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Mechanisms of ATF4-mediated neuronal apoptosis

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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MECHANISMS OF ATF4-MEDIATED NEURONAL APOPTOSIS

by

Patrick Swan

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Unmitigated cellular stress is known to activate apoptosis, an active form of cell death requiring the coordinated expression of pro-death factors belonging to the Bcl-2 family of proteins. Once a critical threshold is reached, the pro-apoptotic effectors, BH3-only proteins, facilitate mitochondrial permeabilization and downstream caspase activation, culminating in cell death. A growing body of evidence suggests that key hallmarks of apoptosis are present in Alzheimer and Parkinson disease, and ischemic *post-mortem* brain tissue. Because of the delayed onset of the apoptotic process, recent efforts have been directed towards intervention strategies to reduce tissue loss and the concurrent symptomatic burden. Previous work from our group has demonstrated that the BH3-only protein, p53 Up-regulated Modulator of Apoptosis (PUMA), is critically important during neuronal apoptosis. In the current study, we investigated potential upstream regulators of PUMA in response to sustained neuronal stress conditions. Surprisingly, we found that ER-stress triggered PUMA expression independently of p53 in primary cortical cultures. Instead, we identified ATF4 and CHOP, key transcription factors of the integrated stress response, as crucial regulators of PUMA expression in response to ER dysfunction. Importantly, CHOP was found to directly transactivate the PUMA promoter. We also established that ATF4 played a significant role in the regulation of neuronal apoptosis triggered by sodium arsenite, an oxidative stressor capable of eliciting p53-independent cell death. ATF4 deletion strongly attenuated PUMA expression and caspase-3 activation induced by arsenite stress. Lastly, we subjected primary cortical cultures to continuous hypoxia, an important physiological stress involving ER dysfunction and reactive oxygen species generation, to identify critical apoptotic regulators. Reduced oxygen was found to be a potent stimulus driving PUMA expression however in the absence of p53 we did not observe any differences in PUMA levels or neuronal apoptosis. Rather, we found that chronic hypoxia induced ATF4, and ATF4 depletion significantly reduced PUMA mRNA and attenuated neuronal death. Because of the growing economic and familial impact of chronic neurodegenerative conditions and ischemic incidents, identifying viable therapeutic targets is critical to preserving functional integrity. We suggest that inhibiting ATF4 may be a valid candidate to consider.

Keywords

Bcl-2 family, Puma, apoptosis, neurodegeneration, ER-stress, ATF4, oxidative stress, p53

Co-Authorship Statement

Some results and text presented in Chapter 3 were sourced from the following Journal of Neuroscience paper published in December 2010:

Galehdar, Z.*, **Swan, P.***, Fuerth, B., Callaghan, S.M., Park, D.S., and Cregan, S.P. (2010). Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *J. Neurosci.* 30, 16938-16948.

* = co-first author

Figures 3.13a, b and 3.15a, b were generated during my enrolment in the Ph.D. program.

Figure 3.1d and Appendices Figures A1, B2 and C1 were generated prior to my enrolment in the Ph.D. program.

Cortical Neuron dissections were largely performed by Meera Karajgikar and Dr. Jenn Guadagno.

Nucleofection and ChIP experiments were performed by Dr. Sean Cregan

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List of Abbreviations

α S	Alpha synuclein
A β	Amyloid Beta
Ac-DEVD-AFC	N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin
AD	Alzheimer Disease
AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator Protein-1
APAF-1	Activating Protease Activating Factor-1
APP	Amyloid Precursor Protein
ASK1	Apoptosis Signal-regulating Kinase-1
ATF3	Activating Transcription Factor-3
ATF4	Activating Transcription Factor-4
ATF6 α/β	Activating Transcription Factor-6 alpha/beta
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
β -TrCP	Beta-Transducin repeats Containing Protein
BAK	Bcl-1 homologous Antagonist Killer
BAX	Bcl-2-Associated X-protein

BCL-2	B-cell Lymphoma -2
BCL-xL	Bcl-2 extra long
BH3	Bcl-2 homology domain-3
BID	BH3-Interacting-domain Death agonist
BIM	Bcl-2-Interacting Mediator of cell death
BiP	Binding Immunoglobulin Protein
bZIP	Basic-region leucine zipper
C/EBP β	CCAAT/Enhancer Binding Protein-beta
CPT	Camptothecin
CHOP	CCAAT-homologous Protein
CK1	Casein Kinase-1
CK2	Casein Kinase-2
CLOCK	Circadian Locomotor Output Cycles Kaput
CREB	cAMP Response Element Binding protein
DNA	Deoxyribonucleic Acid
eIF2 α	eukaryotic Initiation Factor 2 alpha
ERAD	Endoplasmic Reticulum Associated Degradation
ER	Endoplasmic Reticulum
ERSE	Endoplasmic Reticulum Stress Response Element
ETS	Electron Transport System

ERO1 α	Endoplasmic Reticulum Oxidoreductin-1 alpha
FOXO3a	Forkhead box O3a
GADD34	Growth Arrest and DNA Damage-inducible protein 34
GADD45 α	Growth Arrest and DNA Damage-inducible protein 45 alpha
GCN2	General Control Nonderepressible-2
GFP	Green Fluorescent Protein
GSH	Glutathione
GSSG	Oxidized Glutathione
H ₂ O ₂	Hydrogen Peroxide
HRI	Heme-regulated Inhibitor kinase
IRE1 α	Inositol Requiring Enzyme-1 alpha
ISR	Integrated Stress Response
JNK	c-Jun-N-terminal Kinase
LIP	Liver-enriched Inhibitor Protein
MEF	Mouse Embryonic Fibroblast
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger Ribonucleic Acid
NAC	N-acetyl cysteine
NAD ⁺	Nicotinamide Adenine Dinucleotide oxidized

NADH	Nicotinamide Adenine Dinucleotide reduced
NMDA	<i>N</i> -Methyl- <i>D</i> -Aspartate
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOX	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
NRF2	Nuclear factor (erythroid-derived 2)-like 2
O ₂ ⁻	Superoxide Anion
OMM	Outer Mitochondrial Membrane
ONOO ⁻	Peroxynitrite
P53	Tumor Protein p53
P-eIF2 α	Phosphorylated eukaryotic Intiation Factor 2 alpha
PARP-1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate Buffered Saline
PERK	PKR-like ER kinase
PD	Parkinson Disease
PDI	Protein Disulfide Isomerase
PHD3	Prolyl-4-Hydroxylase Domain 3
PKR	Protein Kinase RNA-activated
PP1	Protein Phosphatase-1

PUMA	P53-Up-regulated Modulator of Apoptosis
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT-PCR	Real Time-Polymerase Chain Reaction
S1P/S2P	Site 1 protease/Site 2 protease
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SERCA	Sarco-Endoplasmic Reticulum Ca ²⁺ -ATPase
SN _{pc}	Substantia Nigra <i>pars compacta</i>
SOD1	Superoxide Dismutase-1
TBI	Traumatic Brain Injury
TBS-T	Tris Buffered Saline-Tween20
TG	Thapsigargin
TM	Transmembrane domain
TU	Tunicamycin
UPR	Unfolded Protein Response
UPRE	Unfolded Protein Response Element
uORF	upstream Open Reading Frame
XBP1	X-Box-binding Protein-1
XO	Xanthine Oxidase

Chapter 1

1 Introduction

1.1 The current impact of acute neuronal injury and chronic neurodegenerative diseases

The lasting impact of acute neuronal injury such as stroke and chronic neurodegenerative diseases is considerable and may persist long after the initial diagnosis. For instance, the cost of care incurs a significant financial and psychological burden on caregivers. Recent estimates have shown that stroke costs the Canadian economy upwards of \$3 billion per year. Additionally, if left unchanged the cost of Alzheimer disease is projected to exceed \$240 billion in 2040. Moreover, caregivers themselves are susceptible to developing psychological illnesses, most commonly depression. Further complicating the matter is the growing population of seniors, who are particularly vulnerable to developing cognitive illnesses. Over 80% of Parkinson disease patients are over the age of 65 and treatments directed at managing its symptoms can reach a monthly cost of \$1,000. Taken together, the innovation of new treatments that can improve the quality of life for the patient is a primary initiative. This is particularly important for Amyotrophic Lateral Sclerosis, who on average have a limited life expectancy between 2 to 5 years, following diagnosis (Als.ca; Alzheimer.ca; heartandstroke.ca; Parkinson.ca).

The goal of this thesis is to nominate candidate genes that might play a key role in mediating neuronal death after acute neuronal injury and during chronic neurodegenerative illnesses, thus becoming potential therapeutic targets.

1.2 Pathways of Cell Death

1.2.1 Apoptosis

Apoptosis is an active and regulated form of programmed cell death requiring the induction and interaction of various proteins in order to maintain cellular homeostasis. This process plays a key role in neurodevelopment during synaptogenesis as well as in the removal of damaged, cancerous or senescent cells. Key hallmarks of apoptosis include DNA fragmentation, chromatin condensation, cleavage of cellular components, and the appearance of apoptotic bodies (Buss et al., 2006). The targeted disposal of redundant cells is resolved via phagocytosis of apoptotic debris, thus preventing an inflammatory response (Savill et al., 1989). Despite the necessity for homeostatic apoptosis, a growing body of evidence suggests that apoptotic cell death plays a critical role in the pathology associated with chronic neurodegenerative conditions such as AD (Overmyer et al., 2000), PD (Hartmann et al., 2000; Viswanath et al., 2001), traumatic brain injury (TBI) (Smith et al., 2000), and acute neuronal injury (Rami et al., 2003). Moreover, similar findings have been demonstrated using *in vitro* and animal models of ALS (Kieran et al., 2007), AD (Xie et al., 2013), brain ischemia (Plesnila et al., 2001; Gibson et al., 2001), and PD (Ryu et al., 2002; Perier et al., 2007).

1.2.2 Necrosis

In contrast to the ordered and energy-dependent process of apoptosis, necrotic cell death is characterized as a passive, rapid and deregulated form of cellular injury that is consistently harmful. The loss of membrane integrity facilitates ion fluxes and the escape of cellular contents into the extracellular environment, triggering widespread inflammation and immune cell infiltration (Sauter et al., 2000; Fink and Cookson, 2005). Morphologically, necrosis also displays non-specific DNA cleavage due to the absence of coordinated enzymatic nuclear fragmentation, a notable feature of apoptotic cell death (Fink and Cookson, 2005).

Due to the accelerated pace of necrosis, the goal of this thesis is to elaborate the signaling components regulating the staggered and deliberate process of apoptotic cell death, an execution program that is susceptible to therapeutic intervention.

1.3 The BCL-2 Family

Apoptosis is regulated by the B cell lymphoma-2 (Bcl-2) family of proteins, which is comprised of two classes: pro- and anti-apoptotic effectors. Bcl-2 members are distinguished by the varying presence of 4 unique Bcl-2 homology (BH) domains that permit the binding of death-inducing and survival molecules via their BH3 domain. In healthy cells, BH3-only pro-apoptotic proteins, BID and BAD, require post-translational modifications in order to gain lethality, while *Puma*, *Noxa* and *Bim* are regulated primarily via transcriptional mechanisms. Conversely, anti-apoptotic proteins, Bcl-2, Bcl-xL, MCL-1, are abundantly expressed in unstressed cells. Pro-survival proteins function to sequester the pro-apoptotic, BAX, from interacting with other BAX molecules. In response to apoptotic stimuli, stressed cells induce BH3-only proteins to activate BAX (Figure 1.1). The manner by which this is achieved is controversial although, two competing theories exist to further this notion. In the direct activation model, BH3-only ligands are subdivided into two groups: sensitizers or activators. Sensitizers, like BAD and NOXA, engage pro-survival members to prevent their binding to Activators, PUMA, BIM or BID, which can directly associate with BAX. The indirect model suggests that BH3-only molecules do not interact with BAX. Instead, each BH3-only protein can bind to a varying spectrum of anti-apoptotic proteins and induce the dissociation of BAX, facilitating its oligomerization at the outer mitochondrial membrane (OMM) (Figure 1.2) (Kim et al., 2006; Willis et al., 2007; Youle and Strasser, 2008).

The BCL-2 Family

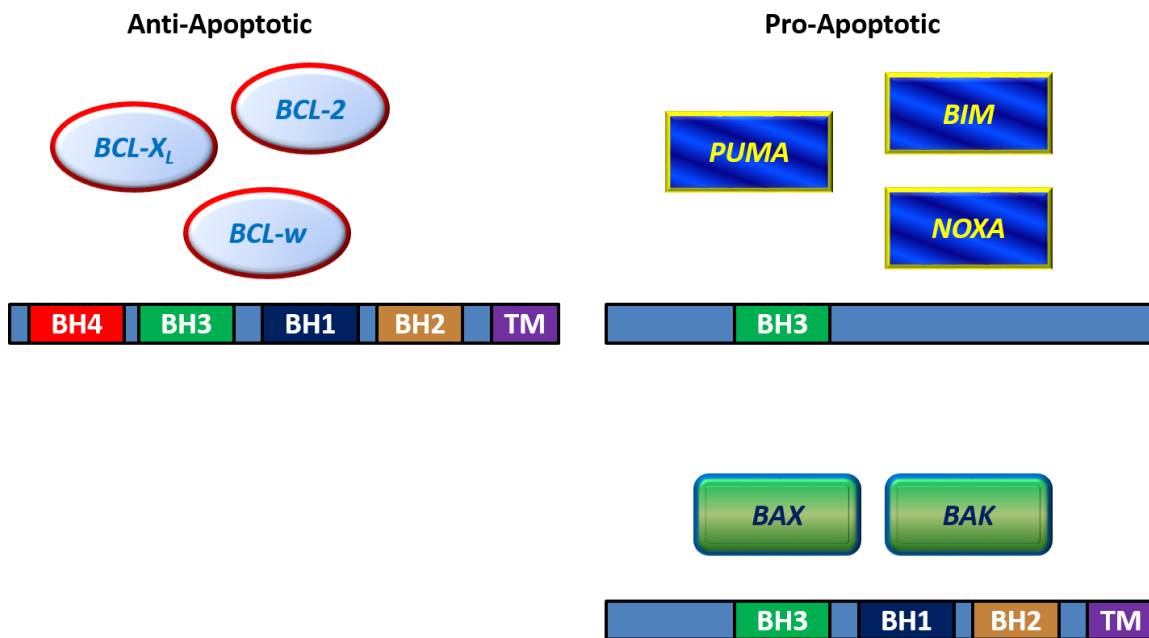
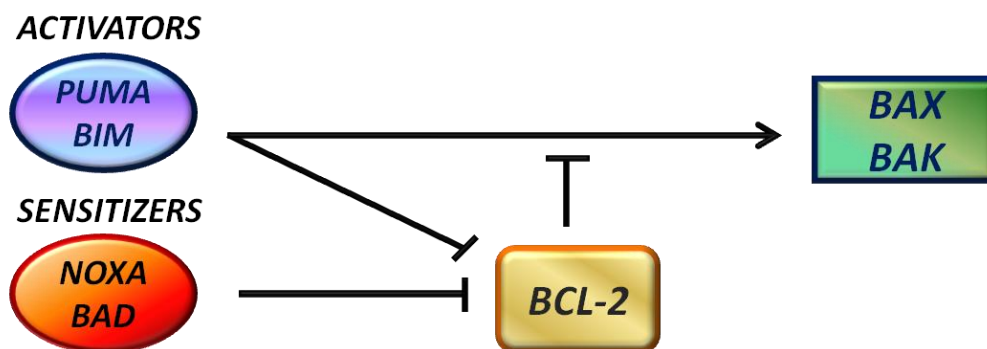


Figure 1.1. The BCL-2 family of proteins.

Apoptosis is regulated by the interplay of pro- and anti-apoptotic proteins. Anti-apoptotic members are comprised of 4 BH domains and a unique C-terminal transmembrane domain (TM), which facilitates organelle docking. Pro-apoptotic variants consist of BH3-only proteins such as PUMA, BIM and NOXA, which harbor a single BH domain. Additionally, BAX and BAK are classified as pro-apoptotic multidomain members that express BH domains 1-3 and a TM (Youle and Strasser, 2008).

Direct Activation Model



Indirect Activation Model

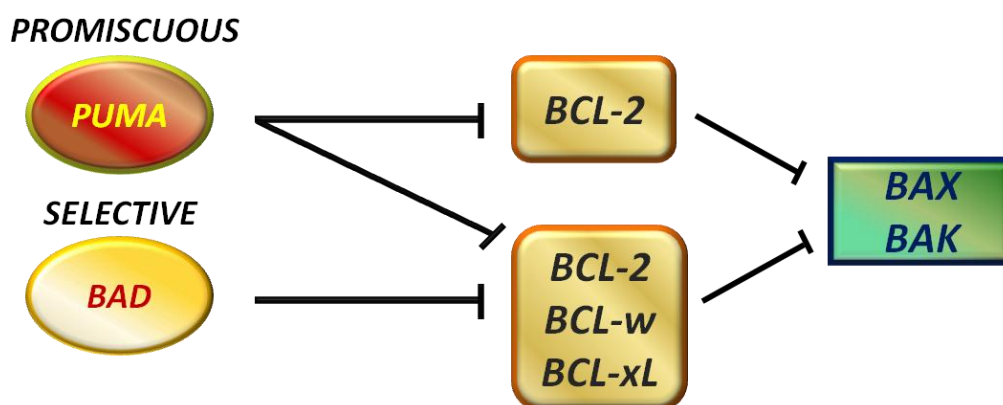


Figure 1.2. Proposed models of BAX activation.

In the direct activation model, PUMA, BIM and BID are ascribed as activators, which implies their ability to engage and activate BAX directly or through the uncoupling of BAX bound to an anti-apoptotic member. In contrast to activators, NOXA and BAD function as sensitizers by dissociating BAX and anti-apoptotic proteins thereby facilitating BAX activation. In the indirect activation model, PUMA can promiscuously disengage any pro-survival variant from its quiescent complex with BAX to trigger apoptosis. As a selective BH3-only protein, BAD is limited to bind specific anti-apoptotic ligands to enable BAX activation (Adams and Cory, 2007).

1.3.1 Pathways of Apoptosis

The extrinsic pathway of apoptosis, also known as the death-receptor pathway, is a critical signaling axis utilized by immune cells, which is modulated by extracellular signaling molecules that bind to cell surface death receptors.

The intrinsic pathway of apoptosis or the mitochondrial pathway, responds to intracellular perturbations such as, disruptions in Ca^{2+} flux, oxidative dysfunction or DNA damage. These apoptotic stimuli shift the balance of Bcl-2 expression favoring the augmentation of BH3-only pro-death ligands. BH3-only proteins can participate in either the direct or indirect activation of BAX. The key function of BAX is to oligomerize at the OMM, forming a pore that connects the intermembrane space to the cytoplasm, facilitating mitochondrial membrane permeabilization and the subsequent release of cytochrome c. In the cytosol, cytochrome c associates with pro-caspase-9, apoptotic protease-activating factor-1 (APAF-1) and dATP, in a complex known as the apoptosome. In the final stage of the apoptotic process, the apoptosome activates a specific family of cysteine-aspartate proteases (caspases) that sequentially deconstruct the cellular architecture (Adams and Cory, 2007; Green and Kroemer, 2004). The resulting cellular debris is phagocytized by circulating immune cells, which prevents widespread inflammation (Figure 1.3) (Fink and Cookson, 2005).

Caspases are classified into 2 categories: initiator (e.g. caspases-2, -8, -9, -10) and executioner (e.g. caspases-3, -6, -7), and are largely expressed as inert zymogens that require proteolysis to become fully functional. Although the extrinsic and intrinsic pathways are distinctly activated, both signaling cascades converge upon caspase-3. Moreover, both pathways have the potential to intersect following the activation of caspase-8, of the extrinsic pathway, which can cleave the BH3-only protein, BID, to its active form, truncated BID (tBID). The conversion of BID enables the intrinsic pathway, facilitating BAX activation and homodimerization at the OMM (Strasser et al., 2000).

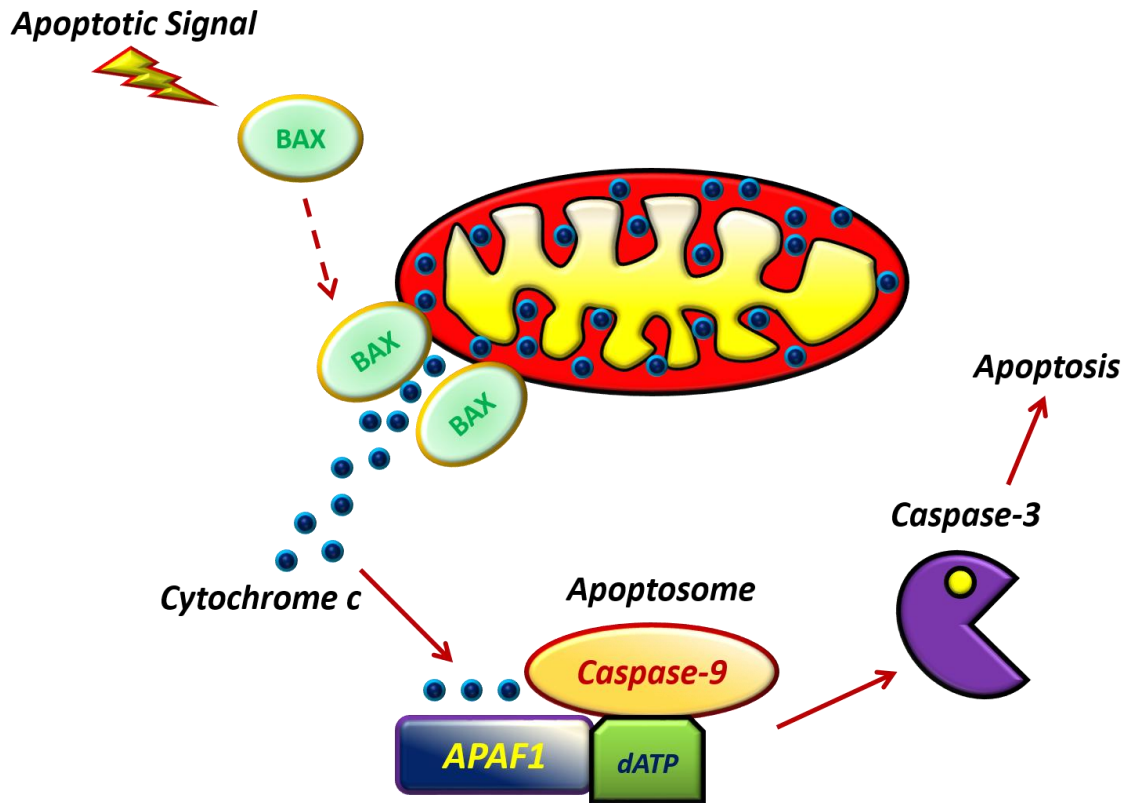


Figure 1.3. The intrinsic pathway of apoptosis.

Upon a fatal stress signal, BAX becomes activated and oligomerization takes place at the OMM. Embedded BAX oligomers form a pore connecting the cytoplasm with the intermembrane space, which allows cytochrome c to escape into the intracellular environment. Cytochrome c then pairs with pro-caspase-9, APAF-1 and dATP, forming the apoptosome, a complex necessary to convert the zymogen, pro-caspase-3, to its active cleaved form. Caspase-3 serves as a key executioner protease that disassembles cellular components (Youle and Strasser, 2008).

1.4 Cell Stress Pathways

1.4.1 Oxidative Stress

Oxidative stress is defined as the imbalance of reactive oxygen species (ROS) generation relative to the detoxifying actions of intrinsic antioxidant enzymes. ROS can cause damage to cellular lipids, proteins and DNA, and this is sufficient to induce apoptosis. The mitochondrion is central to the generation of ROS due to the process of oxidative phosphorylation that yields the vital energy currency of the cell, ATP, and the detrimental byproduct, superoxide anion (O_2^-). Oxidative phosphorylation occurs by the sequential cycling of electrons over mitochondrial complexes (I-IV) of the electron transport system (ETS), embedded in the inner mitochondrial membrane, culminating in the reduction of O_2 to water, at complex IV, and the creation of ATP at complex V. Over the course of electron transfer, leakage may occur at complexes I and III enabling electrons to interact with O_2 , generating of O_2^- , a volatile free radical capable of damaging other molecules. Intrinsic antioxidant defenses such as superoxide dismutase (SOD) can convert O_2^- to hydrogen peroxide (H_2O_2), which can be detoxified by catalase, producing water. In order to bolster further protection, cells also contain non-enzymatic ROS scavengers like vitamin E, vitamin C and glutathione (GSH) (Figure 1.4) (Uttara et al., 2009; D'Autreaux and Toledano, 2007; Perier and Vila, 2012).

ROS can also be generated via enzymatic sources, such as NADPH oxidases (NOX), xanthine oxidase (XO) and neuronal nitric oxide synthase (nNOS), which play key roles in modulating tissue damage following seizures and ischemic insults (Sattler et al., 1999; Luo et al., 2014; Bagetta et al., 2002; Suzuki et al., 2012; Kovac et al., 2014). Although ROS are typically associated with pathological functions, endogenous production of the reactive nitrogen species (RNS) free radical, nitric oxide (NO), can perform important physiological functions such as vasodilation (Quillon et al., 2015).

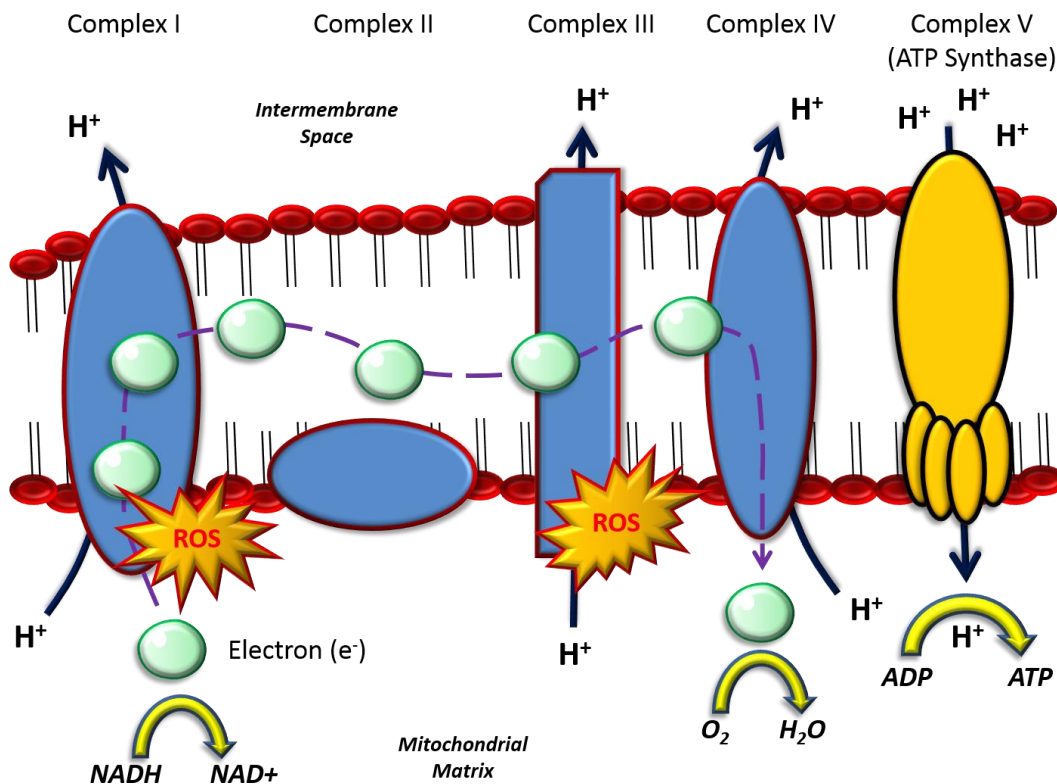


Figure 1.4. The mitochondrial electron transport system.

The mitochondrion relies on the efficient shuttling of electrons through the ETS in order to produce ATP via oxidative phosphorylation. Initially, reduced nicotinamide adenine dinucleotide (NADH) is oxidized to NAD⁺, in the mitochondrial matrix. The liberated electron (e⁻) is then transferred through mitochondrial complexes I-IV, which are dispersed throughout the inner mitochondrial membrane. Protons (H⁺) found in the matrix are also redistributed to the intermembrane space at complexes I, III and IV. As a consequence of e⁻ transport, reactive oxygen species (ROS) formation can occur at complexes I and III. The final step of the ETS involves passing e⁻ through complex IV into the matrix where O₂ is reduced to form water (H₂O). At complex V or ATP synthase, the accumulated H⁺ within the intermembrane space form a significant concentration gradient with the matrix, and diffuse through complex V into the matrix. The kinetic energy generated through the excessive H⁺ transfer powers the enzymatic complex V to generate ATP (Perier and Vila, 2012).

1.4.2 The Endoplasmic Reticulum (ER) stress response

Under normal conditions, the ER directs lipid biosynthesis, Ca^{2+} homeostasis and the modification, transport, and development of nascent proteins. The ER also modulates the degradation of misfolded proteins via a regulated process known as Endoplasmic Reticulum-Associated Degradation (ERAD), which involves cytoplasmic export and disposal by the ubiquitin-proteasome system. During periods of stress, where normal ER function is impaired and improperly folded proteins accumulate beyond the homeostatic threshold, an ER-stress response is triggered. In order to restore balance, the ER implements a specific signaling cascade, the unfolded protein response (UPR), to arrest further protein synthesis thereby reducing the protein load within the ER lumen. UPR signaling also results in the up-regulation of genes involved with the trafficking and degradation of misfolded aggregates (Hetz, 2012).

1.4.2.1 The Unfolded Protein Response (UPR)

The initial phase of the UPR involves the activation of 3 transmembrane sensors: Activating Transcription Factor-6 (ATF6), PKR-like ER kinase (PERK), and Inositol Regulating Kinase 1 (IRE1). Under quiescent conditions, these sensors are held inactive via bound molecules of the ER chaperone, Binding Immunoglobulin Protein (BiP). In response to the increasing presence of misfolded proteins, BiP disengages the luminal border of the ER sensors and binds unfolded aggregates, targeting them for ERAD (Hetz, 2012).

ATF6 is a Basic-region leucine zipper (bZIP) member of the ATF/CRE family of transcription factors and is expressed as 2 distinct isoforms, ATF6 α (90kDa) and ATF6 β (110kDa) that exist as ER membrane-bound proteins (Yoshida et al., 2001). In response to ER-stress, ATF6 α and ATF6 β are released from inhibition via BiP, translocate to the Golgi Apparatus where they are processed by Site 1 and Site 2 Proteases (S1P/S2P), yielding 50-60 kDa transcriptionally active fragments, ATF6 α_f /ATF6 β_f . Although both ATF6 α_f and ATF6 β_f can transactivate the same promoters consisting of ER-Stress Elements (ERSE) or Unfolded Protein Response Elements (UPRE), it is believed that ATF6 α drives transcription whereas ATF6 β represses ER-stress-related gene expression

(Thuerauf et al., 2004). Additionally, it was demonstrated that the cleavage of ATF6 α appears prior to the accumulation of ATF6 β_f , supporting the notion of an endogenous deactivating signal (Haze et al., 2001). The role of ATF6 α is primarily to facilitate ERAD via ER chaperone expression (Haze et al., 1999; Ye et al., 2000).

The induction of the ubiquitous isoform of IRE1, IRE1 α requires homodimerization and autophosphorylation (Bertolotti et al., 2000). Upon activation, IRE1 α can promote the expression of chaperones to facilitate ERAD, and has the potential to modulate apoptosis. Following the dissociation of BiP, the cytoplasmic domain of IRE1 α employs its endoribonuclease activity to cleave a 26-nucleotide sequence from an unspliced *X-box binding protein-1 (Xbp1)* mRNA, yielding the bZIP transcription factor, XBP1 $_s$, which regulates the expression of ER chaperones independently or cooperatively with its potential binding partner, ATF6 α_f (Lee et al., 2003). XBP1 is thought to play a key role during brain development as evidenced in neurons lacking XBP1, which displayed poor neurite outgrowth and reduced axonal branching (Hayashi et al., 2007). IRE1 α can also mediate apoptosis via the activation of Apoptosis Signaling Kinase-1 (ASK1), which in turn induces Jun N-terminal Kinase (JNK) (Tobiume et al., 2001). JNK has been linked with BIM activation, and the down-regulation of Bcl-2 (Putchu et al., 2003; Yamamoto et al., 1999).

The final ER-stress sensor, PERK is activated by mounting levels of protein aggregates in the ER compartment and the release of inhibition by BiP (Bertolotti et al., 2000). Once BiP has dissociated from the luminal domain of PERK to engage misfolded proteins, PERK dimerizes and undergoes autophosphorylation, targeting eukaryotic Initiation Factor-2 α (eIF2 α) for phosphorylation at Serine 51 (P-eIF2 α). This results in a global attenuation of protein translation to ease the load of unfolded proteins in the ER. The process of eIF2 α phosphorylation in response to stressful stimuli is known as the integrated stress response (ISR). Other cellular stresses capable of inducing the ISR are mediated by specific eIF2 α kinases such as, Protein Kinase RNA-activated (PKR) in response to dsRNA, General Control Nonderepressible 2 (GCN2) activated by amino acid deprivation, and Heme-Regulated Inhibitor Kinase (HRI) triggered by heme-deficiency (Baird and Wek, 2012). Although protein synthesis is reduced under these

conditions, a specific subset of mRNAs are preferentially translated, such as Activating Transcription Factor-4 (ATF4), C/EBP Homologous Protein (CHOP), and Growth Arrest and DNA damage-inducible 34 (GADD34) (Figure 1.5). This specialized mechanism of translation is thought to involve the presence of inhibitory uORFs that are bypassed during translational arrest, allowing for the efficient ribosomal scanning of the coding region (Vattem and Wek, 2004; Lee et al., 2009; Palam et al., 2011).

ATF4 is a critical downstream effector of the ISR regulating a variety of responses including antioxidant gene expression, amino acid synthesizing enzymes and pro-apoptotic machinery (Ameri and Harris, 2008; Hetz and Mollereau, 2014). CHOP is an ATF4-target gene known to facilitate the apoptotic process via the down-regulation of Bcl-2 (McCullough et al., 2001). GADD34 encodes a regulatory subunit of the Protein Phosphatase-1 (PP1) -containing complex that is essential in modulating the phosphorylation status of eIF2 α , and thus serves as a negative regulator of translational arrest (Novoa et al., 2003).

The failure to resolve ER-stress due to prolonged UPR activation, leads to an adaptive switch from homeostatic restoration to an apoptotic profile, for reasons that remain unclear, but may be due to the accumulation of ATF4 and CHOP, which can induce pro-apoptotic mRNAs (Rutkowski et al., 2006; Hetz and Mollereau, 2014). For example, CHOP has been shown to up-regulate PUMA and BIM expression while suppressing Bcl-2 mRNA in response to ER dysfunction (McCullough et al., 2001; Cazanave et al., 2010; Puthalakath et al., 2007).

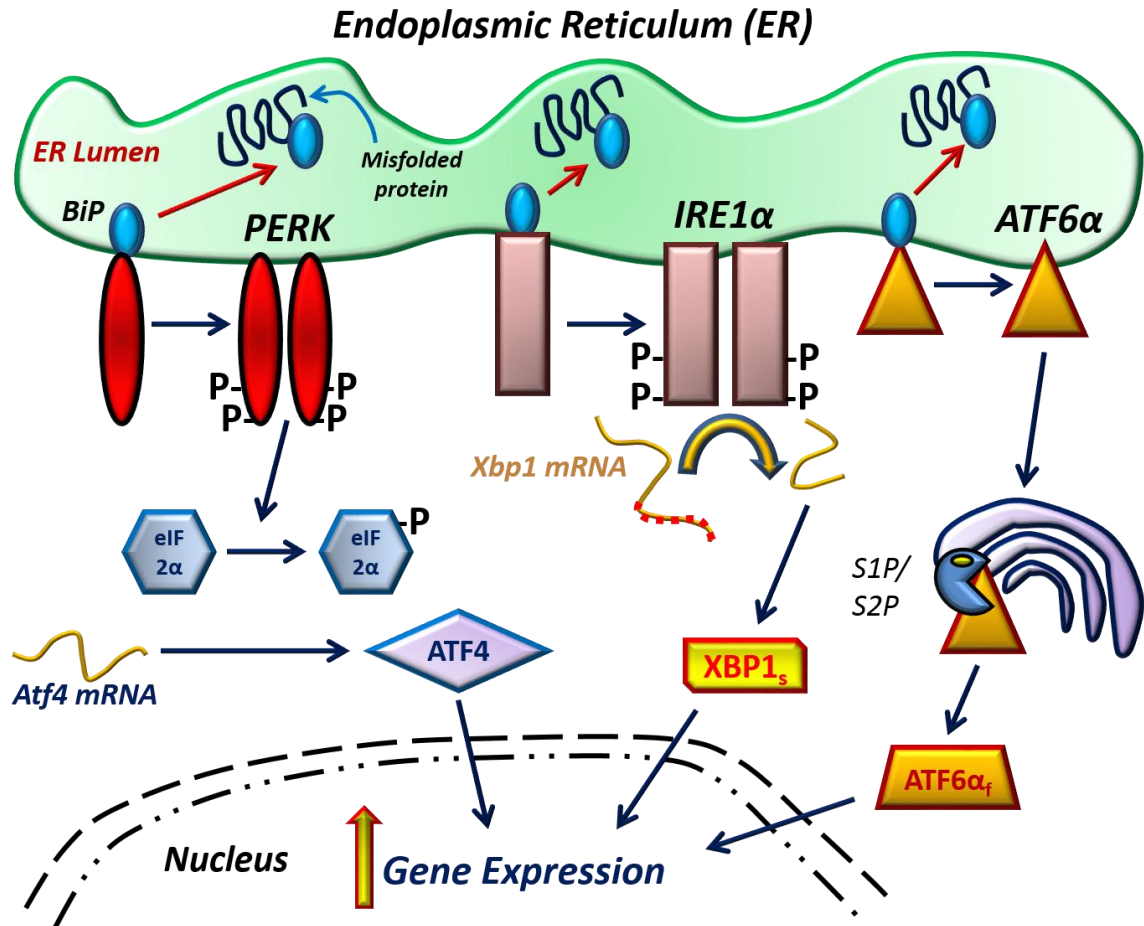


Figure 1.5. The Endoplasmic Reticulum stress response.

The accumulation of misfolded proteins within the ER lumen induces the ER chaperone, BiP, to disengage the luminal domains of ER-membrane bound sensors: PERK, IRE1 α and ATF6 α , and sequester the newly formed aggregates. Upon dissociation from BiP, all the ER sensors are activated. PERK dimerizes and is autophosphorylated, which leads to the phosphorylation of eIF2 α and a reduction in protein synthesis. Under this condition, *Atf4* mRNA is uniquely translated and generates a potent transcriptional regulator. IRE1 α activation also requires dimerization and autophosphorylation, which results in the processing of *Xbp1* mRNA into a truncated and transcriptionally active species, XBP1_s. Finally, ATF6 α localizes to the Golgi Apparatus where it undergoes enzymatic cleavage via Site 1 and Site 2 proteases (S1P/S2P), generating the transcription factor, ATF6 α_f (Hetz, 2012).

1.4.2.2 The ATF/CREB Family of Transcription Factors

ATF4 is a critical bZIP transcriptional regulator of the ISR that directs the expression of amino acid synthesizing enzymes, antioxidant and chaperone responses, and pro-apoptotic genes (Baird and Wek, 2012). Members of the ATF/CREB family are composed of a DNA-binding basic region and a promiscuous leucine zipper dimerization domain that enables several combinations of homo- and hetero-dimers to drive transcription (Ameri and Harris, 2008). The composition and function of the dimers is strongly dependent upon the nature of the stress and will dictate gene repression or activation (Lassot et al., 2005; Soda et al., 2013). For example, ATF4 can interact with Activator Protein-1 (AP-1) and other C/EBP family members that recognize and bind to the CRE consensus site (TGACGTCA). Levels of ATF4 have been demonstrated to accumulate in response to a wide array of cellular stresses, such as ER and oxidative dysfunction, hypoxia, A β toxicity and nutrient deprivation (Armstrong et al., 2010; Ryu et al., 2002; Halterman et al., 2008; Baleriola et al., 2014; Lange et al., 2008). Although little is known regarding ATF4 transcription, *Atf4* mRNA is thought to be induced by the transcription factors, NRF2 and CLOCK, whereas the liver-enriched inhibitory protein (LIP) isoform of CCAAT-enhancer-binding protein- β (C/EBP β) has been shown to antagonize its expression (Afonyushkin et al., 2010; Miyamoto et al., 2011; Igarashi et al., 2007; Dey et al., 2012). In order for the *Atf4* message to be efficiently translated, eIF2 α must be phosphorylated at serine 51, a modification indicative of translational suppression. Interestingly, *Atf4* mRNA exhibits two upstream open reading frames (uORF) 1 and 2 that pose as positive and negative-acting elements, respectively. Stimuli that induce the ISR will promote P-eIF2 α and delay the reacquisition of the translational complex as a manner to bypass uORF 2, leading to the enhanced translation of the *Atf4* coding region (Vattem and Wek, 2004).

ATF4 protein stability is thought to be modulated by several mechanisms. Firstly, by the ATF4-inducible GADD34, a negative regulator of P-eIF2 α , a condition associated with optimal ATF4 translation and the resumption of protein synthesis (Novoa et al., 2003). Second, ATF4 has been shown to be phosphorylated on serine residues by the protein kinases, CK1 and CK2, spanning amino acids 215-219, a region thought to facilitate the

interaction with the F box protein, β -TrCP leading to the ubiquitination and degradation of ATF4 (Frank et al., 2010; Ampofo et al., 2013). Finally, ATF4 stability was shown to be heavily dependent upon the activity of the oxygen sensor, prolyl-4-hydroxylase 3 (PHD3), and the hydroxylation of prolyl residues within the oxygen degradation domain of ATF4. During periods of normoxia, PHD3 function is robust and suppresses ATF4 accumulation. Conversely, hypoxia impairs PHD3 activity thus allowing the enhancement of ATF4. Whether PHD3-mediated hydroxylation of ATF4 involves β -TrCP-induced ubiquitination, and subsequent degradation, remains unclear (Köditz et al., 2007).

1.4.3 ER-Stress and Oxidative Stress are linked

Unmitigated ER-stress leads to the resumption of protein translation and the accumulation of pro-death modulators of the UPR such as, ATF4 and CHOP. The termination signal that recapitulates translation is carried out by the joint action of PP1/GADD34 complex leading to the dephosphorylation of eIF2 α (Hetz, 2012). Both ATF4 and CHOP play a pivotal role in the resumption of translation via the regulation of GADD34 expression (Han et al., 2013). Because the rate of protein synthesis is increased, there is a corollary incline in the formation of disulfide bonds within the ER lumen. This is achieved by the cooperative functioning of the critical foldases, ER oxidoreductin-1 α (ERO1 α) and Protein Disulfide Isomerase (PDI), which are induced as a result of prolonged UPR stimulation. In the ER lumen, ERO1 α oxidizes PDI, which then transfers its electrons to client thiol proteins enabling disulfide bond formation. As a by-product of continuous ERO1 α -PDI functioning, ERO1 α can eventually reduce O₂ to H₂O₂, thereby generating ROS indirectly through enhanced translation. One of the endogenous coping mechanisms relies on the scavenger GSH, which is converted to oxidized GSH (GSSG) throughout the folding process. Even though GSH is abundant in multiple cellular compartments, the strongly oxidizing environment within the ER can act as a sink to GSH defenses. The depletion of GSH pools to GSSG impairs ROS scavenging within the cell, in particular, at the mitochondrion during oxidative phosphorylation where electrons can escape the ETS to generate ROS (Tu and Weissman et al., 2004). It was recently demonstrated that targeted knockdown of ERO1 α enhanced

survival in response to either ATF4 or CHOP overexpression (Han et al., 2013). Moreover, cell death triggered by prolonged ER-stress requires mitochondrial involvement, as demonstrated in p^0 cells, which lack mitochondria (Haynes et al., 2004). This finding strongly implicates mitochondrial ROS generation as a key contributor to apoptosis triggered by extended ER dysfunction.

1.4.4 DNA damage

1.4.4.1 P53

The tumor suppressor, p53, is a critical transcriptional activator capable of mounting cell death responses, attenuating cell division and inducing DNA repair. Collectively, these functions have labeled p53 as, “the guardian of the genome”, and are beneficial against the cycling of damaged cells harboring DNA mutations with the potential of becoming neoplastic. Indeed, p53 is mutated in 50% of all human cancers largely within its DNA-binding domain, which impairs its transcriptional function. P53 is maintained at low levels throughout the body by the protein MDM2, which facilitates its ubiquitination and degradation, until it is stabilized by stressful stimuli, like DNA damage. Other stresses capable of accumulating p53 include oxidative stress, severe hypoxia and chemotherapeutic agents (Perier et al., 2007; Hammond et al., 2002; Haupt et al., 2003). Under conditions where too much damage has accrued, p53 can induce several pro-death genes linked to both the extrinsic and intrinsic pathways of apoptosis. P53 can up-regulate key death-receptor associated genes, Fas and DR5, that play an essential role during the initial phase of the extrinsic pathway. The intrinsic pathway can be facilitated by p53-dependent increases in BAX, NOXA, PUMA and APAF-1 (Haupt et al., 2003). The adaptive switch from a restorative signaling axis converting to an apoptotic program is speculated to involve shifting levels between the cell cycle inhibitor, p21 and rising amounts of PUMA (Yu et al., 2003).

1.5 Chronic Neurodegenerative Disease

Alzheimer Disease (AD) is a chronic neurodegenerative condition resulting in the gradual decline of cognitive and executive functions. A hallmark of the disorder is the accumulation of toxic plaques consisting of amyloid-beta ($A\beta$) deposits and neurofibrillary tangles composed of hyperphosphorylated tau. $A\beta$ is produced by the sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretase enzymes. The most common cleavage variant of APP is a 40-amino acid species however it is the 42-amino acid product that is a key initiator of $A\beta$ aggregation and neuronal cell death (Perrin et al., 2009; Palop and Mucke, 2010; Nakagawa et al., 2000; Biswas et al., 2007; Feng et al., 2014). It was recently demonstrated that amyloid plaques contribute to the induction of neuronal apoptosis *in vivo* (Xie et al., 2013).

Parkinson Disease (PD) is the second most common neurodegenerative condition after AD, and involves a gradual loss of neuronal tissue in the midbrain. Symptoms of PD include deficits in motor control due to the progressive ablation of dopaminergic neurons of the *substantia nigra pars compacta* (SN_{pc}) located in the mesencephalon. Other distinguishing pathological features include the presence of intraneuronal inclusions comprised of aggregated alpha-synuclein (αS), known as Lewy bodies, and mitochondrial deficits. The exact mechanism leading to dopaminergic cell death is still unclear but may involve oxidative dysfunction originating from a breakdown in the shuttling of electrons within mitochondria. Indeed it has been shown that mitochondrial complex I deficiency is pronounced in autopsied PD brains (Mizuno et al., 1989). Moreover it is known that exogenous exposure to 1-methyl-4-phenyl-1, 2, 3, 6-tetra-hydropyridine (MPTP), which is converted intrinsically to 1-methyl-4-phenylperidinium (MPP^+), is a potent inhibitor of complex I function and induces Parkinsonism in humans and rodents.

Amyotrophic Lateral Sclerosis (ALS) or Lou Gehrig's disease is a progressive neurodegenerative disorder specifically affecting motor neuron survival resulting in profound skeletal muscle disruptions ultimately becoming fatal. Although most cases of ALS are sporadic in origin, a smaller portion is inherited arising from mutations in the superoxide dismutase-1 (SOD1) gene, an important component of the body's antioxidant defense system. It is suspected that impaired SOD1 function facilitates oxidative stress, which if improperly buffered can trigger apoptosis (Kieran et al., 2007; An et al., 2014).

1.5.1 The BCL-2 Family and Neuronal Cell Death

A substantial body of work has shown that the Bcl-2 family plays an essential role in the pathogenesis associated with acute neuronal injury (ischemia, epilepsy, TBI) and chronic neurodegenerative conditions (PD, AD, ALS). As such, many groups have employed the use of gene-specific knockout mice to evaluate their influence in this context.

1.5.2 The BCL-2 Family and Chronic Neurodegenerative Disease

Within the context of chronic neurodegeneration, the pro-apoptotic Bcl-2 member, BAX, was found to be significantly enriched after A β treatment *in vitro*, and in autopsied AD brain tissue, relative to age-matched controls (Paradis et al., 1996; Su et al., 1997; Lu et al., 2005). Similar findings were demonstrated in human PD nigral neurons and neurotoxin-induced experimental PD in the mouse midbrain, where BAX expression was robustly accumulated (Tatton, 2000; Vila et al., 2001). Because BAX is an essential gateway of the apoptotic process, the targeted disruption of BAX was pursued to establish a critical link between neuronal death and chronic neurodegenerative pathology. This notion was supported by recent work indicating that BAX-depleted neurons were significantly protected against A β toxicity, a modulator of caspase-dependent neuronal apoptosis *in vivo* (Kudo et al., 2012; Xie et al., 2013). Additionally, the neurotoxic effects of drug-induced PD were completely mitigated in the SN_{pc} of BAX^{-/-} mice, in contrast to wild type controls (Vila et al., 2001). In an animal model of ALS, BAX^{-/-}-SOD1^{G93A} mice showed enhanced motor neuron survival and lifespan compared with SOD1^{G93A} littermates (Gould et al., 2006; Reyes et al., 2010). Interestingly, BAX oligomerization

was profoundly increased in human *post-mortem* ALS brains, relative to control cases (Martin, 1999).

Knockdown of the BH3-only protein, BIM reduced neuronal death triggered by A β toxicity *in vitro*, while autopsied AD brain tissue has identified BIM as a potential contributor to neuronal loss *in vivo* (Biswas et al., 2007). BIM was also noted as a key participant in the modulation of dopaminergic cell loss in an animal model of PD, and disease onset and survivability in ALS model mice (Perier et al., 2007; Hetz et al., 2007). Similarly, double mutant SOD1^{G93A}-PUMA^{-/-} mice showed delayed motor neuron death, relative to SOD1^{G93A}-PUMA^{+/+}, in an ALS animal model of disease (Kieran et al., 2007). Moreover, PUMA has been implicated as a key mediator of MPP+ and A β -induced neuronal apoptosis, and was strongly up-regulated within the brains of AD model mice, compared with littermate controls (Steckley et al., 2007; Akhter et al., 2014; Feng et al., 2014). Taken together, there is sufficient evidence to suggest the involvement of the intrinsic pathway in the pathogenesis of several progressive neurodegenerative conditions.

1.5.2.1 PUMA and Neuronal Cell Death

PUMA was originally described as a p53-target gene in response to DNA damaging agents, but could also be induced independently of p53 by serum-withdrawal and dexamethasone treatment, in non-neuronal systems (Nakano and Vousden, 2001; Yu et al., 2001; Han et al., 2001). The discovery of p53-independent apoptotic gene expression was critical for the design of chemotherapeutic agents that could circumvent p53 mutations in cancer, which impair its transcriptional function and facilitate cellular transformation. Although some of the difficulty surrounding the treatment of cancer involves a lack of p53-mediated apoptosis, it is rather the heightened response of p53 that is problematic in acute neuronal injury and in chronic neurodegenerative conditions. For instance, p53-null mice show reduced infarct sizes compared with wild type, in an *in vivo* stroke model (Crumrine et al., 1994). p53 has also been identified in *post-mortem* human AD, PD and ALS nervous tissue (de la Monte et al., 1998; Kitamura et al., 1997). Consistent with this, the Parkinsonian mimetics, MPP+ and 6-OHDA, have been shown to induce PUMA in a p53-dependent manner (Bernstein et al., 2011; Bernstein and

O'Malley, 2013). Spinal tissue from ALS transgenic mice expressing SOD1^{G93A} showed a progressive increase in *Puma* mRNA, relative to wild type. Moreover, PUMA^{-/-}-SOD1^{G93A} mice displayed significantly longer motor neuron survival (Kieran et al., 2007). Also, it was recently demonstrated that A β treatment *in vitro* can trigger PUMA expression, which is partially dependent upon p53 (Akhter et al., 2014; Feng et al., 2014). This notion was extended in neuronal cells when it was later demonstrated that PUMA could be induced in p53-deficient neurons treated with ER- and oxidative stressors (Fricker et al., 2010; Wong et al., 2005). Some proposed p53-independent transcriptional activators of PUMA include, p73, FOXO3a, ATF4, CHOP, AP-1 and E2F1 (Fricker et al., 2010; Melino et al., 2004; Akhter et al., 2014; Feng et al., 2014; Qing et al., 2012; Cazanave et al., 2010; Hershko and Ginsberg, 2004).

1.5.3 Oxidative Stress and Chronic Neurodegenerative Disease

Oxidative dysfunction was recently implicated as a probable mechanism instigating caspase-dependent neuronal loss *in vivo*, in an animal model of AD. Healthy neurons within close proximity to amyloid plaques were found to undergo oxidative stress and subsequent neuronal death (Xie et al., 2013). Although AD, PD and ALS are unique with respect to their distinctly affected neuronal populations, these conditions share a similar trait throughout their pathologies in that they are proteopathies. Specifically, A β production can be furthered by ROS leading to enhanced plaque deposition, α S can be modified into a more aggressively aggregating species and nitration of SOD1 impairs its antioxidant capacity, indirectly facilitating toxic ROS modifications to other proteins (Giasson et al., 2000; Aoyama et al., 2000; Liu et al., 2007; Leuner et al., 2012).

Although oxidative stress is thought to play a significant role in the pathogenesis of several neurodegenerative diseases, little is known regarding the signaling events regulating neuronal death. In order to elucidate those mechanisms, multiple *in vitro* models have been employed using synthetic inducers of oxidative dysfunction capable of replicating the phenotypes observed *in vivo*. For example, PD is characterized by the gradual depletion of dopaminergic neurons within the *substantia nigra pars compacta*

(SN_{pc}), a region of the midbrain responsible for motor control tuning. One of the *in vitro* modeling systems involves the use of animal-sourced mesencephalic neurons treated with the toxic metabolite of MPTP, MPP⁺, a synthetic inducer of oxidative stress capable of selectively destroying the SN_{pc}. Similar to the observed deficit in human PD *post-mortem* brain tissue involving reduced amounts of mitochondrial complex I, MPP⁺ functions to specifically inhibit complex I and impair electron flow through the ETS (Mizuno et al., 1989). Because the mitochondrion is a primary hub for the generation of ROS, perturbations of the ETS can overwhelm intrinsic antioxidants, leading to oxidative stress and apoptosis. It has been suggested that oxidative stress-induced neuronal apoptosis triggered by MPP⁺ requires *de novo* gene expression and this likely involves key contributions from the Bcl-2 family (Choi et al., 1999; Steckley et al., 2007). Although oxidative dysfunction can induce the expression of multiple BH3-only genes, only PUMA was critical for neuronal apoptosis whereas BIM and NOXA were dispensable (Steckley et al., 2007). One of the key transcriptional regulators of PUMA is the tumor suppressor, p53, which has been shown to mediate PUMA expression in response to specific oxidative stressors and not with others, indicating the existence of p53-independent pathways (Nakano and Vousden, 2001; Yu et al., 2001; Steckley et al., 2007; Fricker et al., 2010). One of those agents is the environmental toxicant, sodium arsenite (arsenite), which has been shown to induce a potent pro-oxidant function in part via protein oxidation and lipid peroxidation (Samuel et al., 2005; Lin et al., 2007). Moreover, arsenite has been widely used experimentally in a variety of *in vitro* and *in vivo* models of neurotoxicity (Fricker et al., 2010; Mengesdorf et al., 2002; Wong et al., 2005; Namgung and Xia, 2000; Lin et al., 2007; Fan et al., 2010). Furthermore, arsenite has been shown to mediate apoptosis in a PUMA-dependent but p53-independent manner, suggesting that other factors compensate for the absence of p53, in this context (Fricker et al., 2010).

1.5.4 ER-stress and Chronic Neurodegenerative Disease

Protein aggregation is a hallmark feature in a variety of neurodegenerative conditions such as, A β peptides in AD, α -synuclein (α S) in PD, and mutant SOD-1 in ALS (Taylor et al., 2002; Soto, 2003; Kikuchi et al., 2006). Similarly, autopsied human AD brains showed a pronounced up-regulation of ER-stress-related factors, compared with control brains (Yoon et al., 2012; O'Connor et al., 2008; Baleriola et al., 2014). Baleriola and others (2014) recently demonstrated that targeted knockdown of ATF4 was able to significantly reduce A β -induced neurotoxicity *in vivo*. Consistent with this, current evidence suggests that pro-apoptotic factors linked with the ER-stress pathway, PUMA and BAX, play key roles in the modulation of neuronal death triggered by A β stress (Akhter et al., 2014; Feng et al., 2014; Kudo et al., 2012).

A growing body of work suggests that the UPR is robustly activated in PD and ALS *in vivo*, although its involvement as a deleterious component in the progression of disease is controversial (Kikuchi et al., 2006; Hoozemans et al., 2007; Atkin et al., 2008; Sun et al., 2013). As suggested previously, ATF4 was associated with a pro-death role in AD whereas its role in PD seems to favor a protective function. For example, ATF4 was detected in human *post-mortem* midbrain sections, and ATF4 knockdown sensitized neuronal cells to MPP⁺ due to a lack of Parkin expression, a transcriptional target of ATF4 (Bouman et al., 2011; Sun et al., 2013). Another ATF member, ATF6 α was recently shown to play a key role in the preservation of dopaminergic neurons against MPTP neurotoxicity *in vivo* (Egawa et al., 2011). Data from ATF6 α -null mice exposed to chronic ER-stress *in vivo* suggests that ATF6 α plays a key role in longevity by alleviating continuous stress (Wu et al., 2007). Additionally, knockdown of XBP1 within the adult mouse SN_{pc} was found to induce extensive neurodegeneration, as evidenced by the pronounced reduction in dopaminergic neurons, the enhanced expression of ER chaperones and the pro-apoptotic factor, CHOP (Valdés et al., 2014). Conversely, overexpression of XBP1_s into the mouse SN_{pc} preserved dopamine-producing neurons challenged with a PD-inducing neurotoxin (Valdés et al., 2014). Taken together, these data suggest that consistent chaperone production throughout life, mediated by ATF6 α and XBP1_s, is essential for long-term survival.

Some recent findings have supported the notion that interference in chaperone signaling can trigger disease course. For instance, α S is known to be significantly enriched in human PD brains, and knock-in mice carrying a missense mutation (A53T) in α S showed a pronounced interaction between A53T α S and BiP, which is thought to sensitize neurons to ER-stress (Colla et al., 2012).

The persistent activation of the UPR has been observed in autopsied ALS tissue and is suspected to precede disease onset and reduce survivability, as evidenced in ALS model mice, compared to mutant $SOD1^{G86R}$ - $ATF4^{-/-}$ littermates (Atkin et al., 2008; Matus et al., 2013). Moreover, both $SOD1^{wt}$ and mutant $SOD1$ can localize to the ER however, only $SOD1^{G93A}$ interacted with BiP, which facilitated the increased expression of pro-apoptotic UPR factors detected in ALS transgenic mice (Kikuchi et al., 2006). Similarly, $XBP1^{-/-}$ - SOD^{G86R} mutant mice had greater longevity and showed a significant reduction in neuronal apoptosis in the ventral horn, compared to ALS transgenic control mice (Hetz et al., 2009).

1.5.5 DNA damage and Chronic Neurodegenerative Disease

Although the function of p53 is to prevent cellular transformation, p53 can be inappropriately activated in the central nervous system to modulate the death of post-mitotic neurons (Haupt et al., 2003; Enokido et al., 1996; Sakhi et al., 1994). DNA strand breaks are a potent DNA damaging stimulus capable of inducing apoptosis, and have been detected in autopsied AD, PD and ALS tissues (Enokido et al., 1996; Mullaart et al., 1990; Zhang et al., 1999; Fitzmaurice et al., 1996). A unifying factor linking DNA damage to chronic neurodegenerative illness is the persistent presence of ROS, a known modulator of genotoxic stress and a key component in neurodegenerative pathologies (Yu and Anderson, 1997; Andersen, 2004). For example, mitochondrial complex I dysfunction in PD leads to a surplus of O_2^- production, a free radical capable of extensive DNA damage (Rowley and Halliwell, 1983; Perier and Vila, 2012). Moreover, p53 was found to be enriched in *post-mortem* human AD, PD, ALS brains, after traumatic brain injury, and in response to MPP⁺ and $A\beta$ treatment *in vitro* (de la Monte et al., 1998;

Napieralski et al., 1999; Kitamura et al., 1997; Bernstein and O'Malley, 2013; Akhter et al., 2014). Studies employing the use of p53-knockout mice have demonstrated that apoptosis is reduced in models of excitotoxicity and ischemic neuronal injury, relative to wild type (Morrison et al., 1996; Crumrine et al., 1994). A substantial body of work has shown that in neuronal systems, p53 effects its pro-apoptotic function through a PUMA-BAX-mediated pathway (Cregan et al., 1999; Cregan et al., 2004; Steckley et al., 2007). This is supported by studies that have detected PUMA in ischemic brain tissue, and *in vitro* following exposure to A β , DNA damaging agents and MPP⁺, stimuli known to be p53-dependent (Reimertz et al., 2003; Cregan et al., 2004; Perier et al., 2007; Feng et al., 2014; Steckley et al., 2007). Similarly, PUMA-deficient neurons show a complete attenuation of neuronal apoptosis triggered by a wide spectrum of neurotoxins, an observation that is not recapitulated in other BH3 knockout mice (Steckley et al., 2007; Wong et al., 2005; Bernstein et al., 2011). Although PUMA is a p53-target gene, some stressors are able to drive its expression regardless of p53 status, indicating the existence of p53-independent pathways (Han et al., 2001; Jeffers et al., 2003; Fricker et al., 2010).

1.6 Acute Neuronal Injury

Interrupted blood flow within the central nervous system can trigger rapid necrotic cell death in the core region of the insult, while the surviving post-ischemic tissues in the penumbral zone are vulnerable to apoptosis over an extended time frame. The occlusive nature of the insult reduces oxygen delivery, impairing ATP production, and the subsequent maintenance of ionic gradients that are essential to neuronal functioning. One key ion that becomes imbalanced is Ca²⁺, which leads to the asymmetrical distribution of Ca²⁺ within the intracellular compartment. Increased cytosolic Ca²⁺ levels facilitate exocytosis within presynaptic terminals leading to the release of glutamate, the most abundant excitatory neurotransmitter. Glutamate transmits its excitatory impulse by interacting with *N*-methyl-*D*-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, enhancing Ca²⁺ influx. Supra-physiological concentrations of intracellular Ca²⁺ cause a myriad of deleterious effects collectively referred to as excitotoxicity. Excitotoxic neuronal death is thought to proceed via

necrosis, whereas the surviving peripheral post-ischemic tissue is susceptible to apoptosis. This appears to be linked to the presence of functional mitochondria in the peri-infarct region, an area of reduced oxygen saturation that maintains cellular respiration (Broughton et al., 2009). Interestingly, blocking NMDA receptors with MK-801 did not reduce hypoxia-induced neuronal apoptosis (Haltermann et al., 2008). Similarly, antagonizing caspase activity had little effect attenuating excitotoxic neuronal injury (Glassford et al., 2002). NMDA receptor activation also leads to an interaction with neuronal Nitric Oxide Synthase (nNOS), which overproduces NO using infiltrated Ca^{2+} as a required substrate (Sattler et al., 1999). Disrupting the association of NMDA and nNOS has been shown to reduce neurotoxicity and improve stroke outcome (Sattler et al., 1999; Luo et al., 2014).

Excessive cytosolic Ca^{2+} also exerts toxic effects at the mitochondria by permeabilizing the OMM, causing a breakdown in the ETS and ATP production, leading to energy failure. Escaping electrons transition to ROS, such as O_2^- that can interact with nNOS-generated NO to form the highly poisonous free radical, peroxynitrite (ONOO^-) (Broughton et al., 2009). The importance of ROS in the modulation of ischemic tissue injury is well documented. For instance, a study involving transgenic mice that overexpressed the antioxidant enzyme, SOD1, showed reduced infarct volumes in a model of cerebral ischemia (Kinouchi et al., 1991). Conversely, mice lacking SOD1 showed enhanced neuronal loss under similar conditions (Kim et al., 2002). Mitochondria also contain an essential flavoprotein, Apoptosis-Inducing Factor (AIF), which is capable of nuclear translocation and DNA fragmentation upon mitochondrial breakdown. The importance of AIF as a potent modulator of ischemic cell death has been demonstrated *in vivo* using Harlequin mutant mice, which express substantially reduced amounts of AIF (Culmsee et al., 2005).

Although many cellular macromolecules are susceptible to ROS toxicity, the resulting genotoxic stress can potentiate ATP depletion via excessive activity of the DNA-damage sensing enzyme, Poly (ADP-ribose) Polymerase 1 (PARP-1). Indeed cerebral ischemia has been shown to potently induce DNA strand breaks (Chen et al., 1997). In order to facilitate DNA repair, PARP-1 utilizes the essential metabolic co-enzyme, NAD^+ , to

signal the recruitment of restorative enzymatic complexes. Diminishing levels of NAD⁺ indirectly impairs ATP production, further increasing energy failure (Pieper et al., 1999). Interestingly, PARP-1 knockout mice are resistant to excitotoxic neuronal death, and show a significant reduction in AIF nuclear translocation (Wang et al., 2004).

Ischemic neuronal injury can also trigger ER-stress by disrupting Ca²⁺ handling through the Sarco-Endoplasmic Reticulum Ca²⁺-ATPase (SERCA) pump. For example, ONOO⁻ can directly modify and inhibit SERCA activity, which leads to deregulated Ca²⁺ levels within the ER lumen (Gutiérrez-Martín et al., 2004). Impaired ER-Ca²⁺ homeostasis results in defective chaperone functioning, and protein folding deficits, culminating with ER-stress (Figure 1.6) (Ashby and Tepikin, 2001). The initial phase of ER-stress signaling involves a reduction in protein translation to prevent a potentially toxic overload of unfolded proteins within the ER. Indeed, it has been demonstrated that ischemic neuronal injury activates the PERK-P-eIF2 α axis (Kumar et al., 2001). Moreover, knockout studies involving pro-apoptotic downstream effectors of the PERK pathway, ATF4 and CHOP, showed reduced tissue loss following ischemic insults (Lange et al., 2008; Tajiri et al., 2004). Conversely, the pharmacological induction of the ER chaperone, BiP, significantly reduced CHOP expression and cerebral infarct volume (Kudo et al., 2008).

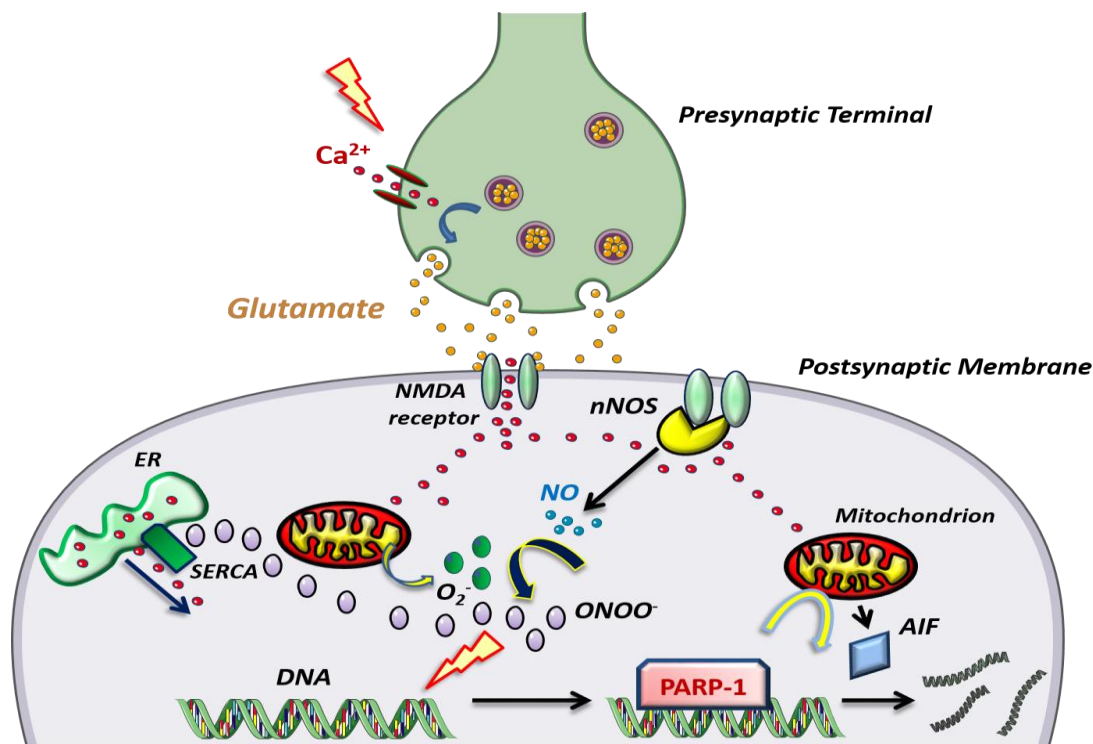


Figure 1.6. Acute excitotoxic neuronal injury.

Following stroke, oxygen delivery is impaired and as a consequence, ionic gradients are susceptible to fluctuations. One of the key ions that can perpetuate post-ischemic damage is calcium (Ca^{2+}). Uncontrolled increases in cytosolic Ca^{2+} in the presynaptic terminal can facilitate exocytosis of glutamate, an abundant excitatory neurotransmitter. Glutamate is known to bind the NMDA receptor, located on the postsynaptic membrane, which triggers an influx of Ca^{2+} . The NMDA receptor can also couple with neuronal Nitric Oxide Synthase (nNOS), which uses Ca^{2+} to overproduce the free radical, nitric oxide (NO). Ca^{2+} toxicity also permeabilizes the outer mitochondrial membrane, allowing the outflow of ROS such as O_2^- , due to disruptions in the ETS. The combination of NO and O_2^- yields peroxynitrite (ONOO^-), a harmful ROS that inhibits enzyme activity and induces DNA damage. One key target of ONOO^- modification is the enzymatic ER Ca^{2+} pump, SERCA. Alterations in ER Ca^{2+} can induce ER-stress. Extensive genotoxic damage signals the DNA repair enzyme, PARP-1, which indirectly depletes ATP stores and recruits the mitochondrial protein, AIF. Excitotoxicity can lead to DNA fragmentation via AIF activity (Broughton et al., 2009; Servier medical art).

1.6.1 The BCL-2 Family and Acute Neuronal Injury

The importance of apoptotic cell death as a critical pathway driving ischemic neuronal death was demonstrated in Bcl-2-depleted mice, which exhibited substantially larger infarct volumes compared to wild type (Hata et al., 1999). Conversely, gain-of-function mice overexpressing Bcl-2 were significantly protected when subjected to cerebral ischemia or TBI (Martinou et al., 1994; Raghupathi et al., 1998; Nakamura et al., 1999). In 2001, Plesnila and colleagues showed that BID knockout mice sustained reduced infarct volumes following an *in vivo* model of brain ischemia (Plesnila et al., 2001). Similarly, other BH3-only members, PUMA and BIM, were robustly expressed following ischemic neuronal damage and TBI (Kuan et al., 2003; Reimertz et al., 2003; Niizuma et al., 2009; Sabirzhanov et al., 2014). In a model of seizure-induced neuronal death, PUMA-depleted mice significantly resisted hippocampal damage, compared to littermate controls (Engel et al., 2010). Further, BAX-deficient mice showed significantly reduced caspase-3 activation and tissue loss in response to TBI and hypoxic-ischemic brain injury, relative to wild type (Gibson et al., 2001; Tehranian et al., 2008). Consistent with this, pharmacological antagonism of caspase activity has proven effective, a notion that was recapitulated in caspase-3 knockout mice (Endres et al., 1998; Le et al., 2002). Interestingly, active caspase-3 was identified in rodent brain tissue following seizures and TBI, and in the post-ischemic human brain (Henshall et al., 2000; Clark et al., 2000; Rami et al., 2003).

Other studies have investigated targeted inhibition strategies in pathways downstream of mitochondrial permeabilization preceding caspase activation, which have produced similar results. For example, pharmacological inhibition of the apoptosomal complex member, caspase-9, significantly reduced infarct volume relative to untreated control mice (Mouw et al., 2002). Similarly, overexpression of APAF-1-interacting protein (AIP) into the rat brain strongly attenuated caspase-3 activation, and remarkably enhanced neuronal survival following cerebral ischemia (Cao et al., 2004).

1.7 Models of Neurodegeneration

In order to understand the key signaling events leading to the induction of pro-apoptotic effectors at the cellular level, considerable attention has been focused on specific stress signals, in particular, DNA damage, oxidative dysfunction and ER-stress, to extrapolate their relative contribution to the progression of chronic neurodegenerative and ischemic injury models. Moreover, these stress pathways have been shown to robustly induce the expression of pro-apoptotic genes in neuronal cells (Ryu et al., 2002; Reimertz et al., 2003; Lange et al., 2008; Cregan et al., 2004; Steckley et al., 2007). Consistent with this is the reliance upon *de novo* gene expression, which is required for DNA damage, ER- and oxidative stress-induced neuronal apoptosis (Nath et al., 1997; Morris and Geller, 1996; Choi et al., 1999).

1.7.1 DNA damage

Since DNA strand breaks have been detected in *post-mortem* AD, PD and ALS tissues, extensive research has focused on the use of genotoxic stressors as tools to investigate the signaling mechanisms that perpetuate neuronal loss *in vitro* (Enokido et al., 1996, Morris and Geller, 1996; Cregan et al., 2004). For example, the DNA-damaging agents, Camptothecin (CPT) and Etoposide (ETP) have been shown to trigger DNA strand breaks and apoptosis in neuronal cells (Enokido et al., 1996; Morris and Geller, 1996).

1.7.2 ER-stress

It is widely accepted that ER- and oxidative stress are involved throughout the disease course of many neurodegenerative conditions (Andersen, 2004; Hetz and Mollereau, 2014). Therefore, to dissect the individual contributions of ER dysfunction to cell death signaling, specific inhibitors of critical ER functions have been employed such as, Thapsigargin (TG) and Tunicamycin (TM). The ER is major cellular compartment for Ca^{2+} storage and any perturbations involving its trafficking can elicit apoptosis. TG inhibits the SERCA pump, which functions as a vital ER- Ca^{2+} intake mechanism (Treiman et al., 1998). Another essential ER function is the maintenance of proper protein folding and packaging, including any post-translational modifications. TM is a

glycosylation inhibitor that prevents the successful preparation of proteins, facilitating an accumulation of misfolded aggregates in the ER lumen (Duksin and Bornstein, 1977).

1.7.3 Oxidative stress

In vitro modeling of oxidative stress is largely focused on disrupting electron flow throughout the mitochondrial ETS, yielding the excessive production of ROS beyond the antioxidant buffering capacity. For example, MPP⁺ and rotenone are known to induce experimental Parkinsonism due their neurotoxicity in the SN_{pc} through a mechanism involving the inhibition of mitochondrial complex I (Mizuno et al., 1987; Betarbet et al., 2000). Alternatively, treatment with exogenous ROS donors such as, tert-butyl hydroperoxide (*t*-BH), H₂O₂ and arsenite, have been demonstrated to achieve similar results in neuronal systems (Wong et al., 2005; Steckley et al., 2007; Lin et al., 2007).

1.7.4 Hypoxic stress

Following acute neuronal injury triggered by an ischemic insult, the surviving tissue surrounding the infarct core is susceptible to apoptotic cell death. This is due to the reduced oxygen supply and the diminished capacity to generate ATP, making neuronal tissue in the penumbral zone sensitive to hypoxic stress. Although penumbral tissue is vulnerable to apoptosis, the presence of functional mitochondria allows the existing neuronal subpopulation some resistance to cell death and therefore, occurs over an extended time period. In this way, targeting the penumbral zone as a salvageable area following ischemic injury is an attractive prospect ideal for therapeutic consideration. *In vitro* modeling of delayed hypoxic neuronal apoptosis involves exposing primary cortical cultures to 0.5% O_{2(g)} rather than the endogenous 3% O_{2(g)} saturation, which yields a primarily apoptotic phenotype (Halterman et al., 2008; Halterman et al., 2010).

1.8 Rationale

DNA damage, oxidative stress and ER-stress are key signaling pathways regulating neuronal death following both acute neuronal injury and chronic neurodegenerative diseases. This is thought to occur by shifting the balance between pro- and anti-apoptotic members of the Bcl-2 family, favoring an accumulation of pro-death BH3-only factors that are capable of inducing BAX, a critical convergence point in the apoptotic process. The delineation of the critical BH3-proteins involved in mediating ER- and oxidative stress-induced neuronal apoptosis is not currently resolved but may be related to ISR signaling. The ISR is central to a variety of cellular perturbations and initially manages a cytoprotective function favoring restoration, whereas prolonged activation of the ISR can result in apoptosis. A master regulator of the ISR is ATF4, a transcriptional regulator capable of inducing pro-survival and pro-death factors. However, its role and mechanism of action in regulating neuronal cell death remains unclear.

1.8.1 Hypothesis

Prolonged activation of the integrated stress response factor, ATF4, triggers neuronal apoptosis via the transcriptional regulation of pro-apoptotic Bcl-2 family proteins.

1.8.2 Objectives

1. Determine whether ATF4 is required for oxidative stress and ER-stress-induced neuronal apoptosis.
2. Determine whether ATF4 regulates the expression of pro-apoptotic Bcl-2 family proteins in neurons during oxidative- and ER-stress.
3. Determine whether the ISR regulates hypoxia-induced neuronal apoptosis.

Chapter 2

2 Materials and Methods

2.1 Animals

P53 mice, Chop and Bax mice were obtained from Jackson Laboratories (Bar Harbor, ME), mice harbouring null mutation for Puma were generated in the laboratory of Dr. Andreas Strasser (WEHI, Victoria, Australia), ATF3-depleted mice were generously donated by Dr. Tsonwin Hai (Ohio State University, Columbus, OH), and mice carrying an ATF4 null mutation were obtained from Drs. Tim Townes and Joe Sun (University of Alabama at Birmingham, AL). Timed-pregnant CD1 and C57/BL6 mice were obtained from Charles River Laboratories (Sherbrooke, QC, CA). All transgenic strains were maintained on a C57BL/6 background and genotyped as previously described (Cregan et al., 1999; Fortin et al., 2001; Zinszner et al., 1998; Hartman et al., 2004; Masuoka and Townes, 2002; Villunger et al., 2003). Wildtype and knockout littermates (experimental mice) were generated by breeding heterozygous mice.

2.2 Primary cortical cultures

Cortical neurons were dissociated from E14.5-E15.5 male and female mouse embryos and cultured in Neurobasal media containing N2 and B27 supplements (Invitrogen, Burlington, ON) as previously described (Cregan et al., 2002). Drug treatments were initiated 4 days after plating. Stock solutions of the PERK inhibitor, GSK2606414 (EMD Millipore), tunicamycin (TM), thapsigargin (TG), camptothecin (CPT), and etoposide (ETP) were prepared in DMSO (all from Sigma Aldrich, Oakville, ON, Canada) and diluted in culture media immediately before adding to neuronal cultures. Stock solutions of sodium arsenite and N-acetyl cysteine (NAC) (both from Sigma), were prepared in water and diluted in culture media immediately before adding to neuronal cultures.

2.3 Hypoxic conditions

After 7 days *in vitro*, healthy primary cortical neuron cultures were transferred to a humidified hypoxic glove box (Model No. HYGB 42", Serial No. 054520, COY Lab Products, Grass Lake, MI, USA) and continuously exposed to 0.5% O₂ and 5% CO₂, while being maintained at 37°C. Control cultures were held in a standard incubator at 37°C, while infused with 21% O₂ (normoxic) and 5% CO₂.

2.4 Nucleofection

Cortical neurons were transiently transfected using the Nucleofection system according to the manufacturers' instructions (Amaxa Biosystems, Cologne, Germany). Briefly, 0.25µg of pGFP (as a reporter) and 0.75µg of either pcDNA3-CHOP, or pcDNA3 as an empty vector control, was added to freshly dissociated cortical neurons suspended in Amaxa Mouse Neuron Nucleofector solution (5×10^7 cells/ml). The cell suspension was then electroporated with the Nucleofector device using programme setting O-05 and then cells were immediately transferred to tubes containing DMEM supplemented with 10% FBS. Neurons were plated at a cell density of 1×10^6 cells/ml in DMEM/FBS and the following day the media was changed to standard Neurobasal media containing N2/B27 supplements.

2.5 Cell death determination

Neuronal apoptosis was assessed by examining nuclear morphology in Hoechst 33258 stained cells as previously described (Cregan et al., 2002). Briefly, neurons were fixed in 4% paraformaldehyde (containing 0.2% picric acid in 0.1M phosphate buffer, pH 7.1) for 30 minutes, washed in PBS and stained with Hoechst 33258 (0.25µg/ml) dye. Neurons were visualized by fluorescence microscopy and images were captured using a CCD camera (Q-imaging, Burnaby, BC, Canada) and Northern Eclipse software (Empix imaging, Mississauga, ON, Canada). The fraction of cells exhibiting an apoptotic nuclear morphology characterized by pyknotic and/ or fragmented nuclei containing condensed chromatin was scored by an individual blinded to the treatments.

2.6 Caspase-3 activity assay

Neurons were harvested in caspase lysis buffer (10 mM Hepes, pH 7.4, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 10% glycerol, 5µg/ml leupeptin and 2 µg/ml aprotinin) and 10 µg of protein was used in caspase-3-like activity assay as previously described (Cregan et al., 1999). Briefly, protein samples were added to caspase reaction buffer (25 mM Hepes, pH 7.4, 10mM DTT, 10% sucrose, 0.1 % CHAPS, and 10 µM caspase-3 substrate Ac-DEVD-AFC) and fluorescence produced by cleavage of Ac-DEVD-AFC substrate was measured on a SpectraMax M5 spectrofluorimeter (excitation 400 nm, emission 505 nm) over a 1 hour interval. Caspase activity is reported as ratio of fluorescence output in treated samples relative to corresponding non-treated controls.

2.7 Quantitative reverse transcription-PCR

RNA was isolated using Trizol reagent as per manufacturers' instructions (Invitrogen) and RNA concentration was measured on a spectrophotometer. Quantitative RT-PCR was performed using the QuantiFast SYBR Green RT-PCR kit (#204154, Qiagen, Mississauga, ON, Canada) and 20 ng of RNA as previously described (Steckley et al., 2007). RT-PCR was carried out on a Chromo4 detection system (MJ Research/ Bio-Rad, Mississauga, ON, Canada) and changes in gene expression were determined using the $\Delta(\Delta C_t)$ method using the ribosomal S12 transcript for normalization. Data is reported as fold increase in mRNA levels in treated samples relative to corresponding untreated control samples for each transcript. All PCRs exhibited high amplification efficiencies (>90%) and the specificity of the PCR products were confirmed by sequencing. Primer sequences used for gene specific amplification are available upon request.

2.8 XBP1 splicing

RNA was isolated using Trizol reagent as per manufacturers' instructions (Invitrogen) and RNA concentration was measured on a spectrophotometer. Semi-quantitative RT-PCR was performed using the OneStep RT-PCR kit (#210212, Qiagen, Mississauga, ON, Canada) and 20 ng of RNA was reverse-transcribed into cDNA. RT-PCR was carried out using an Eppendorf AG Mastercycler 22331 (Hamburg, Germany). Full length *Xbp1* and *Xbp1_s* were resolved by electrophoresis using a 2% agarose gel and visualized using

ethidium bromide and UV light. S12 was used as a loading control. Primer sequences are available upon request.

2.9 Western blot analysis

Whole cell extracts (50 µg protein) were loaded and separated on 10-12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were probed with primary antibodies to ATF4/CREB-2x (Santa Cruz, 1:5000), Gadd153/CHOP (Santa Cruz, 1:500), ATF3 (Santa Cruz, 1:200), ATF6 (Imgenex, 1:500), p53 (Cell Sign, 1:1000), BAX (Santa Cruz, 1:500), PUMA (Sigma, 1:500), Cleaved Caspase-3 (Cell Sign, 1:1000), total eIF2 α (Cell Sign, 1:1000), Phospho-eIF2 α (Invitrogen, 1:1000) and total PERK (Sigma, 1:1000) in blocking buffer overnight. Membranes were washed in TBST buffer, probed with HRP secondary (1:10,000), and immunoreactive bands proteins were visualized using SuperSignal ECL (Pierce). Blots were stripped and reprobed for GAPDH (Cell Sign, 1:5000), α -tubulin (Cell Sign, 1:5000), or actin (Santa Cruz, 1:10,000) as loading controls.

2.10 Chromatin Immunoprecipitation (ChIP) assay

Neuronal cultures (2×10^7 cells) were crosslinked with 0.5% formaldehyde and then harvested in SDS lysis buffer and sonicated to fragment DNA. Extracts were incubated with antibodies to ATF4/CREB-2 (C-20X, Santa Cruz), Gadd153/CHOP (sc-575, Santa Cruz), or rabbit IgG control and immunoprecipitated with protein-G sepharose beads. Immunocomplexes were eluted, decrosslinked at 65°C, and DNA was recovered by phenol-chloroform extraction. Quantitative PCR was performed using the Quantitect SYBER Green PCR kit according to manufacturer's instructions (Qiagen, Mississauga, ON, Canada). Relative DNA binding of ATF4 and CHOP was determined as the ΔC_t values between treated and untreated samples immunoprecipitated with the same antibody. ΔC_t values were corrected for any changes in nonspecific interactions as determined by corresponding IgG pulldown samples and data is reported as fold enrichment ($2^{\Delta C_t}$) in DNA binding. Primer sequences used for amplification of the PUMA promoter were 5'-CTGTCCCCACGCTGTC (forward) and 5'-GCTTGCTTGCTGGTGTCTG (reverse) and for the CHOP promoter were 5'-

GACAAGTTCAGGAAGGACAGC (forward) and 5'-CGGAGGAGGTGAGTGAGTCA (reverse).

2.11 Data analysis

Data is reported as mean and SEM. The value n represents the number of independent neuron cultures or number of embryos of indicated genotype from which independent neuron cultures were prepared involving at least 3 independent experiments. Differences between groups were determined by ANOVA and *post hoc* Tukey test and were considered statistically significant when $p < 0.05$.

Chapter 3

3 Results

The gene expression experiments represented in Figures 3.10a, b and Fig. 3.12b, c were performed concurrently but are presented separately to enhance the flow of the results. The cell death assessments represented in Figures 3.11a, b and Fig. 3.14b, c are depicted in the same way.

3.1 ER and oxidative dysfunction trigger neuronal apoptosis through a PUMA-BAX-mediated pathway that is activated independently of p53

Oxidative dysfunction and ER-stress are known to trigger apoptosis via both p53-dependent and -independent pathways that require the intrinsic/mitochondrial Bax-mediated pathway (Li et al., 2006; Wei et al., 2001; Smith and Deshmukh, 2007; Fricker et al., 2010; Wong et al., 2005; Bernstein et al., 2011; Perier et al., 2007; Steckley et al., 2007; Wang et al., 2007). Pro-apoptotic family members of the Bcl-2 family, BH3-only proteins, serve as direct or indirect regulators of BAX-mediated apoptosis (Youle and Strasser, 2008). Previous work has indicated that BH3-only members, Puma, Bim and Noxa, are induced following exposure to several inducers of ER- and oxidative stress (Reimertz et al., 2003; Li et al., 2006; Puthalakath et al., 2007; Steckley et al., 2007; Bernstein et al., 2011; Wong et al., 2005). However, only the deletion of PUMA was sufficient to effectively mitigate neuronal cell death in response to oxidative dysfunction (Steckley et al., 2007). Since ER- and oxidative stress can induce PUMA expression independently of p53, and because PUMA is a dominant regulator of Bax-mediated neuronal apoptosis, we sought to determine the p53-independent signaling pathways regulating its induction.

To do so, we examined the expression of *Puma* in primary cortical neuron cultures exposed to sodium arsenite (arsenite), an oxidative stressor, and thapsigargin (TG), an inhibitor of the SERCA pump. We found that *Puma* was robustly expressed following exposure to arsenite, and to the ER-stressor, TG (Fig. 3.1a). Similarly, we detected

PUMA protein levels were strongly accumulated following 8h of arsenite treatment (Fig. 3.1b). We also detected the enrichment of PUMA protein that was maintained after 20h, and correlated with a marked enhancement of cleaved caspase-3, an executioner caspase associated with the late stages of apoptosis (Fig. 3.1c). However, in the ER-stress paradigm this was only evident in BAX-depleted neurons, presumably due to the rapid cell death occurring in wild type neurons (Fig. 3.1d) (Galehdar et al., 2010).

To ascertain whether the increase of caspase-3 cleavage enhanced apoptosis, we assessed the appearance of chromatin condensation and nuclear fragmentation and observed that arsenite and TG increased the incidence of condensed chromatin, indicative of a primarily apoptotic phenotype (Fig. 3.1e-f). The cell death response was quantified and demonstrated that arsenite or TG-treated neuronal cultures showed a substantial increase in the fraction of cells exhibiting apoptotic nuclei (Fig. 3.1g).

We next assessed the importance of Bax as a key gateway to the apoptotic process by quantifying the appearance of pyknotic nuclei (Fig. 3.2a) and caspase-3 activity (Fig. 3.2b) in BAX-deficient neurons exposed to ER-stress. Similarly, we observed that BAX-deletion strongly attenuated caspase-3 activation (Fig. 3.2c) and neuronal apoptosis (Fig. 3.2d) in response to arsenite stress, relative to BAX^{+/-} littermates.

Consistent with the diminished levels of caspase-3 activation and neuronal apoptosis detected in BAX-null neurons, we found that PUMA-deficiency similarly abrogated the expression of active caspase-3 (Fig. 3.3a) and neuronal apoptosis (Fig. 3.3b) following exposure to arsenite stress. Interestingly, PUMA-depleted neurons treated with TG or the *N*-glycosylation inhibitor, tunicamycin (TM), showed a drastic reduction (~40%) in neuronal apoptosis extending up to 48h (Appendix A Figure 1). Although BIM is known to be up-regulated following arsenite treatment and ER-stress, BIM-depletion does not recapitulate the protective phenotype observed in PUMA-null neurons (Wong et al., 2005; Galehdar et al., 2010). Taken together, our results suggest that PUMA plays a dominant role in the modulation of neuronal apoptosis in response to ER dysfunction and arsenite stress.

We and others have previously demonstrated that the transcriptional activator p53 is essential for PUMA induction and neuronal apoptosis induced by DNA damage (Cregan et al., 2004; Wytttenbach and Tolkovsky, 2006; Uo et al., 2007). Accordingly, we next investigated whether a p53-PUMA pathway is also involved in ER-stress-mediated neuronal death. To address this we first determined the expression of p53 protein following DNA damage and ER-stress. Interestingly, p53 was not stabilized by ER-stress whereas p53 was gradually accumulated in response to the DNA damaging agent, camptothecin (CPT) (Appendix B Figure 1).

Finally we examined *Puma* expression in p53^{+/+} and p53^{-/-} cortical neurons following treatment with the ER stressors TM and TG. *Puma* mRNA levels were increased by the ER stressors to a similar extent in p53^{+/+} and p53^{-/-} neurons while, as expected, DNA damage-induced *Puma* expression was abolished in the absence of p53 (Appendix B Figure 2). Moreover, we demonstrated that neurons lacking p53 were equally sensitive to neuronal apoptosis as wild type cultures, following exposure to TM (Galehdar et al., 2010).

Taken together, current evidence suggests that ER-stress induces neuronal apoptosis through a PUMA-mediated pathway that is activated independently of p53.

Figure 3.1. ER dysfunction and arsenite stress induce key hallmarks of apoptosis.

A. Total RNA was isolated from wild type cortical neurons 8h post-treatment with 5 μM sodium arsenite (NaAsO_2^- , oxidative stress) or 3 μM thapsigargin (TG, ER-stress), as a positive control, and mRNA level of *Puma* was determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3; **p < 0.01). **B.** Wild type cortical neurons were exposed to 5 μM NaAsO_2^- , and after 8h, protein levels of PUMA and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **C.** Wild type cortical neurons were exposed to 5 μM NaAsO_2^- , and after 20h, protein levels of PUMA, Cleaved Caspase-3, and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **D.** Bax-deficient cortical neurons were treated with TM and Puma protein was assessed by Western blot at 24 hours. **E-F.** Representative panels of control (left panel), 5 μM NaAsO_2^- -treated (middle panel) and TG-treated (right panel) wild type cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-treatment. **G.** The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (400X magnification).

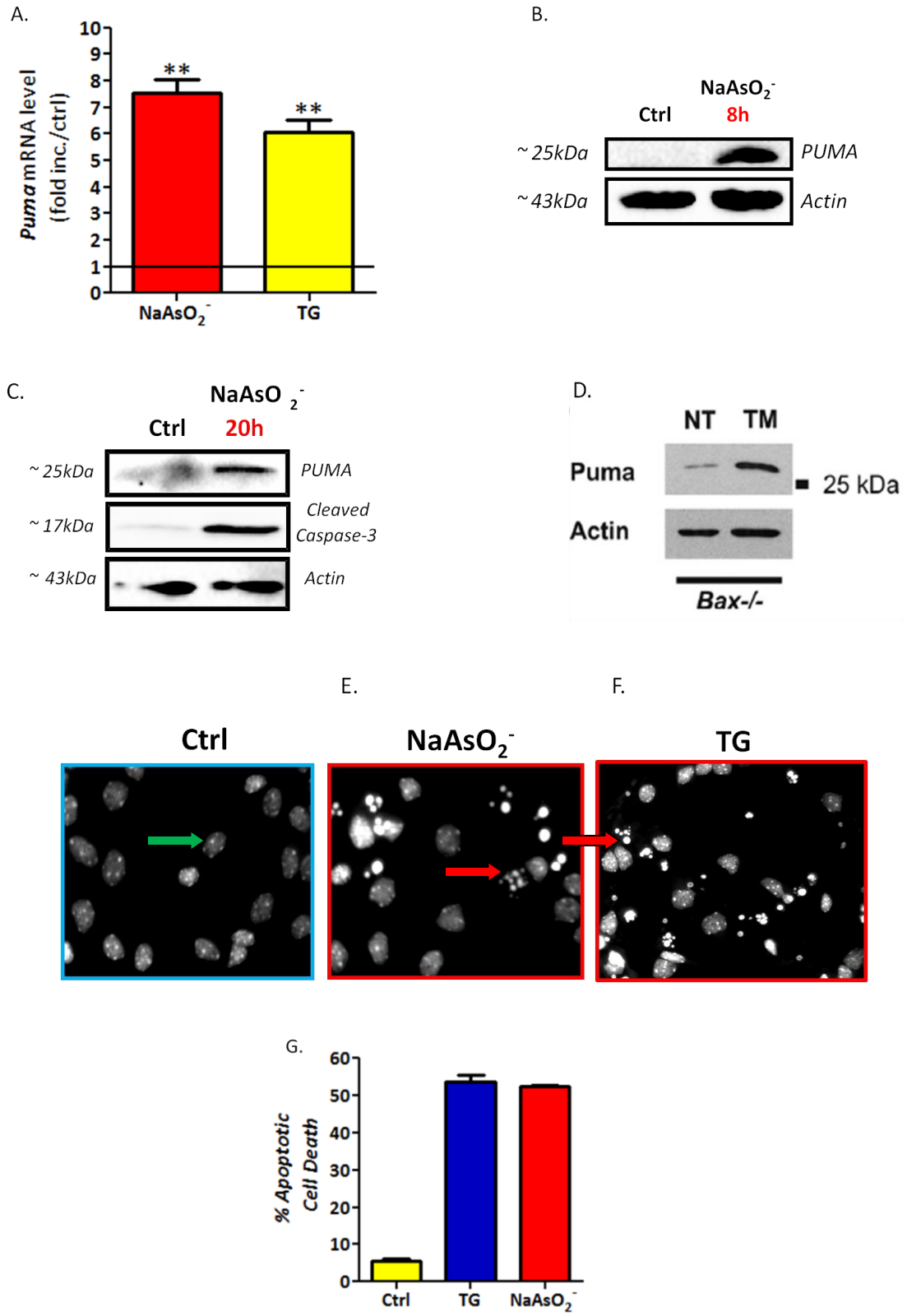
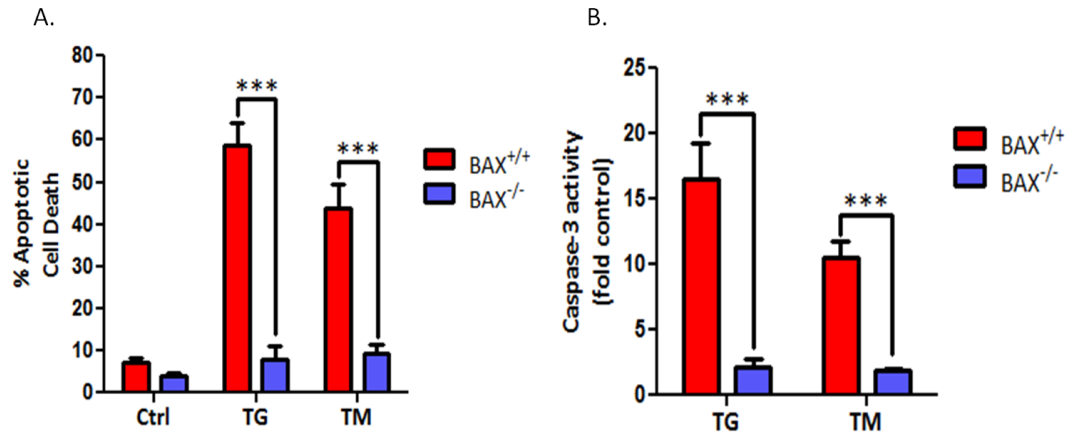
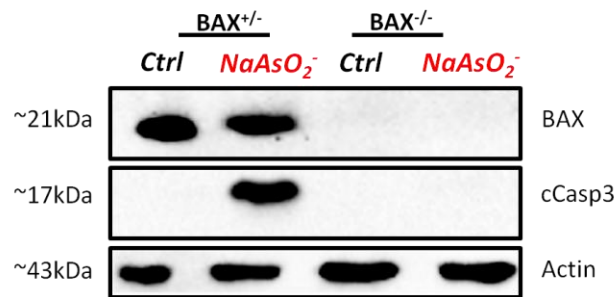


Figure 3.2. ER- and arsenite stress promote neuronal apoptosis through a BAX-mediated pathway.

A. Cortical neurons derived from Bax wild type and knockout littermates were treated with 3 μM TG, 4 μM TM, and the fraction of apoptotic cells was determined at 30h by assessing nuclear morphology following Hoechst staining ($n \geq 3$; *** $p < 0.001$). **B.** Bax wild type and knockout cortical neurons were treated with ER stressors as above. Cell lysates obtained 20 hours after treatment were assayed for caspase-3 activity ($n \geq 3$; *** $p < 0.001$). **C.** $\text{BAX}^{+/-}$ and $\text{BAX}^{-/-}$ cortical neurons were treated with 5 μM NaAsO_2^- , and after 20h, protein levels of BAX, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **D.** Representative panels of $\text{BAX}^{+/-}$ and $\text{BAX}^{-/-}$ cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-treatment with 5 μM NaAsO_2^- . The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined ($n = 3$; *** $p < 0.001$) (400X magnification).



C.



D.

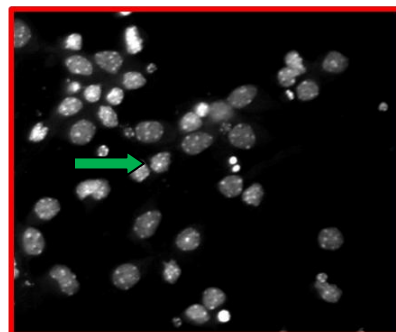
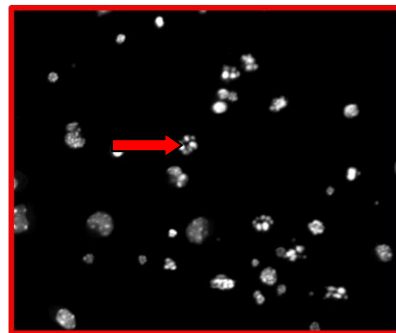
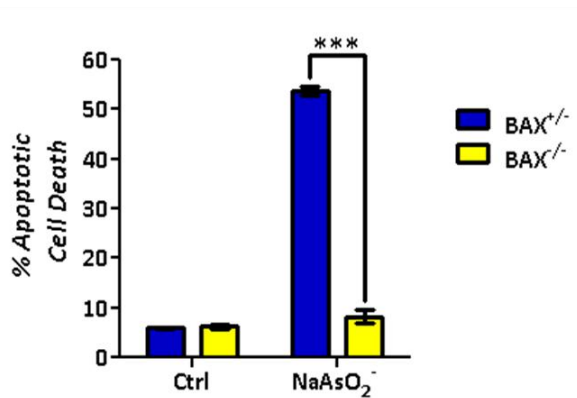
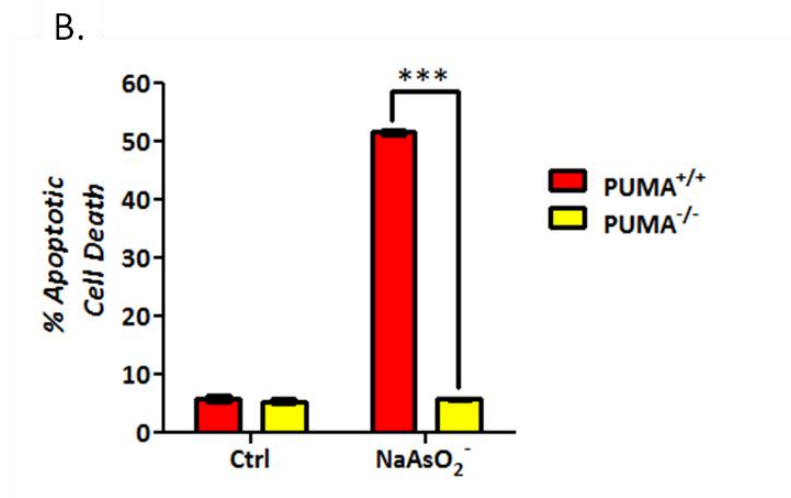
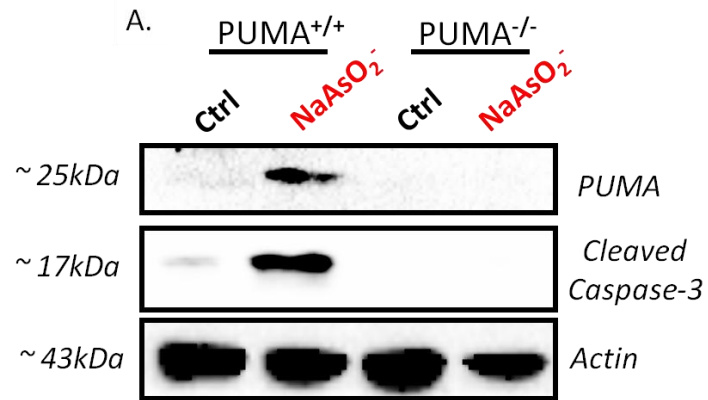
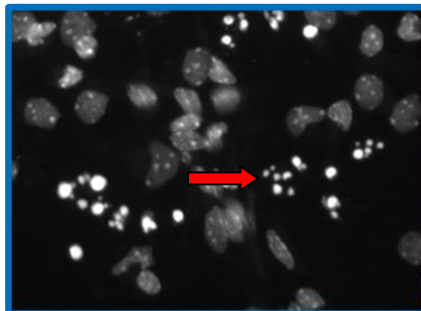


Figure 3.3. PUMA is critical for neuronal apoptosis triggered by arsenite stress.

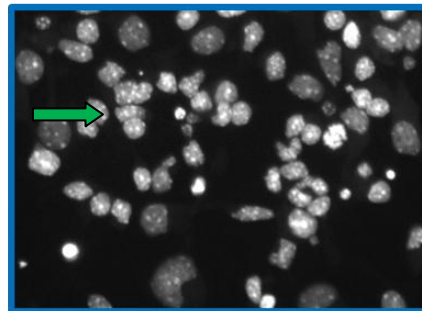
A. PUMA^{+/+} and PUMA^{-/-} cortical neurons were treated with or without 5 μ M NaAsO₂⁻, and after 20h, protein levels of PUMA, Cleaved caspase-3, and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **B.** Representative panels of PUMA^{+/+} and PUMA^{-/-} cortical neurons 24h post-treatment with 5 μ M NaAsO₂⁻, were fixed and stained with Hoechst stain (1:1000). The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (n=3; ***p < 0.001) (400X magnification).



PUMA^{+/+}



PUMA^{-/-}



3.2 ER-dysfunction and arsenite stress differentially activate components of the UPR

Since PUMA plays a critical role in mediating arsenite-induced neuronal death and arsenite is known to induce PUMA independently of p53 (Fricker et al., 2010), we next surveyed other stress pathways to determine their involvement in the regulation of PUMA. Indeed, previous reports have indicated that arsenite can induce the expression of genes associated with the UPR in non-neuronal cells (Jiang et al., 2007; Weng et al., 2013). Therefore, we examined the activation of the UPR by arsenite, in wild type cortical cultures. Firstly, we determined the activation of the ER-stress sensor, IRE1 α , which can be accomplished by measuring the degree of *Xbp1* processing from its unspliced form to its transcriptionally active, spliced variant. Using semi-quantitative PCR, we showed that TG but not arsenite induced *Xbp1* processing to its active, spliced form (Fig. 3.4a). Second, we analyzed the expression of the ER chaperone, *BiP/Grp78*, by qRT-PCR and observed a disparate expression pattern, where *BiP/Grp78* was differentially activated by ER-stress but not by arsenite treatment (Fig. 3.4b).

Conversely, when we determined ATF6 activation, we found a significant up-regulation of *Atf6* mRNA, by qRT-PCR, in response to oxidative stress (*p < 0.05) and ER-stress (*p < 0.05; **p < 0.01) (Fig. 3.5a-b). We then confirmed the activation of ATF6 α protein by indicating the fraction of full-length ATF6 α and that of the transcriptional activator, protease cleaved ATF6 α_f (Fig. 3.5c).

Finally, we evaluated the activation of the PERK-eIF2 α pathway by determining the gene expression changes of its key downstream targets: *Atf3*, *Atf4* and *Chop*. All three UPR-associated genes were profoundly induced in response to arsenite and TG treatment, as measured by qRT-PCR (Fig. 3.6a). We then performed a time-course experiment for up to 8 hours measuring the protein expression of ATF3, ATF4, CHOP, P-eIF2 α /eIF2 α . Because PUMA expression was strongly induced 8 hours post-treatment, we reasoned that the critical transcription factor(s) necessary for its induction would be required within that limited time frame. In support of this, we found an initial increase in P-eIF2 α at 1h that was persistent, indicating general translational arrest (Fig. 3.6b). However, certain mRNAs such as *Atf4* and *Chop* are known to be preferentially

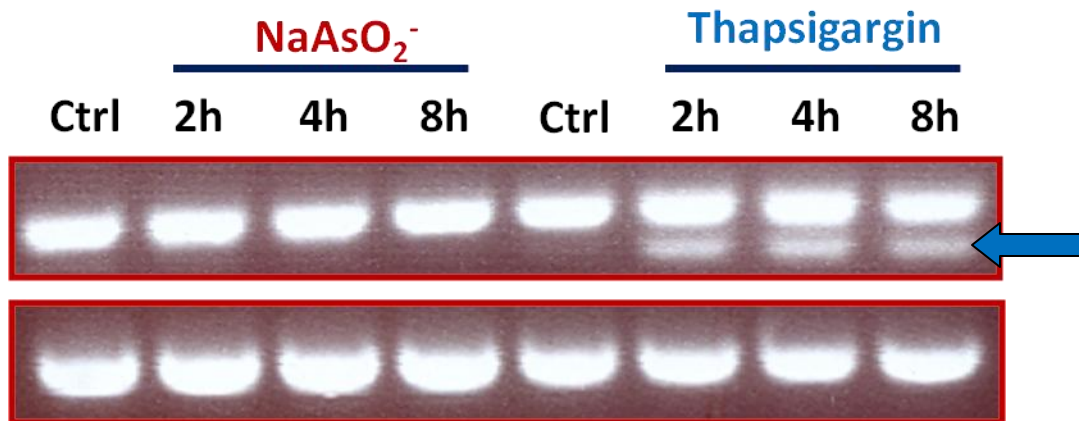
translated under these conditions (Vattem and Wek, 2004; Palam et al., 2011). Following the detection of P-eIF2 α , at 2 hours we found that arsenite treatment markedly enhanced ATF4 levels at 2h, followed by ATF3 at 4 hours, and CHOP at 8 hours. The intensity of their expression was similar in magnitude to TG treatment.

In summary, we found that arsenite triggers a selective activation of the UPR leading to the induction of ATF6 α and downstream targets of the PERK-eIF2 α pathway, ATF3, ATF4 and CHOP. Conversely, *Xbp1* splicing and the expression of *BiP/Grp78* were not detected under these conditions.

Figure 3.4. *Xbp1* mRNA splicing and *BiP* expression are activated by ER-stress but not by arsenite.

A. Total RNA was isolated from wild type cortical neurons at 2h, 4h and 8h post-treatment with 5 μM NaAsO_2^- or 3 μM TG, and mRNA levels of unspliced (240bp) and spliced (218bp) *Xbp1* mRNA (blue arrow) were determined by semi-quantitative RT-PCR. Expression was normalized to *s12* mRNA (350bp) levels (n=3). **B.** Total RNA was isolated from wild type cortical neurons 8h post-treatment with 5 μM NaAsO_2^- or 3 μM TG, as a positive control, and mRNA levels of ER chaperone, *BiP/Grp78* was determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3; **p < 0.01).

A.



B.

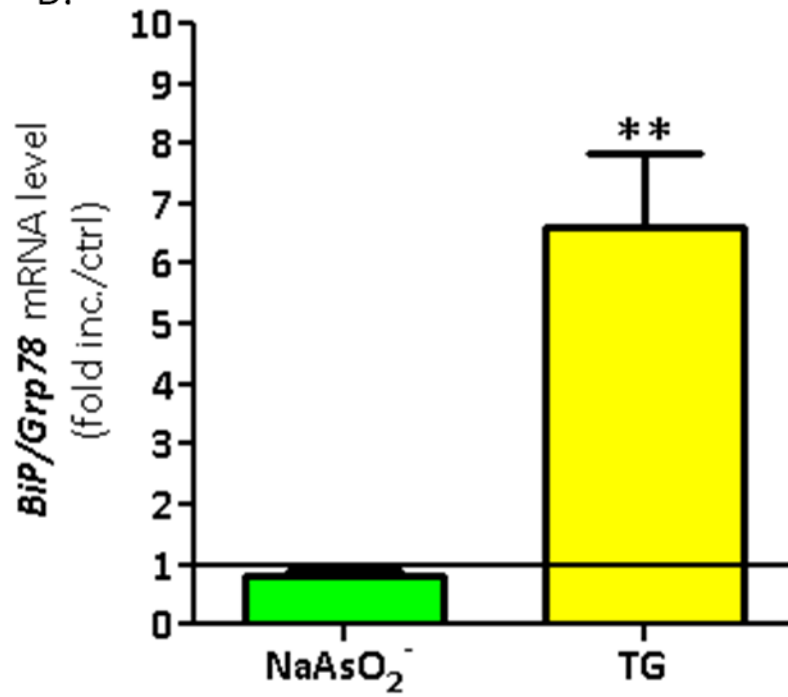


Figure 3.5. The ATF6 arm of the UPR is activated by ER-stress and in response to arsenite toxicity.

A-B. Total RNA was isolated from wild type cortical neurons at 2h, 4h and 8h post-treatment with 5 μM NaAsO_2^- (green panel) or 3 μM TG (orange panel), then mRNA levels of *Atf6* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3; *p < 0.05; **p < 0.01). **C.** Wild type cortical neurons were exposed to 5 μM NaAsO_2^- or 3 μM TG, and after 8h, ATF6 α and ATF6 α_f protein levels and the loading control, GAPDH, were assessed as a whole cell extract by Western blot analysis.

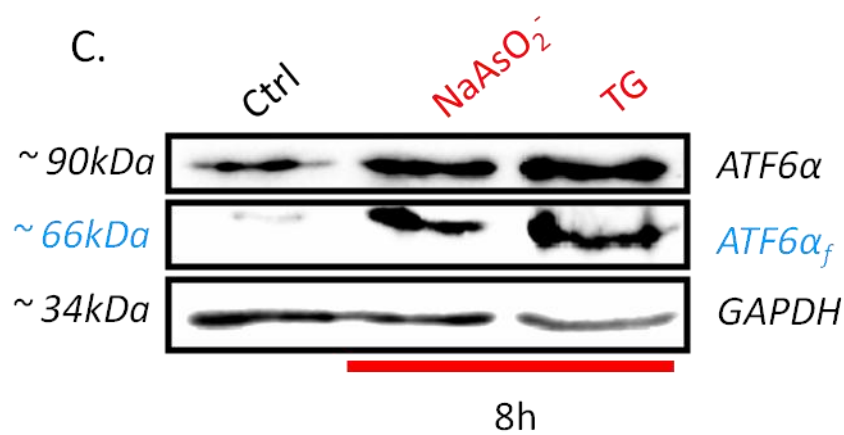
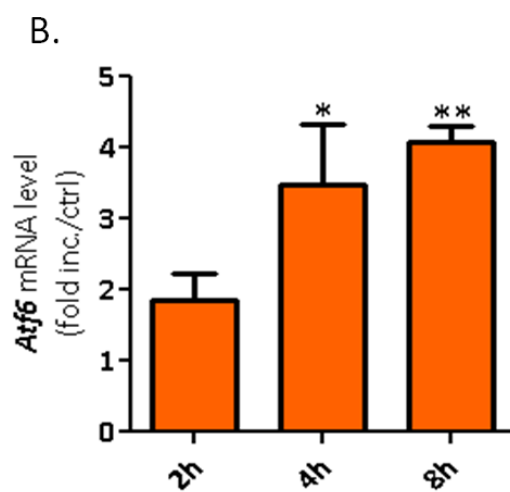
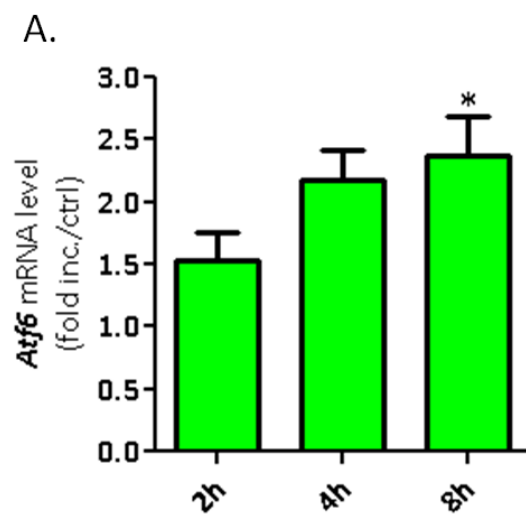
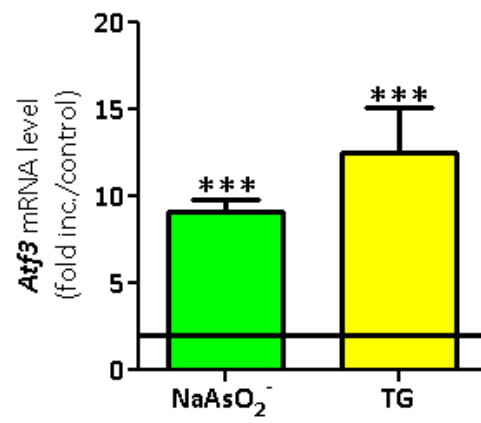
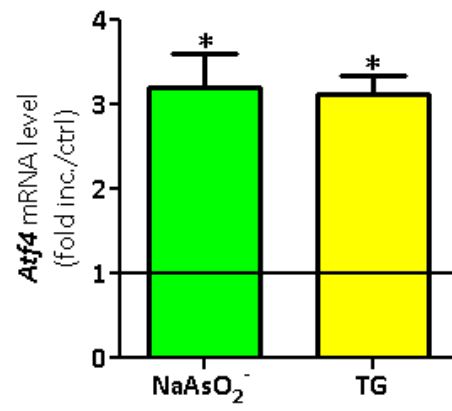
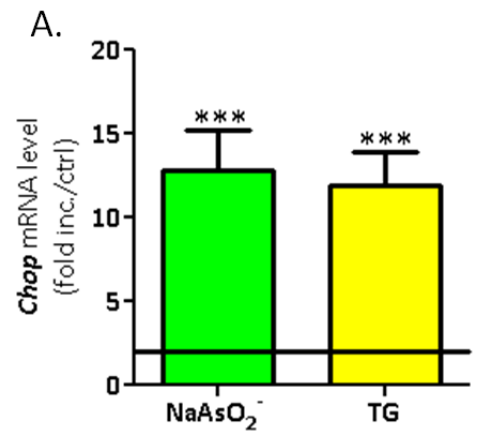


Figure 3.6. Downstream proteins of the PERK branch of the UPR are similarly activated by ER- and arsenite stress.

A. Total RNA was isolated from wild type cortical neurons 8h post-treatment with 5 μM NaAsO_2^- or 3 μM TG and mRNA levels of *Chop*, *Atf4* and *Atf3*, were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3; *p < 0.05; ***p < 0.001). **B.** Wild type cortical neurons were exposed to NaAsO_2^- and after 1h, 2h, 4h and 8h, or after 8h of TG treatment, and protein levels of ATF4, CHOP, ATF3, P-eIF2 α /total eIF2 α , and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis.



3.3 Arsenite and ER-stress activate ATF4 expression by different mechanisms

We then defined whether arsenite triggers the activation of PERK to maintain ATF3, ATF4 and CHOP expression. This can be accomplished by monitoring changes in the electrophoretic mobility of PERK as an indication of P-PERK, with an antibody directed against the C-terminus (Bertolotti et al., 2000). As expected, TG induced an appreciable shift in PERK mobility whereas the PERK inhibitor (Pi), GSK2606414, and untreated control were indifferent, after 8h. The supershift of PERK was abolished when TG was paired with Pi, resembling untreated control or Pi alone (Fig. 3.7a). Similar to previous work using non-neuronal cells (Harding et al., 1999), PERK activation was detected only in neuronal lysates treated with TG after 4 and 8h, relative to untreated control or arsenite-treated samples (Fig. 3.7b). We found that ATF3, ATF4 and CHOP protein levels were attenuated by TG in combination with Pi, relative to the profound induction by TG alone. Conversely, we observed that ATF3, ATF4 and CHOP were similarly expressed in response to arsenite and Pi, or arsenite stress alone (Fig. 3.7c). These findings indicate that oxidative dysfunction mediated by arsenite stress triggers a PERK-independent mechanism to robustly accumulate ATF3, ATF4 and CHOP.

3.3.1 N-acetyl cysteine (NAC) reduces ATF4 levels induced by oxidative stress but not by ER dysfunction

Since we have previously demonstrated that both oxidative and ER-dysfunction lead to the enrichment of ATF4 in neuronal cells, we addressed whether or not both stresses share a common pathway regulating ATF4 expression. To do this we pretreated cortical neurons with the antioxidant, N-acetyl cysteine (NAC), 1h prior to arsenite or TG treatment to evaluate the potential disparity of each pathway. We observed that the addition of NAC does not activate ATF4 protein expression, while exposure to arsenite or TG robustly accumulated ATF4 levels. However, pre-incubation with NAC suppressed arsenite-mediated ATF4 expression, while having only a mild effect on ATF4 levels induced by ER dysfunction (Fig. 3.8). These data suggest the presence of mutually exclusive signaling mechanisms by which unique cellular stresses are interpreted.

Figure 3.7. Arsenite activates ATF3, ATF4 and CHOP independently of PERK.

A. Wild type cortical neurons were pre-treated with or without 1 μM of the PERK inhibitor, GSK2606414, (Pi) for 1h, and then in conjunction with 3 μM TG or 3 μM TG alone, and after 8h, protein levels of PERK and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **B.** Wild type cortical neurons were treated with or without 5 μM NaAsO_2^- or 3 μM TG, and after 4 and 8h, protein levels of PERK and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **C.** Wild type cortical neurons were pre-treated with or without 1 μM Pi for 1h, followed by 5 μM NaAsO_2^- or 3 μM TG, and after 8h, protein levels of ATF4, CHOP, ATF3, and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. n.s. = non-specific.

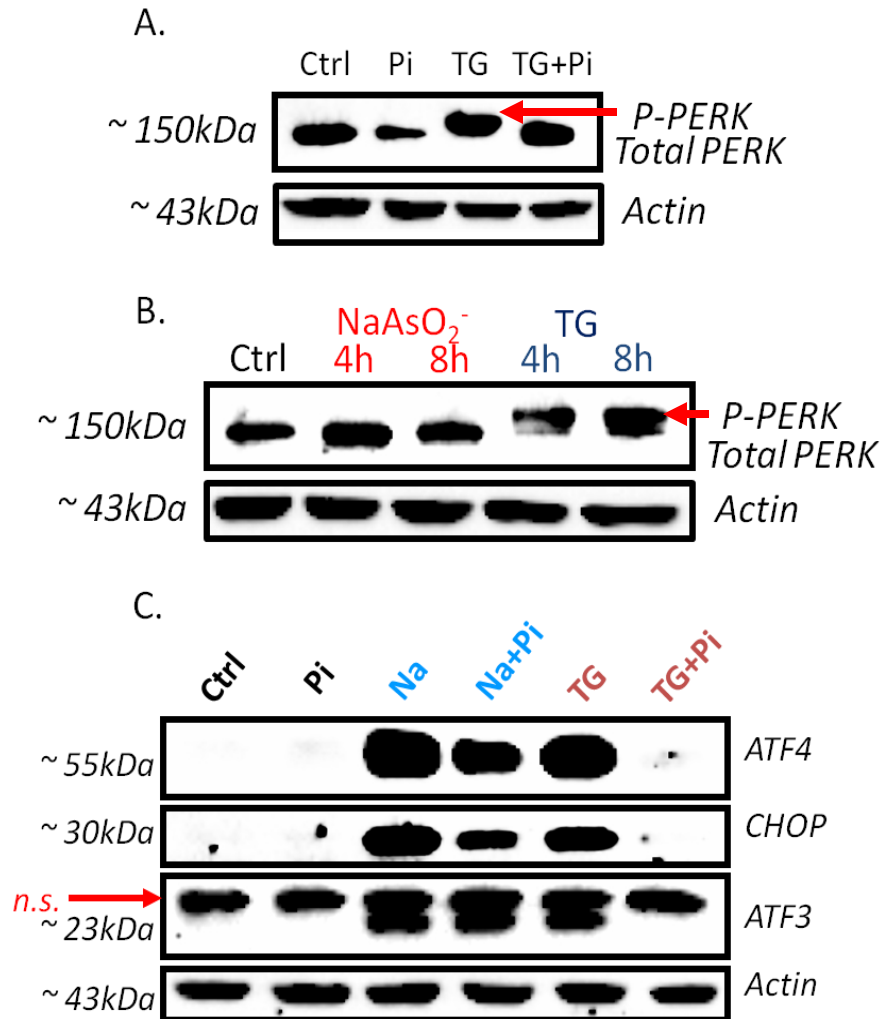


Figure 3.8. NAC attenuates ATF4 expression in response to oxidative stress but not ER dysfunction.

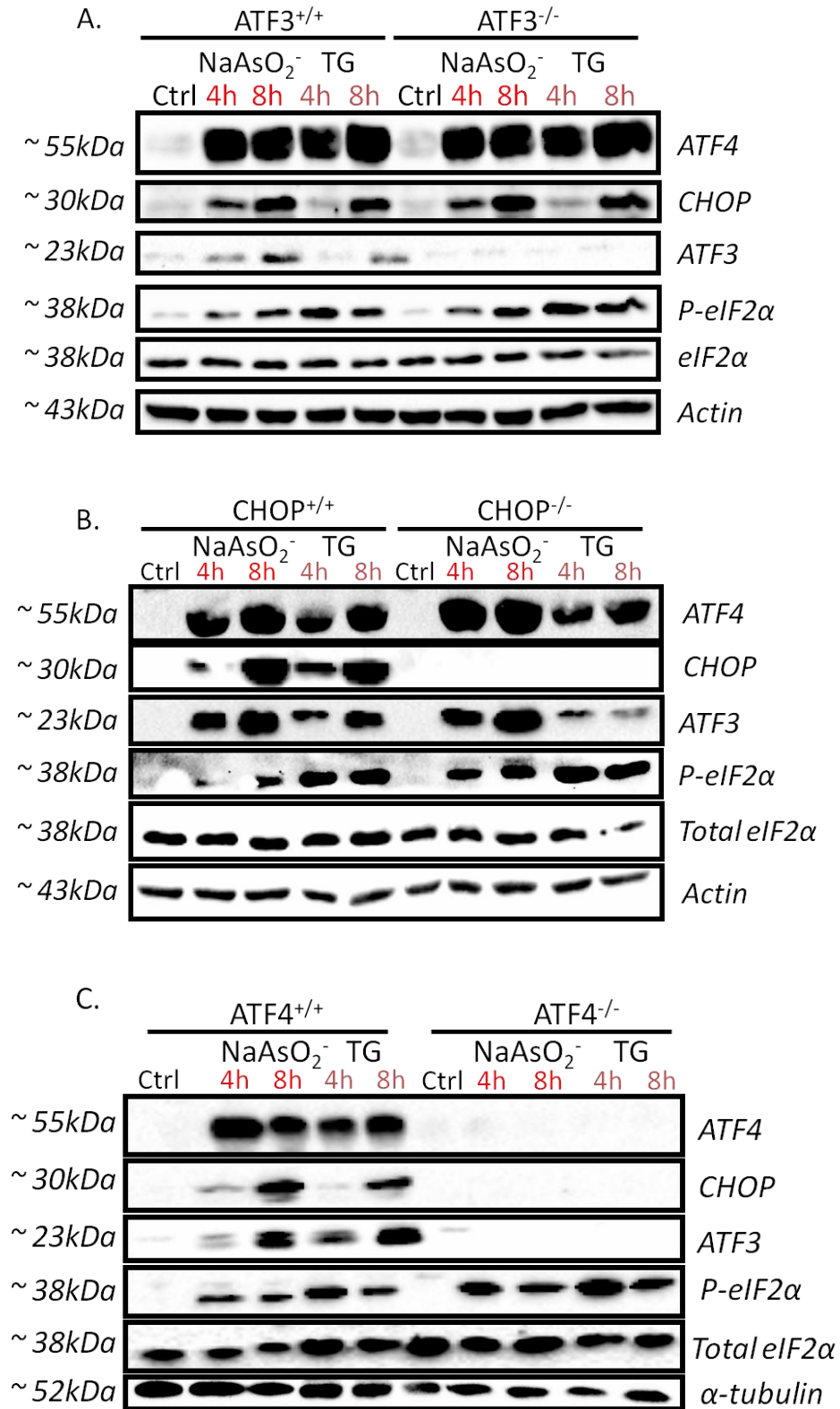
Wild type cortical neurons were pre-treated with or without 5 mM N-acetyl cysteine (NAC) for 1h prior to additional treatment with or without 5 μ M NaAsO₂⁻ (Na) for 1h, 4h and 8h, or after 8h with or without 3 μ M TG. Whole cell lysates were collected at the indicated times and levels of ATF4 and the loading control Actin, were assessed by a Western blot.

3.4 ATF4 regulates CHOP and ATF3 during arsenite stress and ER dysfunction

Since ATF3, ATF4 and CHOP are transcriptional regulators, we aimed to show the hierarchy of their induction using gene-specific knockout mice. We found that ATF3-deficiency does not contribute to the regulation of ATF4 or CHOP, in response to arsenite or TG treatment (Fig. 3.9a). Moreover, CHOP-deletion did not reduce ATF4 protein levels due to arsenite or TG exposure. However, we did notice the differential regulation of ATF3 by CHOP, which was stress-dependent. We observed no difference between ATF3 protein expression in CHOP^{+/+} or CHOP^{-/-} neurons treated with arsenite stress however we detected an appreciable reduction of ATF3 protein in CHOP-depleted neurons treated with TG, relative to their wild type counterparts (Fig. 3.9b). Interestingly, both CHOP and ATF3 protein levels were completely abolished in arsenite- and TG-treated ATF4^{-/-} neurons, relative to wild type (Fig. 3.9c).

Figure 3.9. Arsenite stress and ER dysfunction activate ATF3 and CHOP in an ATF4-dependent manner.

A. ATF3^{+/+} and ATF3^{-/-} cortical neurons were exposed to 5 μ M NaAsO₂⁻ or 3 μ M TG, and after 4h and 8h, protein levels of ATF4, CHOP, ATF3, P-eIF2 α /total eIF2 α , and Actin, as a loading control, were assessed as a whole cell extract by Western blot analysis. **B.** CHOP^{+/+} and CHOP^{-/-} cortical neurons were exposed to 5 μ M NaAsO₂⁻ or 3 μ M TG, and after 4h and 8h, protein levels of ATF4, ATF3, P-eIF2 α /total eIF2 α , and Actin, as a loading control, were assessed as a whole cell extract by Western blot analysis. **C.** ATF4^{+/+} and ATF4^{-/-} cortical neurons were exposed to 5 μ M NaAsO₂⁻ or 3 μ M TG, and after 4h and 8h, protein levels of ATF4, CHOP, ATF3, and α -tubulin, as a loading control, were assessed as a whole cell extract by Western blot analysis.



3.5 ATF4 promotes oxidative-stress-induced PUMA expression and neuronal apoptosis

Because ATF3, ATF4 and CHOP have been implicated as pro-death transcription factors (Hartman et al., 2004; Silva et al., 2005; Lange et al., 2008; Han et al., 2013), we examined their potential roles as regulators of PUMA expression in response to arsenite toxicity. Interestingly, both ATF4 and CHOP have been shown to transactivate the PUMA promoter, in non-neuronal cells (Cazanave et al., 2010; Qing et al., 2012). However, we found that only ATF4 deletion significantly attenuated *Puma* mRNA levels (* $p < 0.05$), relative to wild type, whereas ATF3 or CHOP depletion did not significantly reduce *Puma* expression (Fig. 3.10a-c). Moreover, ATF4-deficiency resulted in a profound reduction of PUMA protein, relative to wild type neurons exposed to 8h of arsenite stress (Fig. 3.10d). This effect was persistent and mitigated both PUMA and caspase-3 cleavage following 20 hours of arsenite exposure (Fig. 3.10e). Conversely, ATF3 or CHOP-depleted neurons treated with arsenite showed a similar degree of cleaved caspase-3, relative to wild type and heterozygous littermates (Fig. 3.10f-g). Additionally, only ATF4 null cortical cultures showed significantly reduced levels of apoptotic cell death, compared to wild type, when challenged with arsenite stress for 24 hours ($n=3$; *** $p < 0.001$), whereas ATF3 or CHOP-deficiency did not attenuate arsenite-induced apoptosis (Fig. 3.11a-c). Together, our results indicate that ATF4 plays a key role in modulating PUMA expression, caspase-3 activation, and neuronal cell death mediated by arsenite stress.

Figure 3.10. ATF4 drives PUMA expression and caspase-3 cleavage during arsenite stress.

A. Total RNA was isolated from ATF4^{+/+} and ATF4^{-/-} 8h post-treatment with 5 μ M NaAsO₂⁻ and mRNA levels of *Puma* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=4; *p < 0.05). **B.** Total RNA was isolated from ATF3^{+/+} and ATF3^{-/-} neurons 8h post-treatment with 5 μ M NaAsO₂⁻, and mRNA levels of *Puma* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3). **C.** Total RNA was isolated from CHOP^{+/+} and CHOP^{-/-} neurons 8h post-treatment with 5 μ M NaAsO₂⁻, and mRNA levels of *Puma* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to untreated control cells (n=3). **D.** ATF4^{+/+} and ATF4^{-/-} cortical neurons were treated with or without 5 μ M NaAsO₂⁻ for 8h, and protein lysates were assessed for levels of ATF4, PUMA, and Actin, as a loading control, by Western blot analysis. **E.** ATF4^{+/+} and ATF4^{-/-} cortical neurons were treated with or without 5 μ M NaAsO₂⁻ for 20h, and protein lysates were assessed for levels of ATF4, PUMA, cleaved caspase-3, and Actin, as a loading control, by Western blot analysis. **F.** ATF3^{+/+} and ATF3^{-/-} cortical neurons were treated with or without 5 μ M NaAsO₂⁻ for 20h, and protein lysates were assessed for levels of ATF3, cleaved caspase-3, and Actin, as a loading control, by Western blot analysis. **G.** CHOP^{+/+} and CHOP^{-/-} cortical neurons were treated with or without 5 μ M NaAsO₂⁻ for 20h, and protein lysates were assessed for levels of CHOP, cleaved caspase-3, and Actin, as a loading control, by Western blot analysis.

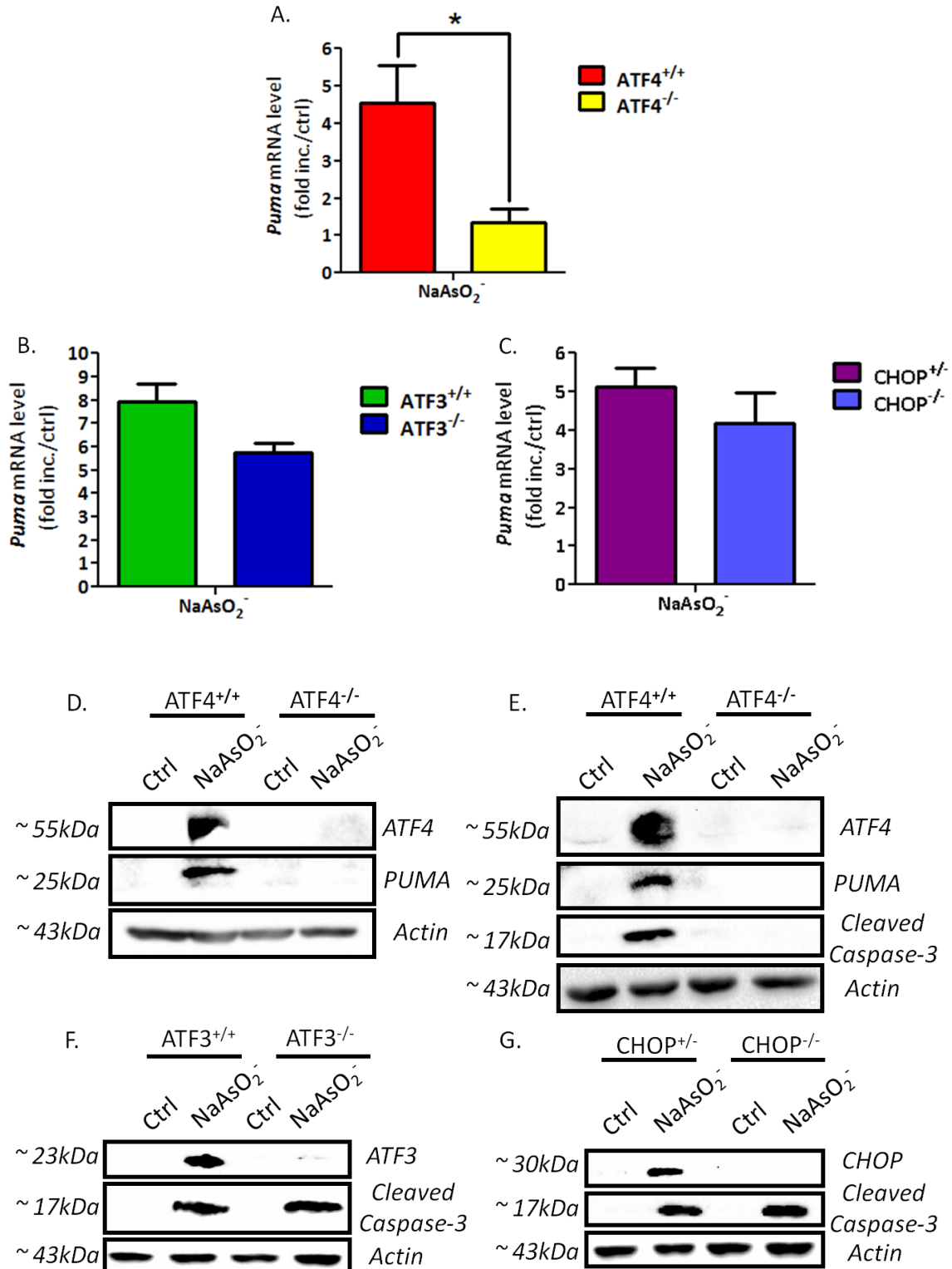
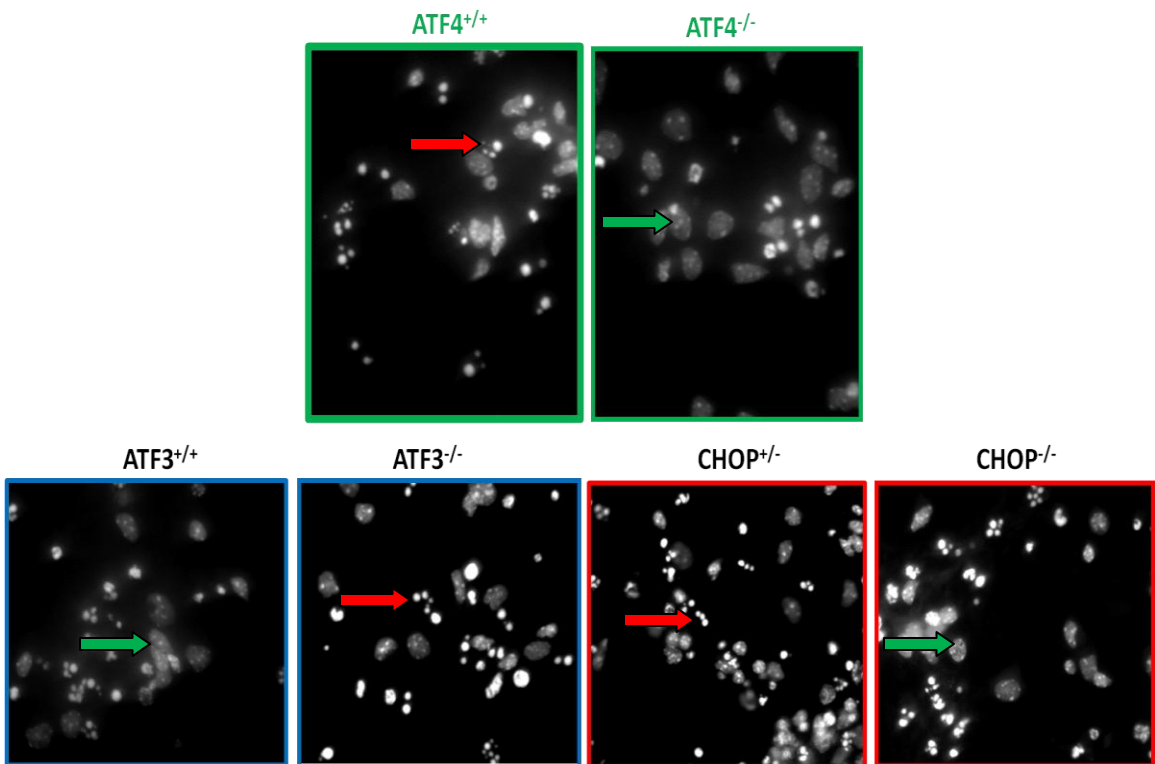
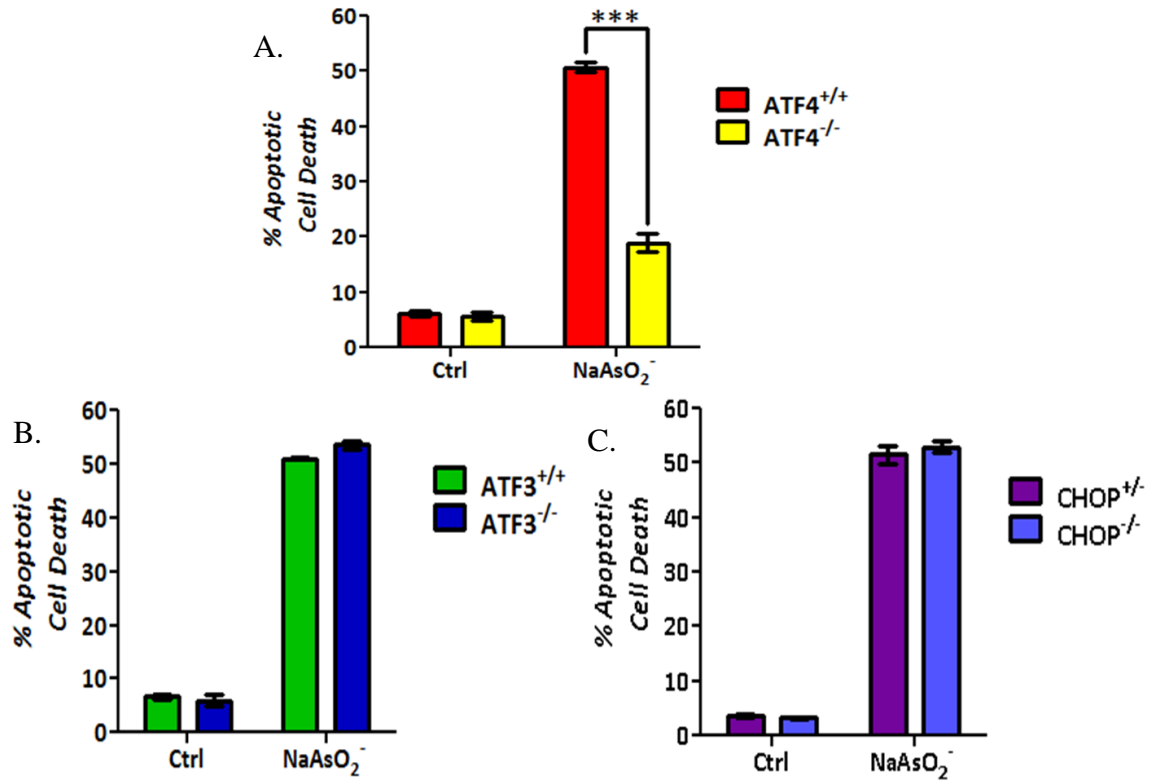


Figure 3.11. ATF4-deficiency reduces arsenite-mediated neuronal apoptosis.

A-C. The fraction of apoptotic cells showing chromatin condensation and fragmented nuclei relative to healthy cells was determined in ATF4, ATF3 or CHOP-deficient cortical neurons (n=3; ***p < 0.001). Representative panels of wild type, heterozygous and ATF3-, CHOP- or ATF4-null cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-treatment with 5 μ M NaAsO₂. The red arrow indicates apoptotic nuclei, and the green arrow indicates healthy nuclei (400X magnification).



3.6 ATF4 regulates PUMA expression via an indirect mechanism during ER-stress

We have previously indicated that a transcriptional activator other than p53 is required for *Puma* induction during ER-stress (Appendix B Figure 2). The transcription factor ATF4 has been implicated in ER-stress, however its functional role in the ER-stress response and relevance to cell death remain unclear. Indeed it has been shown that ATF4 can favor a pro-survival or a pro-death response in a variety of experimental systems (Lange et al., 2008; Armstrong et al., 2010; Harding et al., 2003; Jiang et al., 2007). Interestingly, ATF4 was recently demonstrated to transactivate the PUMA promoter following nutrient deprivation, in non-neuronal cells (Qing et al., 2012). Accordingly, we examined the potential role of ATF4 in ER-stress-mediated death pathways. Indeed, we observed a robust induction in ATF4 protein levels following treatment with the ER stressor TM, but not in response to the DNA damaging agent, CPT. Conversely, p53 was enriched only after exposure to CPT and not TM (Fig. 3.12a). We next examined the endogenous role of ATF4 using gene-specific knockout mice. As shown in Figure 3.12b, *Puma* induction and neuronal apoptosis (Fig. 3.12c) induced by TG were significantly reduced in ATF4-deficient neurons as compared to wild type cultures. Thus, ATF4 promotes *Puma* induction and neuronal death during ER dysfunction, whereas p53 is expendable (Galehdar et al., 2010).

We next examined whether ATF4 can activate the PUMA promoter. To address this we generated reporter constructs to identify the ATF4 responsive region present in the PUMA promoter, which corresponded to a region ~90-210 base pairs upstream of the transcription start site of *Puma* (Galehdar et al., 2010). Therefore, we examined this region for potential ATF4 binding sites using the Genomatix promoter analysis software and identified a conserved element exhibiting significant homology to the consensus ATF/CRE binding sequence and located approximately 25bp downstream of the previously identified p53 binding site (Fig. 3.13a) (Yu et al., 2001; Han et al., 2001). To determine whether ATF4 binds to the PUMA promoter during ER-stress *in situ* we performed quantitative chromatin immunoprecipitation (ChIP) assays using PCR primers flanking the ATF4 responsive region and encompassing the putative ATF4-binding sites.

Curiously, we did not detect an increase in ATF4 binding in this region following either DNA damage or ER-stress treatments. However, ATF4 binding was significantly enriched at a previously described ATF4 response element located in the CHOP promoter (Ma et al., 2002) confirming that the ATF4 ChIP assay worked (Fig. 3.13b). These results suggest that ATF4 likely regulates *Puma* expression during ER-stress via an indirect mechanism.

Figure 3.12. ATF4 facilitates ER-stress-induced neuronal apoptosis.

A. Wild type cortical neurons were treated with 10 μ M camptothecin or 4 μ M tunicamycin (TM) for 1h, 2, 4h and 8h, and protein levels of ATF4, p53 and the loading control, GAPDH, were then assessed by Western blot. **B.** Cortical neurons derived from ATF4^{+/+} and ATF4^{-/-} littermates were treated with the ER stressor TG (3 μ M), and RNA was extracted after 8 hours. *Puma* mRNA levels were quantified by real-time PCR and are reported as fold increase over corresponding untreated controls (n=4; *p<0.05). **C.** Cortical neurons derived from ATF4^{-/-} embryos and wild type littermates were treated with the TG (3 μ M). At the indicated time, neurons were stained with Hoechst dye 24h after treatment and the fraction of apoptotic (red arrow) and healthy (green arrow) cells was determined by assessing nuclear morphology (400x magnification) (n=3; ***p<0.001).

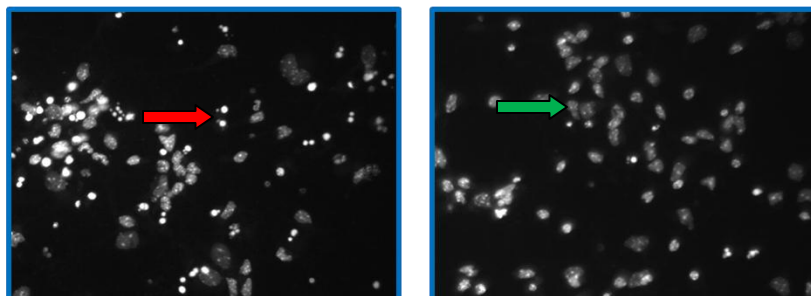
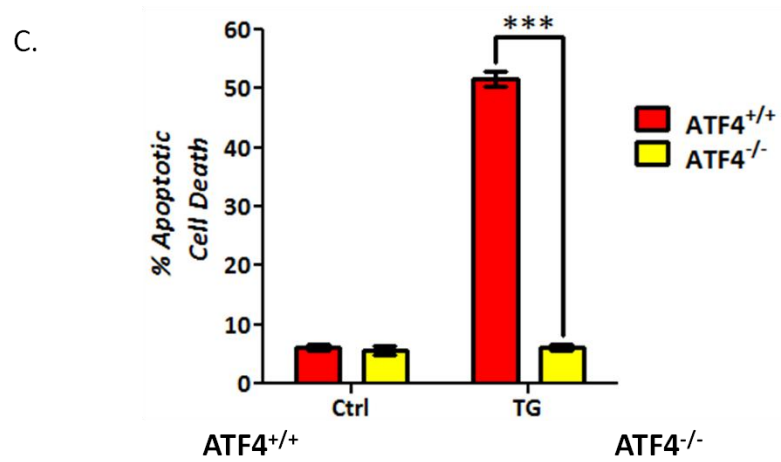
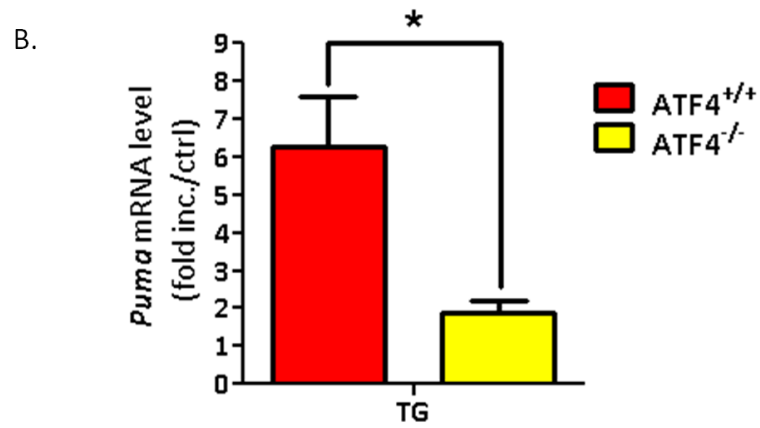
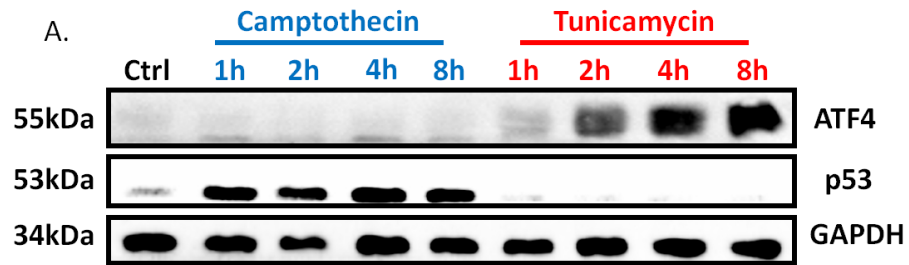
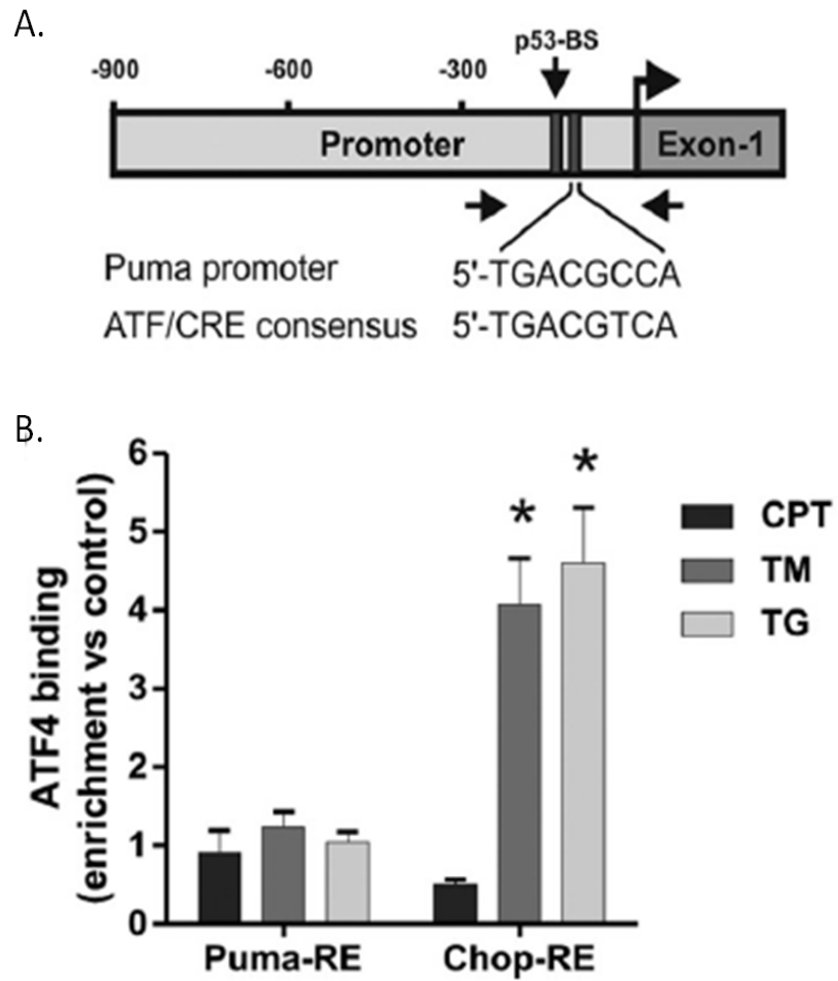


Figure 3.13. ATF4 does not directly activate the Puma promoter during ER-stress.

A. Schematic of the Puma promoter showing the location of the putative ATF/CRE and p53-binding sites. Arrows indicate the approximate positions of the PCR primers used in the ChIP assays. **B.** Cortical neurons were treated with DNA damage, CPT (10 μ M) or ER-stressors, TM (4 μ M) or TG (3 μ M), and binding of ATF4 was assessed by ChIP assay and real-time PCR using primers specifically targeting the putative ATF4 response elements (RE) in the Puma and Chop promoters. Data is reported as fold increase over untreated control samples for each promoter region (n=5; *p<0.01).



3.6.1 ATF3 does not regulate PUMA expression or neuronal apoptosis during ER dysfunction

The above results suggest that an intermediary factor is involved in ATF4-mediated *Puma* induction. Previous studies have suggested a relationship between ATF4 and the transcription factors, ATF3 and CHOP (Jiang et al., 2004; Jiang et al., 2007). Therefore, we first considered the involvement of ATF3 by confirming its gene expression levels in ATF4-null neurons following TM exposure. In agreement with our previous results, in Figure 3.9c, that determined ATF3 protein expression was strongly ATF4-dependent up to 8h, we found that ATF4-deficiency severely impaired *Atf3* mRNA expression 12h post-treatment with TM (Fig. 3.14a). We next assessed the contribution of ATF3 in the regulation of *Puma* expression and neuronal cell death during ER-stress. As shown in Figure 3.14b, after 8h of TG treatment, cortical neurons lacking ATF3 did not show reduced levels of *Puma* mRNA compared with wild type littermates. Additionally, ATF3-deleted neurons showed no observable difference in the degree of ER-stress-mediated neuronal apoptosis relative to wild types (Fig. 3.14c).

3.6.2 ATF4-CHOP drives PUMA induction and neuronal apoptosis in response to ER-stress

Since we did detect the enhanced binding of ATF4 to the CHOP promoter in neurons during ER-stress (Fig. 3.13b), we hypothesized that ATF4 may regulate *Puma* induction through CHOP. Indeed CHOP protein levels were strongly enhanced in wild type cortical neurons following treatment with TG or TM, and dramatically reduced in ATF4-deficient neurons, consistent with its activation primarily driven by ATF4 in this paradigm (Fig. 3.9c and Appendix C Figure 1). Interestingly, CHOP was originally described as a DNA-damage-sensitive gene in non-neuronal systems (Luethy et al., 1990), however we did not detect *Chop* mRNA or protein expression in cortical neurons exposed to DNA damaging agents (Galehdar et al., 2010). We next determined whether CHOP can activate the PUMA promoter from the ATF4 responsive region. To do so, we performed ChIP assays to determine whether CHOP binds to the PUMA promoter *in situ* during ER-stress. As shown in Figure 3.15a, CHOP binding to the PUMA promoter increased approximately 3-fold following treatment with TM or TG, but was not increased by CPT treatment.

These results suggest that CHOP can directly regulate the PUMA promoter during ER-stress, but not in response to DNA damage. Interestingly, we found that the enforced expression of CHOP was sufficient to induce apoptosis in wild type neurons but not in PUMA^{-/-} neurons suggesting that PUMA is required for CHOP induced cell death (Fig. 3.15b). Moreover, the targeted knockdown of CHOP was sufficient to significantly attenuate ER-stress-induced *Puma* expression and neuronal apoptosis (Galehdar et al., 2010).

Figure 3.14. ATF3 does not mediate *Puma* expression or neuronal apoptosis during ER-stress.

A. Total RNA was isolated from ATF4^{+/+} and ATF4^{-/-} neurons 12h post-treatment with 4 μ M TM, and mRNA levels of *Atf3* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels (n=3; ***p<0.001). **B.** Total RNA was isolated from ATF3^{+/+} and ATF3^{-/-} neurons 8h post-treatment with 3 μ M TG, and mRNA levels of *Puma* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels (n=3). **C.** The fraction of apoptotic cells showing chromatin condensation and fragmented nuclei relative to healthy cells was determined in ATF3^{+/+} and ATF3^{-/-} cortical neurons (n=3). Representative panels of wild type and ATF3-null cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-treatment with 3 μ M TG. The red arrow indicates apoptotic nuclei, and the green arrow indicates healthy nuclei (400X magnification).

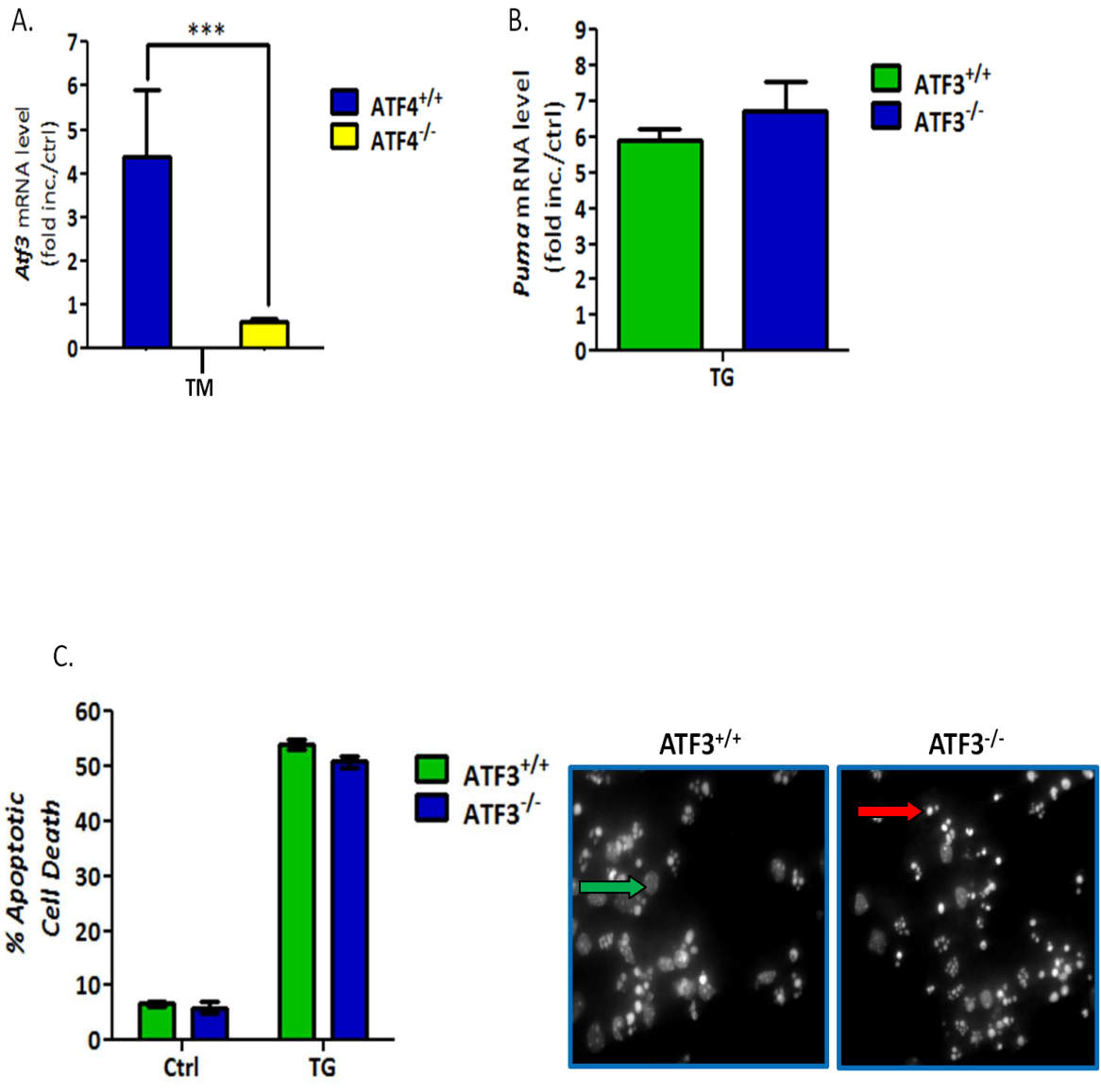
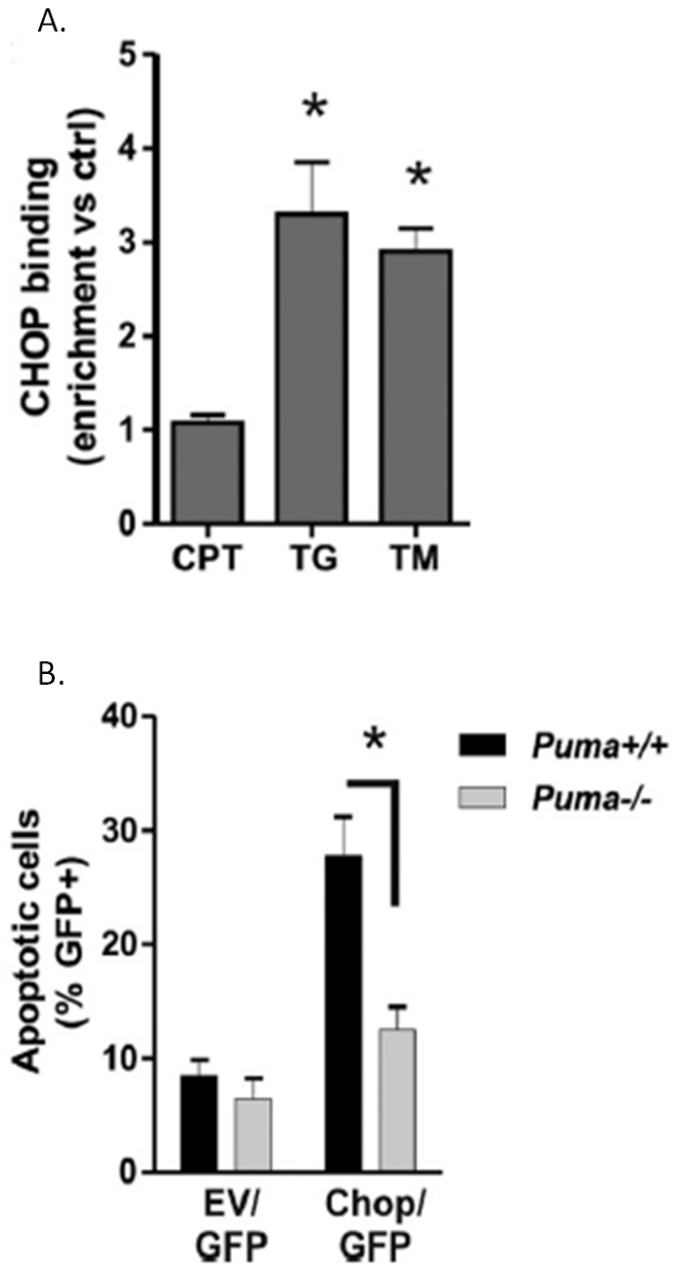


Figure 3.15. CHOP activates the Puma promoter and induces neuronal apoptosis in a PUMA-dependent manner in response to ER-stress.

A. Cortical neurons were treated with CPT (10 μ M), TU (4 μ M) or TG (3 μ M) and CHOP binding to the Puma promoter was assessed after 12 hours by ChIP assay. The level of CHOP binding was quantified by real-time PCR and is reported as fold enrichment over untreated controls (n=4; *p<0.05). **B.** Cortical neurons derived from Puma^{+/+} and Puma^{-/-} littermates were nucleofected with pGFP and either pcDNA3 (empty vector (EV)) or pcDNA3-CHOP. Neurons were Hoechst stained 48 hours post-transfection and the fraction of GFP positive neurons exhibiting an apoptotic nuclear morphology was determined (n=3; *p<0.05).

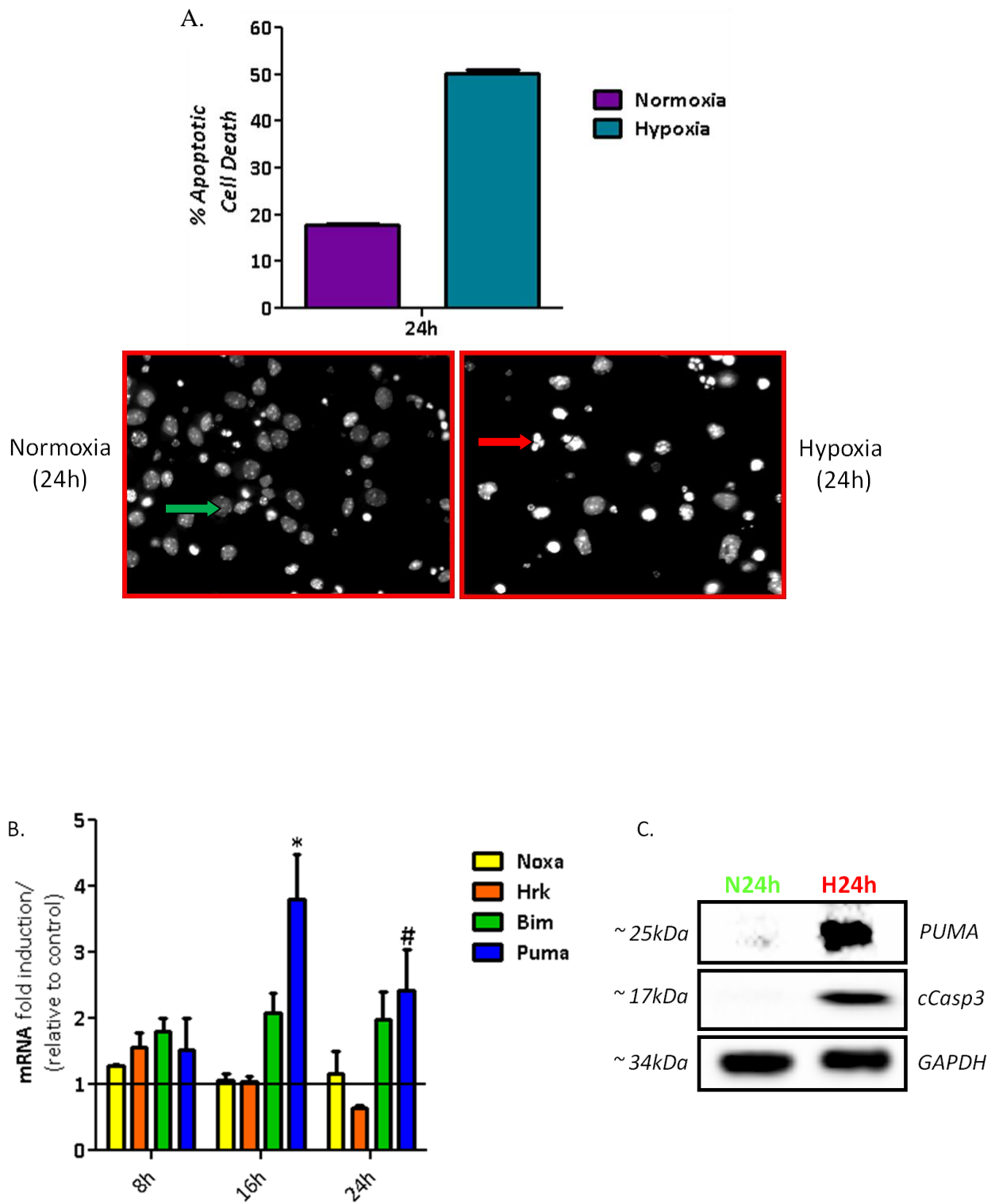


3.7 Hypoxic stress triggers PUMA expression, caspase-3 activation, and neuronal apoptosis

Hypoxic stress plays a key role in modulating neuronal death in the ischemic penumbral zone following stroke. Interestingly, cell death in this region is believed to be apoptotic in nature, occurring over an extended time frame after the initial insult (Banasiak et al., 2000; Nakajima et al., 2000). The prolonged aspect of neuronal death in the peri-infarct tissue offers an intriguing opportunity for therapeutic intervention. Therefore we proposed identifying molecular targets that are essential for delayed neuronal death using an *in vitro* model of hypoxia-induced neuronal apoptosis. Firstly, we compared the amount of apoptotic cell death between 7-DIV wild type cortical neuron cultures under normoxic conditions versus those exposed to continuous hypoxia (0.5% O_{2(g)}), for 24h, using Hoechst nuclear staining. Continuous hypoxia (0.5% O_{2(g)}) induced key hallmarks of apoptosis including chromatin condensation and nuclear pyknosis in ~50% of neurons (Figure 3.16a). Because hypoxic neuronal death can be antagonized by inhibitors of *de novo* gene synthesis (Halterman et al., 2008), we next determined the time-course expression of selected BH3-only genes, *Puma*, *Bim*, *Hrk* and *Noxa* in response to continuous hypoxia. In Figure 3.16b, we show that only *Puma* mRNA was significantly enhanced after 16 and 24h of hypoxic stress. Moreover, we show that protein levels of PUMA and cleaved caspase-3 were robustly accumulated 24h post- hypoxia (0.5% O_{2(g)}) (Fig. 3.16c).

Figure 3.16. Hypoxic stress triggers PUMA expression, caspase-3 activation, and neuronal apoptosis.

A. Representative panels of control (normoxia, N) and hypoxia (0.5% O_{2(g)}) (H) wild type cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-hypoxia. The fraction of healthy (green arrow) and apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei was determined (400X magnification). **B.** Total RNA was isolated from wild type cortical neurons 8h, 16h and 24h post-hypoxia, and the mRNA levels of *Puma*, *Noxa*, *Bim* and *Hrk* were determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to normoxic control cells (n=3; #p < 0.05; *p < 0.001). **C.** Wild type cortical neurons were exposed to hypoxic stress, and after 24h, protein levels of PUMA, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis.



3.8 Hypoxic stress triggers caspase-3 activation and neuronal apoptosis in a PUMA- and BAX-dependent manner

We and others have shown that PUMA is a critical mediator of neuronal apoptosis triggered by pharmacological inducers of oxidative dysfunction and ER-stress (Steckley et al., 2007; Bernstein et al., 2011; Galehdar et al., 2010). Interestingly, recent evidence supports that continuous hypoxia is capable of eliciting markers of both oxidative- and ER-stress (Liu et al., 2008; Halterman et al., 2008). Therefore, we wanted to address whether PUMA-deficiency could also confer significant protection against hypoxic stress-induced neuronal death. To do this, we subjected PUMA^{+/+} and PUMA^{-/-} cortical neurons to either normoxic conditions or continuous hypoxia, for 24h, and assessed apoptotic cell death via nuclear staining. In Figure 3.17a, we show that PUMA-null neurons were significantly protected (**p < 0.001), relative to wild type littermates. We next confirmed by Western blotting that PUMA-depleted neurons did not show any accumulation of cleaved caspase-3 in response to hypoxic stress, relative to the pronounced levels observed in PUMA^{+/+} (Fig. 3.17b).

The pro-death function of PUMA is generally understood to be a key catalyst towards the activation of BAX, coupled with the subsequent permeabilization of mitochondria. Because BAX plays a crucial role in this process, we aimed to determine whether BAX-depletion would abrogate hypoxia-induced neuronal apoptosis to a similar extent. We determined by Western blotting in Figure 3.18a that BAX-deficiency effectively mitigated the cleavage of caspase-3, relative to the enriched amount present in BAX^{+/-} neurons. We also determined, that the amount of chromatin condensation and pyknotic nuclei is significantly reduced (**p < 0.001) in BAX^{-/-} relative to BAX^{+/-} littermates (Fig. 3.18b).

Figure 3.17. Hypoxic stress triggers caspase-3 activation and neuronal apoptosis in a PUMA-dependent manner.

A. PUMA^{+/+} and PUMA^{-/-} cortical neurons were exposed to either normoxia (N) or hypoxia (0.5% O_{2(g)}) (H) conditions, and after 24h, protein levels of PUMA, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **B.** Representative panels of PUMA^{+/+} and PUMA^{-/-} cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-hypoxia. The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (n=3; ***p<0.001) (400X magnification).

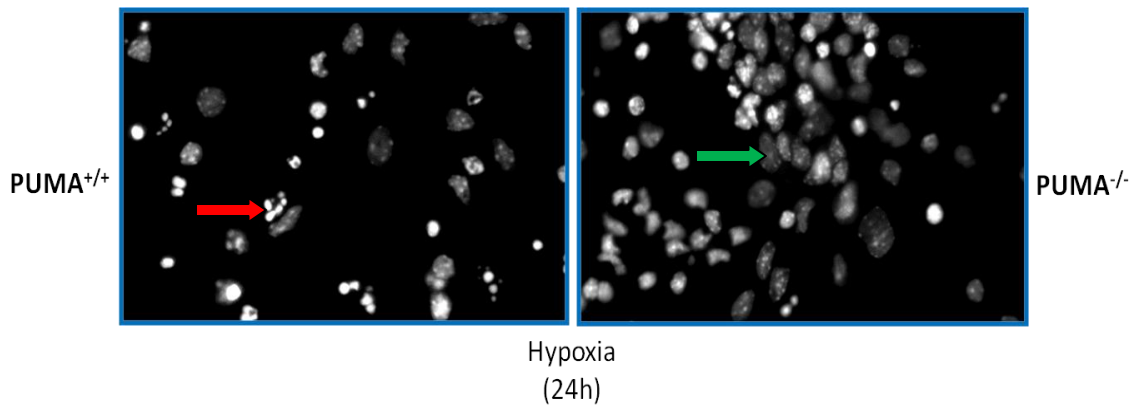
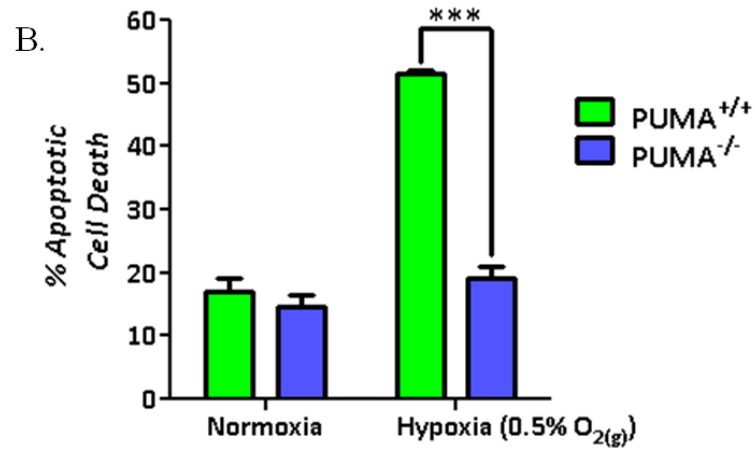
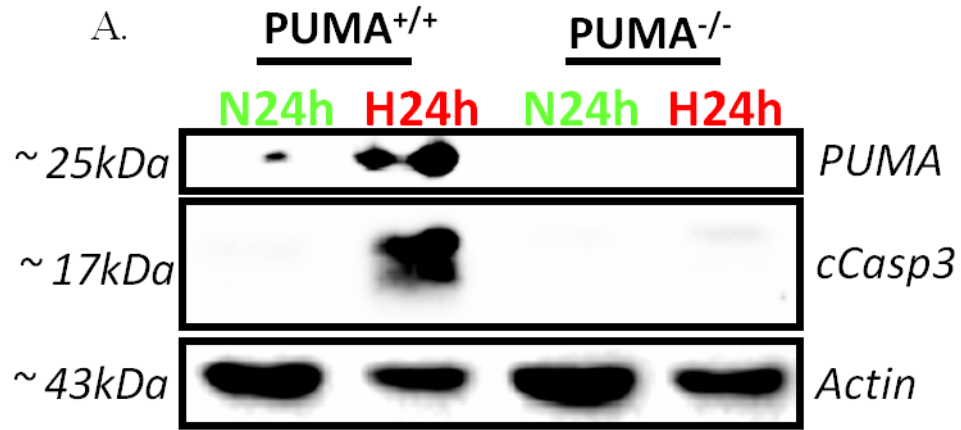
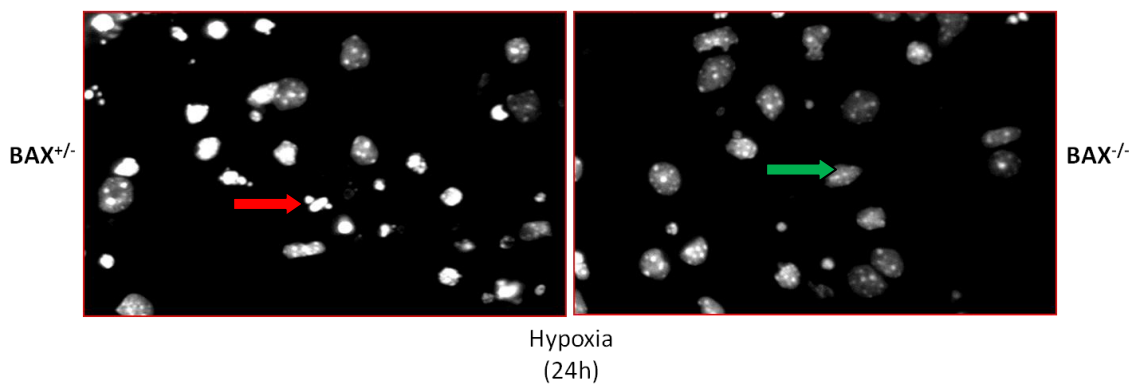
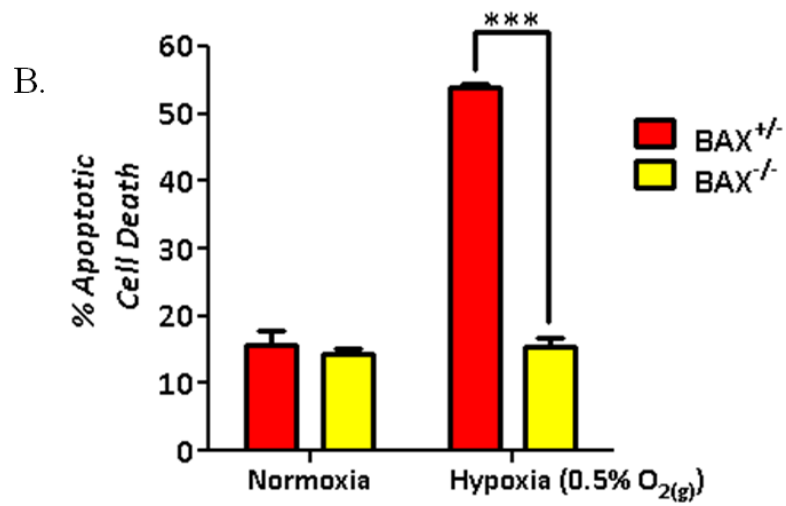
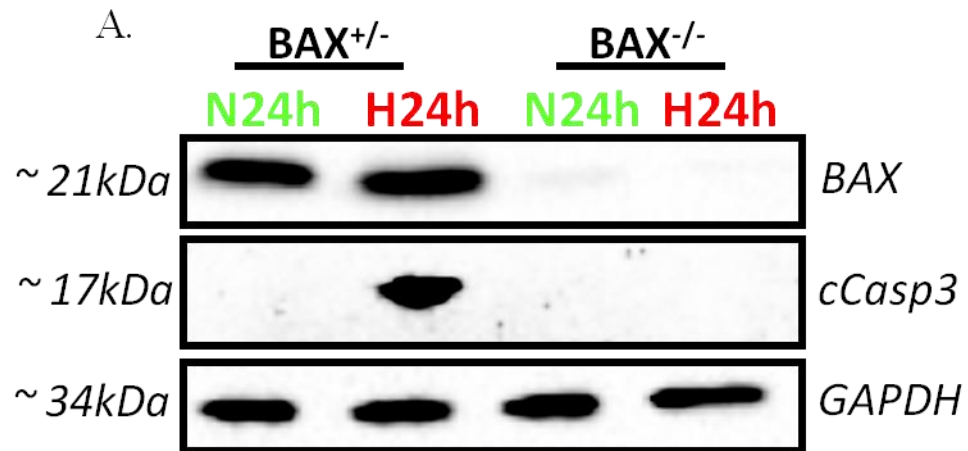


Figure 3.18. BAX is critical during hypoxia-induced caspase-3 activation and neuronal apoptosis.

A. BAX^{+/-} and BAX^{-/-} cortical neurons were exposed to either normoxia (N) or hypoxia (0.5% O_{2(g)}) (H) conditions, and after 24h, protein levels of BAX, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. **B.** Representative panels of BAX^{+/-} and BAX^{-/-} cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-hypoxia. The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (n=3; ***p<0.001) (400X magnification).



3.9 Hypoxia-induced PUMA expression is p53-independent

PUMA is a critical downstream effector of p53 in response to DNA damaging agents and some oxidative stressors however, cytokine deprivation, serum withdrawal, ER-stress and arsenite toxicity have been reported to activate p53-independent pathways (Nakano and Vousden, 2001; Yu et al., 2001; Han et al., 2001; Jeffers et al., 2003; Villunger et al., 2003; Steckley et al., 2007; Bernstein et al., 2011; Fricker et al., 2010; Galehdar et al., 2010). In order to determine if p53 plays a role in hypoxia-induced PUMA expression, we examined the time-course accumulation of p53, PUMA and cleaved caspase-3 protein in wild type neuronal lysates. In Figure 3.19a, we show that p53 protein is not enriched in response to continuous hypoxia (0.5% O_{2(g)}), as compared to the robust stabilization induced by the DNA damaging agent, camptothecin (CPT). Interestingly, we observed increases in both PUMA and activated caspase-3 protein levels, suggesting a p53-independent mechanism. Next we considered the impact of p53-deletion on *Puma* expression triggered by hypoxic stress. When p53^{+/-} and p53^{-/-} cortical neurons were exposed to continuous hypoxia for 8, 16 and 24h, we observed no significant difference in *Puma* expression between genotypes (Fig. 3.19b). Moreover, the degree of apoptotic neuronal death between p53^{+/-} and p53^{-/-} was almost identical (Fig. 3.19c).

Since our model of hypoxia-induced neuronal apoptosis indicated the involvement of a p53-independent pathway, we next assessed the expression of the pro-death factor, ATF4. Consistent with this, recent evidence suggests that hypoxic stress increases reactive oxygen species production leading to the induction of the ISR, an arm of the unfolded protein response geared towards the adaptation to ER-stress (Liu et al., 2008). Indeed, ATF4 is known to modulate p53-independent apoptosis in a variety of stress paradigms (Galehdar et al., 2010; Qing et al., 2012). Firstly, we analyzed *Atf4* mRNA levels in wild type neuronal cultures following 8, 16 and 24h post-hypoxia (Fig. 3.20a). Although we did not detect any significant changes at the gene expression level we did find that ATF4 protein and P-eIF2 α were enhanced after 20h of continuous hypoxic stress (Fig. 3.20b). Consistent with this, Blais and colleagues (2004) reported a similar trend favouring a translational induction of ATF4 over a transcriptional response. Lastly, we aimed to investigate the p53-independent pathway by establishing a link

between ATF4-PUMA in p53-depleted neurons. To do this, we determined PUMA, cleaved caspase-3, p53 and ATF4 protein levels in p53^{+/-} and p53^{-/-} cortical cultures exposed to hypoxic stress over 24h. In agreement with our previous data, we did not observe p53 stabilization after hypoxic stress however, we did detect ATF4 protein expression, which closely mirrored the up-regulation of PUMA and activated caspase-3 (Fig. 3.20c).

3.10 Hypoxia induces PUMA expression, caspase-3 activation, and neuronal apoptosis in an ATF4-dependent manner

ATF4 has recently been implicated as a critical mediator of neuronal death triggered by ischemia, amyloid-beta toxicity and ER dysfunction (Lange et al., 2008; Baleriola et al., 2014; Galehdar et al., 2010). Since ATF4 is known to directly regulate *Puma* expression independently of p53 during amino acid deprivation (Qing et al., 2012), we aimed to show whether ATF4-deficiency would reduce hypoxia-induced *Puma* expression. Firstly, we analyzed *Puma* mRNA in ATF4^{+/-} and ATF4^{-/-} cortical cultures over 8 and 16h of continuous hypoxia and found a significant reduction after 16h (*p < 0.05) (Fig. 3.21a). Similarly, we observed that ATF4-null neurons displayed significantly less neuronal apoptosis than their ATF4^{+/-} counterparts 24h post-hypoxia (**p < 0.01) (Fig. 3.21b). Finally, we found that ATF4 status was critical to caspase-3 activation following 24h of hypoxic stress, as evidenced in ATF4-depleted neurons that showed minimal cleaved caspase-3, relative to wild type (Fig. 3.21c).

Figure 3.19. Hypoxia-induced PUMA expression is p53-independent.

A. Wild type cortical neurons were exposed to either normoxic conditions for 0h or hypoxic stress, and after 2, 4, 8, 16 and 24h, protein levels of p53, PUMA, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis. A positive control lane was included using wild type cortical neuron lysates treated with the DNA damaging agent, Camptothecin (CPT), to estimate p53 levels. **B.** Total RNA was isolated from p53^{+/-} and p53^{-/-} cortical neurons 8h, 16h and 24h post-hypoxia, and the mRNA level of *Puma* was determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to normoxic control cells (n=5). **C.** Representative panels of p53^{+/-} and p53^{-/-} cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-hypoxia. The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (n=3) (400X magnification).

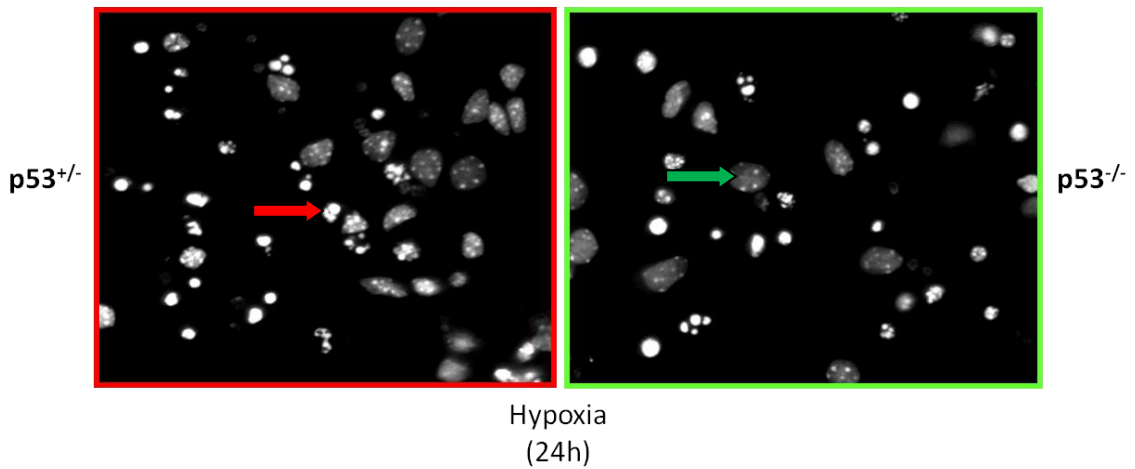
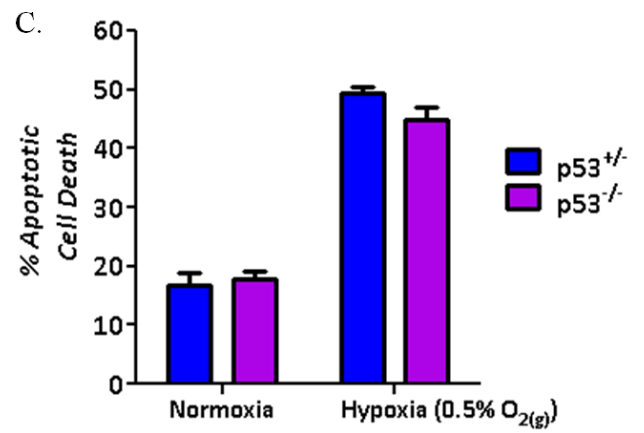
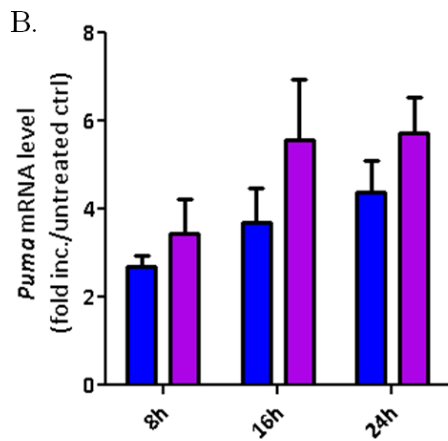
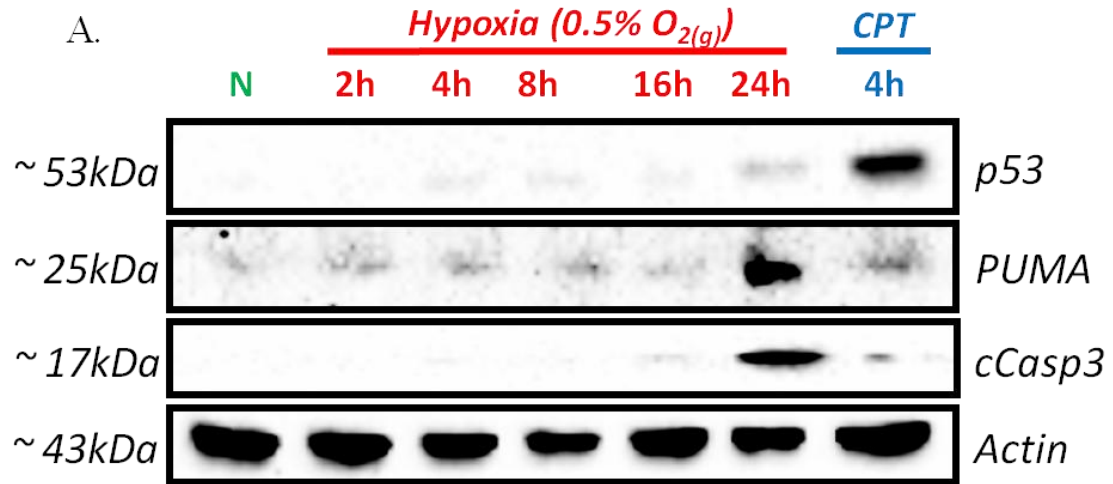
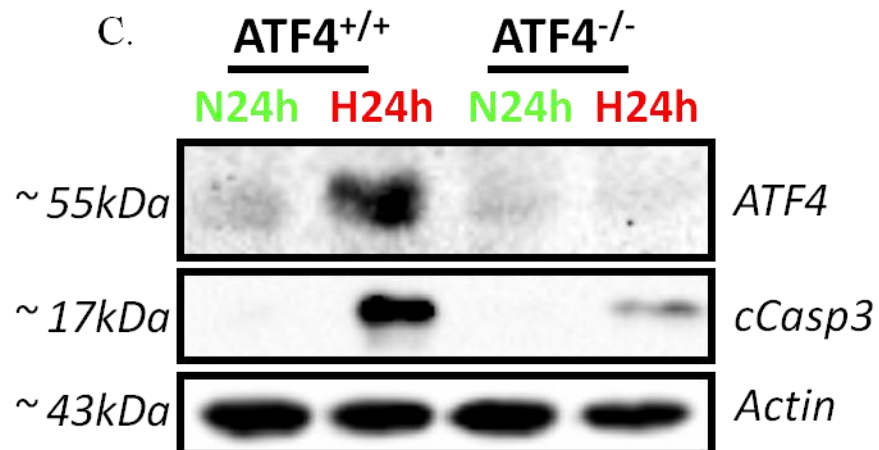
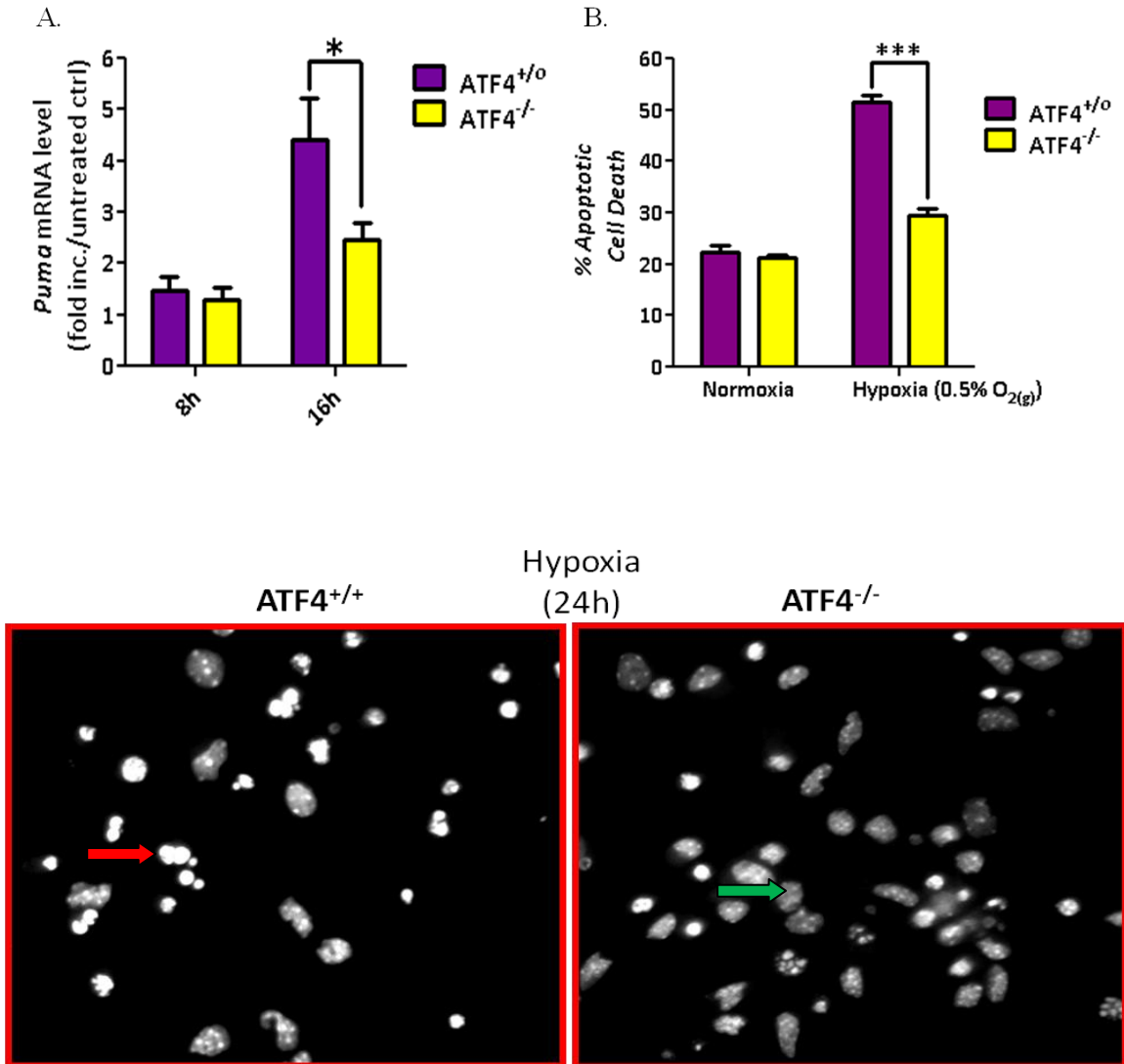


Figure 3.20. Hypoxia induces the enrichment of ATF4, PUMA and cleaved caspase-3 in p53-deficient neurons.

A. Total RNA was isolated from wild type cortical neurons 8h, 16h and 24h post-hypoxia (0.5% O_{2(g)}), and the mRNA level of *Atf4* was determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to normoxic control cells (n=3). **B.** Wild type cortical neurons were exposed to either normoxic (N) conditions or hypoxic stress (H), and after 20h, protein levels of ATF4, P-eIF2 α were assessed as a whole cell extract by Western blot analysis. The membrane was then stripped and re-probed for the loading control, total eIF2 α . A positive control lane was included using a wild type cortical neuron lysate treated with the ER-stress agent, Thapsigargin (TG), for 24h, to stimulate an ER-stress response. **C.** p53^{+/-} and p53^{-/-} cortical neurons were exposed to either normoxic or hypoxic conditions, and after 24h, protein levels of p53, ATF4, PUMA, cleaved caspase-3 (cCasp3), and the loading control, total eIF2 α , were assessed as a whole cell extract by Western blot analysis. CPT was included as a positive control, as described above.

Figure 3.21. Hypoxia induces PUMA expression, caspase-3 activation, and neuronal apoptosis in an ATF4-dependent manner.

A. Total RNA was isolated from ATF4^{+/-} and ATF4^{-/-} cortical neurons 8h, 16h post-hypoxia, and the mRNA level of *Puma* was determined by qRT-PCR. Expression was normalized to *s12* mRNA levels and is reported as fold increase relative to normoxic control cells (n≥3; *p < 0.05). **B.** Representative panels of ATF4^{+/+}/ATF4^{+/-} (ATF4^{+/-}) and ATF4^{-/-} cortical neurons fixed and stained with Hoechst stain (1:1000) 24h post-hypoxia. The fraction of apoptotic cells (red arrow) showing chromatin condensation and fragmented nuclei relative to healthy cells (green arrow) was determined (n≥3; ***p < 0.001) (400X magnification). **C.** ATF4^{+/+} and ATF4^{-/-} cortical neurons were exposed to either normoxic conditions (N) or hypoxic stress (H), and after 24h, protein levels of ATF4, cleaved caspase-3 (cCasp3), and the loading control, Actin, were assessed as a whole cell extract by Western blot analysis.



Chapter 4

4 Summary and Discussion

The goal of this work was to identify candidate genes that play a critical role in modulating neuronal death in response to stress pathways intimately associated with neurodegenerative pathologies. Ideally, the long term goal would be the implementation of targeted therapeutics designed to inhibit the expression of pro-death mediators to preserve brain function and structural integrity. To this end we have identified the transcription factors, ATF4 and CHOP, and the pro-death genes, PUMA and BAX, as key players associated with neuronal apoptosis driven by ER-stress, oxidative dysfunction and delayed neuronal death triggered by hypoxic stress (Figure 4.1).

4.1 Summary of ER-stress-mediated neuronal apoptosis

Indeed ER-stress has been linked with the disease course of AD, PD and ALS, and acute neuronal death induced by cerebral ischemia (Hoozemans et al., 2005; Hoozemans et al., 2007; Atkin et al., 2008; Morimoto et al., 2007). The manifestation of ER dysfunction is carried out through the concerted effort of the UPR to restore homeostatic balance to the ER lumen. Protein translation is arrested and ER chaperones are redistributed to coordinate the systematic degradation of misfolded aggregates thereby alleviating the toxic protein load. If the intensity of the stress proves too great and cannot be resolved, the gradual accumulation of pro-death effectors drives the cell to commit suicide (Hetz, 2012). The relative importance and contribution of ER-stress related proteins to neuronal apoptosis is not completely resolved. Under pathological conditions, the UPR is paradoxically associated with facilitating recovery after spinal cord injury (Valenzuela et al., 2013), and enabling disease course via increased BACE1 expression in AD (O'Connor et al., 2008).

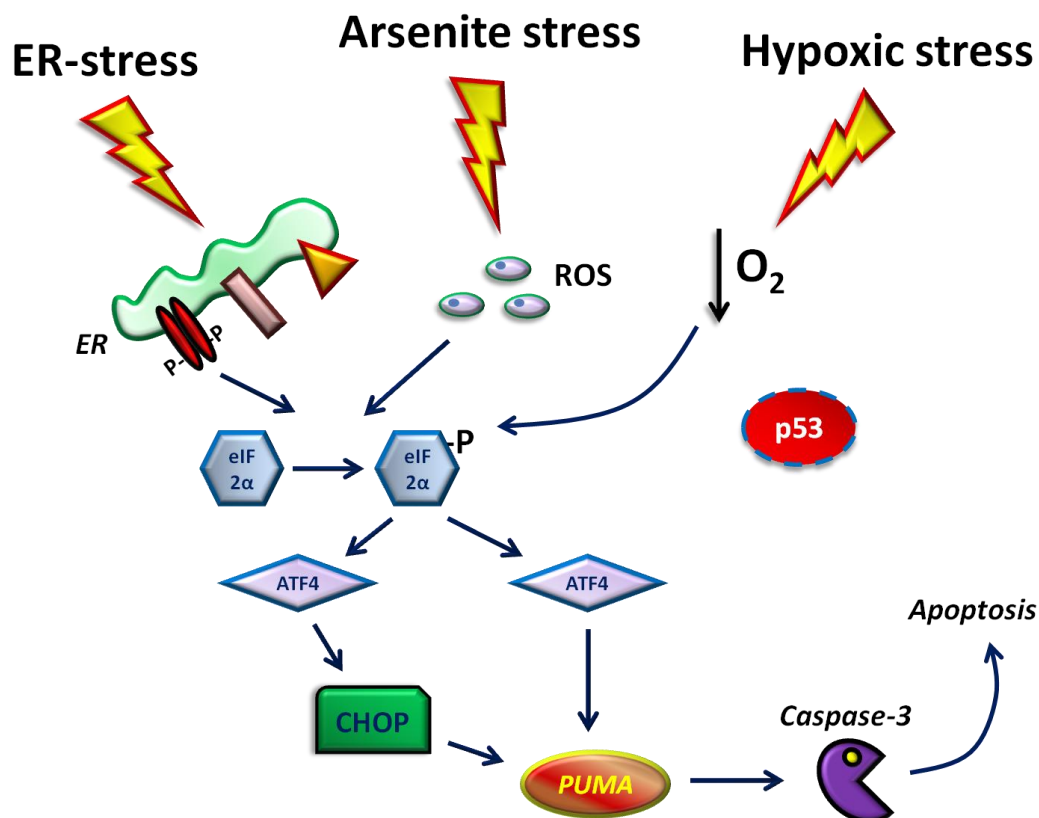


Figure 4.1. Summary of findings.

From this work we have shown that ER-stress induced the sequential activation of ATF4 followed by the stabilization of CHOP, which was required to directly induce PUMA expression and neuronal apoptosis. Second, we demonstrated that the oxidative stressor, arsenite, selectively enhanced UPR factors, of which ATF4 played a dominant role in the modulation of PUMA, caspase-3 activity and neuronal death. In our final aim, we employed a physiological model of oxygen deprivation and found that ATF4 played an essential role in regulating PUMA expression and caspase-3 activity. Remarkably, we and others (Fricker et al., 2010) have demonstrated that p53 does not regulate neuronal apoptosis under each of our experimental parameters.

Our results have shown that ATF4, an essential modulator of the ISR, plays a critical role in the regulation of neuronal apoptosis in response to ER-stress. We have also demonstrated that ATF4 regulates *Puma* mRNA levels induced by ER-stress, whereas p53 is dispensable in this context. Conversely, ATF4 does not dictate *Puma* expression or neuronal apoptosis triggered by DNA damage, which we showed to be strongly p53-dependent (Galehdar et al., 2010). We further elaborated that ER dysfunction led to the sequential enhancement of ATF4 and CHOP, of which the latter was directly responsible for the induction of PUMA and neuronal cell death.

In non-neuronal systems, ER-stress was shown to promote apoptosis in a partially p53- and NOXA-dependent manner (Li et al., 2006). Conversely, we found that in post-mitotic neurons, p53 was not required for this process, which may be attributable to ER-stress-mediated degradation of p53 (Galehdar et al., 2010; Qu et al., 2004). Similarly, ATF4 does not modulate DNA damage-induced neuronal apoptosis rather this may be due to an accumulation of the LIP isoform of C/EBP β , a known transcriptional repressor of ATF4, which can be regulated by genotoxic stress (Galehdar et al., 2010; Dey et al., 2012). Importantly, we considered the contribution of another BH3-only gene, BIM, as BIM was induced at the mRNA and protein level following ER-stress. Although PUMA-deletion rescued neurons from ER-stress toxicity, this phenomenon was not recapitulated in BIM-null neurons, suggesting that PUMA plays a dominant role in the regulation of stress-induced neuronal death (Steckley et al., 2007; Galehdar et al., 2010).

We next determined whether ATF4 mediated PUMA expression via a direct transcriptional mechanism by performing a chromatin immunoprecipitation. Our results indicated that ATF4 did not directly induce PUMA but rather, we detected an enrichment of the ATF4-target gene, CHOP, at the PUMA promoter. CHOP has also been shown to modulate BIM expression in non-neuronal cells (Puthalakath et al., 2007). Interestingly, ATF4 was recently ascribed as a direct regulator of PUMA in response to nutrient deprivation (Qing et al., 2012). Although ATF4-deletion and CHOP knockdown afforded significant protection from ER-stress-mediated neuronal apoptosis, the survival benefit was incomplete. This suggests the involvement of alternative mechanisms possibly attributable to other UPR factors like, XBP1 or ATF6. Interestingly, the enforced expression of ATF6 can induce CHOP however XBP1 does not appear to influence

CHOP levels (Yoshida et al., 2000; Lee et al., 2003). Moreover, ATF6 α -deficient mice are sensitized to ER-stress *in vivo*, and the endogenous knockdown of XBP1 within the midbrain enhances dopaminergic neuronal death (Wu et al., 2007; Valdés et al., 2014). Another potential candidate is the transcriptional regulator, FOXO3a, which has been shown to activate PUMA expression in neuronal and non-neuronal systems (Zou et al., 2009; You et al., 2006). FOXO3a was recently demonstrated to regulate PUMA independently of p53 in response A β treatment, a stimulus capable of inducing ER-stress (Yoon et al., 2012; Akhter et al., 2014; Feng et al., 2015).

4.2 Summary of arsenite-mediated neuronal apoptosis

Oxidative stress occurs when the amount of ROS produced through endogenous or hazardous mechanisms cannot be efficiently detoxified by our intrinsic antioxidants, thereby making cellular biomolecules increasingly vulnerable to the abundance of toxic modifiers. Although a substantial body of work has linked oxidative dysfunction with the etiology of several neurodegenerative illnesses, the mechanisms regulating cell death are poorly understood.

Our second aim elaborated the signaling pathway regulating PUMA expression and neuronal apoptosis induced by the oxidative stressor, sodium arsenite, a stimulus capable of driving p53-independent neuronal death (Fricker et al., 2010). Arsenite toxicity is thought to arise from the generation of several free radicals and non-radicals such as, NO, ONOO $^-$, H $_2$ O $_2$ and hydroxyl (Chattopadhyay et al., 2002; Guidarelli et al., 2005; Chen et al., 1998; Garcia-Chavez et al., 2003). Chronic exposure to arsenite can also further reduce the brain's limited antioxidant defenses by lowering SOD and GSH levels (Garcia-Chavez et al., 2003; Namgung and Xia, 2001).

Importantly, components of the UPR can be induced by oxidative dysfunction, independently of ER-stress and PERK activation. For instance, ATF4 requires both stress-induced transcriptional control and favorable translational conditions via P-eIF2 α to accumulate to appreciable levels. Interestingly, both ER-stress and UV irradiation can induce P-eIF2 α , however only ER-stress robustly enhanced ATF4 levels while UV irradiation inhibited its transcription (Dey et al., 2010; Dey et al., 2012). Since we and others have demonstrated that ATF4 plays a critical role in regulating neuronal apoptosis,

it is imperative to define what other ER-stress-independent pathways can accumulate ATF4 (Lange et al., 2008; Galehdar et al., 2010, Armstrong et al., 2010).

The interpretation of cellular stresses by eIF2 α kinases, heme-regulated inhibitor (HRI), protein kinase RNA-activated (PKR), PKR-like ER kinase (PERK), and general control nonderepressible 2 (GCN2), is termed, the Integrated Stress Response (ISR), an adaptive pathway regulating antioxidant, chaperone and apoptotic gene expression, largely through the activity of ATF4. Although our data supports the pronounced activation of notable transcriptional regulators of the ISR like, ATF3, ATF4 and CHOP, we have not identified the putative kinase that generates P-eIF2 α , a condition that paradoxically favors the translation of unique mRNAs. Several reports have suggested that GCN2 is abundantly expressed in the brain and may influence ATF4 levels, and modulate P-eIF2 α in response to oxidative stress (Sood et al., 1998; Costa-Mattioli et al., 2005; Zhan et al., 2004; Roff   et al., 2013). However, to our knowledge, there is no specific inhibitor of GCN2 currently available.

Other oxidative stressors such as Parkinsonian mimetics, 6-OHDA and MPP+, are known to induce ER-stress and the UPR. However, in this scenario the UPR is thought to play a pro-survival role in response to 6-OHDA as evidenced by the enhanced degree of apoptosis observed in PERK^{-/-} neurons relative to wild type (Ryu et al., 2002). Moreover, MPP+ and 6-OHDA have been shown to trigger neuronal apoptosis in a p53-dependent manner, whereas p53 is dispensable for arsenite-mediated neuronal apoptosis (Perier et al., 2007; Bernstein et al., 2011; Fricker et al., 2010).

Although there was no evidence of ER-stress, arsenite activated selected components of the UPR to promote the induction of PUMA and neuronal apoptosis. Specifically, downstream effectors of the PERK pathway, ATF3, ATF4 and CHOP were robustly expressed by arsenite in a PERK-independent manner. Using gene-specific knockout mice, we analyzed the contribution of ATF3, ATF4 and CHOP in the regulation of arsenite-mediated PUMA expression and neuronal survival. We determined that only the deletion of ATF4 was sufficient to significantly attenuate *Puma* mRNA, caspase-3 activation and neuronal apoptosis, in response to arsenite stress. In contrast to our initial finding, where we identified CHOP as a key regulator of ER-stress-induced PUMA

expression and neuronal apoptosis, we found that in the context of arsenite toxicity, CHOP-deletion did not mitigate *Puma* levels or neuronal apoptosis. Other distinctions between ER- and oxidative stress signaling involved the differential regulation of ATF3. In the ER-stress paradigm, the initial up-regulation of ATF4 was followed by CHOP and then ATF3. Conversely, arsenite exposure induced ATF4, and then incurred the expression of ATF3 and CHOP simultaneously. We also indicated that ROS generated by arsenite toxicity played a crucial role in stabilizing ATF4 protein, as this effect was abrogated by the antioxidant, NAC. However, pretreatment with NAC did not reduce ER-stress-mediated ATF4 expression.

Another important distinction in our findings included the observation that neurons lacking ATF3- or CHOP did not show significant protection from arsenite stress, whereas MEFs harboring deletions for ATF3 or CHOP exhibited a strong reduction in apoptosis triggered by arsenite toxicity (Jiang et al., 2007). Moreover, ATF4-depleted MEFs were sensitized to apoptotic cell death (Jiang et al., 2007), while our results from ATF4^{-/-} neurons were consistent with previous data that showed the opposite phenotype (Lange et al., 2008). One key difference from our work relates to the unequal contribution of CHOP towards the regulation of PUMA in the context of ER dysfunction and its expendable role in modulating PUMA expression during arsenite stress. This potentially reflects a dichotomous posttranslational regulation of CHOP and ATF4 by the ubiquitous kinase, casein kinase-2 (CK2). Indeed, it has been reported that arsenite stress can stimulate CK2 leading to both an enhancement and a reduction in the transcriptional activities of ATF4 and CHOP, respectively (Sayed et al., 2000; Ubeda and Habener, 2003; Ampofo et al., 2013).

We also proposed that the hierarchical regulation between the transcription factors ATF3, ATF4 and CHOP was distinct between stress paradigms. During ER-stress, we elaborated the sequential activation of ATF4, which induced CHOP followed by ATF3, whereas during arsenite treatment, ATF3 and CHOP were similarly expressed after ATF4 stabilization. Our results are consistent with previous reports that also demonstrated a stepwise pattern of CHOP preceding ATF3 expression, following ER-stress (Zinszner et al., 1998; Jiang et al., 2004). However during arsenite exposure, ATF4-null neurons showed no residual induction of ATF3 or CHOP, whereas in MEFs, other transcription

factors compensate for the absence of ATF4 to modestly increase their expression (Jiang et al., 2007). Moreover, intervention with the antioxidant, NAC, attenuated ATF4 levels induced by arsenite, a finding that is supported in non-neuronal cells (Roybal et al., 2005; Jiang et al., 2007). Interestingly, Malhotra and others (2008) showed that the lipid-soluble antioxidant, butylated hydroxyanisole, reduced ER-stress by suppressing multiple UPR-related transcripts. In contrast to our results, NAC did not mitigate ATF4 protein induced by ER dysfunction. Although we have associated ATF4 with a pro-death function, it was recently reported that ATF4 regulates both ATF6 expression and processing, indicating a disparate and potentially protective role, as ATF6 α -null mice are sensitized to oxidative stress *in vitro* and *in vivo* (Teske et al., 2011; Egawa et al., 2011; Hashida et al., 2012).

Since we have not associated a direct transcriptional function to ATF4 driving PUMA expression, we have considered an alternative mechanism involving an intermediate, GADD45 α . Indeed recent reports have suggested that GADD45 α knockdown significantly attenuated oxidative stress- and glutamate-induced apoptosis (Gao et al., 2013; Choi et al., 2011). Moreover, GADD45 α is an ATF4-target gene that can be up-regulated independently of p53 (Jiang et al., 2007; Gao et al., 2013). Interestingly, GADD45 α expression is substantially increased in AD *post-mortem* brain tissue relative to age-matched control brains (Torp et al., 1998). Future studies would include a targeted knockdown approach using a lentiviral vector expression system.

4.3 Summary of hypoxia-induced neuronal apoptosis

In the current study, we investigated the molecular events leading to neuronal apoptosis during continuous hypoxic stress. We propose that hypoxic neuronal injury triggers the expression of the BH3-protein, PUMA, in an ATF4-dependent and p53-independent manner. Although we and others have reported that hypoxia and ischemic neuronal injury can induce PUMA, some recent data suggested that PUMA is not expressed following oxygen-glucose deprivation (OGD) in cortical cultures (Halterman et al., 2008; Niizuma et al., 2009; Pfeiffer et al., 2014). Rather, OGD induced the BH3 gene, *Bmf*, and *Bmf*-depletion was sufficient to significantly reduce infarct size after transient middle cerebral artery occlusion (tMCAO) (Pfeiffer et al., 2014). Similarly, BID^{-/-} mice were able to resist significant neuronal loss following tMCAO (Plesnila et al., 2001). These data

confirm that apoptosis plays a critical role in neuronal death after ischemic insults. Thus it would be important to identify whether our *in vitro* observations in PUMA-null mice can be translated to *in vivo* models. Functionally, BH3-only family members are known to facilitate apoptosis either through the direct or indirect activation of BAX (Kim et al., 2006; Willis et al., 2007; Du et al., 2011). Because BAX serves as a key gateway to the initiation of mitochondrial permeabilization and caspase activation, we tested the impact of BAX deletion on neuronal survival after hypoxic stress. Similar to our data from PUMA^{-/-} neurons, apoptosis was thoroughly compromised in cortical cultures lacking BAX. Consistent with this, neonatal BAX-deficient mice that were subjected to hypoxic-ischemic (HI) brain injury showed reduced caspase activation and tissue damage relative to wild type (Gibson et al., 2001). Interestingly, the pharmacological inhibition of BAX activation after HI has been demonstrated with promising results extending towards functional recovery (Wang et al., 2010b). This was an important finding because of the profound and persistent behavioral changes that can surface as collateral damage after stroke.

In order to investigate BH3 expression, we elected to survey two known transcriptional activators of PUMA, p53 and ATF4, in a model of hypoxia-induced neuronal apoptosis. Initially we analyzed whether p53 levels could be stabilized over a time-course of hypoxic stress. Strikingly, we found that not only was p53 largely absent over that period but there was a substantial enrichment of PUMA and activated caspase-3. The lack of p53 accumulation could be due to the oxygen concentration employed in our model. Specifically, we used 0.5% O_{2(g)} whereas p53 levels are known to be stabilized at 0.2% but not at 2%, indicating a highly specific regulatory mechanism (Hammond et al., 2002). We further validated the contribution of p53 using cortical neuronal cultures devoid of p53 and analyzed *Puma* mRNA levels after hypoxia. Our data suggest that p53 does not regulate PUMA transcription in this context. Similarly we did not observe any significant reduction in neuronal apoptosis between p53 genotypes.

We next considered if ATF4, a key player in the ER-stress response, could be modulating PUMA levels in the absence of p53 involvement. As supported by Blais and others (2004), we also showed that hypoxia preferentially stabilizes ATF4 post-translationally.

Moreover, Halterman and colleagues (2008) demonstrated that the bZIP transcriptional regulator, C/EBP β , dissipated over the course of hypoxic stress and that decrease correlated with an enhancement of ATF4 and the apoptotic response. This distinction is supported by the notion that C/EBP β functions as a repressor of ATF4 expression (Dey et al., 2012). Another potential mechanism of ATF4 stabilization involves the oxygen sensor, prolyl-4-hydroxylase domain 3 (PHD3). Under normoxic conditions, PHD3 suppresses ATF4 levels via proteasomal degradation however, during conditions of reduced oxygen availability, PHD3 function decreases and ATF4 becomes enriched. This was further elaborated when ATF4 accumulated during normoxia, after PHD3 knockdown (Köditz et al., 2007).

Our data support that hypoxia stimulates the phosphorylation of eIF2 α , which leads to a refined increase in ATF4 protein synthesis. The process of eIF2 α modification by cellular perturbations is termed the Integrated Stress Response (ISR). It is generally understood that hypoxia triggers the ISR via the ER membrane kinase, PERK, to phosphorylate eIF2 α leading to the selective induction of ATF4, as evidenced from PERK-depleted cells (Blais et al., 2004). We then demonstrated that ATF4 protein was enhanced in both p53-heterozygous and -null neurons, with the concurrent expression of PUMA and cleaved caspase-3. Moreover we showed that ATF4-deficiency lead to significantly reduced *Puma* levels and neuronal apoptosis after continuous hypoxia. Conversely, Bi and others (2005) reported that impairments throughout the ISR lead to the consistent hyperactivation of caspase-3 in non-neuronal cells. The consensus on whether ATF4 is pro-survival or pro-death is controversial and appears to be both context and cell type specific. For instance, in non-neuronal cells hypoxia can trigger the production of ROS, a potent stimulus that can lead to enhanced levels of apoptosis in ATF4-deficient MEFs (Liu et al., 2008; Jiang et al., 2007). Moreover, ATF4^{-/-} MEFs require supplementation with reducing agents to lower the endogenous levels of oxidative stress (Harding et al., 2003). Conversely, neurons lacking ATF4 have been shown to resist cell death triggered by ER-stress, amyloid beta toxicity, ischemic injury, and show increased survivability in ALS model mice (Galehdar et al., 2010; Baleriola et al., 2014; Lange et al., 2008; Matus et al., 2013).

Although we have implicated ATF4 as a key component regulating PUMA expression in response to hypoxic stress, the reduction in *Puma* mRNA observed in ATF4-null neurons appears to be incomplete. Moreover we cannot discount that other BH3-only members or unrelated pro-death modulators might be involved since the protection afforded by the deletion of ATF4 is not absolute. It is possible that E2F1 could impact PUMA expression and neuronal apoptosis during hypoxia, as E2F1^{-/-} mice showed reduced infarct sizes following a cerebral ischemic insult (MacManus et al., 2003). Moreover, E2F1 has been shown to directly associate with the PUMA promoter, and the enforced expression of E2F1 is sufficient to mediate neuronal apoptosis (Hou et al., 2000; Hershko and Ginsberg, 2004). Other BH3-only genes that could be playing a compensatory role in this paradigm are, Hrk, Bim, Bid, Bmf and Bnip3 (Pike et al., 2012; Plesnila et al., 2001; Kuan et al., 2003; Li et al., 2013; Pfeiffer et al., 2014). Interestingly, ATF4 can directly induce Hrk expression during severe hypoxia (Pike et al., 2012).

In summary we propose that ATF4 plays a crucial role in the regulation of delayed neuronal death during hypoxic stress *in vitro*. In our model, ATF4 contributes significantly in the regulation of pro-apoptotic gene, PUMA, a known facilitator of BAX activation, mitochondrial permeabilization and caspase-3 activation. A growing body of evidence from knockout mice of the Bcl-2 family have suggested that apoptosis plays a considerable role in the modulation of neuronal loss after ischemia. Moreover, ATF4 is known to directly modulate PUMA expression and ATF4-null mice displayed reduced infarct volumes after tMCAO (Lange et al., 2008, Qing et al., 2012). Therefore the pharmacological inhibition of ATF4 could prove a valuable therapeutic target to mitigate delayed neuronal death and preserve functional viability after stroke.

4.4 A potential mechanism linking ER-stress and oxidative dysfunction with neurodegenerative disease progression

The deleterious contribution of environmental stressors to neurological impairments is well-studied (Betarbet et al., 2000; Sherer et al., 2002; Giasson et al., 2000; Lin et al., 2007). For instance, the pesticide, rotenone and the contaminant, arsenite are known to generate oxidative stress, and contribute to the toxic gain-of-function of α S into a more aggressively aggregating species (Sherer et al., 2002; Lin et al., 2007; Giasson et al., 2000). The cellular hub for the generation of oxidative stress is the mitochondrion, and deficits in electron transport through the ETS are strongly linked to excessive ROS production, and the disease course of AD, PD and ALS (Lin and Beal, 2006). It was recently demonstrated that mitochondrial-derived ROS can enhance the amyloidogenic processing of APP, yielding the neurotoxic oligomer, A β (Leuner et al., 2012). Similarly, the enhanced expression of α S can increase ROS production, and nitrosative stress arising from ONOO \cdot can deactivate SOD1 function, increasing cellular susceptibility to ROS (Aoyama et al., 2000; Hsu et al., 2000). Indeed both aggregated α S, A β and misfolded SOD1 are known to localize in the ER and associate with the chaperone, BiP, which is thought to sensitize cells to ER-stress (Kikuchi et al., 2006; Colla et al., 2012; Soejima et al., 2013). Interestingly, the α S-BiP interaction was detected in presymptomatic PD-model mice (Colla et al., 2012). Consistent with this, UPR sensors and BiP were shown to be activated in ALS- and AD-model mice prior to disease onset (Atkin et al., 2008; Soejima et al., 2013).

Taken together, current evidence suggests that excessive ROS can profoundly modify wild type proteins into toxic disease enablers and this may have to do with a detrimental association with the ER chaperone, BiP. By removing BiP from the luminal domains of ER-stress sensors, the UPR is inappropriately activated for a sustained duration. This could explain why the UPR has been observed early on in young AD-, PD- and ALS-model mice before and during symptom onset, and remains induced until death, as evidenced in human *post-mortem* tissues (Colla et al., 2012; Soejima et al., 2013; Hoozemans et al., 2005; Hoozemans et al., 2007; Atkin et al., 2008) (Figure 4.2).

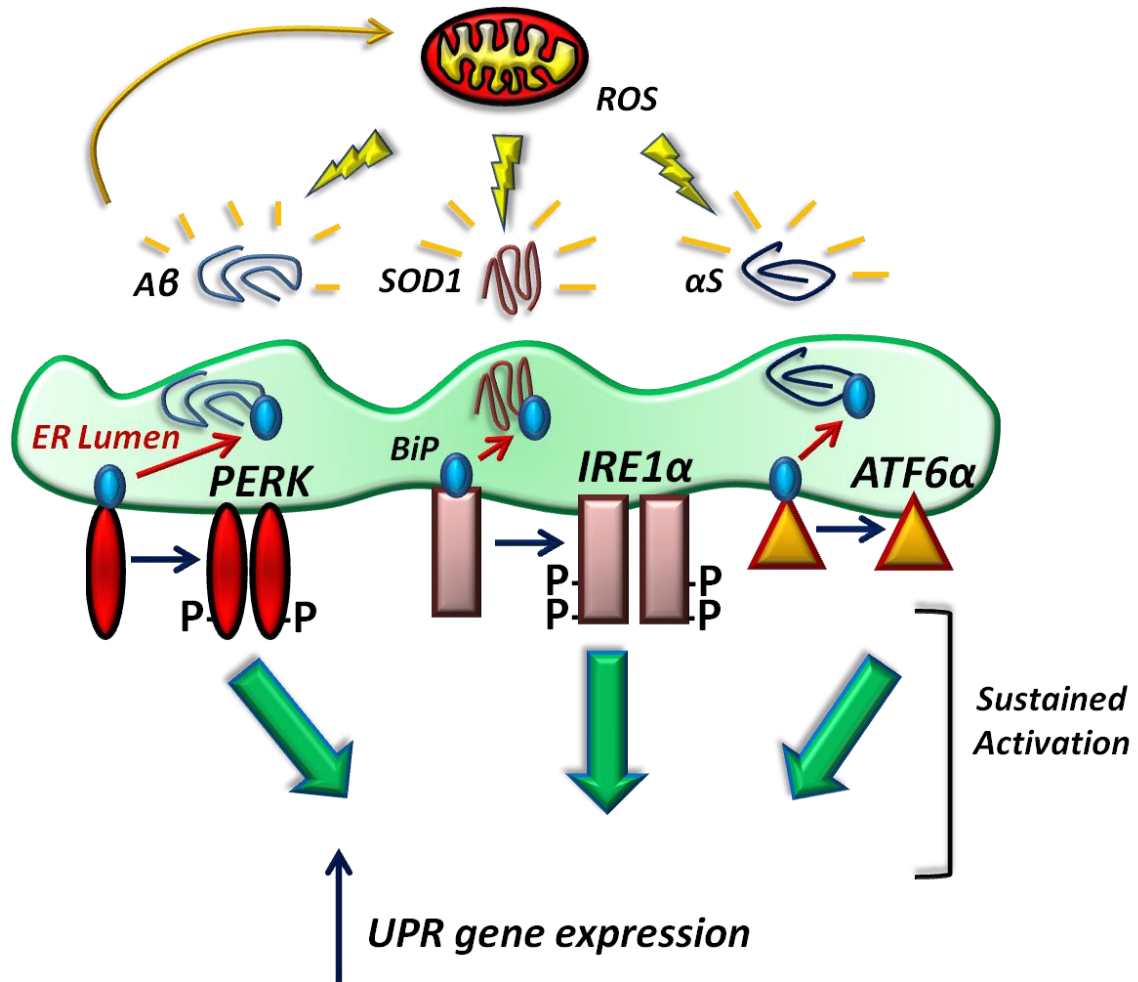


Figure 4.2. ER and oxidative dysfunction can potentiate neurodegenerative disease.

Mitochondria are central to the generation of ATP via oxidative phosphorylation. However, ROS escaping from the ETS can detrimentally alter cellular macromolecules. Alpha synuclein (αS) and superoxide dismutase-1 (SOD1) can acquire toxic attributes through ROS-mediated alterations. Moreover, amyloid beta ($A\beta$) generation can be induced by ROS, which instigates a vicious cycle furthering mitochondrial impairments. The newly accumulated toxic proteins can also localize within the ER where they may interact with the ER chaperone, BiP. It is possible that this interaction sensitizes the cell to a persistent induction of the UPR, as markers of the ER-stress response have been detected throughout the life of animal and human neurodegenerative disease-affected tissues.

The importance of BiP in neurons was demonstrated *in vivo* using a conditional BiP knockout in the Purkinje cells (PC) of the cerebellum. PC lacking BiP showed enhanced CHOP expression, caspase activation and a significant increase in neuronal apoptosis, relative to wild type (Wang et al., 2010a). Finally, pretreatment with an exogenous BiP inducer significantly attenuated CHOP levels and infarct size, compared with untreated mice subjected to cerebral ischemia (Kudo et al., 2008).

4.5 The role of ATF4 in chronic neurodegenerative disease

Given the recent evidence suggesting that the UPR is profoundly activated before disease-related symptoms arose, the next consideration was to analyze downstream effectors of the UPR to evaluate their importance in the determination of cell fate. Within the context of ALS, this was achieved by crossing mice expressing mutant SOD1 with mice carrying a targeted deletion for the pro-death gene, ATF4, which resulted in their increased survival, relative to wild type littermates (Matus et al., 2013). The pro-apoptotic function of ATF4 is believed to be executed either through the intermediate expression of CHOP, followed by the induction of the BH3-only genes, BIM and PUMA, or via the direct transcription of PUMA (Galehdar et al., 2010; Qing et al., 2012; Matus et al., 2013; Puthalakath et al., 2007). Consistent with this, PUMA, BIM and CHOP were found to be robustly expressed in SOD1^{G93A} mice prior to the development of the ALS phenotype, and motoneurons derived from SOD1^{G93A}-PUMA^{-/-} mice showed enhanced survival (Kieran et al., 2007). This protective benefit was also observed in BAX-depleted-SOD1^{G93A} animals (Gould et al., 2006). Interestingly, BAX oligomerization was detected in autopsied ALS tissue whereas control cases did not display signs of BAX activation (Martin, 1999).

The prolonged induction of the UPR and ATF4 is a prominent feature in AD, as observed in animal models of disease and *post-mortem* AD brain tissue (Ma et al., 2013; O'Connor et al., 2008; Lewerenz and Maher, 2009; Yoon et al., 2012; Hoozemans et al., 2005). Moreover, exogenous treatment with A β can induce ER- and oxidative stress in neuronal cells, and ATF4 knockdown rescued selected neuronal populations from A β -mediated toxicity *in vivo* (Leuner et al., 2012; Yoon et al., 2012; Baleriola et al., 2014). In agreement with our results, ATF4-depletion also attenuated neuronal apoptosis induced by ER and oxidative dysfunction, and this is thought to be mediated through a PUMA

and BAX-dependent pathway (Galehdar et al., 2010). Indeed A β stress has been shown to drive neuronal apoptosis through an intrinsic mitochondrial route, as evidenced in PUMA- and BAX-deficient neurons, and also through caspase activation *in vivo* (Feng et al., 2014; Akhter et al., 2014; Kudo et al., 2012; Xie et al., 2013).

In contrast to the pro-death function of ATF4 in AD, ALS and ischemic neuronal injury, its role in PD appears to favor survival. For example, it was recently shown that ATF4 knockdown sensitized neuronal cells to PD-inducing neurotoxins due to the reduction of Parkin (Sun et al., 2013). Interestingly and in the context of experimental PD, p53 appears to play a critical role in the regulation of neuronal death *in vitro* and *in vivo* (Perier et al., 2007; Bernstein et al., 2011; Bernstein and O'Malley et al., 2013). Conversely, ATF4 directs Parkin expression to reverse MPP⁺-induced neurotoxicity, suggesting that ATF4 observed in the PD brain plays a preservative role (Bouman et al., 2011; Sun et al., 2013). It is interesting to note that despite the fact that ATF4 and p53 have largely opposing functions in response to specific stimuli (i.e. DNA damage and ER-stress, PD-neurotoxins), sufficient evidence exists to indicate that each master regulator can influence the stability of the other (Jiang et al., 2007; Horiguchi et al., 2013).

4.6 Conclusion

In summary, we suggest that ATF4 plays a pivotal role in determining neuronal survival in response to ER dysfunction, oxidative stress triggered by arsenite toxicity, and oxygen deprivation. The executive pro-death function of ATF4 is carried out through the expression of PUMA to modulate arsenite- and hypoxia-induced neuronal apoptosis, or through the intermediate CHOP, which in turn drives PUMA during ER-stress. Importantly, these stress paradigms are known to accumulate PUMA in a p53-independent manner. This work provides viable therapeutic targets that could be considered to assist in the mitigation of chronic neuronal death observed after acute ischemic injury and in neurodegenerative conditions.

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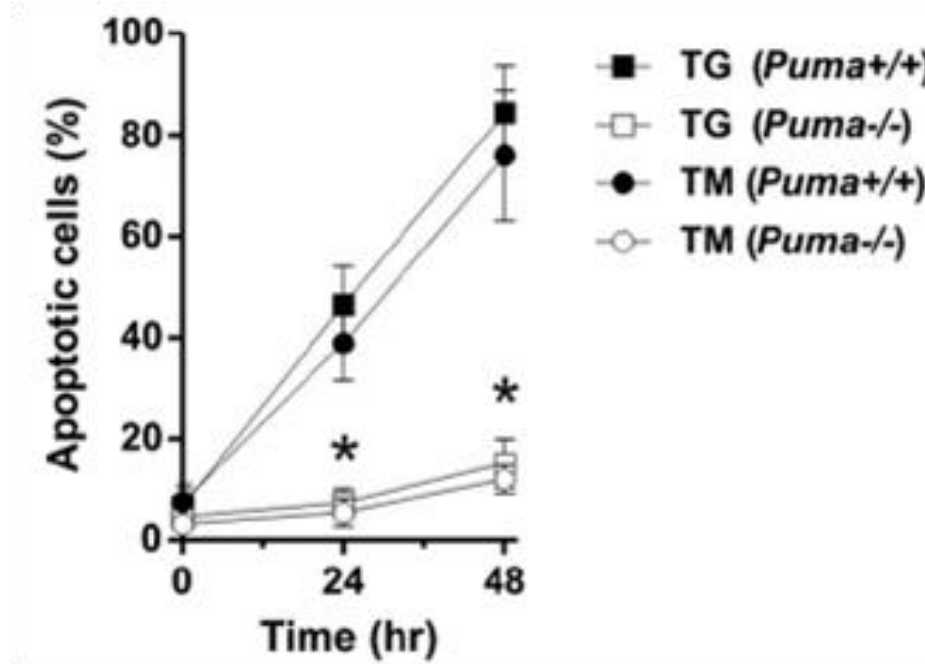
Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L., and Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* 12, 982-995.

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Appendices

Appendix A: PUMA-deficiency mitigates ER-stress-induced neuronal apoptosis

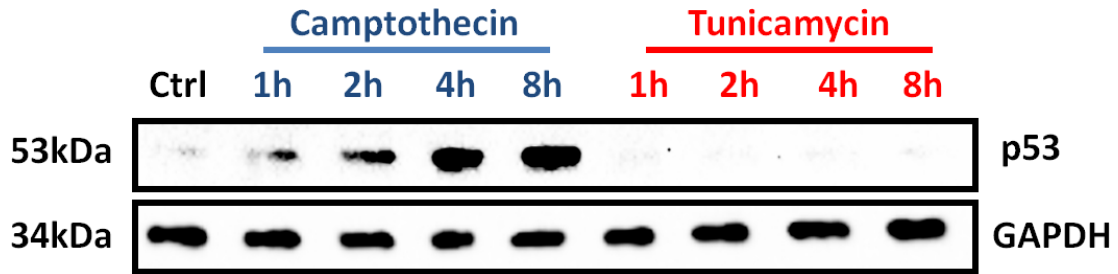
1.



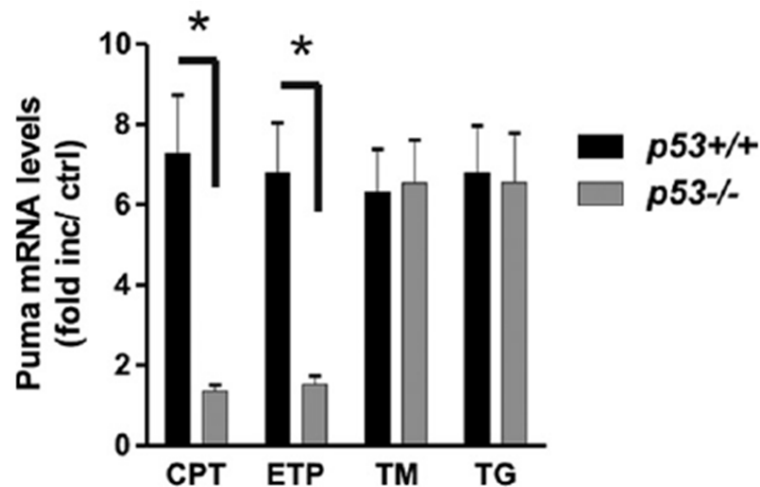
1. Cortical neurons derived from Puma wild type and knockout littermates were treated with 3 μ M TG or 4 μ M TM, and the fraction of apoptotic cells was determined at 24h and 48h by assessing nuclear morphology following Hoechst staining. The fraction of apoptotic neurons was significantly decreased in Puma^{-/-} versus Puma^{+/+} cultures ($n \geq 7$; $*p < 0.001$) (Galehdar et al., 2010).

Appendix B: p53 does not regulate *Puma* during ER-stress

1.



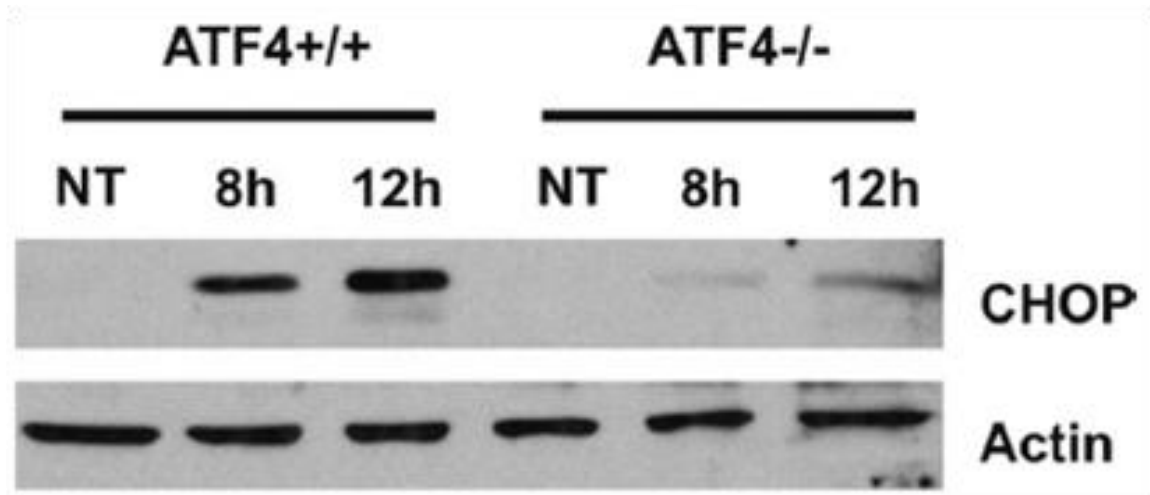
2.



1. Wild type cortical neurons were treated with 10 μ M camptothecin (CPT) or 4 μ M tunicamycin (TM) for 1h, 2, 4h and 8h, and protein levels of p53 and GAPDH were then assessed by Western blot. 2. Cortical neurons derived from p53 wild type and p53 knockout littermates were treated with TG (3 μ M), TM (4 μ M), CPT (10 μ M) or etoposide (ETP) (10 μ M). RNA was extracted 12h after treatment and *Puma* mRNA levels were quantified by RT-PCR. *Puma* expression was normalized to *S12* levels and is reported as fold increase over untreated controls ($n \geq 6$; * $p < 0.01$) (Galehdar et al., 2010).

Appendix C: ATF4 regulates CHOP expression during ER dysfunction

1.



1. ATF4^{+/+} and ATF4^{-/-} cortical neurons were treated with 4 μ M TM and after 8 and 12 hours CHOP and Actin protein levels were assessed by Western blot (Galehdar et al., 2010).

Curriculum Vitae

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