein Signaling (RGS) 16 among others, which have previously been shown to be induced by ER stress [12, 40-43]. To identify the general pathways regulated by JAK1, we used Ingenuity Pathway Analysis (IPA). As
Figure 2.3 JAK1 is required for full engagement of PERK-dependent gene expression.

A and B. Astrocytes were isolated from PERKfl/fl mice without or with tamoxifen-inducible cre (CAGG-CreERTM). Cells were treated with tamoxifen for 48 h to delete PERK fold by thaps (1 μM) treatment for 4 h and RT-qPCR analysis. Astrocytes were transfected with JAK1 siRNA #1 for 48 h, treated with thaps (1 μM) for 4 h, and analyzed by RT-qPCR. C. Astrocytes were transfected with a second JAK1 siRNA, and ER stress was induced by treating with thaps (1 μM) or tunicamycin (tunic) (5 μM) for 4 h, followed by RT-qPCR analysis. Data are represented as means ± standard deviation. N = 3. *p ≤ 0.05.

shown in Figure 2.2E, growth arrest and DNA damage (GADD) 45α signaling and other stress-responsive pathways, including the UPR, were significantly regulated by JAK1.
These data indicate that JAK1 has a central role in the regulation of transcriptional reprogramming induced by ER stress.

We have previously shown PERK-dependent activation of JAK1 [12]. Therefore, we expected that JAK1 would be important for PERK-dependent transcriptional responses. To test this, we selected several genes including IL-6 and CCL2 that we know to be PERK and JAK1 dependent. We also selected, based on the RNA-seq data, the DNA damage induced protein GADD45α and the pseudokinase TRIB3. As shown in Figure 2.3A, ER stress induces the expression of IL6, CCL2, GADD45α, and TRIB3. Genetic deletion of PERK significantly reduced ER stress induced expression of each of these genes, indicating they are PERK dependent. JAK1 knockdown also significantly suppressed each of these genes, indicating they are JAK1 dependent (Figure 3A). Importantly, not all PERK-dependent gene expression relies on JAK1. As shown in Figure 2.3B, ER stress-induced expression of ATF4, CHOP and the chemokine C-X-C motif ligand 1 (CXCL1) are PERK dependent but are unaffected by JAK1 knockdown. These data demonstrate that JAK1 is essential for full engagement of PERK-dependent gene expression in response to ER stress. These data suggest, for the first time, that GADD45α and TRIB3 are JAK1 dependent. GADD45α and TRIB3 are known to be induced by ER stress, however, have not been previously associated with JAK-STAT signaling. To confirm that ER stress upregulates GADD45α and TRIB3 expression in a JAK1-dependent manner, we utilized a different ER stress-inducing agent (tunicamycin) and a second distinct JAK1 siRNA. In Figure 2.3C, we corroborated that JAK1 knockdown reduces ER stress-induced expression of IL-6, GADD45α, and TRIB3, providing further evidence that these genes are JAK1-dependent.
Next, we tested if a JAK1-activating cytokine could also drive GADD45α and TRIB3 expression. We used the IL-6 family cytokine, oncostatin M (OSM), which signals through JAK1-STAT3-dependent mechanisms in astrocytes [33]. JAK1 siRNA knockdown in astrocytes led to an abrogation of OSM-mediated phosphorylation of STAT3, confirming the requirement of JAK1 (Figure 2.4A). Stimulation of astrocytes with OSM induced a concentration-dependent increase of IL-6, as expected. However, OSM had no effect on GADD45α or TRIB3 expression (Figure 2.4B). Next, we took a transcriptome-wide approach to compare the set of JAK1-dependent genes in response to OSM versus ER stress (Figure 2.2B). Here, we used RNA-seq to identify significantly induced (EDGE test p value < 0.05) genes by OSM. These genes had a fold change of 1.5 or greater when compared to untreated samples. Next, we identified genes that were significantly downregulated (EDGE test p value < 0.05, fold change < -1.5) with JAK1 knockdown. These criteria allowed us to identify the 183 OSM-induced JAK1-dependent genes. We then compared the genes that are induced by ER stress and OSM in a JAK1-dependent fashion. This revealed strikingly disparate gene expression profiles, with only four genes in common (Figure 2.4C). The genes that are JAK1 dependent in response to both OSM and ER stress are pentraxin 3 (Ptx3), nuclear protein 1 (Nupr1), Regulator of G Protein Signaling (Rgs) 16, and chemokine (C-C motif) ligand (CCL) 7. These data suggest that, in astrocytes, cytokines and ER stress induce distinct JAK1-dependent gene expression changes. Next, we performed gene ontology analysis which assigns genes to groups based on their molecular and functional characteristics previously defined in the literature. Gene ontology showed JAK1 regulates gene expression corresponding to different biological process depending on the stimulus (OSM or ER stress). (Figure 2.4D).
OSM-induced JAK1 dependent genes generally induce immune and inflammatory related genes. However, ER stress-induced JAK1 dependent genes are related to cell death and apoptosis. This highlights that ER stress engages JAK1 to control a distinct transcriptional

Figure 2.4 ER stress induces a unique JAK1-dependent gene expression profile that is distinct from OSM-induced JAK1-dependent gene expression.

A. Primary astrocytes were transfected with Control (CTL) or one of two distinct JAK1 siRNAs for 48 h and stimulated with OSM for 30 minutes followed by immunoblotting. B. Astrocytes were stimulated with OSM at the indicated concentrations for 4 h. Gene expression was measured by RT-qPCR. Data are represented as means ± standard deviation. N = 3. *p ≤ 0.05. C. Astrocytes were transfected with CTL or JAK1 siRNA#2 and treated with OSM (2.5 ng/ml) for 4 h. Gene expression was then measured by RNAseq to identify the JAK1-dependent genes. Venn diagram of JAK1 dependent genes in response to ER stress or OSM. D. Gene ontology analysis of the genes represented in (C).
profile in comparison to the well-established role of JAK1 downstream of cytokine receptors that we have modeled using OSM stimulation.

Figure 2.5 JAK1 and ATF4 cooperatively regulate a subset of ER stress-induced genes.

A. List of genes that are both JAK1 and ATF4 dependent in response to ER stress as determined by RNA-seq and reported by others. B. Astrocytes were transfected with control (CTL) or ATF4-targeting siRNA for 48 h and treated with thapsigargin for 4 h. Indicated gene expression was analyzed by RT-qPCR. UT = untreated. C. Astrocytes were transfected with CLT or one of two JAK1 siRNAs and immunoblotted for ATF4. Immunoblots were quantified and normalized to GAPDH expression. D. Astrocytes were transfected with CTL or JAK1 siRNA for 48 h and then treated with thapsigargin for 4 h. Cytosolic and nuclear fractions were isolated and analyzed by immunoblot. E. Primary astrocytes were treated with thaps (1 µM) for 4 h. Protein lysates reserved or immunoprecipitated with α-ATF4 antibody before immunoblotting. F. Quantification of JAK1 co-immunoprecipitated with ATF4 as shown in the top panel of (E). Data are represented as means ± standard deviation. N = 3. *p ≤ 0.05.
Previous work has shown that GADD45α and TRIB3 are ATF4 dependent [44, 45]. ATF4 is a transcription factor known to be induced by cell stress, including ER stress. Expression of ATF4 is initiated downstream of PERK activation, requiring the phosphorylation of eIF2α [46]. Further, we determined that many genes are regulated by both JAK1 and ATF4 in response to ER stress and identified that 12 (out of 56) ER stress-induced JAK1-dependent genes have been previously reported as ATF4-dependent [40, 41, 47-51] (Figure 2.5A). To confirm that GADD45α and TRIB3 are ATF4-dependent, we used siRNA to knockdown ATF4. As shown in Figure 2.5B, ATF4 knockdown abrogated ER stress-induced GADD45α and TRIB3, but failed to reduce expression of IL-6, consistent with our previous work [52]. These findings were confirmed using a second, distinct siRNA targeting ATF4 (Figure 2.5C). This suggests that JAK1 and ATF4 may cooperatively regulate gene expression. To determine if JAK1 regulated protein expression of ATF4, we quantified ATF4 immunoblots of thaps-treated astrocytes with or without JAK1 knockdown. JAK1 knockdown had no significant effect on ATF4 protein levels in response to ER stress (Figure 2.5C). Previously, proteins related to JAK1 and ATF4, JAK2, and CREB, respectively, have been shown to interact and translocate to the nucleus [53]. Although JAK1 knockdown does not affect ATF4 expression, we next tested if JAK1 expression is required for nuclear translocation of ATF4. We found that ATF4 is expressed in the nucleus in response to thaps treatment independent of JAK1 expression, indicating that JAK1 does not influence the expression or nuclear translocation of ATF4 (Figure 2.5D). Although JAK1 appeared in the nuclear fraction under these conditions, analyzing cytosolic, nuclear, and plasma membrane markers indicated that the nuclear fraction also contained plasma membrane (detected by the presence of transferrin
receptor, **Figure 2.S3**). JAK1 is largely associated with the plasma membrane [54]. To test if JAK1 and ATF4 physically interact, protein lysates from thaps-treated astrocytes

![Image of Western Blot](image)

**Figure 2.6 A subset of ER stress-induced JAK1-dependent signaling is insensitive to kinase inhibition of JAK1.**

A. Astrocytes were pretreated with JAK1/2 kinase inhibitor AZD1480 (1 μM) for 1 h before 0.5 h treatment with oncostatin M (OSM). Protein lysates were collected and analyzed by immunoblot. B. Astrocytes were pretreated with AZD1480 for one hour and then treated with thaps for 4 h. Gene expression was analyzed by RT-qPCR. Data are represented as means ± standard deviation. N = 3. *p < 0.05.

were immunoprecipitated using anti-ATF4 antibody and immunoblotted for ATF4 and JAK1. Here, we found that JAK1 coimmunoprecipitates with ATF4, suggesting a physical
interaction between these two molecules (Figure 2.5E & 2.5F). Altogether, these data suggest that JAK1 and ATF4 cooperatively regulate ER stress-induced gene expressions.

The role of JAK1 to direct transcription factor activity in response to cytokines and growth factors is well established to rely on tyrosine phosphorylation. JAKs phosphorylate STATs to induce dimerization and translocation to the nucleus to initiate gene expression [55]. Because we have shown that JAK1 and ATF4 regulate common genes and coimmunoprecipitate, we hypothesize that ATF4 could be an alternative transcription factor that JAK1 can phosphorylate to alter activity in response to UPR activation. To determine if the kinase activity of JAK1 is necessary to promote ER stress-induced ATF4-dependent gene expression, astrocytes were treated with the JAK1/2 kinase inhibitor AZD1480 and thaps. As shown in Figure 2.6A, AZD1480 effectively abrogates OSM-induced phosphorylation of STAT3. Expression of known JAK1/STAT3-dependent genes IL-6 and CCL2 were increased by thaps and kinase inhibition of JAK1 attenuated expression of these genes (Figure 2.6B). However, GADD45α and TRIB3 were not sensitive to JAK1 kinase inhibition (Figure 2.6B). These results imply that JAK1 elicits noncanonical signaling in response to ER stress that may not rely on the kinase activity of JAK1. These data suggest that JAK1, through physical interaction, can influence ATF4-dependent gene expression.

Discussion

In this study, we have shown that JAK1 controls the expression of an unexpectedly large number of genes in response to ER stress. Many of the genes regulated are associated with inflammation, consistent with the critical and well-established role of JAK1
in immune function [19, 20]. We and others have shown that UPR signaling integrates with multiple pathways regulating inflammation [3, 13]. We have established that the PERK-JAK1 axis drives inflammatory gene expression including IL-6 in murine astrocytes and other cell types [12, 52] while IRE1 drives IL-6 through a nucleotide binding oligomerization domain 1/2 (NOD1/2) dependent mechanism in macrophages and in the periphery in vivo [18]. JAK1 also regulated genes involved in the ER stress response including the key signal transducing molecules PERK and ATF6. Further, our previous work has shown that JAK1 interacts with PERK and that PERK is phosphorylated by JAK1. Our current work demonstrates that JAK1 is a critical mediator of PERK-dependent gene expression but does not regulate phosphorylation of eIF2α or subsequent attenuation of protein translation. These data suggest a reciprocal interaction in which PERK drives JAK1 activation, which in turn, drives PERK expression. This work also suggests that PERK-dependent activation of JAK1 and phosphorylation of eIF2α are distinct signaling branches. While PERK appears to initially stimulate independent pathways through JAK1 and eIF2α, we have shown that ER stress-induced IL-6, CCL2, and CCL20 expression require both JAK1 and translational attenuation independent of ATF4 [52]. Further, we have shown that JAK1 mediates synergistic gene expression between ER stress and the proinflammatory cytokine TNF-α. Our findings, here, are summarized in Figure 2.7. This is consistent with other reports that ER stress is able to augment ongoing inflammatory responses [18, 56-58], and for the first time, have shown that JAK1 is integral for this synergy in astrocytes. We have now identified that JAK1 modulates ATF4-dependent gene expression, indicating that JAK1 integrates at multiple points downstream from PERK. In some contexts, ER stress has been reported to inhibit
JAK/STAT signaling [59, 60]. However, additional studies are needed to determine if JAK1 also drives noncanonical gene expression in those cell types and conditions.

Our current work demonstrates that JAK1 is a critical signaling node in response to ER stress in astrocytes. However, we cannot distinguish if the JAK1-dependent effects are immediate to ER stress signaling or result from basal regulation in the expression of critical signaling molecules. The data indicate that JAK1 can drive stimulus-dependent gene expression programs. We have shown that JAK1 dependent genes in response to cytokine (OSM) stimulation is distinct from ER stress driven JAK1-dependent gene expression. We show this JAK1 promoted the expression of genes such as GADD45α.
and TRIB3 in response to ER stress but not following cytokine stimulation. It is currently unknown if this differential JAK1-dependent gene expression program is an adaptive response or part of a maladaptive response to ER stress and potentially other pathogenic stimuli. Moreover, pathway analysis implicated JAK1 in the regulation of several stress-activated pathways not investigated in the present study, such as sirtuin signaling, PI3K/Akt signaling, aryl hydrocarbon receptor signaling, and protein ubiquitination pathways (Figure 2.2E). JAK1 has broad involvement in mediating the biological actions of many cytokines such as the IL-6 family, IL-10 family, and interferons [61-64]. Additionally, JAK1 inhibitors are under investigation for treatment of cancers and autoimmunity [55]. However, this treatment may promote immunosuppression; upper respiratory tract and urinary tract infections were among the most common side effects reported in psoriasis patients using JAK1 inhibitors [65]. Therefore, a complete understanding of the JAK1-dependent mechanisms induced by both cytokines and cellular stress may provide broad insight into the mechanisms that underlie pathology-associated signaling pathways.

The nature of the novel JAK1 signaling activity is currently unknown but, as we have shown, may involve interaction with the stress-inducible transcription factor ATF4. We have shown that JAK1 and ATF4 regulate many of the same genes in response to ER stress and that JAK1 coimmunoprecipitates with ATF4. However, as suggested in Figure 2.6, the JAK1-mediated regulation of ATF4 may not involve the well-characterized kinase activity of JAK1. Although we used a kinase inhibitor of JAK1 that also inhibits kinase activity of JAK2, we do not believe that JAK2 plays an appreciable role in regulated ER stress-induced gene expression (Figure 2.1A). Expanded studies to confirm this
kinase-independent interaction between JAK1 and ATF4 are currently underway. Other potential mechanisms include JAK1 nuclear localization [66] and modulation of gene expression or a structural/adaptor function to facilitate key signaling events such as the activation of other transcription factors, like ATF4. JAK1 contains functional domains including a FERM domain and a pseudokinase domain which may mediate important non-catalytic functions of JAK1 [67]. In models of diffuse large B cell lymphoma, others have recently elucidated that JAK1 has a classical nuclear localization sequence between its FERM and SH2 domains, demonstrating that JAK1 may influence transcriptional changes using various mechanisms that are independent of STAT phosphorylation at the site of cytoplasmic cytokine receptors [66]. Further, a noncanonical role for JAK1 has been described in epigenetic modulation of gene expression. JAK1 has been shown to directly phosphorylate the histone protein, H3, to promote STAT-independent gene expression [68].

While we have revealed an important and previously unknown role for JAK1 in response to ER stress, there are several caveats. First, this work was completed using a single type of cultured cells (primary astrocytes) and high concentrations of pharmacological agents to induce ER stress. It is unknown from these data if JAK1 has a similarly important role in vivo under physiological conditions. These studies are currently underway. Additionally, we focused on measuring gene expression at the mRNA level because of the tools available for whole genome transcriptomics. Considering that most translation is inhibited by ER stress-induced eIF2α phosphorylation [8], it is likely that many of the transcripts we have measured are not translated into proteins. Nonetheless, this transcriptional reprogramming may be important following resolution of
ER stress and resumption of translation. Overall, our data indicate that JAK1 is a central mediator of transcriptional reprogramming during ER stress.
## Table 2-1 Top ER stress induced JAK1 dependent genes.

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Top 50 ER stress induced genes and full list of genes induced by ER stress in a JAK1-dependent fashion.
Figure 2.S2 Confirming that ATF4 is necessary for ER stress-induced gene expression.

Astrocytes were transfected with control (CTL) or ATF4 siRNA #2 and treated with thapsigargin (1 µM) for 4 h. Gene expression was analyzed by RT-qPCR. Data are represented as means +/- standard deviation. N=3. *p < 0.05.

Figure 2.S3: Subcellular markers for cytosolic and nuclear isolation.

Astrocytes were transfected with control (CTL) or JAK1 siRNA #2 and treated with thapsigargin (1 µM) for 4 h. Whole cell lysates, cytoplasmic, and nuclear fractions were isolated and immunoblotted for various subcellular markers. Transferrin receptor (TfR) is a plasma membrane marker. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a cytoplasmic marker. Lysine-specific histone demethylase (LSD) 1 is a nuclear marker.
Bibliography


Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability Statement

The RNA-sequencing datasets for the ER stress studies can be found in the NCBI Sequence Reads Archive #SRP129889 at this location https://www.ncbi.nlm.nih.gov/sra/SRP129889. Any raw data, including the OSM RNA-sequencing datasets, supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.
JAK1 directs ATF4-dependent gene expression

Savannah G. Sims, Claire O. Kisamore, Rylee N. Cisney, Nikki Cannon, Michael Hu, and Gordon P. Meares

Currently unpublished as of September 2021

Abstract

Neurodegenerative diseases are heterogeneous, have poor prognosis and limited treatment options. Many neurological diseases are associated with protein misfolding and inflammation. Accumulated unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen results in ER stress. When ER stress occurs, the cell activates a conserved adaptive response deemed the Unfolded Protein Response (UPR). The UPR is, in part, activated by the protein kinase R (PKR) – like ER kinase (PERK). PERK is activated and phosphorylates the eukaryotic initiation factor (eIF) 2α. Phosphorylated eIF2α selectively attenuates translation to hinder nascent polypeptides entering the ER lumen to reduce the protein folding demand in the ER. However, some proteins are selectively translated under these conditions. This includes activating transcription factor (ATF) 4. Previously, we have linked cellular stress initiated by protein misfolding to astrocyte-driven inflammatory signaling that is dependent on Janus kinase (JAK) 1. We found that ER stress induced noncanonical JAK1-dependent signaling that is distinct from cytokine-induced JAK1-dependent signaling. Further, we demonstrated that JAK1 and ATF4 coimmunoprecipitate, suggesting there is a physical interaction between these two proteins. In the present study, we focused on understanding the mechanisms that drive noncanonical JAK1-dependent signaling. We demonstrate that a portion of ER stress-induced JAK1-dependent gene expression does not rely on STAT transcription factors and can utilize ATF4 as an alternative. Further, JAK1 is required for ATF4-promoter
interactions in response to ER stress and JAK1 is expressed in the nucleus in these conditions. Here, we have described a novel mechanism by which JAK1 controls ER stress-induced signaling in astrocytes.

Introduction

Neurodegenerative diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), and Multiple Sclerosis (MS) are progressive diseases associated with protein misfolding and inflammation [1-3]. It is now accepted that a properly functioning immune system is paramount for normal neurological function, however, during neurodegenerative states, aberrant and chronic inflammation may drive disease pathology. Astrocytes contribute to the overall inflammatory milieu of the CNS. Astrocytes are the most populous glial cell in the CNS and respond to insult or injury by producing chemokines and cytokines including interleukin (IL) - 6, interferon (IFN) -γ, tumor necrosis factor (TNF)-α, IL-1β, chemokines C-C motif (CCL) 2, CCL3, CCL20, among many others [1, 4-7] that can promote activation of brain resident macrophages (microglia) and initiate infiltration of peripheral immune cells to the CNS [8-10]. Many of the previously mentioned cytokines and chemokines that astrocytes produce are dependent on the Janus Kinase (JAK) – Signal Transducer and Activator of Transcription (STAT) pathway controls signaling of many of these cytokines.

JAK-STAT signaling is a ubiquitous inflammatory signaling pathway that controls the function of many cytokines and chemokines. There are four JAKs which are typically associated with the cytoplasmic tail of cytokine receptors and are activated upon ligand binding and oligomerization of the cytokine receptor. There are seven STATs (STAT1,
STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) that are recruited to the phosphorylated cytokine receptors by recognizing phosphotyrosine residues using SH2 domains. STATs are then phosphorylated by JAKs to be activated and hetero- or homo-dimerize. This reveals a nuclear localization sequence (NLS). STATs then translocate to the nucleus where they bind promoter regions to initiate gene expression of a wide variety of inflammatory-related genes.

The critical activation of JAK-STAT signaling is the phosphorylation of JAKs on their activation loop, catalyzed by the dimerization of cytokine receptors which increases their intrinsic kinase activity of JAKs are trans- and auto-phosphorylated and the adjacent cytokine receptors are phosphorylated which initiates downstream signaling and transcriptional changes. However, we have previously shown a specific role for JAK1 signaling in response to endoplasmic reticulum (ER) stress in astrocytes, highlighting that JAK-dependent signaling can be activated by cellular homeostasis disruption in addition to cytokines [11-13]. Like inflammation, UPR activation is ubiquitous amongst neurological diseases and has been proposed to drive pathogenesis [14]. The UPR is, in part, activated by the protein kinase R like endoplasmic reticulum kinase (PERK) [15].

PERK, in response to misfolded proteins accumulate in the ER lumen, phosphorylates the eukaryotic initiation factor (eIF) 2α. The UPR selectively attenuates translation via phosphorylation of serine 51 on eIF2α [16]. Phosphorylation of eIF2α inhibits the binding of methionyl tRNAs to the initiation complex, thereby inhibiting translation [15, 17]. During ER stress, it is advantageous to attenuate translation in order to eliminate nascent polypeptides that would enter the ER, reducing demand for protein
folding. However, some proteins are selectively translated under these conditions. Typically, proteins that are translated while the UPR is active are genes that encode for protein chaperones that aid in protein folding mechanisms to help restore homeostasis by limiting the demand on the ER [17]. Certain genes, like activating transcription factor (ATF) 4 are selectively translated due to the presence of upstream open reading frames (uORF) in the 5’ region of the ATF4 mRNA [18]. The ATF4 mRNA contains two uORFs. Under conditions of phosphorylated eIF2α in response to cellular stress, “leaky scanning” facilitates increased expression of ATF4 by allowing the ribosome to bypass the regulatory uORFs [19]. If cellular homeostasis cannot be reached, the cell will initiate apoptotic mechanisms. Some reports conclude that this is through ATF4-dependent regulation of CHOP [20].

Previous work has shown that, in response to ER stress, PERK can directly bind and activate JAK1. This initiates JAK1-STAT3 dependent activation of inflammatory genes, such as interleukin (IL) – 6 as well as the chemokines chemokine CCL2 and CCL20 [11, 12]. These studies provided the first evidence that UPR signaling can directly initiate inflammatory responses in astrocytes. Other groups have demonstrated that UPR-reactive astrocytes produce soluble inflammatory signaling molecules in a PERK-dependent manner, and that targeting PERK signaling in astrocytes is protective in a model of prion disease [21]. This work provides evidence that the UPR signaling in astrocytes could be a mechanism to selectively attenuate pathogenic inflammation while preserving the normal immune responses.
Previously, we showed that JAK1 is a major driver of transcriptional adaptation to ER stress. Approximately 10% of the ER stress-induced genes in astrocytes were dependent on JAK1 [13]. A subset of these genes had not been previously associated with JAK-STAT signaling. This included genes such as growth arrest and DNA-damage-inducible protein (GADD45) α and tribbles (TRB) 3, which we found to be dependent on both JAK1 and ATF4 [13]. In this study, we hypothesized that astrocytic JAK1 can use additional transcription factors to modulate gene expression without STAT transcription factors in response to ER stress. Here, we have demonstrated that JAK1 can utilize ATF4 as an alternative transcription factor under conditions of ER stress in addition to STATs. Using immunoprecipitation, we identified that JAK1 binds ATF4 at a specific location, and JAK1 is expressed in the nucleus. Further, using ChIP, we determined that JAK1 expression is necessary for ATF4 to bind transcription start sites.

Methods

CHIP

Primary astrocytes were isolated wild type mice and ER stress was induced using 1 µM of thapsigargin for 4 h. Cells were crosslinked and immunoprecipitated using an isotype control (IgG) or anti-ATF4 antibody. DNA was eluted and RT-qPCR was performed using primers designed to target the most-likely ATF4 binding regions of the Tribbles3 promoter, based on previously published ChIP-seq data. DNA samples were performed as described in panel. Reads were aligned to the mouse genome. Redundant reads were removed. To determine DNA binding locations based on enrichment of reads in a single location, peak calling was performed using Model-based Analysis of ChIP-seq.
(MACS) 3 algorithm. ChIP-seq data represented as a profile plot to read density across all transcription start sites in the genome.

Mice and Primary Cell Preparations

C57Bl/6 mice were purchased from The Jackson Laboratory and bred and housed in the animal facility at West Virginia University under the care of the animal resources program. Primary murine astrocytes were prepared as previously described [22]. Astrocytes were cultured in Dulbecco’s modified eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 16 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES; Gibco), 1X non-essential amino acids (Corning), 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco), and 50 µg/ml gentamicin (Lonza). Astrocytes were separated from microglia by shaking at 200 RPM for 1.5 h. Cells were then trypsinized (0.05%, Gibco) for 5 min at 37°C, collected in media and centrifuged for 5 min at 300g. Cells were then seeded into multi-well plates and stimulated after 48 – 72 h.

Antibodies and Reagents

Primary antibodies used were: Anti JAK1 (3344), JAK2 (3230), P-eIF2α (3398), eIF2α (5324), P-STAT3 (9145), STAT3 (12640), Lysine-specific histone demethylase 1 (LSD1) (2184), ATF4 (11815) from Cell Signaling; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (MAB374) from Millipore; JAK1 (610231) from BD Transduction Laboratories; transferrin receptor (TfR) H68.4 (13-6800) from Thermo Fisher; and CCAAT-enhancer-binding protein homologous protein (CHOP) (sc-7351)
from Santa Cruz. Cytokines used were: oncostatin (OSM) M and tumor necrosis factor (TNF) – α from R&D systems. Thapsigargin and tunicamycin used were from EMD Millipore and AZD1480 was supplied from Santa Cruz Biotechnology. Puromycin was supplied from Fisher Scientific.

Immunoblotting

Cells were washed twice with phosphate buffered saline (PBS) and lysed with lysis buffer (20 mM 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 7.5; 150 mM NaCl; 2 mM Ethylenediaminetetraacetic acid (EDTA); 2 mM Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA); 0.5% Nonidet P-40 (NP-40)) containing 1X phosphatase and protease inhibitor cocktail (Pierce) as previously described [23]. Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Equal amounts of protein from each sample were solubilized in Laemmli sample buffer and heated for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the membranes were blocked in 5% milk/tris buffered saline with tween-20 (TBST), followed by an overnight incubation at 4°C with primary Ab diluted in 5% bovine serum albumin (BSA) or milk in TBST, according to the manufacturer’s recommendation. Horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse (1:4000 dilution) secondary Ab (Jackson Immuno Research) were incubated for 1 h at room temperature, followed by detection with enhanced chemiluminescence. Membranes were imaged digitally using a ChemiDoc Touch (Biorad). Immunoblot images were analyzed using ImageLab software (BioRad). When
applicable, quantification of immunoblot images were quantified by obtaining volumetric measurements in ImageLab.

**qRT-PCR**

RNA was isolated using 1 ml of TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions. RNA was quantified using a NanoDrop (NanoDrop Technologies), and 1 µg of RNA was used for cDNA synthesis using Moloney Murine Leukemia Virus reverse transcriptase (Promega). The cDNA was analyzed by quantitative PCR performed using probe-based gene expression assays (IDT or Thermo Fisher) in a Stratagene MX3005P or Applied Biosystems Quant Studio 3. Reactions were carried out in 20 µL and analyzed using the ΔΔCt method.

**ChIP sequencing and Bioinformatics**

Wild type astrocytes were transfected with control or JAK1 siRNA and treated with thapsigargin and analyzed by CHIP-seq to characterize differential regulation of ATF4-dependent transcription in response to JAK1 knockdown. IP and DNA isolation from 1×10^7 astrocytes per condition (N = 2). Library preparation and sequencing was performed by Admera Health BioPharma Services. Reads were aligned to the mouse genome. Redundant reads were removed. To determine DNA binding locations based on enrichment of reads in a single location, peak calling was performed using model-based analysis of ChIP-Seq (MACS) 3 algorithm.

**Immunoprecipitation**
Protein lysates were collected in lysis buffer. Anti-rabbit Dynabeads (15 µl per sample, Invitrogen) were coated with 1 µg of α-ATF4 antibody overnight. Beads with the α-ATF4 antibody were washed with PBS with 0.1% BSA 3 times. Protein (750 µg) was then incubated with the Dynabeads for 3 h and washed 2 times with 0.5% NP-40 lysis buffer and 2 times with PBS with 0.1% BSA. Protein was eluted by incubating the Dynabeads in 1X Laemmelli Buffer at 95°C for 5 minutes.

siRNA Transfections

Primary astrocytes were transfected with the indicated small interfering (si) RNA (50 pmols per 35 mm well) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol. Cells were used for experiments 48–72 h after transfection. The siRNAs used in this study include Control (non-targeting) siRNA, JAK1 siRNA #1 (sequence: GCUCGAACCGAAUCAUCA), JAK1 siRNA #2 (sequence: CACUGAUUGUCCACAAUAUUTT), JAK2 siRNA (sequence: GGACUAUAUGUGCUACGAUTT), ATF4 siRNA #1 (sequence: GCUGCUUACAUUACUCUAATT), ATF4 siRNA #2 (sequence: GCCUAGGUCUCUUAGAUGATT).

Statistics

Data are the means of at least three independent experiments. Significance, indicated by * where p < 0.05, was determined by one-way analysis of variance (ANOVA) with post hoc analysis or by Student’s T test. RNA-seq significance was determined using Empirical Analysis of Differential Gene Expression (EDGE) test [24, 25].
RNA Seq and Gene ontology

RNA was quantified by Qubit fluorometer. RNA quality was assessed by Bioanalyzer Nano chip. All RIN values were greater than 8. Libraries were built using 750 ng RNA and KAPA stranded mRNA kit as per manufacturers protocol. The libraries were then quantified with the Qubit and run on the Bioanalyzer using a High Sensitivity DNA chip to determine average size. They were then pooled at an equimolar ratio and sequenced (paired end (PE) 100bp) on the HiSeq 2500 at Marshall University. RNA seq was also performed externally by Genewiz. Analysis was performed using CLC Biomedical Genomics Workbench and Ingenuity Pathway Analysis (Qiagen). Non-coding or non-annotated genes were not included in analysis.

Subcellular Cell Fractionation

To isolate whole cell lysate, nuclei, and cytoplasmic fractions, cells were washed with PBS and collected in nuclei wash buffer (5 mM HEPES pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 250 mM sucrose, 0.1 % BSA with protease and phosphatase inhibitors). Cellular contents were fractionated using nitrogen cavitation. Briefly, cell lysates were placed in a nitrogen cavitation device pressurized to 200 psi and incubated on ice for 5 minutes before releasing contents. Whole cell lysate was collected at this step and processed with 2X RIPA buffer. Cytosol was separated from nuclei by centrifugation (2,700 g for 5 minutes). Nuclei was isolated using nuclear extraction buffer (20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 100 mM β-glycerophosphate, 300 mM NaCl with protease and phosphatase inhibitors).
Results

Here, we investigated the mechanisms by which JAK1 controls the transcriptional adaptation to ER stress in astrocytes. We previously identified JAK1 as a major driver of ER stress-induced inflammation, demonstrating that JAK-STAT signaling functions

![Diagram showing gene expression changes](image)

Figure 3.1 JAK1 kinase inhibition does not ablate most JAK1-dependent gene expression in response to ER stress.

A) Primary astrocytes were transfected with control or JAK1 siRNA for 48 h. AZD1480 (2 µM) was added to cells transfected with control siRNA 1 h before thapsigargin (1 µM) treatment (4 h). RNA was isolated and sequenced. B) Primary astrocytes were transfected with control or JAK1 siRNA for 48 h. AZD1480 (2 µM) was added to cells transfected with control siRNA 1 h before OSM (2.5 ng/ml) treatment (4 h). RNA was isolated and sequenced. C) Gene ontology (GO) was performed on the ER stress-induced, JAK1-dependent genes as well as the ER stress-induced, AZD1480 inhibited genes. Top 5 most significant GO groups are shown. D) Gene ontology (GO) was performed on the ER stress-induced, JAK1-dependent genes as well as the OSM-induced, AZD1480 inhibited genes. Top 5 most significant GO groups are shown.
outside of its traditional role downstream of cytokine receptors. Previously, we showed that JAK1 controls approximately 10% of ER stress-induced gene expression in primary astrocytes, including inflammatory genes such as IL-6 and chemokines CCL2 and CCL20 as well as ER stress-related genes that had not previously been associated with JAK1 signaling such as GADD45α and TRIB3. We showed ER stress related genes that are dependent on both JAK1 and on ATF4 expression are insensitive to JAK1 kinase inhibition using a pharmacological agent that targets JAK1 and JAK2 kinase activity, AZD1480. Based on these findings, we wanted to determine if there were more genes that were ER stress-induced in a JAK1-dependent fashion that were not reduced by inhibiting the kinase activity of JAK1. To test this using an unbiased approach, we used bulk RNAseq to determine the number of ER stress induced JAK1 dependent genes that are sensitive to inhibition by the JAK1/2 kinase inhibitor AZD1480. We performed differential gene expression on untreated and thapsigargin treated groups as well as on thapsigargin and JAK1 siRNA thapsigargin treated groups from our previously published dataset [13]. The overlapping genes were compared to differential gene expression of thapsigargin and AZD1480 thapsigargin treated genes. Surprisingly, we found that expression of most ER stress induced JAK1 dependent genes cannot be reduced by inhibiting JAK1 with AZD1480. Only 4 of the 50 total genes were ER stress induced and inhibited by AZD1480 (Figure 3.1A). We compared these results to the transcriptome of oncostatin M (OSM) stimulated astrocytes. OSM is an IL-6 family cytokine that signals through gp130 in a JAK1-STAT3 dependent manner [26]. Astrocytes robustly express the OSM receptor [27]. In contrast to the thapsigargin stimulated astrocytes, we found that
expression of the majority of the OSM-induced JAK1 dependent genes can be reduced using AZD1480 (Figure 3.1B). Overall, less than 20% of the ER stress induced genes can be inhibited using AZD1480 compared to approximately 80% of the OSM induced

**Figure 3.2 ER stress-induced gene expression does not require STAT3.**

A) Primary astrocytes were treated with thapsigargin (1 µM) for 4 h. RNA was isolated and sequenced. RPKM values for 3 technical replicates is shown. B) Primary astrocytes were transfected with control (CTL), STAT1, or STAT1 and STAT3 siRNA for 48 h and treated with IFN-γ (10 ng/ml) for 0.5 h. Protein lysates were collected and evaluated by immunoblotting. C) Primary astrocytes were transfected with control (CTL), STAT3, or STAT1 and STAT3 siRNA for 48 h and treated with OSM (2.5 ng/ml) for 0.5 h. Protein lysates were collected and evaluated by immunoblotting. D) Primary astrocytes were transfected as in (B) and (C) using STAT1 and STAT3 siRNA. Cells were treated with thapsigargin (1 µM) for 4 h. RNA was collected for analysis via RT-qPCR. N = 3.
genes. Further, we performed gene ontology on the ER stress induced genes that are downregulated using JAK1 siRNA and the genes that are downregulated using both JAK1 siRNA and AZD1480. Here, the top 3 gene ontology groups are different for each group. Expectedly, the genes that are sensitive to inhibition to AZD1480 belong to gene ontology groups related to immune processes. The ER stress-induced JAK1-dependent genes belonged to functional gene ontology categories that include type I interferon signaling and the unfolded protein response, as anticipated (Figure 3.1C). The ER stress-induced, AZD1480-inhibited genes do not belong to these categories. Further, the OSM-induced JAK1-dependent genes and OSM-induced AZD1480-inhibited genes belong to functional gene ontology categories associated with inflammatory and immune signaling. This is in line with the well-established role of JAK1-dependent gene expression. This suggests that JAK1 utilizes different signaling mechanisms to regulate gene expression using different stimuli.

JAK-STAT signaling is well-established to rely on JAK-mediated tyrosine phosphorylation of STATs. Phosphorylated STATs dimerize to form an active transcription factor complex. We found that most of the ER stress-induced genes cannot be inhibited by targeting JAK kinase activity. Therefore, this led us to hypothesize that JAK1 can regulate a subset of gene expression, in response to ER stress activation, without STAT proteins.

To test if ER stress induced JAK1 dependent genes require STAT expression, we first analyzed basal expression of STATs in astrocyte cultures by RNAseq. STAT3 is well-established to contribute to JAK-STAT signaling in astrocytes [28-32]. We confirmed via
RNAseq that STAT3 is the most highly expressed STAT in astrocytes and is induced by thapsigargin (Figure 3.2A). We proceeded to knockdown STAT1 and STAT3 in primary astrocytes using siRNA and treated with thapsigargin for 4 h and analyzed gene expression of JAK1 and ATF4 dependent genes. We knocked both STAT1 and STAT3 down simultaneously because previous reports demonstrate that STAT1 and STAT3 can

Figure 3.3 JAK1 and ATF4 interact between amino acids 248 and 275 of ATF4, and this interaction does not require ER stress.

A) Graphical representation of ATF4 truncation mutants. B) Primary astrocytes were transfected with 6 ug of full length ATF4 or truncated mutants of ATF4 using lipofectamine 3000 for 48 hours. Protein lysates were collected and immunoprecipitated using a JAK1-targeting antibody and immunoblotted for ATF4 using 2 ATF4 antibodies with different epitopes to detect all ATF4 mutants. C) Primary astrocytes were transfected with wild type ATF4 vector as in (B) or treated with thapsigargin for 4 h. RNA was isolated and used for RT-qPCR analysis.
compensate for the expression of the other. STAT1/3 knockdown effectively eliminates
the STAT1 and STAT3 protein expression, as shown my immunoblotting (Figure 3.2B, C). Interestingly, we found that STAT1/3 knockdown did not reduce thapsigargin-induced expression of IL-6, GADD45α, or TRIB3, suggesting that JAK1 regulates genes in response to ER stress without utilizing STAT transcription factors (Figure 3.2D).

We determined that STAT3 is not necessary for expression of JAK1-dependent genes in astrocytes in response to ER stress. Therefore, we hypothesized that JAK1 can utilize alternative transcription factors under conditions of cellular stress. Our previous studies found that JAK1 and the transcription factor activating transcription factor (ATF) 4 coimmunoprecipitate under conditions of ER stress. ATF4 is selectively translated under conditions of phosphorylated eIF2α. It is possible that JAK1 is driving gene expression via ATF4 specifically under conditions of ER stress because ATF4 is not basally expressed and JAK1 would not have the opportunity. To expand upon these findings, we utilized vectors to express ATF4 truncation mutants to determine specifically where JAK1 binds ATF4. We expressed the ATF4 mutants in primary astrocytes in vitro and used JAK1 immunoprecipitation strategies. Here, we expressed the wild type, full length ATF4, amino acids 1 – 275 of ATF4, amino acids 111 – 248 of ATF4, and amino acids 248 – 351 (Figure 3.3A). Importantly, there is no induction of ER stress in these conditions. ATF4 is expressed lowly at basal conditions, therefore, there should be little contribution of the endogenous ATF4 protein in these samples. These vectors were expressed in primary astrocytes and, using immunoprecipitation for JAK1, we identified that JAK1 bound all four of the ATF4 mutants. Because of this, we infer that JAK1 binds ATF4 in a location between amino acids 248 and 275 (Figure 3.3B). Importantly, this also
implies that ER stress is not necessary for the JAK1 and ATF4 interaction. To determine if ATF4 expression alone is sufficient to induce gene expression of JAK1 and ATF4-dependent genes, we overexpressed wild type ATF4 in primary astrocytes or treated astrocytes with thapsigargin for 4 h. This demonstrates that ATF4 expression is sufficient to induce gene expression of TRIB3, however, at a lower magnitude than thapsigargin treatment (Figure 3.3C). We hypothesized that this physical interaction leads to ATF4-dependent gene expression in response to ER stress and that JAK1 binding is essential for ATF4 to be recruited to promoters in a gene-specific fashion. However, these findings were surprising because JAK1 has been reported to be constitutively associated with cytokine receptors and ATF4 should be expressed in the nucleus where it drives transcription.

To determine the subcellular localization of the JAK1-ATF4 interaction, we treated primary astrocytes with thapsigargin for 4 hours to induce expression of ATF4 and then isolated whole cell lysates (WCL), cytosol, and nuclei protein lysates from these cells. We immunoblotted each subcellular fraction and confirmed selectivity of these fractions by blotting for the Transferrin Receptor 1 (TfR1) as a plasma membrane marker, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a cytosolic marker, and lysine-specific histone demethylase (LSD) 1 as a nuclear marker. Then, we determined the location of ATF4 and JAK1. ATF4 is exclusively expressed in the nucleus. JAK1 was detected in each fraction, indicating that JAK1 is not exclusively associated with the plasma membrane, as some studies have suggested [33] (Figure 3.4A). However, recent reports have also suggested that JAK1 is expressed in the nucleus [34, 35], corroborating our findings. Further, we quantified these immunoblots. In response to thapsigargin
treatment and induction of ER stress, less JAK1 is in the cytosol and there is an increase of

![Diagram showing protein expression in WCL, Cyto, and Nuc](image)

**Figure 3.4 JAK1 is expressed in the nucleus in response to ER stress.**

A) Primary astrocytes were treated with thapsigargin (1 µM) for 4 h. Nuclear isolates were isolated via nitrogen cavitation. Whole cell lysates (WCL), cytoplasm (cyto), and nucleus (nuc) were collected and immunoblotted. B) Three immunoblots were quantified. ATF4 protein expression was normalized to expression of either GAPDH (for WCL and cyto) or to LSD1 (for nuclear lysates). C) Three immunoblots were quantified. JAK1 protein expression was normalized to expression of either GAPDH (for WCL and cyto) or to LSD1 (for nuclear lysates).

JAK1 in the nucleus (**Figure 3.4B**). This suggests that JAK1 may be translocating to the nucleus in response to ER stress and/or is sequestered in the nucleus by ATF4. Overall, these data suggest that JAK1 and ATF4 are interacting in the nucleus. We hypothesized that this interaction between JAK1 and ATF4 drives ATF4-dependent gene expression in response to ER stress.
To test this hypothesis, we expanded our studies to determine the impact of JAK1 on ATF4-dependent gene expression on a genome wide level. We used ChIP-seq to characterize differential regulation of ATF4-dependent transcription in response to JAK1 knockdown. We used primary astrocytes with control (CTL) or JAK1 targeting siRNA and treated with (or without) thapsigargin. We crosslinked the cells and immunoprecipitated using an isotype control (IgG) or α-AF4 antibody. To identify how JAK1 influences ATF4 binding, we compared the mapped chromatin locations in the ER stress-treated condition.

**Figure 3.5 JAK1 is required for ATF4-promoter interactions.**

A) Primary astrocytes were isolated wild type mice and ER stress was induced using 1 µM of thapsigargin for 4 h. Cells were crosslinked and immunoprecipitated using an isotype control (IgG) or anti-AF4 antibody. DNA was eluted and RT-qPCR was performed using primers designed to target the most-likely ATF4 binding regions of the Tribbles3 promoter, based on previously published ChIP-seq data. (B) DNA samples were performed as described in panel (A). Reads were aligned to the mouse genome. Redundant reads were removed. To determine DNA binding locations based on enrichment of reads in a single location, peak calling was performed using Model-based Analysis of ChIP-seq (MACS) 3 algorithm. (C) ChIP-seq data represented as a profile plot to read density across all transcription start sites in the genome.

To test this hypothesis, we expanded our studies to determine the impact of JAK1 on ATF4-dependent gene expression on a genome wide level. We used ChIP-seq to characterize differential regulation of ATF4-dependent transcription in response to JAK1 knockdown. We used primary astrocytes with control (CTL) or JAK1 targeting siRNA and treated with (or without) thapsigargin. We crosslinked the cells and immunoprecipitated using an isotype control (IgG) or α-AF4 antibody. To identify how JAK1 influences ATF4 binding, we compared the mapped chromatin locations in the ER stress-treated condition.
in comparison to an untreated control to determine ATF4 DNA binding locations in response to ER stress. We then compared these mapped DNA sequences to a JAK1 knockdown ER stress treated group. Here, we were able to identify locations in the genome where JAK1 knockdown interrupts ATF4 binding. ATF4 is not basally expressed. Therefore, as expected, ChIP targeting ATF4 resulted in low numbers of mapped reads in both the isotype control samples as well as the untreated samples where ATF4 expression is not stimulated. Thapsigargin treated groups had much higher reads, as expected. We then performed motif analysis this demonstrated that much of the sequenced DNA included the known ATF4 DNA binding motif, suggesting that ChIP successfully selected for ATF4-bound DNA sequences (Figure 3.5A). These analyses suggest that JAK1 is required, globally, for ATF4-promoter interactions (Figure 3.5B). To test this empirically, we detected via ChIP-qPCR that ATF4 is recruited to the promoter of TRIB3, a gene that we have demonstrated to be induced upon ER stress in astrocytes and that is dependent on both JAK1 and ATF4. JAK1 knockdown interrupts the ability of ATF4 to bind the TRIB3 promoter, suggesting that JAK1 is required for ATF4 to bind the TRIB3 promoter (Figure 3.5C). Overall, these data suggest that JAK1 is required for ATF4 to bind promoter regions and drive gene expression in astrocytes in response to ER stress.

Discussion

This study has identified a novel role for JAK1 signaling in response to cellular stress in astrocytes. To our knowledge, this is the first study to demonstrate that JAK1 can initiate gene expression using transcription factors in addition to STATs. Here, we have identified that JAK1 interacts with ATF4. The interaction between JAK1 and ATF4
is necessary for ATF4 to bind transcription start sites and regulate gene expression in astrocytes. ATF4 is lowly expressed at basal conditions, therefore, under conditions of low phosphorylated eIF2α, JAK1 would be expected to drive gene expression primarily through STATs. However, we suggest that ER stress, which induces high levels of eIF2α phosphorylation, provides JAK1 with the opportunity to modulate gene expression using additional transcription factors, like ATF4. For example, PERK-mediated phosphorylation of eIF2α is one part of the integrated stress response (ISR). There are other eIF2α kinases that are activated upon limited heme availability, viral infection, or limited amino acid deprivation. ATF4 overexpression is sufficient to induce expression of TRIB3, without ER stress (Figure 3.3C). The interaction between JAK1 and ATF4 is dependent on ATF4 expression, not ER stress (Figure 3.3B). Therefore, we anticipate that other types of cell stress that induce ATF4 would also be regulated by JAK1. This is currently the scope of ongoing studies.

ATF4 is expressed in many CNS cells [27] and has been reported to impact neurological behavior and is implicated in neurodegenerative disease [36]. For example, ATF4 is upregulated in the substantia nigra in some Parkinson’s disease (PD) patients. In the same study, cultured neurons are protected from toxin-induced neurotoxicity by overexpressing ATF4. This suggests that ATF4 may be protective by promoting dopaminergic neuron survival in PD [37]. In in vivo mouse models and in post-mortem brain tissue of AD patients demonstrates an upregulation of ATF4 [38, 39]. Further, ATF4 deficient mice are protected against ALS progression in mutant SOD1 transgenic animals [40]. ATF4 is also necessary for normal synaptic plasticity and memory in hippocampal neurons [41, 42]. Taken together, this suggests that ATF4 plays an important role in
proper neuronal function, however, there is a paucity of studies that focus on the cell-specific contribution of ATF4-regulated gene expression.

Here, we provide evidence that JAK1 uniquely controls gene expression in astrocytes in response to ER stress. Further, JAK1 is required for ATF4 to bind promoter regions and regulate gene expression. We determined this by performing ChIP-seq on primary astrocytes. Previously, ATF4 ChIP studies have been completed using immortalized cell lines and have not been completed in CNS cells. Most of the ATF4 target genes in the CNS that have been unveiled have relied heavily on in vitro systems. Therefore, in vivo models of neurodegenerative disorders using conditional ATF4 knockouts are needed to solidify the role of ATF4 in these diseases.

There are currently 3 FDA approved pharmacological agents that target activation of JAK1 and additional inhibitors undergoing clinical trials. These inhibitors target the kinase activity of JAK1. In this study, we have unveiled a role for JAK1 that does not require JAK1-mediated phosphorylation of STATs. Therefore, we would anticipate that JAK1 inhibitors in patients would not impact ATF4-dependent gene expression. For example, we hypothesize that patients on JAK1-targeting drugs would not have altered expression of JAK1-dependent genes like GADD45α and TRIB3. Further, we do not anticipate that inhibitors targeting JAK1 will have impacts on the homeostatic function of the UPR. JAK1 inhibitors target the well-characterized kinase activity of JAK1. Here, we demonstrate that this kinase activity is not necessary for ATF4-dependent gene expression. Functional contributions of this STAT-independent gene expression in physiological and disease conditions are not known. Previously, we showed that JAK1
knockdown did not influence phosphorylation of eIF2α or expression of CHOP [13]. The UPR is beneficial for adapting to acute, transient demands on the ER when there is large demand for protein synthesis. However, chronic activation of the UPR, as demonstrated in a variety of neurological diseases, is thought to be maladaptive. Overall, we have described a novel JAK1-dependent signaling mechanism. Here, we found that, in astrocytes, JAK1 can specifically bind ATF4 when it is expressed, JAK1 is expressed in the nucleus, and that JAK1 is necessary for ATF4 to bind genetic promoters.

Bibliography


Conclusions and Perspectives

Conclusions and Summary

Our work here has identified that JAK1, an immune-related tyrosine kinase, plays a novel role in regulating responses to endoplasmic reticulum stress in the glial cell type astrocytes. This work built upon previous reports from our lab that found PERK, a UPR activator, drives inflammatory gene expression. This work sought to explore the possibility of targeting ER stress to selectively attenuate inflammation associated with pathology while retaining inflammatory responses that are restorative and promote homeostasis.

The Unfolded Protein Response has been well-characterized in peripheral cells harboring high secretory capacity such as plasma cells and pancreatic β-cells. In the CNS, the UPR has been studied in oligodendrocytes (due to their production of myelin) and neurons. Astrocytes make up the largest proportion of cells in the CNS, therefore, we believe that they are uniquely positioned to influence the trophic and inflammatory environment. Astrocytes are physically proximal to blood vessels and neuronal synapses. Although microglia are the bona fide immune cell of the brain and may produce higher amounts of soluble inflammatory molecules on a per cell basis, astrocytes, due to their large number, are likely to greatly contribute to the inflammatory milieu that influences neurological diseases.

Using primary cultures of astrocytes, we found that astrocytes respond to ER stress by producing the cytokine, IL-6. Here, we treated astrocytes with the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor, thapsigargin. Treatment with this non-competitive SERCA inhibitor causes rapid depletion of ER calcium stores,
inducing ER stress. As expected, induction of ER stress in astrocytes is associated with enhanced gene expression of known ER stress induced genes CHOP, ATF4, and GADD45α. Previous work in our lab demonstrated that PERK drives expression of those genes, as well as expression of inflammatory mediators such as IL-6, CCL2, and CCL20. IL-6, CCL2, and CCL20 production is driven by the JAK-STAT signaling pathway. Therefore, we questioned if JAK-STAT signaling was required to drive ER stress-induced IL-6 production. We knocked down JAK1 and JAK2 because these are the JAKs that are most appreciably expressed in astrocytes. JAK3 and Tyk2 expression is restricted to cells of the hematopoietic lineage. We used siRNA to knockdown JAK1 and JAK2 in primary astrocytes and treated these astrocytes with thapsigargin to induce ER stress. We found that JAK1 knockdown substantially ablated ER stress-induced IL-6 production. Further, this is an effect that is specific to JAK1. JAK2 knockdown had no impact on ER stress-induced expression of IL-6. We treated astrocytes with thapsigargin. The astrocytes responded with expression of ATF4 and CHOP, and eIF2α was phosphorylated. JAK1 knockdown had no impact on these expression levels indicating that canonical ER-stress induced signaling molecules are not JAK1 dependent. Therefore, we hypothesized that JAK1 uniquely drives transcriptional adaptation to ER stress in astrocytes.

To test this, we used RNAseq to understand the global impact that JAK1 has on the ER stress induced transcriptome. We once again knocked down JAK1 and treated with thapsigargin. Then, we isolated RNA and performed RNAseq. We determined all the genes that are ER stress induced in astrocytes by performing EDGE analysis on the RNAseq data [1]. We focused on genes that were at least 1.5-fold upregulated in comparison to the untreated samples. Further, we ensured that there was not significant
variation between technical replicates. After this analysis, we found a markedly large number of genes are JAK1 dependent in response to ER stress. We identified over 400 genes to be significantly upregulated in astrocytes in response to ER stress, and 51 of these genes were JAK1 dependent, suggesting that JAK1 plays a large role in controlling the transcriptional adaptation to ER stress. Further, 15 of the top 50 (30%) ER stress induced genes are JAK1-dependent, suggesting a vital role for JAK1 in response to cellular stress.

We performed gene ontology to understand the biological functions of the JAK1-dependent ER-stress induced genes. Many of the genes controlled by JAK1 in response to ER stress had not been previously associated with canonical cytokine induced JAK-STAT signaling. To test this, we also performed RNAseq on primary astrocytes with or without JAK1 knockdown and treated with the IL-6 family cytokine Oncostatin M (OSM). As anticipated, most of the OSM induced genes were also JAK1 dependent. However, the genes that were dependent on JAK1 in response to OSM were a distinct subset of genes than those that were JAK1 dependent in response to thapsigargin. This suggests that JAK1 controls a different subset of gene expression in response to different stimuli (cell stress vs. cytokine stimulation).

To understand how this occurs, we hypothesized that JAK1 may be able to coordinate gene expression in response to cell stress using additional molecules that are not typically expressed under non-stress conditions. For example, since genes that are JAK1-dependent in response to ER stress are different genes than those that are JAK1-dependent in response to OSM, we hypothesized that other transcription factors may be
utilized by JAK1 in addition to STATs. Further, this hypothesis was strengthened because many of the ER stress induced JAK1 dependent genes did not have predicted STAT binding motifs in their promoter regions. We examined the genes that are JAK1 dependent in response to ER stress and determined that many of these genes have been reported in the literature to express ATF4, a transcription factor that is induced upon active PERK signaling and expressed in response to thapsigargin treatment in astrocytes. We chose to evaluate the PERK-dependent transcription factor ATF4 because it is lowly expressed under basal conditions. ATF4 is only expressed under conditions of phosphorylated eIF2α. eIF2α can be phosphorylated by PERK, under conditions of ER stress, by GCNI, under conditions of amino acid deprivation, PRK, during viral infection, or by HRI, under conditions where heme is limited. We hypothesized that, because ATF4 is selectively expressed under these conditions, this would provide JAK1 with an opportunity to utilize ATF4 during conditions of ER stress. We found that many of the JAK1-dependent genes are also dependent on ATF4. This included GADD45α and TRIB3. We further evaluated genes that are classic inflammatory genes as well as genes that have been associated with ER stress, but not JAK-STAT signaling. The candidate genes we selected for further molecular analysis were IL-6 and CCL2 (inflammatory genes) and GADD45α and TRIB3 (ER stress-related genes). Here, we confirmed IL-6, CCL2, GADD45α, and TRIB3 are all JAK1-dependent in astrocytes treated with thapsigargin. Further, the induction of IL-6 and CCL2 expression in response to ER stress could be abrogated using the JAK1/2 inhibitor AZD1480, as expected. However, GADD45α and TRIB3 were insensitive to this inhibition. This indicates that JAK1/ATF4 dependent genes may not rely on the characterized JAK1 kinase activity.
To determine if JAK1 has a direct impact on ATF4 signaling, we tested if JAK1 knockdown reduces overall ATF4 expression in response to ER stress. JAK1 does not regulate ATF4 expression. Next, we hypothesized that there may be a protein-protein interaction between JAK1 and ATF4. We demonstrated that ATF4 and JAK1 coimmunoprecipitate, suggesting there is a physical interaction between these proteins. Further, we have seen that ATF4 is expressed specifically in the nucleus, leading us to hypothesize that JAK1 also can be expressed in the nucleus in astrocytes under conditions of endoplasmic reticulum stress.

We next hypothesized that the JAK1 and ATF4 interaction may influence the ability of ATF4 to bind promoter regions and drive gene expression. To test this, we used ChIP-qPCR and ChIP-seq immunoprecipitating for ATF4. First, we pulled down ATF4-bound DNA sequences and ChIP-seq was used to expand these studies as an unbiased, genome-wide fashion. We confirmed that ChIP-seq was successful by identifying the known ATF4 binding motif in our sequences. Further, preliminary analysis suggests that JAK1 is required, globally, for ATF4-promoter interactions. This suggests a unique role for JAK1 in regulating transcription factor-DNA binding. To empirically confirm these findings, we performed ChIP-RT-qPCR targeting predicted ATF4-binding regions in the promoter of TRIB3. Here, we demonstrated that JAK1 is necessary for ATF4 to bind the TRIB3 promoter.

This work has established JAK1 as a major regulator of ER-stress induced gene expression in astrocytes. JAK1 has canonically been studied downstream of cytokine receptors. Here, we show that endoplasmic reticulum stress can directly activate JAK1-
dependent signaling. To our knowledge, this work is the first to demonstrate noncanonical JAK1 signaling in the CNS. Further, we have identified that JAK1 regulates global promoter binding of the transcription factor ATF4 and is expressed in the nucleus. Additionally, JAK1 controls transcription of different genes dependent on the stimulus that activates JAK1. Therefore, we hypothesized that JAK1 utilizes alternative transcription factors in addition to STATs. We found that JAK1 is necessary for ATF4 to bind promoter regions in DNA, suggesting a significant impact on ER stress-induced transcription.

Discussion and Future Directions

Canonical and Noncanonical JAK1 signaling

We define canonical JAK1-dependent signaling as the activation of JAK1 upon receptor ligation that creates a conformational change to increase JAK1’s tyrosine kinase activity that facilitates recruitment of STATs to be phosphorylated by JAKs and activated to drive gene expression. Our work has defined a noncanonical role for JAK1 signaling in response to cell stress. Here, JAK1 can be activated by ER stress in a PERK-dependent fashion. Further, JAK1 can cooperatively regulate expression via a novel mechanism dependent on ATF4. Excitingly, we also identified JAK1 in the cytosol and nucleus, challenging other reports that state JAK1 is constitutively associated with plasma membrane-spanning receptors.

Our finding that 10% of the genes upregulated by ER stress are JAK1-dependent leads to additional research questions. For example, two genes induced by JAK1 that we did not focus on are NUAK1 and NUAK2 which are AMPK-related kinases that have been implicated in neurological processes. NUAK1 and NUAK2 both contribute to neural tube formation in development [2]. NUAK2 reportedly plays a role in neurodevelopment [3] and
some types of cancer [4]. NUAK1 and NUAK2 are homologous [5], and NUAK1 has been reported to play a role in neurodegeneration. In post-mortem brain tissue of AD and progressive supranuclear palsy cases, there was an increased association of NUAK1 with tau protein in comparison to age-matched controls. The same study found that NUAK1 phosphorylates tau, providing a mechanism for the link between NUAK1 activity and tau accumulation. Finally, this study crossed heterozygous NUAK1 knockout mice with the P301S model of tauopathy. Partial loss of NUAK1 rescued some of the pathologies seen in the P301S model including reduced hyperphosphorylated tau and fewer neurofibrillary

Figure 4.1 JAK1 regulates basal and inflammation-induced C3.

A) Primary astrocytes were transfected with control or JAK1 siRNA and treated with thapsigargin for 4 h. RNA was isolated and RNaseq was performed. B) Primary astrocytes were treated with thapsigargin (1 µM) or tunicamycin (5 µM) for 24 h. Protein lysates were collected and immunoblotted for C3 and ATF4 to confirm ER stress induction. C) Primary astrocytes were transfected with siRNA as in (A) and treated with neurotoxic oligomers (500 pg/ml) for 24 h. RNA was isolated for qPCR. N = 3. D) Astrocytes were transfected with siRNA as in (A) and treated with the TNF-α (5 ng/ml), OSM (2.5 ng/ml) or IL-1β (500 pg/ml) for 24 h. Protein lysates were collected for immunoblotting.
tangles [6]. Therefore, this provides a hypothetical mechanism that could link endoplasmic reticulum stress-mediated activation of JAK1 to hyperphosphorylation of tau and subsequent tauopathy-associated neurodegeneration [2].

Another gene we identified in our RNAseq data as a JAK1-target gene is the complement component C3. C3 has recently gained much attention as a marker of reactive astrogliosis. C3-expressing astrocytes are implicated in a variety of neurological and neurodegenerative diseases including PD, AD, MS, and stroke [7]. It is thought that C3 expressing astrocytes aberrantly prune synapses, leading to neurodegeneration [7-12]. Thapsigargin does not appreciably induce C3 expression at the RNA level, but JAK1 siRNA abrogates basal C3 expression (Figure 4.1A). However, thapsigargin and tunicamycin induce C3 protein expression (Figure 4.1B). Further, we found that neurotoxic oligomers can induce C3 expression in a JAK1-dependent manner (Figure 4.1C). Proinflammatory cytokines also induce protein levels of C3, and this can be reduced by siRNA-mediated JAK1 knockdown (Figure 4.1D). Although these findings are outside the scope of the current project, this provides evidence that targeting JAK1 could rescue the astrocyte-mediated synaptic loss and dysfunction in neurodegenerative diseases.

Additionally, using RNAseq data generated in our lab and published elsewhere, we found that JAK1 and ATF4 in astrocytes regulate genes related to histone 1. This included genes such as Hist1h1d, Hist1h1c, and Hist1h1b [13]. Currently, there is little known about the regulation of histone 1 related genes in astrocytes, and functional consequences are yet to be explored. However, this information taken together with the
previous reports that JAK1 and JAK2 can influence epigenetic modifications and phosphorylate histones, we hypothesize that, in astrocytes, JAK1 may contribute to epigenetic regulation.

Although these studies are outside of the scope of this project, this could be a future direction of this project to expand on the mechanistic insights of JAK-dependent signaling. Histone 1 molecules make up the linker portion of chromatin structure and control DNA entry and exit sites. Interestingly, H1 protein knockouts in various model organisms are viable and show that H1 does not act as a general suppressor of transcription but impacts regulation of specific genes [14-17]. For example, knockdown of H1-related genes has been demonstrated to upregulate expression of interferon-stimulated genes (ISG) and induce interferon – β to limit viral replication [18]. Therefore, although empirical evidence is needed, we suggest that JAK1 may promote basal expression of ISGs via maintaining histone 1 gene expression. Further, overexpression of Hist1h1c has been shown to induce autophagy in retinal ganglion cells [19]. Here, Hist1h1c promotes cell death. It is possible that Hist1h1c could also mediate the JAK1-dependent ER stress-induced transcriptional response. In addition to controlling histone-related genes, our findings that JAK1 can be expressed in the nucleus support other reports that suggest JAK1 regulates histones post-translationally at the epigenetic level.

The first discovery that JAKs may be able to function in the nucleus was the finding that JAKs are associated with cytokine receptors. Some cytokine receptors that are known to be associated with JAK-STAT signaling have been demonstrated to translocate to the nucleus. Some of these cytokine receptors include growth hormone [20, 21], insulin
[22-24], and the type I angiotensin receptor [25, 26]. Therefore, these reports suggest that JAK1 could translocate to the nucleus with associated cytokine receptors. These findings and others challenge reports that JAK1 is constitutively expressed near the plasma membrane, including a 2004 study that demonstrated that the subcellular localization of JAK1 is restricted to the plasma membrane. However, this was demonstrated in HepG2, HeLa, A375, HUVEC, U4C, 2C4 cell lines [27], not primary cells.

JAKs have been reported to function non-canonically in the nucleus. However, we are the first to report that this occurs in brain cells. In 2009, Dawson et. al found that JAK2 is present in the nucleus and can utilize its tyrosine kinase activity to phosphorylate histones and modulate heterochromatin. Here, JAK2 was visualized using immunofluorescence in the hematopoietic cell lines HEL, UKE1, and K562 cell lines [28]. Additionally, cellular fractionation confirmed these results, demonstrating for the first time that JAKs have roles in the nucleus. Previously, JAKs were thought be constitutively in the cytosol to initiate STAT translocation to the nucleus. Here, in the nucleus, JAK2 tyrosine phosphorylated histone 3 at tyrosine 41 (H3Y41). This phosphorylation was demonstrated to be required for the protein HP1α to bind histone 3 and regulate expression of the gene LIM domain only 2 (also known as rhombotin-like 1) (lmo2). ChIP-seq analysis revealed that lmo2 is required for yolk sac production of erythrocytes and is considered an acute T-lymphoblastic leukemia-related oncogene. This provides a model for JAK proteins to have a role in epigenetic regulation [28].

More recently, a similar phenomenon was demonstrated with JAK1. JAK1, in B cell lymphoma cells, was found to be imported to the nucleus. Here, the authors identified
a classical nuclear localization sequence (NLS) in JAK1. The NLS of JAK1 is a cluster of basic amino acids located at amino acids 342 – 345. In HBL1 and OCI-Ly1 cell lines that represent a type of B cell lymphoma (activated B cell diffuse large B cell lymphomas (ABC-DLBCL)), JAK1 was found in the nucleus regardless of activation status of JAK1 by cytokine receptor ligation using constitutively active mutants of JAK1 often associated with ABC-DLBCL.

We demonstrated, in astrocytes, that JAK1 is in the nucleus and that JAK1 physically interacts with ATF4, which is also expressed in the nucleus. Further, we have demonstrated that JAK1 influences an entirely distinct subset of gene expression in astrocytes in response to ER stress. ATF4 regulated genes have been linked to ER stress induced cell death and apoptosis. For example, the pseudokinase TRIB3 that we have identified to be both JAK1 and ATF4-dependent in astrocytes was first described in Drosophila and has been reported to modulate glucose signaling by binding and inhibiting protein kinase B (Akt) [29]. This results in a reduction in insulin-dependent phosphorylation of GSK-3β [29]. This phosphorylation is important to inhibit glycogenolysis. Therefore, these findings link ATF4-dependent expression of TRIB3 to glucose output from the liver under fasting conditions [29]. Additionally, in cell lines derived from hepatocarcinoma, TRIB3 inhibits Akt and mTORC1 and this was shown to promote both autophagy and apoptosis, corroborating the previous findings. Further, in aged rats, TRIB3 expression is increased in the liver. Here, this is associated with insulin resistance [30]. Taken together, this suggests that, in response to ER stress, JAK1 could drive inhibition of Akt in astrocytes in a TRB3-dependent mechanism.
Mechanistic studies utilizing HEK293T cells showed that TRIB3 can interact with SMAD3, an important downstream signaling molecule of the TGFβR1. This interaction retains SMAD3 in the nucleus. The same study used trans-well migration assay to demonstrate that HepG2 (representing carcinoma) cells with stable TRIB3 knockdown failed to migrate to the same extent as control cells, indicating that TRIB3 may play a role in tumor cell migration [31]. This still needs to be confirmed in vivo. However, in support of these findings, the drug ABTL0812 that binds and activates transcriptional activity of PPARα and PPARγ has been demonstrated to activate TRIB3 and promote cytotoxic autophagy in cancer cells [29, 32, 33]. ABTL0812 has begun clinical testing and Phase I study results show that it is well-tolerated in patients and will continue to Phase II testing.

**Figure 4.2 Development of BioID - a potential future direction to identify novel JAK1-interacting molecules.**

Primary astrocytes were transfected with a DNA construct encoding the promiscuous biotin ligase, BirA or a BirA-JAK1 fusion protein. 100 µM of biotin was added to the media 24 h after transfection. Protein lysates were collected 48 h post-transfection and immunoprecipitated with streptavidin beads. These data demonstrate, as a proof of concept, that the JAK1-BirA construct can be utilized to immunoprecipitate novel JAK1-interacting proteins.
Our findings suggest that JAK1 could similarly influence cancer cell migration through regulation of ATF4 and subsequently, TRIB3.

Therefore, we believe our finding that JAK1 and ATF4 cooperatively regulate TRIB3 expression in astrocytes via previously unknown mechanisms may promote crosstalk with additional signaling pathways. For example, our work implies that ER stress may, in a JAK1 and ATF4 dependent mechanism, inhibit AKT phosphorylation. However, our work suggests that current therapeutics that target JAK1 would not impact TRIB3-dependent consequences because JAK1-dependent expression of TRIB3 does not require the phosphorylation of STATs.

Additional evidence that JAK1 signaling and metabolism could be linked is that AMPK, a central regulator of metabolic signaling, can phosphorylate JAK1 and inhibit JAK-STAT induced inflammation. This suggests that low nutrient status can be linked to inflammatory activation of cells. For example, activating AMPK resulted in lower levels of JAK1-mediated IL-6 production [35]. Although these links have not been fully investigated in the CNS, and little is known about the immunometabolism of astrocytes, this is likely an important study. The brain utilizes the majority of the body’s glucose, and astrocytes play a vital role in nutrient processing for the CNS. Our work raises the possibility that, under conditions of low nutrients, AMPK could inhibit JAK1-dependent production of cytokines through traditional mechanisms. However, under these conditions, ATF4-dependent genes may still be transcribed downstream of JAK1. For example, under conditions of phosphorylated AMPK, JAK1-driven expression of TRB3 may inhibit
glycogenolysis in astrocytes or impact other metabolic processes [36]. This suggests that our findings, in addition to ER stress, may have broader applications in metabolic stress.

Our work has mechanistically linked the Unfolded Protein Response in astrocytes to immunological signaling cascades. We have identified that JAK1 can utilize ATF4 to regulate gene expression, however, there are many proteins that bind or interact with JAK1 that could facilitate noncanonical signaling in addition to ATF4. One additional approach that could address this possibility is using a proximity biotinylation approach to identify novel proteins that interact with JAK1. Our lab has begun to optimize the BioID approach, a type of proximity biotinylation technique (45). We have generated a JAK1 construct fused to a promiscuous biotin ligase, allowing biotinylation of interacting and proximal proteins to JAK1 in experimental conditions (Figure 4.2). Biotinylation of JAK1 binding partners allows for streptavidin-dependent isolation and identification using mass spectrometry.

We plan to strengthen the findings that JAK1 is in the nucleus and can bind ATF4 to modulate ATF4-promoter interactions. We will perform RNAseq on primary astrocytes with or without ATF4 siRNA-mediated knockdown with thapsigargin treatment. We predict that the thapsigargin induced genes that are JAK1 dependent, but insensitive to inhibition by AZD1480 will have significant overlap with thapsigargin induced ATF4-dependent gene expression. We have found this to be true with genes like GADD45α and TRIB3 and anticipate that more genes will fall into this category.

To test the hypothesis that ATF4 sequesters JAK1 in response to ER stress, we will knockdown ATF4 in primary astrocytes, treat with thapsigargin for 4 hours, and collect
cellular fractions of whole cell lysate, cytoplasm, and nucleus. Like we have demonstrated previously, we expect that JAK1 will be present primarily in the whole cell lysate and enriched in the nucleus under ER stress. If ATF4 is required for this process, we expect that in an ATF4 siRNA thapsigargin treated condition, there will be a loss of JAK1 accumulation in the nucleus in comparison to nontargeting control siRNA. If there is no difference between nuclear JAK1 in the control siRNA and ATF4 siRNA thapsigargin treated groups, the translocation of JAK1 to the nucleus could be dependent on additional unknown factors.

**The in vivo role of JAK1 in astrocytes**

To understand the overall contribution of JAK1 signaling in astrocytes to neuroinflammatory disease, we sought out to generate conditional astrocyte-specific JAK1 knockouts in mouse models. JAK1 controls the signaling activity of many cytokines including the IL-6 family, interferons, and the IL-10 family [37]. Many of these cytokines are well-established to play roles in neurological diseases [38]. JAK1, JAK2, and STAT3 are the most highly expressed JAKs and STATs, respectively, in astrocytes. Both JAK1 and JAK2 can phosphorylate STAT3 to drive gene expression. Additionally, JAK inhibitors, such as the JAK1/2 kinase inhibitor AZD1480, have been shown to ameliorate disease progression in EAE and this is associated with lower numbers of infiltrating Th1 and Th17 cells, but the cell specific contributions are unknown [39, 40]. JAK1 knockout mice are perinatal lethal. JAK1 knockout mice are born, but in fewer numbers and fail to nurse so understanding the role of JAK1 in vivo has been limited [41]. Recently, a JAK1 floxed animal was generated by the group of Dr. Ross Levine. We obtained these mice and crossed the JAK1<sup>fl/fl</sup> animals to the astrocyte specific GFAP-Cre (line 77.6) [42].
However, no viable pups were generated from the GFAP-Cre JAK1<sup>fl/fl</sup> mice. Therefore, we assume that astrocyte specific JAK1 knockout results in deleterious developmental impacts. This is in line with previous literature demonstrating that global JAK1 deletion in mice is perinatal lethal and that STAT3 is required for astrocyte development [41, 43].

An alternative approach that we have developed to understand the cell-specific role of JAK1 in vivo is using a newly available tamoxifen inducible astrocyte specific cre line (ALDH1L1 Cre<sup>EERT2</sup>). We plan to use this line to temporally control when JAK1 is knocked out – for example, after the mice have reached adulthood. To test the in vivo response of astrocyte selective JAK1 deletion, experimental autoimmune encephalomyelitis (EAE) could be used to model neuroinflammatory disease. Although these studies are outside of the scope of this current project, the generation of these animal lines opens a multitude of future investigations evaluating the in vivo role of JAK1 signaling. Future studies will involve inducing EAE by immunizing mice (12 – 18 weeks old) with an emulsion of CNS-specific protein, myelin oligodendrocyte glycoprotein (MOG), and Complete Freund’s Adjuvant. This model will be used because it is accompanied by excessive neuroinflammation, including upregulated cytokines and chemokines, many of which are known to be JAK1-dependent. EAE is a model of MS, but also will allow evaluation of astrocyte derived JAK1 signaling in both neuroinflammatory and neurodegenerative phases of disease. Here, we will induce EAE in control (JAK1<sup>fl/fl</sup> Cre negative) animals and JAK1<sup>Astro-KO</sup> animals. We will monitor disease state, characterized by ascending paralysis, over a period of 45 days. We will record mean onset day of disease, percent incidence, and mean peak score of disease. We anticipate that JAK1<sup>Astro-KO</sup> mice will have later disease onset, lower percent
incidence, and a lower mean peak disease score. At pre-onset (~day 8), onset (~day 10), peak (~day 14), and chronic (~day 21), we will collect cerebellum and spinal cord tissues, isolate RNA, and protein to evaluate inflammatory and ER stress markers via RT-qPCR. Proinflammatory cytokine expression will be measured for the following cytokines: IL-6, CCL2, CCL20, TNF-α, IFN-γ, IL-17, and IL-1β. We will also analyze activation (phosphorylation) of STAT3 via western blotting. Infiltrating lymphocytes and monocytes are key drives of neuroinflammation and neural injury in EAE and are recruited, in part, by astrocyte-derived chemokines [44]. Therefore, flow cytometry will be used to analyze infiltrating inflammatory cell populations including TH1 cells, TH17 cells, and macrophages in control and JAK1 Astro-KO tissue (cerebellum and spinal cord). We anticipate that JAK1 will appreciably lower the overall inflammatory profile in the brains and spinal cord in mice afflicted with EAE. This will show that astrocytes play a central role in directing neuroinflammation. However, because JAK1 also is important in signaling for the IL-10 family of cytokines, it is possible that astrocyte-specific knockdown will worsen EAE. This may be because the IL-10 receptor will be unable to initiate expression of immunomodulatory genes to quell the neuroinflammation. This would also be interesting, because this would demonstrate an important cell-autonomous immunomodulatory role for astrocytes in the context of EAE. We have generated the first inducible, CNS-specific JAK1 knockouts. Therefore, there is a variety of neurological disease models, in addition to EAE, in which astrocyte dependent JAK1 signaling mechanisms can now be studied. We have generated this mouse line and mice are viable and show no overt phenotype following tamoxifen injections.
In parallel to developing the ALDH1L1 Cre JAK1\textsuperscript{fl/fl} line, we have crossed JAK1\textsuperscript{floxed} [45] to a global inducible Cre line (CAGG-Cre\textsuperscript{ERT\textsuperscript{TM}}), isolated primary astrocytes, and confirmed that we can successfully knockout JAK1 (JAK1\textsuperscript{cKO}) as shown by immunoblotting. We also showed by qPCR that Cre positive, JAK1\textsuperscript{fl/fl} animals have reduced cytokine-induced gene expression. Here, JAK1 can be conditionally deleted in all cells. Isolating specific cell types will also allow us to knockout JAK1 \textit{ex vivo} to understand mechanisms of JAK1 signaling in a variety of cell types of interest. We have established a role for JAK1/ATF4 dependent gene expression in astrocytes in response to ER stress. Using these models, we will be able to translate this to various non-CNS

\textit{Figure 4.3 JAK1 global inducible in vivo knockouts.}

JAK1 is knocked out in peripheral and central tissues in vivo using the inducible CAGG-Cre\textsuperscript{ERT\textsuperscript{TM}} mice crossed to JAK1\textsuperscript{fl/fl} mice. A) Represents the overall experimental schema. Mice were injected with 75 mg/kg of tamoxifen suspended in corn oil intraperitoneally (i.p.) for 7 consecutive days. After a 10-day washout period, mice were injected with 2 mg/ml of tunicamycin i.p. Tissues were harvested 12 or 24 hours later, and protein and RNA were isolated. Protein lysates were evaluated by immunoblotting and RNA was used for RT-qPCR.

In parallel to developing the ALDH1L1 Cre JAK1\textsuperscript{fl/fl} line, we have crossed JAK1\textsuperscript{floxed} to a global inducible Cre line (CAGG-Cre\textsuperscript{ERT\textsuperscript{TM}}), isolated primary astrocytes, and confirmed that we can successfully knockout JAK1 (JAK1\textsuperscript{cKO}) as shown by immunoblotting. We also showed by qPCR that Cre positive, JAK1\textsuperscript{fl/fl} animals have reduced cytokine-induced gene expression. Here, JAK1 can be conditionally deleted in all cells. Isolating specific cell types will also allow us to knockout JAK1 \textit{ex vivo} to understand mechanisms of JAK1 signaling in a variety of cell types of interest. We have established a role for JAK1/ATF4 dependent gene expression in astrocytes in response to ER stress. Using these models, we will be able to translate this to various non-CNS
cell types as well that are associated with ER stress including β cells, plasma cells, macrophages, and T cells. This will be a powerful tool to analyze JAK1 signaling in primary cells.

We have also tested this system in vivo. As described in Figure 4.3A, CAGG-Cre negative or positive JAK1fl/fl mice were injected with tamoxifen to delete JAK1. In the liver, JAK1 was effectively deleted (Figure 4.3B). To replicate our in vitro findings in vivo, we also treated mice with tunicamycin for 12 or 24 h. Here, we found that tunicamycin induces expression of IL-6 and TRIB3 in the liver (Figure 4.3C). TRIB3 is JAK1-dependent in the liver in vivo (Figure 4.3C). In the brain, ER stress induces both IL-6 and TRIB3 and both of these genes are JAK1-dependent in vivo, confirming our in vitro studies. Overall, this suggests that the JAK1fl/fl animal model will be an effective tool in studying JAK1 in vitro.

This will also allow us to expand upon mechanistic understanding of JAK1 in astrocytes and a variety of other cell types. For example, in astrocytes, expressing kinase dead mutants of JAK1 is difficult because JAK1 is endogenously expressed. However, this experiment will be extremely important to demonstrate that JAK1 can initiate gene expression without using its characterized kinase activity to phosphorylate STAT transcription factors, initiating STAT-dependent expression. Therefore, we will utilize CAGG-Cre positive, JAK1fl/fl astrocytes to express wild type and kinase dead mutants of JAK1. Subsequently, we will treat these astrocytes with thapsigargin to induce ER stress. We anticipate that genes such as IL-6 will be induced upon thapsigargin treatment. This induction will be ablated when JAK1 is knocked out but rescued when wild type JAK1 is expressed. However, we hypothesize that rescuing expression with the JAK1 kinase dead
mutant will fail to rescue IL-6 expression because IL-6 is dependent on JAK1-dependent phosphorylation of STATs. However, we hypothesize that GADD45α and TRIB3 will be induced upon thapsigargin stimulation and their expression will be similar in both the wild type and kinase dead expression mutants. This experiment will provide empirical evidence that the JAK1 and ATF4-dependent genes are not regulated by JAK1 kinase activity.

Overall, our work here has established JAK1 as a central molecular regulator of cellular stress in astrocytes. We are the first to identify novel mechanisms for JAK1 involving additional transcription factors (ATF4). This opens the possibility that additional transcription factors may also control JAK1-directed gene expression in various states of cellular stress.

**Figure 4.4 Summary of JAK1-dependent signaling in response to ER stress.**
cell stress. Our work has provided important insight to the role of the UPR signaling in astrocytes. This is summarized in Figure 4.4. Although our work does not provide a causal link to neurotoxicity, our work along with other work where ER stress has been linked to neurotoxicity in models of neurodegeneration. However, neurotoxicity may be the result of multiple unconfirmed mechanisms including: 1) initiation of apoptosis by the UPR in neurons, 2) loss of supportive function in other CNS cells, such as glia, 3) UPR signaling in glial cells have a gain of neurotoxic function, or 4) a combination of these possibilities. Altogether, an accumulation of evidence suggests that the duration, cell type, and inflammatory environment of the CNS dictates the consequences of active UPR signaling. Therefore, careful studies examining cell-specific contributions of the UPR in neurological disease models are necessary to form a complete picture.

In this work, we have provided evidence that activation of the UPR greatly alters the transcriptome of murine astrocytes. We have provided molecular evidence that, although the UPR attenuates translation, many secreted inflammatory molecules such as IL-6 are still expressed under these conditions. We have demonstrated that JAK1 is a major factor in driving adaptation to ER stress in astrocytes. JAK1 may have similar mechanisms in response to other types of cell stress such as oxidative stress, nitric oxide-induced stress, or depletion of oxygen or resource availability. Now that JAK inhibitors have been on the market for a few years, an interesting observational study to conduct, clinically, would be to examine the neurological outcomes of patients taking JAK inhibitors for other conditions, such as rheumatoid arthritis. This could provide proof of principle that JAK inhibition has an impact on neurological outcomes in diseases such as ischemic stroke, Alzheimer’s disease, among others.
Here, we provide evidence that UPR signaling could substantially contribute to astrocyte-driven neuronal dysfunction. To gain complete understanding of the UPR in the CNS, more advanced cell-specific studies must be completed. Gain and loss of function studies in vivo in astrocyte conditional models are necessary and attainable future directions for the field. Recent studies have established that substantial regional heterogeneity of astrocytes exists [46]. Currently, astrocyte specific UPR signaling has not been characterized or compared across regional locations of the CNS. The location and composition of misfolded proteins is known to vary amongst each neurodegenerative disease. Therefore, this prompts the possibility that UPR signaling in astrocytes can greatly differ based on location, disease state or stage.

Overall, we have found that JAK1 plays a major role in the transcriptional reprogramming of astrocytes in response to endoplasmic reticulum stress. We have demonstrated that endoplasmic reticulum stress drives JAK1-dependent gene expression. Approximately 10% of the ER stress induced genes in astrocytes are JAK1-dependent. This includes genes that are traditionally associated with JAK-STAT signaling, such as IL-6. However, JAK1 uniquely regulates stress-induced genes such as TRIB3 and GADD45α in an ATF4-dependent manner. Under conditions of ER stress, JAK1 is expressed in the nucleus, and is required for ATF4 promoter interactions.


Appendix I: Review Article Full Text

Title: The role of endoplasmic reticulum stress in astrocytes

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Short Running Title: ER stress signaling in astrocytes

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Abstract

Astrocytes are glial cells that support neurological function in the Central Nervous System (CNS), in part, by providing structural support for neuronal synapses and blood vessels, participating in electrical and chemical transmission, and providing trophic support via soluble factors. Dysregulation of astrocyte function contributes to neurological decline in CNS diseases. Neurological diseases are highly heterogeneous but share common features of cellular stress including the accumulation of misfolded proteins. Endoplasmic reticulum (ER) stress has been reported in nearly all neurological and neurodegenerative diseases. ER stress occurs when there is an accumulation of misfolded proteins in the ER lumen and the protein folding demand of the ER is overwhelmed. ER stress initiates the unfolded protein response (UPR) to restore homeostasis by abating protein translation and, if the cell is irreparably damaged,
initiating apoptosis. Although protein aggregation and misfolding in neurological disease has been well described, cell-specific contributions of ER stress and the UPR in physiological and disease states are poorly understood. Recent work has revealed a role for active UPR signaling that may drive astrocytes towards a maladaptive phenotype in various model systems. In response to ER stress, astrocytes produce inflammatory mediators, have reduced trophic support, and can transmit ER stress to other cells. This review will discuss the current known contributions and consequences of activated UPR signaling in astrocytes.

Keywords:

astrocytes, endoplasmic reticulum, cell signaling, protein folding, translation, glia
Main Points:

- Astrocytes experience endoplasmic reticulum (ER) stress during neuroinflammatory and neurodegenerative diseases.

- ER stress activates the unfolded protein response (UPR) in astrocytes and drives the production of inflammatory molecules including IL-6, CCL2, CCL20, CCL3, CXCL1, CXCL10, Lcn2, VEGF, and C3.

- Astrocytes under ER stress can induce UPR and inflammatory signaling in neighboring cells.

- Activated UPR in astrocytes may contribute to neurotoxicity and/or reduced neuronal and synaptic support.

Introduction

Endoplasmic Reticulum Stress and the Unfolded Protein Response

Secreted and membrane bound proteins are translated and processed in the endoplasmic reticulum (ER). Within the ER, proteins mature by folding into the proper tertiary and quaternary structure and acquire necessary post-translational modifications.
The ER is also critical for membrane lipid production and for the regulation of intracellular Ca\(^{2+}\) [47]. Often, proteins within the ER fail to fold into the correct form. Fortunately, the cell has intrinsic quality control mechanisms that eliminate misfolded proteins, such as chaperone-mediated folding [48] and ER associated degradation (ERAD) [49, 50]. However, when these control mechanisms are overwhelmed, misfolded proteins accumulate in the ER lumen. The aberrant accumulation of misfolded proteins and concomitant induction of ER stress has been observed in many diseases and cell types [51, 52]. ER stress occurs when a cell can no longer keep up with the demand to fold proteins due to the number of misfolded proteins in the ER lumen. ER stress initiates a highly conserved adaptive mechanism called the unfolded protein response (UPR). The intracellular signaling stimulated by ER stress is aimed at restoring homeostasis; however, if the stress is not alleviated, prolonged ER stress can drive cell death and inflammation which may contribute to pathology [52].

ER stress can occur transiently in physiological conditions when there is an increased demand for protein secretion, or in pathogenic states where ER stress occurs due to genetic mutations, oxidative stress, ischemia, or other maladaptive cellular states. Physiological ER stress has been best characterized in cells harboring high secretory capacities such as pancreatic β cells and antibody-producing B cells [53-59]. Although UPR activation is necessary to maintain homeostasis and clearly plays a role in homeostatic processes, tight regulation of the UPR is paramount for maintaining cellular health. Persistent activation of the UPR is reported in multiple diseases, including diabetes, cancer, and neurodegeneration [60-62].
There are three known proteins which sense the accumulation of misfolded proteins and transmit distinct signals to the cytosol and nucleus to modify transcriptional and translational programs to cope with ER stress. These trans-ER membrane proteins are inositol requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK) and activating transcription factor (ATF) 6. These enzymes are maintained in their inactive state through interaction with the ER-resident protein chaperone glucose regulated protein (GRP) 78 (also known as binding immunoglobulin protein (BiP)) proteins [63]. GRP78 binds broadly to hydrophobic residues that are exposed by misfolded proteins [64]. Excess misfolded proteins recruit GRP78 away from the luminal domains of PERK, IRE1, and ATF6 allowing activation [65, 66]. PERK and IRE1 can also directly interact with misfolded proteins which contributes to its activation via a ligand-receptor type interaction [67-71]. Figure 5.1 provides an overview of the UPR signal transducing molecules.

IRE1 is the most evolutionarily conserved UPR initiator and contains both kinase and endoribonuclease domains. Following release of GRP78, IRE1 oligomerizes in the ER membrane facilitating trans-autophosphorylation of IRE1 which increases RNase activity [72-74]. IRE1 then splices the mRNA of x-box-binding protein (XBP1) to remove a small stop codon-containing intron which allows translation of the functional transcription factor leading to expression of genes encoding molecular chaperones and ERAD. Further, the activation of IRE1 kinase can promote stress signaling pathways such as c-Jun N-terminal kinase (JNK) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) [75, 76] [77]. Additionally, the RNase activity of IRE1 mediates regulated IRE1-dependent decay (RIDD) in which a subset of ER-targeted mRNAs are
degraded [78, 79]. Collectively, IRE1 drives XBP-1-dependent gene expression that includes ER chaperones and, through RNA degradation, reduces nascent polypeptide entry into the ER to reduce the folding demand [79, 80].

PERK is a trans-ER membrane serine/threonine kinase which is activated by misfolded proteins in the ER lumen. Following release of GRP78, PERK dimerizes and trans- and auto-phosphorylates to increase its kinase activity [63, 81]. PERK phosphorylates the eukaryotic initiation factor (eIF) 2α which leads to binding and inhibition of the guanine nucleotide exchange factor (GEF) eIF2B. This prevents formation of the complex needed to load the 43S ribosome with methionine, thus preventing translation initiation [82, 83]. Under these conditions, some proteins are selectively translated. For example, activating transcription factor (ATF) 4 is translated when eIF2α is phosphorylated. ATF4 translation can lead to expression of CHOP (encoded by the gene ddit3). In many cases, CHOP acts as a proapoptotic factor. Overall, PERK activation reduces the protein load on the ER, and if mechanisms fail to restore homeostasis, initiate cell death.

ATF6 is a transmembrane glycoprotein that is a member of the basic leucine-zipper proteins (bZIP) transcription factor family. Upon the accumulation of misfolded proteins and disassociation of GRP78, ATF6 localizes to the golgi apparatus where is cleaved by site-1 and site-2 proteases [84], revealing a nuclear localization sequence. Subsequently, ATF6 translocates to the nucleus and binds promoter sequences to increase gene expression of ER protein chaperones and UPR regulators to increase folding capacity of the ER [85].
Overall, IRE1, PERK, and ATF6 are activated in response to the accumulation of misfolded proteins within the ER lumen to promote efficient protein folding through the upregulation of protein chaperones and by reducing the folding burden on the ER by eliminating influx of mRNA and polypeptides. If these mechanisms are insufficient, persistent UPR activation will promote apoptosis to eliminate the irreparably damaged cell.

Unfolded Protein Response and Astrocytes

The UPR is activated transiently to restore homeostasis, however, chronic UPR activation has been implicated in Central Nervous System (CNS) diseases including, but not limited to, Alzheimer’s disease (AD), Multiple Sclerosis (MS), traumatic brain injury (TBI), stroke, prion disease, Parkinson’s disease (PD), and Huntington’s disease (HD) [86-100]. Many of these CNS disorders include components of misfolded or aggregated proteins.
proteins. However, the UPR has been primarily studied in neurons and oligodendrocytes (recently reviewed in [101-103]).

Astrocytes comprise a large portion of the CNS and are vital for proper neuronal survival and function [104, 105]. Historically, astrocytes were considered a homogenous population that primarily played a role in structural support to the CNS, however, technical advances and meticulous experimentation have shown that astrocytes are a heterogeneous and dynamic population of CNS-resident cells, playing important roles in both homeostasis and disease [106-108]. For example, astrocytes support synapse formation and function through both physical interactions and secreted molecules [109]. Astrocytes play a role in synaptic pruning during development, which is essential for proper neural development [12]. Astrocytes also support synaptic function by regulating ion homeostasis (Ca^{2+}, Cl^-, K^+), water transport, and neurotransmitter reuptake and recycling [110, 111].

In addition to their supportive role, astrocytes respond to insult and injury, can promote neurotoxicity, and direct CNS inflammation by promoting microglial activation and leukocyte trafficking. Inflammation, particularly proinflammatory cytokines such as interleukin (IL) - 6, IL-1, tumor necrosis factor (TNF) -α, and the complement system, play an important role in neurological diseases and are associated with worsened neurological outcomes [112-116]. Astrocytes are key directors of inflammation within the CNS. It is well established that astrocytes undergo transcriptional and phenotypical changes in response to injury, called astrogliosis [111]. During astrogliosis, astrocytes have increased proliferative capacity, glial fibrillary acidic protein (GFAP) expression increases,
signaling molecules and cytokines are upregulated, the extracellular matrix remodels, and changes in ability of astrocytes to properly regulate synapses and the blood brain barrier (BBB) occur. Reactive astrocytes can have differential roles depending on the injury or disease. For example, reactive astrocytes worsen AD, but during ischemia or spinal cord injury, reactive astrocytes promote overall neural recovery [117-125].

Perturbations in astrocyte function are now implicated in many neurological diseases including PD, AD, ischemic stroke, epilepsy, and ALS ([111]. This highlights the importance of astrocytes in maintaining and directing neurological function. However, the mechanisms by which cellular stressors initiate astrocyte dysfunction that contributes to disease are not well understood. Recently, single cell RNA sequencing (scRNAseq) revealed that subpopulations of astrocytes that are expanded during Experimental Autoimmune Encephalomyelitis (EAE) have increased UPR signaling, suggesting that the UPR is associated with EAE [126]. Additionally, overexpression of spliced XBP1 in astrocyte-like glial cells in C. elegans extends life span [127]. Astrocytes express all the initiating sensors of the UPR (IRE1, PERK, and ATF6) and express the ER stress sensitive molecule, old astrocyte specifically induced substance (OASIS). Further, astrocytes are largely resistant to aberrant ER stress-induced cell death [128]. GRP78 is important in protecting astrocytes as shown by overexpression in the in vitro stroke model of oxygen glucose deprivation [129].

Astrocytes have been demonstrated to express many cytokines, chemokines, and reactive species that contribute to the inflammatory environment of the CNS [130]. Table 1 describes molecules that have been demonstrated to be induced by ER stress in
astrocytes. Further, the UPR has been explicitly linked to initiating inflammation in other cell types. For example, UPR activation augments inflammatory responses stimulated by the bacterial cell wall component lipopolysaccharide (LPS) and can directly drive activation of the acute phase response [131, 132]. Taken together, accumulating evidence suggests that chronic ER stress and UPR signaling in astrocytes may play a pathological role in neurological disease.

**IRE1 in astrocytes**

IRE1 signaling has been linked to cell death and inflammation in the CNS. Evidence of active IRE1 signaling has been reported in post-mortem human tissue in clinically confirmed cases of AD, HD, and glioma in addition to many mouse in vivo and in vitro disease models. In an immortalized astrocytic cell line, SVGA, cells infected with HIV-1 require IRE1 signaling to activate JNK and activator protein (AP) -1 to induce cell death [133]. Further, nitric oxide (NO) has also been demonstrated to activate IRE1-dependent signaling in human glioma cell lines. Treating human astrocytoma (CRT-MG) cells with an NO donor and the ER stress inducer, thapsigargin, increased apoptosis that coincided with IRE1 nuclease activity, IRE1/TRAF2 complex formation, and p-JNK1/2 levels, implying that treatment of NO subsequently activates the IRE1-α/TRAF2/JNK pathway. IRE1 knockdown confirmed that intracellular NO affects IRE1-dependent phosphorylation of CREB in human glioma cells [134]. Together, this suggests pathogenic stimuli (viral infection and reactive nitrogen species) can activate the IRE1 arm of the UPR and contribute to cell death in in vitro astrocytoma cell lines.
In vivo evidence suggests that IRE1 signaling in astrocytes is associated with the neurodegenerative diseases AD and MS. In brain tissue from AD patients, phosphorylated IRE1 is increased and correlates with disease severity based on Braak Staging, a pathology-based characterization of AD [90, 135, 136]. To investigate the role of IRE1 in a mouse model of AD, IRE1 was deleted in the nervous system using Nestin-cre and crossed with the 5XFAD genetic model of AD. Genetic deletion of the RNase domain of IRE1 significantly reduced amyloid deposition and astrocyte activation. Further, deficiency of IRE1 signaling improved synaptic function and long-term potentiation, suggesting restored memory and learning capacity of the mice. This led to the amelioration of disease hallmarks including Aβ_{1-42} production, amyloid plaque deposition, and cognitive deficits. Additionally, deletion of IRE1 reduced astrogliosis, based on GFAP staining, in the 5XFAD hippocampus. In this case, attenuation of gliosis may be through a direct effect on astrocytes or due to reduced overall disease burden [90]. Further AD studies are needed to delineate the astrocyte-specific contributions of IRE1 signaling.

In a large pharmacogenetic screen to identify signaling pathways involved in pathogenic neuroinflammation in MS, astrocytes were stimulated in vitro with TNF-α and IL-1β, two cytokines known to be associated with the pathogenesis of EAE and MS. Here, Wheeler et. al determined that IRE1 is phosphorylated and XBP1 was spliced, suggesting activation of IRE1 signaling during astrocyte-mediated neuroinflammation. To confirm this in vivo, this study used cell-specific lentiviral delivery of short hairpin (sh) – RNA targeting the gene that encodes for IRE1 (ern1) to knockdown expression in astrocytes during active EAE, which reduced disease severity. These studies demonstrated that abrogating expression of IRE1 under control of the astrocyte selective GFAP promoter ameliorated
EAE disease course and reduced inflammatory mediators produced by astrocytes [137]. This suggests that IRE1 signaling in astrocytes is pathogenic in the murine EAE model of MS.

These studies, collectively, imply that IRE1 signaling in astrocytes can be activated by various stimuli and that activated IRE1 can integrate with many signaling pathways that promote inflammation or cell death.

PERK in astrocytes

Activated PERK signaling has been reported in a variety of neurological diseases including AD, MS, prion disease, neurotropic viral infection, and ALS [88, 95, 138, 139]. Using immunocytochemistry to analyze brain tissue of human MS samples, the UPR proteins GRP78, XBP-1, and CHOP were increased in acute MS lesions [140]. In models of prion disease, neuronal cell lines were infected with PrP, the misfolded protein associated with prion disease. Prion infected neurons were more susceptible to cell death, and targeting PERK signaling in in vivo models of prion disease is protective [94, 95, 98]. Some reports show that prolonged expression of CHOP is pro-apoptotic, but this has not directly been demonstrated in primary astrocytes or in vivo models [141, 142]. However, the Venezuelan Equine Encephalomyelitis Virus (VEEV) induces apoptosis of the astrocyte-like glioblastoma cell line (U87Mg) through CHOP expression that is activated by PERK [143]. PERK knockdown in primary astrocytes reduces viral load of VEEV, but there is no difference in viral load between U87Mg cells with or without PERK expression [144].
Further, evidence of activated PERK signaling in astrocytes has been reported in neuropathological studies of human AD and PD brains [88, 145]. Additional studies have established that PERK is phosphorylated in glial cells in brains from tauopathy-associated dementias [139]. In a 2014 study, Devi and Ohno examined the role of a hemizygous PERK knockout crossed to the genetic AD model, 5XFAD. Genetic PERK ablation reduces phosphorylated eIF2α and ATF4. PERK haploinsufficiency in 5XFAD mice partially rescued memory loss in a behavioral fear conditioning model. These cognitive improvements coincided with a reduction in amyloid-β plaque burden in hippocampal and cortical regions of 5XFAD mice. Importantly, the maladaptive effects of PERK signaling were specific to onset of AD; there were no measured cognitive changes in unaffected PERK+/− mice compared to control animals [146].

In sporadic ALS and in the transgenic ALS mouse model that expresses mutant superoxide dismutase (SOD)G93A, immunohistochemistry staining of spinal cords demonstrated that many astrocytes, along with other cell types, expressed CHOP, suggesting that PERK signaling is activated in astrocytes in ALS [138]. Another study modeling ALS in mice demonstrated that astrocytes are activated, as quantified by GFAP immunofluorescence staining. Here, mice expressing wild type human SOD, which has been reported to spontaneously aggregate and model spontaneous ALS, were exposed to the pharmacological inhibitor of N-linked glycosylation (tunicamycin) to induce UPR activation, which was shown to increase SOD1 aggregation. Importantly, wild type littermates did not have a significant increase in GFAP staining upon tunicamycin treatment [147]. This suggests that SOD aggregation and UPR activation enhance GFAP
expression, which is associated with a reactive astrocyte phenotype, in a murine ALS model.

PERK is also active in models of acute brain injury. In the TBI model of controlled closed cortical impact, PERK is phosphorylated and colocalizes with GFAP, a marker of reactive astrocytes. *In vitro* analyses identified that the calcineurin isoform β (CNβ) can interact with PERK to drive its oligomerization and downstream activation of the UPR, independent of CNβ phosphatase activity. Here, the authors found that CNβ loss is detrimental in TBI and photothrombotic stroke models. In stroke, this is associated with reduced phosphorylation of eIF2α and increased GFAP expression. Additionally, overexpression of CNβ attenuated astrocyte toxicity from oxidative and hypoxic insults through a PERK dependent mechanism [100]. This suggests a potential role for PERK signaling in promoting astrocyte survival after acute injury. These findings are consistent with other reports using the blast injury model of TBI in which GFAP expression is induced upon injury and is reduced when treated with the phosphatase inhibitor salubrinal to maintain eIF2α phosphorylation [148]. Salubrinal reduced impulsive-like behavior induced by repeated blast injury, suggesting that prolonging eIF2α phosphorylation in acute injury models may be protective [148]. Similarly, p-eIF2α, GRP78, and CHOP expression is increased post TBI and these markers of ER stress colocalize with GFAP-positive astrocytes and multiple other cell types. Here, salubrinal improved motor function and spatial defects as tested by the Morris Water Maze post-TBI [149]. Although these studies did not directly define functional mechanisms in astrocytes, they provide evidence of reactive astrocyte activation and ER stress during acute neuronal injury. Importantly, while enhancing or maintaining eIF2α phosphorylation immediately after injury provides
protection, reversing the p-eIF2α-mediated translational block using the eIF2B agonist ISRIB during the chronic phase (4 weeks post injury) improves cognitive function [150]. These studies indicate that PERK signaling, and potentially other eIF2α kinases, have differential effects during the acute and chronic phases following cerebral injury. The role of PERK signaling in astrocytes in either phase is unknown and warrants additional astrocyte-specific loss- or gain-of-function studies.

Additionally, Vanishing White Matter Disease (VWM) demonstrates the importance of downstream PERK signaling in astrocytes. VWM is a leukoencephalopathy in which dysfunctional astrocytes are thought to drive pathogenesis [151]. VWM is caused by an autosomal recessive mutation in eIF2B, which reduce function and cause prolonged suppression of protein translation in response to stimuli that promote eIF2α phosphorylation [152, 153]. This highlights a role for phosphorylated eIF2α-driven translational repression in preserving astrocyte homeostasis and directly links signaling components downstream of PERK to neurological disease.

To date, multiple reports link UPR-dependent PERK signaling in astrocytes to inflammatory gene expression and/or neurotoxicity [93, 128, 154, 155]. ER stress-inducing pharmacological agents thapsigargin and tunicamycin promote phosphorylation of eIF2α in primary murine astrocytes [93, 154, 156, 157]. A 2014 study demonstrated that gene expression of inflammatory markers (IL-6, CCL2), astrocyte markers (GFAP, OASIS), and ER stress-related genes (GRP78, CHOP, PERK, ATF4) are upregulated throughout the course of EAE in brain and spinal cord tissue. Downstream markers of PERK activation such as phosphorylation of eIF2α and CHOP expression are exhibited
in thapsigargin-treated astrocytes concomitantly with upregulation of IL-6, CCL2, and CCL20. Additionally, ER stress augmented IL-6 expression induced by IL-6 or oncostatin M (OSM) in a PERK-dependent fashion. This suggests that astrocytes may contribute to the UPR and inflammatory response seen in CNS tissue during EAE. It is important to note that these inflammatory proteins are induced at the protein level even under conditions of phosphorylated eIF2α, which functions to attenuate translation, demonstrating that these proteins are translated during UPR activation [128].

PERK is an important driver of inflammatory gene expression in astrocytes in response to ER stress. A partial (heterozygous) or complete (homozygous) genetic loss of PERK in primary astrocytes was associated with a lower astrocyte-driven expression of as IL-6, CCL2, and CCL20 analyzed by qPCR or ELISA. Further, primary astrocytes treated with thapsigargin and a PERK inhibitor, GSK2606414, reduced production of cytokines and chemokines measured by ELISA. This demonstrates that PERK activation contributes to both transcriptional and translational activation of inflammatory mediators in astrocytes [154]. Therefore, unresolved UPR activation may contribute to prolonged, aberrant inflammatory activation via PERK signaling that may contribute to the non-resolving nature of neurological diseases.

Cytokines such as IL-6, which is driven by PERK activation in astrocytes rely on Janus Kinase (JAK) – Signal Transducer and Activator of Transcription (STAT) signaling to exert their effects. JAK-STAT signaling has been directly linked to astrocyte-driven pathology in neurodegeneration. STAT3 activation occurs in astrocytes in response to acute injury and is required for astrocytes to form glial scars and take on a reactive
astrocyte phenotype (astrogliosis) [158]. Astrocytes in models of AD, HD, and MS also express higher levels of phosphorylated STAT3 [159]. Additionally, JAK inhibition ameliorates disease progression in rodent models of PD and MS [40, 160].

PERK signaling activates downstream signaling in a JAK1 dependent mechanism, and inhibiting JAK1 kinase activity reduced ER stress-induced inflammatory gene expression. Importantly, JAK1 inhibition does not impact all ER stress-induced gene expression. Further, it has been shown that PERK activates JAK1 to drive a subset of gene expression that is distinct from those induced by the JAK/STAT activating cytokine OSM [157]. This demonstrates that UPR signaling modulates inflammatory responses in a manner distinct from traditional inflammatory signaling. Taken together, this evidence suggests that PERK and JAK-STAT signaling in neurodegenerative disease models may promote aberrant inflammation. Targeting PERK signaling in astrocytes may be a mechanism to selectively attenuate immune responses in neurological diseases.

Targeting the UPR to selectively attenuate inflammation is supported by work in other cell types. For example, UPR signaling in macrophages activates proinflammatory cytokine signaling via the IRE1 pathway. Here, ER stress activates the nucleotide-binding oligomerization domain-containing protein (NOD) 1/2 and sXBP1 in an IRE1 dependent manner. Contrary to macrophages, PERK drives IL-6 expression in astrocytes. This highlights that the UPR regulates inflammation using distinct mechanisms in different cell types [161, 162]. ER stress-induced IL-6 production in astrocytes differs from macrophages in that it requires PERK and JAK1 but is independent of IRE1 and nuclear factor-κB (NF-κB) [128, 154]. Additionally, endothelial cells produce IL-6 in response to
ER stress, but here, this IL-6 expression is dependent on both ATF4 and sXBP1 [163].

ER stress induced IL-6 expression in astrocytes does not rely on ATF4 signaling, as demonstrated using siRNA-mediated knockdown of ATF4 in primary astrocyte cultures [157]. This illustrates the need for more careful investigations regarding the nuances of UPR signaling in various cell types. For example, the UPR in the CNS literature focuses heavily on neurons and oligodendrocytes, however, these findings may not apply to astrocytes. Although astrocytes induce IL-6 and other inflammatory molecules in a PERK-dependent fashion, this is not the case for other IL-6 family members. Importantly, ciliary neurotrophic factor (CNTF) is downregulated upon ER stress induction in cultured astrocytes [13]. This suggests trophic support from astrocytes can be restricted by the UPR. Indeed, it has been shown that ER stressed astrocytes lose trophic support for neuronal synapse formation [93].

Collectively, multiple studies have demonstrated that PERK signaling promotes an astrocyte-driven inflammatory response. Although inflammation provides a beneficial and restorative role, chronic inflammation is thought to contribute to neurological disease. PERK signaling in astrocytes may be a target to selectively attenuate damaging inflammation while retaining beneficial inflammatory signaling in the CNS. Further studies and conditional deletion of PERK and downstream signaling components in astrocytes are needed to solidify the role in disease models.

ATF6 and OASIS in astrocytes

ATF6 and old astrocyte specifically induced substance (OASIS (CREB3L1)) are bZIP transcription factors similarly activated in response to ER stress. OASIS is a
molecule primarily expressed in astrocytes in the CNS. Upon activation, it is transported to the golgi apparatus, is cleaved, and the N-terminal domain promotes expression of ERAD-associated genes [164]. ATF6 is activated (cleaved) in embryonal astrocytes during differentiation suggesting a role for ATF6 in astrocyte development [165]. OASIS is also important for astrocyte differentiation. In mice lacking OASIS, astrocyte development was impaired. OASIS was shown to bind the promoter of glial cells missing transcription factor 1 (Gcm1) and promote Gcm1 expression. Gcm1 may regulate GFAP promoter methylation allowing transcriptional activation. The reduced expression of Gcm1 in OASIS−/− mice may, in part, underlie the reduced astrocyte differentiation [165].

To date, few studies have been performed examining the role of ATF6 in astrocytes during disease states. In a murine model of ischemic stroke, middle cerebral artery occlusion (MCAO), ATF6 knockout mice exhibited reduced infarct area as analyzed by the metabolic stain triphenyl tetrazolium chloride (TTC). Concomitantly, ATF6α knockout mice had reduced STAT3 activation and expression of GFAP in the ischemic area of the brain 3 days post MCAO as measured by immunoblotting [166]. This study suggests that ATF6 is protective during ischemia via an astrocyte-dependent mechanism.

OASIS activation has also been linked, in astrocytes, to AD disease mechanisms. Apolipoprotein E (ApoE) is a protein involved in catabolizing triglycerides and the ApoE4 allele is strongly associated with the development of AD, although causal associations between ApoE4 and AD are not fully known, reviewed in [167]. Primary astrocytes expressing mutant APOE, to model human ApoE4, exhibit reduced ApoE expression and increased UPR activation, including cleavage of OASIS and genes downstream of the IRE1 and PERK pathways. This suggests that ApoE can induce cleavage of OASIS in
astrocytes and activate the UPR in astrocytes and promote neuronal toxicity [168]. Collectively, there are limited studies on the role of ATF6 and OASIS, however, these studies demonstrate that activation must be well-regulated for proper astrocyte function.

Non-cell autonomous effects of ER stressed astrocytes

In a 2017 study, Sprenkle et. al was the first to describe that astrocytes can transmit ER stress to other cell types. A phenomenon that was previously described in cancer cells and termed transmissible ER stress (TERS) [169]. This suggested that UPR activation in astrocytes can induce UPR signaling in neighboring cells. In this study, astrocyte conditioned media (ACM) collected from astrocytes treated with the ER stress-inducing agent thapsigargin or tunicamycin was transferred to HT-22 hippocampal neuronal cells. The cells that were exposed to thapsigargin treated ACM exhibited higher gene expression and protein levels of GRP78, spliced XBP1, and CHOP, indicating that astrocytes secrete a soluble factor that stimulates an ER stress response. Further, this study showed that neurons experiencing ER stress also secrete a molecule that induces ER stress in cultures of neurons, astrocytes, and microglia [156]. This study identified that UPR activation can be transmitted between cells of the nervous system. These studies are consistent with previous work that demonstrated that ER stress is also transmissible between cancer or myocardial cells and macrophages, which also respond to ER stress by producing inflammatory molecules, albeit these mechanisms are distinct from those identified in astrocytes [13, 170, 171]. ER stressed astrocytes, through a PERK-dependent process, also increase microglial expression of IL-1β and IL-6 [128]. Independently of PERK, ER stressed astrocytes reduce microglial expression of arginase, CD206 and insulin like growth factor 1 [154]. Together, these data indicate that in
response to ER stress, astrocytes can shift microglia to an inflammatory phenotype. Additionally, Wheeler and colleagues demonstrated that XBP1 knockdown in astrocytes decreases the number of monocytes that traffic to the CNS during EAE. Macrophages that trafficked to the CNS during EAE in the GFAP-driven XBP1 knockdown had reduced expression of inflammatory genes involved in IL-6 signaling, NF-κB signaling, and chemokine signaling. Similarly, microglia in the astrocyte specific XBP1 knockdown had reduced proinflammatory gene expression in comparison to EAE animals with XBP1 expression astrocytes [137].

Astrocyte conditioned media from healthy astrocytes is known to support synaptogenesis [172-174]. To determine if UPR activation impacts the ability of astrocytes to support synapses, Mallucci and colleagues collected astrocyte conditioned media (ACM) from thapsigargin-treated astrocytes. By immunostaining pre and post synaptic terminals, ACM from UPR activated astrocytes was shown to reduce synaptogenesis. Further, inhibiting PERK pharmacologically restored the ability of ACM to promote synapse formation, suggesting that UPR activation via PERK inhibits astrocyte-mediated neurotrophic functions. Further, this study tested if targeting PERK-eIF2α signaling in vivo could be neuroprotective. Using mice that over express prion protein (PrP) and succumb to prion infection. Astrocyte specific lentiviral overexpression of GADD34, an eIF2α-specific phosphatase, was markedly protective in prion-infected mice. GADD34 overexpression (to reduce PERK signaling) in astrocytes prevented neurodegeneration in the hippocampus, had an increased number of pyramidal neurons, reduced astrocyte reactivity based on morphology and GFAP staining, and extended the life span of these mice in comparison to control PrP animals. This study shows both in vitro and in vivo that
UPR activation via the PERK pathway alters the transcriptome and secreted molecules of astrocytes and this is linked to a reduction in neuronal synapse formation. [93]. These studies expand upon and corroborate the previous findings that PERK inhibition is protective in prion infection [87, 89, 94, 95]. Further, this suggests that UPR-activated astrocytes have pathogenic roles in prion infection and identifies PERK signaling as a central driver in this process.

Consistent with the discovery that astrocytes have a significant role in directing the milieu of the inflammatory environment in the CNS, viral infections have also shown to induce the UPR in astrocytes, leading to pathogenic non-cell-autonomous astrocyte dependent pathology. The HIV protein Tat has been shown to induce ER stress in astrocytes leading to GFAP-dependent neurotoxicity [175]. Inflammation and expression of the human endogenous retrovirus protein, syncytin-1, promote ER stress in astrocytes in MS [176]. This study demonstrated that ER stress proteins were upregulated in MS patient brains, along with the human endogenous retrovirus protein (HERV) syncytin-1. Syncytin-1 induces splicing of XBP1 and leads to downstream inflammation. These mechanisms were confirmed by transfecting primary human fetal astrocytes with syncin-1. This induced splicing of XBP1, indicating that the IRE1 pathway is activated. Further, Nos2 was concomitantly upregulated and contributed to oligodendrocyte toxicity in the EAE model. Together, this suggests that IRE1 signaling is stimulated by the HERV protein syncytin-1 to initiate a sXBP1-dependent nitric oxide and neuroinflammatory response.
Additionally, Zika virus has been shown to activate the UPR in astrocytes. ZIKV infection of astrocytes caused an over expression of UPR-related genes BiP, XBP1, CHOP, and growth arrest and DNA damage-inducible protein (GADD) 34. Under these conditions, cell viability was decreased, RNA metabolism genes and micro-RNAs were downregulated, however, astrocyte-derived soluble factors glial cell line-derived neurotrophic factor (GDNF) and neuronal growth factor (NGF) were upregulated, highlighting that some molecules were still being translated under ER stress conditions [177]. However, these results are associated with UPR activation, and direct evidence for the non-cell autonomous action of UPR signaling in astrocytes still requires investigation.

**Figure 5.2 Proposed mechanisms by which endoplasmic reticulum stress signaling in astrocytes impacts the overall CNS environment.**

Here, we propose a model where astrocytes experience endoplasmic reticulum stress during disease states, activating the Unfolded Protein Response. UPR-related molecules are upregulated in astrocytes, concomitant with inflammatory genes. Further, UPR-activated astrocytes have reduced capacity to support neuronal synapses and activated UPR in astrocytes is associated with increased synaptic loss and lower numbers of neurons during disease. Further, astrocytes have the capacity to “transmit” ER stress to other neuronal cell types including other astrocytes, microglia, and neurons. Figure was created in Biorender.
These results lay the groundwork for further studies examining the role of ZIKV and other neurotrophic viral infections in astrocytes.

In summary, astrocytes play a critical role at directing the overall CNS environment due to their close physical and trophic connection to other CNS cells as well as blood vessels. UPR activation is emerging as an important process by which astrocytes influence the survival, activation and function of other CNS resident and infiltrating cells.

Discussion and Perspectives

The UPR has been studied in a multitude of disease states and cell types, however, the astrocyte-specific roles of the UPR is a relatively new field. ER stress has been primarily characterized in the CNS focusing on neurons and oligodendrocytes [155, 178]. As more studies are performed, it is evident that UPR activation has diverse roles in each CNS cell type. A working model is proposed in Figure 5.2. For example, EAE is ameliorated by PERK activation in oligodendrocytes, but PERK knockdown in astrocytes had no effect on the development of EAE [137, 179]. Although the UPR is known to activate pathways that have been associated with apoptosis, there is little evidence that UPR signaling in astrocytes induces cell death. Instead, UPR-activated astrocytes are posited to be in a unique position to contribute to the inflammatory environment of the CNS because astrocytes are the most populous glial cell, can be neurotoxic, and direct CNS inflammation by promoting microglial activation and leukocyte trafficking [7, 8, 180]. Inflammatory and reactive astrocytes are attributed to neurotoxicity in many disease models. Understanding how astrocytes are fine-tuned to produce these neurotoxic responses is of vital importance; neuronal loss cannot be overcome and leads to motor
and cognitive decline. Here, we provide evidence that UPR signaling could substantially contribute to astrocyte-driven neuronal dysfunction. To gain complete understanding of the UPR in the CNS, more advanced cell-specific studies must be completed. Gain and loss of function studies in vivo in astrocyte conditional models are underway and will provide a wealth of information on the functional role of ER stress signaling in astrocytes.

Targeting UPR signaling in astrocytes has been tested in select models of neurological diseases. For example, IRE1 knockdown in astrocytes is protective in the EAE model of MS [137]. Therefore, IRE1 signaling may be pathogenic in neurological diseases that are associated with excessive neuroinflammation. Astrocyte-specific overexpression of GADD34, an eIF2α-specific phosphatase, is protective in prion disease [93], consistent with studies using small molecules to target PERK signaling [95]. These studies suggest a pathogenic role for PERK signaling in astrocytes in diseases driven by protein misfolding. Together, with in vitro evidence that PERK signaling in astrocytes promotes inflammation and that ER stress is transmissible [13, 93, 128, 154, 155, 157], these studies suggest that UPR signaling in astrocytes may profoundly impact multiple neurological diseases. Additional studies using astrocyte-selective Cre drivers such GFAP-Cre [42, 181] and ALDH1L1-CreERT2 [182] to target specific UPR components in additional disease models of neural injury and neurodegeneration will ultimately define ER stress signaling in astrocytes and its impact on disease.

Further, recent studies have established that substantial regional heterogeneity of astrocytes exists [46]. Currently, astrocyte specific UPR signaling has not been characterized or compared across regional locations of the CNS. The location and
composition of misfolded proteins is known to vary amongst each neurodegenerative disease. Therefore, this prompts the possibility that UPR signaling in astrocytes can greatly differ based on location, disease state or stage.

Critically, limited therapies and cures exist for most neurological diseases. Therefore, it is logical to assume that identifying novel therapeutic targets to regulate disease-associated signaling cascades is vital for the design of effective treatments. Considering the well-established association of protein aggregation and accumulation in neurological disorders and the recent advances in astrocyte biology, understanding how astrocytes experiencing ER stress influence the CNS environment may be a critical link in understanding signaling pathways that contribute to neurological dysfunction. ER stress has been linked to neurotoxicity in models of neurodegeneration. However, neurotoxicity may be the result of multiple unconfirmed mechanisms including: 1) initiation of apoptosis by the UPR in neurons, 2) loss of supportive function in other CNS cells, such as glia, 3) UPR signaling in glial cells have a gain of neurotoxic function, or 4) a combination of these possibilities. Altogether, an accumulation of evidence suggests that the duration, cell type, and inflammatory environment of the CNS dictates the consequences of active UPR signaling.
Table 0-1 Endoplasmic reticulum (ER) stress-induced changes in astrocytes

<table>
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<th>Factor</th>
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Bibliography


